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AN INVESTIGATION INTO THE USE OF *ROL* GENES TO ALTER ROOT FORMATION AND GROWTH IN TRANSGENIC PLANTS

A thesis submitted for the degree of Doctor of Philosophy

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AMENDMENTS

p72 paragraph 2, line 2: a space should be inserted between the words 'roots' and 'were'.

p79 paragraph 2, line 3: 'Growth of the root systems were increased...' should read 'Growth of these root systems was increased...'.

p80 Figure 3.5 legend: after '...rehybridised with the ubiquitin probe (B).' insert 'Bar graph represents *rol*C transcript levels quantified relative to ubiquitin transcript levels for the Nt*rol*C and Nt35SC lines. Standardisation of unequal RNA loading levels by quantification of ubiquitin expression levels enables comparison of *rol*C expression levels between different samples.'

p86 Figure 3.8 legend: '...Pelosi et al., 1996' should read '...Pelosi et al., 1995'.

p88 paragraph 1, line 6: '...Figures 3.10 – 3.13.' should read '...Figures 3.9 – 3.12.'. Note that error bars in all graphs, representing the standard error of the mean, should be equally spaced above and below the mean.

p91 paragraph 3, line 3: a space should be inserted between the words 'other' and '*rol*'.

p125 §3.5.2.6 heading: 'Analysis of A/35S-*rol*C plants' should read 'Analysis of A/35S-C plants'.

p148 Figure 4.1 legend: 'P, petiole' should read 'Pe, petiole'.

p156 paragraph 1, line 8: After the words 'for white clover improvement' add 'that may be positively influenced by expression of *rol*C'.

p157 Figure 4.2B, diagram of DNA construct: replace 'term' with '*rol*C term'. To Fig. 4.2 (B) legend append 'Native *rol*C promoter and terminator sequences were used in the *rol*C construct'.

p225 paragraph 1: Replace the first sentence with 'Studies described in Chapter 5 of this thesis, while not conclusive, raised the possibility that some soil bacteria containing T-DNA with significant homology to Ri T-DNA from *A. rhizogenes* may be present in soils sampled from various sites in and around Melbourne.'

p225 Insert after paragraph 1: Although *A. rhizogenes* and *A. tumefaciens* are often classed as plant pathogens, the economic burden of these organisms in terms of plant disease and damage is unclear. This aspect would need to be evaluated to weigh up potential gains from the use of *A. rhizogenes* to promote rooting and/or root function against possible negative economic effects. This information may be relevant to current quarantine restrictions relating to *A. rhizogenes* in Australia.

ADDENDUM

Add the following table to page 54:

SUMMARY OF PLANT LINE DESIGNATIONS

Tobacco (*Nicotiana tabacum*) lines	
SR1	Non-transformed control line. This line is a streptomycin-resistant mutant from the variety Petit Havana (Maliga et al., 1973)
A/35S-GUS	Transformed SR1 containing the GUS gene under transcriptional control of the CaMV35S domain A promoter
Nt*rol*A	Transformed SR1 containing *rol*A gene under transcriptional control of native promoter
Nt*rol*B	Transformed SR1 containing *rol*B gene under transcriptional control of native promoter
Nt*rol*C	Transformed SR1 containing *rol*C gene under transcriptional control of native promoter
Nt35SB	Transformed SR1 containing *rol*B gene under transcriptional control of full CaMV35S promoter
Nt35SC	Transformed SR1 containing *rol*C gene under transcriptional control of full CaMV35S promoter
Nt35SB+C	Transformed SR1 containing *rol*B gene under transcriptional control of full CaMV35S promoter in combination with *rol*C gene under transcriptional control of native promoter
A/35S-C	Transformed SR1 containing *rol*C gene under transcriptional control of CaMV35S domain A promoter
White clover (*Trifolium repens*) lines	
WT	Non-transformed wild-type *T. repens* cv Haifa line
Tr*rol*C	White clover lines transformed with *rol*C gene under transcriptional control of native promoter

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ABSTRACT

Effects of *Agrobacterium rhizogenes rol* genes on root initiation and root and shoot growth, particularly the effects of *rol*C, were investigated in transgenic *Nicotiana tabacum* and *Trifolium repens*. Lateral root primordia (LRP) formation was analysed in roots of *N. tabacum* seedlings transformed with single or combined *rol* genes, in response to various concentrations of exogenously supplied auxin. Higher frequencies of LRP initiation relative to controls were observed in roots expressing the *rol*C or *rol*B genes, in response to auxin levels ranging from 10^{-6}M to 10^{-4}M, indicating an increased sensitivity to auxin with respect to LRP formation. The combined expression of both genes was also observed to have a synergistic effect on stimulating LRP initiation in response to auxin. These observations raise the possibility that transgenic plants expressing these genes may have an enhanced capacity for lateral root branching, due to an elevated sensitivity to LRP initiation within pericycle tissues.

Growth measurements were made of *rol* gene transgenic tobacco plants cultured *in vitro*, with or without a sucrose supplement, or in soil under greenhouse conditions. Results suggested that the presence of the *rol*C and *rol*B genes, particularly when in combination, induced alterations in root and shoot growth and also the root-shoot balance of plants. This was particularly evident from results of grafting experiments aimed at modelling the effects of root-specific expression of the *rol*C and *rol*B genes on relative root and shoot growth of plants. The combined expression of these two genes in transgenic rootstocks grafted to wild-type scions produced an increase in the relative root mass of plants and a substantial increase in overall plant biomass.

Transgenic *Trifolium repens* (white clover) plants expressing the *rol*C gene were generated to investigate the potential effects of the gene on growth and phenotype of this important forage crop species. Several independent transgenic clover lines expressing *rol*C were found to display a range of shoot phenotypic alterations and modifications to root growth. Possible advantages of these effects are discussed, with respect to breeding programs aimed at improving certain agronomic features of white clover, such as persistence within pastures.

The possibility that free-living *A. rhizogenes* may be present in Australian soils may be relevant to any future studies involving the release of *rol* gene transgenic plants. It would also be of interest in terms of the potential association of *A. rhizogenes* with growth of both agricultural crops and native plants. The presence of *A. rhizogenes* in soils representing various sites from around Victoria was investigated, surveying both cultivated and natural environments. Initial results suggested the presence of *A. rhizogenes* strains in a number of samples, however subsequent analyses were not conclusive. Further investigation involving more detailed characterisations of putative agrobacteria is merited, particularly to take into account the possible presence of novel strains in uncultivated soil environments.

DECLARATION

This thesis contains no material accepted for the award of any other degree or diploma in any university or other institution and, to the best of my knowledge, contains no material previously written or published by another person, except where due reference is made in the text.

Elaine Chow

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Outside of the department, thanks to friends and family for their patience and understanding during the T-thing - here it is! A special thank-you to Melinda and Matthew for offering constant friendship and vegout companionship and lots of gratitude to Michelle for helping me greatly. Thanks also to Floyd for the music.

I am incredibly grateful for my family, for their love and encouragement and practical support in many different forms. Finally, to my one and only true Scott, thank you for sharing this, and everything, with me (even the Tim Tams).

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CHAPTER 1

INTRODUCTION

Among the diverse and essential functions performed by plant root systems are the acquisition of water and nutrients, plant anchorage and storage of metabolites, as well as the synthesis of plant hormones and secondary metabolites. Although roots may appear structurally and developmentally simpler than aerial plant organs, the processes involved in fulfilling these functions are nevertheless complex. As with all plant organs, roots require mechanisms to respond to many different signals from, and regarding, their environment, in order to adapt and survive. Roots must be responsive to the soil environment, including the perception of nutrient and water levels, soil chemical composition, temperature gradients and oxygen status (Aiken and Smucker, 1996; Atwell et al., 1999a). The aerial plant environment is also crucial to plant growth as root and shoot systems are interdependent. Roots require the capacity to receive and respond to shoot signals - root-shoot feedback mechanisms may involve hormones, transport of photoassimilates and other metabolic compounds (Friend et al., 1994; Atwell et al., 1999a). In addition, roots need to communicate information to shoots regarding stressful or deficient soil conditions such as flooding, drought or salinity. Shoot growth processes (e.g. stomatal responses, rates of leaf expansion or leaf senescence) can be accordingly adjusted to help plants to tolerate the soil stresses. Plant hormones are known to have a key role in root to shoot signalling (Aiken and Smucker, 1996; Jackson, 1997).

In response to environmental and metabolic signals, roots can display considerable plasticity of morphology and architecture by modulating growth patterns via alterations in lateral root branching, elongation of primary and lateral roots, direction of root growth and development of root hairs (Schiefelbein and Benfey, 1991; Lynch, 1995). These mechanisms assist roots in adapting to their environment, facilitating the location and utilisation of scarce resources. Many root systems have also evolved finely-tuned symbiotic interactions with soil microorganisms, well-studied examples including the complex associations between legumes and nitrogen-fixing bacteria (Gualtieri and Bisseling, 2000) and between various plant species and fungal mychorrhizae to assist in uptake of phosphates (Atwell et al., 1999a).

Furthermore, many plants develop various forms of roots in addition to seminal roots (taproots, lateral roots); for example, adventitious (shoot-borne) roots can be

produced in response to waterlogging, forming above or near the flooded soil surface. A diverse array of other types of adventitious root adaptations have been described which modify a plant's ability to survive and proliferate in certain environments (Barlow, 1994). Examples include photosynthetic and storage roots, increasing a plant's capacity to convert and store energy, and various forms of support roots that have evolved to optimise the physical position of some species.

However, despite the plasticity and adaptability of root systems, the availability of water and/or nutrients in the environment are often limiting factors in plant growth and survival (Atwell et al., 1999e). These are important considerations in ecological terms and also crucial to agriculture and horticulture, affecting plant productivity and persistence. Improvement of root system efficiency in obtaining resources may thus be potentially advantageous to the productivity of plants or their capacity to survive in marginal environments. An assessment of the potential for modification of root system growth by genetic means is therefore of interest in terms of practical applications, as well for gaining a deeper understanding of a fundamental plant process. For example, root initiation and growth, interactions between roots and the chemical (nutritional) environment and regulation of the allocation of resources between the root and shoot systems are relevant to root system function. Increasing knowledge of root biology at a molecular level has further advanced understanding of mechanisms involved in root formation, development and growth (Rost and Bryant, 1996; Malamy and Benfey, 1997b; Benfey, 1999).

1.1 ROOT-SHOOT INTERACTIONS

1.1.1. Allocation of biomass to root and shoot

Plant growth is often monitored in terms of the amount of biomass, or dry weight, produced by the plant, during a period of time. Biomass production is dependent on the assimilation and subsequent distribution of carbon throughout the plant, influenced by the source-sink relationships between different plant tissues. Photosynthetically active plant tissues, as net producers and exporters of photosynthate, generally function as source tissues, whereas net importers of photosynthate such as roots or young leaves are usually regarded as sink tissues (Atwell et al., 1999b). Sink tissues can be further classified as either metabolic sinks or storage sinks. The first group includes

metabolically active tissues, such as roots and meristems, which utilise photosynthates mostly for rapid growth. The latter group may include tubers and developing seeds, in which photosynthates are deposited as storage compounds e.g. starch or lipids. Sink-to-source transitions can occur during development of some tissues, particularly leaves. In young rapidly growing leaves, more carbohydrate is utilised than is produced, however as leaves mature, net photosynthesis exceeds the demand for photoassimilates and surplus assimilates are available for export to sink tissues. Roots are generally regarded as irreversible carbon sinks, although in some species they may play an important role as storage sinks for subsequent plant growth (e.g. storage tap roots in carrot and related species) (Atwell et al., 1999b). Conversely, in relation to the acquisition and transport of nutrients and water from the soil, roots act as nutrient source tissues while shoots may be regarded as nutrient sinks in this context. In particular, nitrogen (N), as the major growth-limiting mineral nutrient for higher plants (Ericsson, 1995), is a key below-ground substrate.

Partitioning of dry weight between root and shoot depends on the allocation of assimilates and this is central to plant growth and development and the proportional growth of each component. For optimal plant growth and efficiency, a certain equilibrium of root and shoot growth is required, allowing plant parts to mutually support each other. The equilibrium between root and shoot can be expressed in simple terms, as the ratio of root and shoot masses - the root:shoot (r:s) ratio. Although this term is often used to measure the relationship between the root system and aerial parts of the plant, it is a simplified measure of plant growth, as the grouping of all above-ground organs into one compartment does not necessarily take into account the different functions that these organs may have. Poorter and Nagel (2000), among others, have proposed that plants be considered as at least three separate compartments - leaves, stems and roots - for a more representative analysis of biomass allocation. However, the r:s ratio has probably been the most commonly used parameter to date for assessment of partitioning and plant growth responses to the environment.

1.1.2. Alterations to plant growth and biomass allocation in response to environmental influences

Allocation of resources between root and shoot is generally regulated to maintain an equilibrium between the two components (Poorter and Nagel, 2000). In a constant

environment and at a given developmental stage, the relative growth rates of root and shoot would ideally remain at a certain ratio to each other (the allometric constant, *k*). However, natural plant environments are clearly not constant and, in response to changes in environment, the allocation of biomass may be adjusted to counterbalance any consequent deficiencies or surpluses in root or shoot resources and activity (Wilson, 1988; Ericsson, 1995). This is a key factor in the capacity of a plant to adapt to variations in environment. Allocation of biomass can also be affected by the plant species and ontogeny (developmental stage) (Wilson, 1988; Friend et al., 1994; Gedroc et al., 1996; Poorter and Nagel, 2000).

The influence upon allocation and plant r:s ratio of a plethora of environmental conditions, involving both above-ground and below-ground factors, have been observed and studied in detail. Influences in the aerial environment include air temperature and humidity, CO_2, ozone and light levels. Soil factors may include temperature and water content, availability of particular nutrients, soil chemical composition including trace elements, and interactions with rhizosphere microbes (Atwell et al., 1999a).

A widely supported theory to explain the mechanisms controlling the allocation of resources is the functional equilibrium concept, formulated by Brouwer (1962) and developed further into the transport-resistance (TR) model by Thornley (1972). This model is based on the control of root:shoot balance, by the availability of carbon (C) in the shoot and nitrogen (N) in the root, and the transport of C and N between the root and shoot systems (Thornley, 1972; 1998, Wilson, 1988). Deficiencies in above-ground resources are predicted to lead to preferential allocation of resources to the shoots; conversely, allocation of resources is shifted to roots when below-ground resources are at low levels. Plants are thereby able to obtain more of the resource that is limiting growth and this in turn helps to maintain plant equilibrium by ensuring that all resources are equally limiting.

For example, lower rates of photosynthesis by the shoot, as a result of reduced light levels, can lead to decreased carbohydrate availability. In response, a larger proportion of the available carbohydrate within the plant is allocated to the shoot to compensate for the deficiency, thereby reducing the C allocation to roots and leading to a decrease in r:s ratio. In one experiment, Minchin et al. (1994) shaded barley seedlings so that photosynthetic rate was reduced by about 80% relative to unshaded controls. As a consequence, the fraction of photosynthate partitioned to roots was reduced, from approximately 0.6 to 0.5. Root partitioning continued to be reduced during the three-hour period of shading but began to increase again once shade was removed.

Conversely, the TR model predicts that in conditions of reduced availability of nitrogen to roots, a greater part of the N taken up will tend to be utilised by the root system, reducing the N flow to the shoot and negatively affecting shoot growth. This leads to an increase in r:s ratio (Ericsson , 1995; Poorter and Nagel, 2000). Equivalent effects are predicted in response to water deficiency (Wilson, 1988) or for deficiencies of minerals which are closely involved in structural growth and/or synthesis of DNA or new proteins in a similar manner to nitrogen, including phosphorous (P), and sulfur (S) (Ericsson, 1995). Plants limited in P and S have been reported to have similar physical appearance to N-deficient plants, with reduced leaf area and increased accumulation of non-structural carbohydrates, i.e. starch, in leaves (Ericsson, 1995).

In further experimental evidence supporting this model of resource allocation, r:s ratios of birch (*Betula pendula*) seedlings were measured over a range of nutrient stresses, with concentrations ranging from 20% of the optimal level (i.e. nutrient deficient) to 100% of optimal level (i.e. no stress) (Ericsson, 1995). Root:shoot ratios were demonstrated to be increased in response to correspondingly reduced levels of N, P and S. In contrast, deficiencies in the nutrients potassium (K), magnesium (Mg) and manganese (Mn) have been shown to have the opposite effect on r:s partitioning (Cakmak et al., 1994). This is consistent with the apparent role that each of these minerals plays in enabling carbon fixation, rather than synthesis of new plant tissues (Ericsson, 1995).

Nitrogen interactions

The availability of N can affect r:s partitioning responses to limitations in other mineral nutrients, so that r:s ratios in N-limited plants may be different from that generally predicted by C:N resource models (Ericsson, 1995; McDonald and Davies, 1996). For example, contrasting effects on allocation in Mg-deficient birch seedlings were observed depending on whether N levels were lower than optimal (but not growth-limiting) or in surplus (Ericsson, 1995). In another example, contrary to general predictions (Wilson, 1988), biomass allocation in response to water deficiency in loblolly pine (*Pinus taeda* L.) was shown to be affected in a different manner to N deficiency, with r:s ratio significantly reduced by drought (Green et al., 1994). Furthermore, the interaction of the two stresses also altered allocation patterns - prior to drought conditions, plants grown under low N had higher r:s ratios than plants supplied with high N, however r:s ratios decreased over the course of the 10-day drought period so that significant differences in r:s ratios were no longer apparent as a result of the different N-treatments (Green et al., 1994).

Modifications to the TR resource partitioning model

One simplified aspect of the TR model is the treatment of the shoot only as a carbohydrate source tissue (Wilson, 1988; Minchin et al., 1994). In reality, of course, shoots typically include both carbohydrate source and sinks, with mature leaves primarily responsible for carbon fixation while the vegetative and floral meristems, young leaves, developing seeds and fruit all act as carbohydrate sinks. Carbon fixed by mature leaves is thus transported to both shoot and root tissue sinks. To take account of this biological complexity, Minchin et al. (1993) proposed an expanded TR model, describing the flow of photosynthate from a single source to two separate sinks and the effects of small changes in photosynthate supply to partitioning between the sinks. For example, reduced photosynthesis brought about by shading leads to reduced levels of C fixation and therefore a decrease in the amount of photosynthate available for export to sink tissues. In this model, an equal reduction in photosynthate partitioned to two equivalent sinks would be expected. However, as the shoot and root sinks are not equivalent, the decrease in flow is different for each sink - in the case of shading of shoots, the fraction of photosynthate exported to the root is less than that partitioned to the shoot.

Minchin et al. (1994) suggested that reductions in partitioning to roots in shaded barley seedlings occurred too fast to be properly accounted for by the initial TR model. As the TR model is based on alterations to supply of a substrate source, flowing on to affect function in the substrate sink, the time taken to affect photosynthate storage pools would most likely be longer than that actually observed (Minchin et al., 1994). In comparison, the modified TR model (Minchin et al., 1993) predicts that reductions in C supply will cause a change in partitioning to both sinks, with a greater proportion of the reduced photosynthate levels sent to shoots, a change which can occur rapidly. Other modifications have also been added to the Brouwer/Thornley model, taking into account factors such as ontogeny, adjustments in transport resistances or effects of hormones (Thornley, 1998). However, despite its simplicity, it has been found that plant responses to environmental changes generally concur with the predictions of this C:N balance model (Wilson, 1988; Ericsson, 1995; Thornley, 1998; Poorter and Nagel, 2000).

1.1.3. Effects of ontogeny on root-shoot partitioning

Plant growth characteristics such as biomass, partitioning of biomass and growth rates do not remain static throughout the course of development and may vary depending on

the developmental stage, or ontogeny, of the plant (Wilson, 1988; Gedroc et al., 1996; Gary et al., 1998). An example of ontogenetic changes was demonstrated with plants of two annual species, *Abutilon theophrasti* and *Chenopodium album* (Gedroc et al., 1996). Root:shoot ratios of these plants decreased over the course of development, highlighting the relevance of making comparisons at more than one point in the growth cycle to gain a more complete reflection of growth patterns. Another general example is the redirection of resources away from growth of vegetative organs during flowering or fruit production (Gary et al., 1998; Atwell et al., 1999b).

Modifications to the TR model of r:s partitioning were made by Thornley (1998) to include ontogenetic transitions in growth rates, substrate transport and partitioning fractions. For example, exponential growth (biomass) often occurs in young plants maintained in a constant environment but, as the plant matures, the overall growth rate gradually decreases and reaches a steady state. According to this modified model, the fraction of biomass that is allocated to shoots will increase as plant size increases, with a corresponding decrease in the r:s ratio.

Gedroc et al. (1996) also found that although r:s ratios partially conformed with optimal partitioning models, this was highly subject to ontogenetic constraints. Sampling plants at different stages of growth and under changing nutrient regimes revealed divergences from expected patterns of partitioning. Low-nutrient-grown plants had relatively higher r:s ratios than plants grown with high nutrient levels, as predicted by partitioning models. However, this was shown to be mainly due to an early increase in root biomass; in subsequent stages, the r:s ratios of the high- and low-nutrient plants converged. The rate of root versus shoot growth was less for low-nutrient plants in later stages than at earlier stages, even though biomass of plants under low-nutrient conditions remained lower than with high-nutrient conditions. Thus, plants were not able to completely optimise growth by altering partitioning to root and shoot, partly as a result of developmental limitations on continued allocation to roots.

Ericsson (1995) discussed the concept of steady-state nutrition in growth response experiments, whereby nutrient supplies and uptake are maintained in proportion to increasing plant weight. With internal nutrient status controlled in this way, parameters such as relative growth rate, and length, weight and area ratios will have steady-state values and will thus be generally similar in different plants in a group, regardless of size and age (Ingestad and Ågren, 1992). This approach would allow plant growth responses to environmental influences to be determined, essentially independent of ontogenetic factors.

1.1.4. Root system architecture and efficiency of responses to the environment

As described by biomass allocation models, growth and activity of root systems necessarily incur costs in terms of utilisation of carbohydrates and consequently have effects on the whole plant. In fact, roots have been estimated in some cases to utilise over half of the available photosynthate of mature plants (Fogel, 1985; Atwell et al., 1999a). Evidently, it is advantageous to plants that root functions are carried out as efficiently as possible.

The capacity for soil exploration and exploitation of resources is strongly dependent on the architecture (i.e. spatial configuration) of the root system (Lynch, 1995). Root architecture can also affect mechanical support of plants and influence root-microbe interactions (Lynch, 1995). As root architecture is determined by root initiation and developmental processes, it is therefore subject to an underlying genetic control (Schiefelbein and Benfey, 1991). Genetic variability is also a factor affecting the capacity of plants for resource acquisition and biomass allocation (Gabelman et al., 1986); regulation of allocation and partitioning may vary according to species or genotype (Friend et al., 1994; Poorter and Nagel, 2000).

In addition to intrinsic factors such as genotype, external factors also greatly influence root development, and the overall morphology and function of root systems are ultimately determined by the plant environment. In fact, root architecture is highly plastic and can be altered in response to a large range of physical, chemical and biological soil factors (Schiefelbein and Benfey, 1991). In this way plant root systems can be very responsive to the soil environment. This is an important attribute, as soil resources are often unevenly distributed, with patches or gradients of availability or depletion sometimes occurring over the space of centimetres (Lynch, 1995). In addition, the organisation of the root system influences efficiency of conduction of water and other solutes (Fitter, 1991; Lynch, 1995). Thus, root architecture affects the capacity ofroots for soil exploration, adaptation to changes in soil environment and efficient growth. These are aspects important for economy of root growth, in terms of balancing gains with investment of resources and consequently overall plant productivity and success.

1.1.5. Regulation of root development by nitrate availability

A notable example of plasticity of root growth is the perception of localised nutrient-rich zones in soil by roots of many plant species and the subsequent stimulation of lateral root (LR) formation, specifically within these zones (Gersani and Sachs, 1992; Leyser and Fitter, 1998). These localised regions of increased nutrient availability were initially thought to stimulate root growth due to a nutritional effect. However, this isa reverse effect to that observed in soils generally high in nutrients, in which partitioning of resources to roots, and hence root growth, is usually reduced (Wilson, 1988; Ericsson, 1995). Gersani and Sachs (1992) observed that development of individual roots and LR branching in response to local nutrient availability was also dependent on the nutritional microenvironment encountered by other roots within the same root system. Growth of one part of a root system was generally not as advanced when other parts were in relatively higher nutrient conditions.

Root growth responses to local and uniformly high nitrate (NO_3^-) levels were investigated in detail in *Arabidopsis*, leading to elucidation of some of the molecular signalling processes involved (Zhang and Forde, 1998). A model was proposed whereby NO_3^- regulates LR development via two distinct pathways: a systemic inhibitory effect dependent on the internal accumulation of nitrate ions in the shoot and an external stimulatory effect regulated by the localised NO_3^- concentration at the root tip (Zhang et al., 1999).

The localised stimulatory effect of NO_3^- upon LR formation was shown to be independent from NO_3^- metabolism and is triggered by NO_3^- as a signal molecule, as opposed to NO_3^- acting as a nutritional source. This was evidenced by a proliferation of LR in response to localised NO_3^- even in *Arabidopsis* mutants deficient in nitrate reductase (NR), which have a reduced capacity to use nitrate as a nitrogen source (Zhang and Forde, 1998). In addition, other nitrogen sources (i.e. NO_4^+ and glutamine) did not induce the same LR response as NO_3^- (Zhang et al., 1999).

It was noted that localised increases in NO_3^- concentration specifically affected LR directly exposed to the higher nutrient level and also that growth of primary roots was also not affected. Furthermore, in *Arabidopsis*, effects were limited to LR elongation, with no alterations in the number of LR initiated or in early primordium development (Zhang and Forde, 2000). Stimulatory effects of localised NO_3^- supply on LR were associated with 2- to 3-fold increases in the average elongation rate and were shown to be only elicited in mature LRs, after commencement of elongation. Interestingly, this was noted

to be different from studies involving barley and wheat in which both elongation and initiation were stimulated (Zhang and Forde, 2000).

1.1.5.1. Molecular analysis of root growth response to localised nitrate supply

The *ANR1* gene was identified as a key member of the nitrate signalling pathway, in response to localised NO_3^- supply, in *Arabidopsis* (Zhang and Forde, 1998). Expression of *ANR1* was induced specifically by NO_3^- treatment in N-starved roots; changes in K^+ or inorganic phosphate (PO_3^-) supplies did not similarly affect *ANR1* expression. In *Arabidopsis* lines in which *ANR1* expression was downregulated, by antisense suppression or cosuppression, LR growth was found to be unresponsive to stimulation by NO_3^-. In the most strongly downregulated lines, LR growth was significantly inhibited by NO_3^- at concentrations which did not affect control lines.

The homology of ANR1 to the MADS-box family of transcription factors suggests a role for *ANR1* in regulating transcription of genes involved in activation of LR meristems, in response to altered NO_3^- conditions. Furthermore, a likely overlap of the nitrate and auxin signal transduction pathways in *Arabidopsis* was suggested by the LR response of the auxin-resistant mutant *axr4* (Zhang et al., 1999). In *axr4* mutants exposed to localised NO_3^- supplies, increased elongation of LR was not stimulated. The role of the *AXR4* auxin-sensitivity gene and its position in the signalling pathway relative to *ANR1* are yet to be elucidated (Zhang and Forde, 2000).

1.1.5.2. High systemic nitrate levels

Systemic inhibitory effects of NO_3^- were observed in wild-type (WT) *Arabidopsis* seedlings grown on medium containing high KNO_3 (50 mM). Primary root growth was observed to be normal but LR development was strongly inhibited (Zhang et al., 1999). Further investigations found that LR primordia were initiated at normal frequencies and were able to develop up to approximately 0.2 - 0.5 mm in length. However, continued growth of LR were suppressed close to the point of emergence, which is the approximate time at which LR meristems are activated (Malamy and Benfey, 1997a). Thus, the critical stage at which LR become sensitive to the inhibitory effects of NO_3^- appears to be at, or just prior to, activation of the LR meristem (Zhang et al., 1999;

Zhang and Forde, 2000). These inhibitory effects of high systemic nitrate levels are not related to the actual external concentration of NO_3^- but to the internal levels of accumulated NO_3^- (Scheible et al., 1997b; Zhang et al., 1999). The signal involved in systemic inhibition of LR is not known, although it is thought to originate in the shoot. *ANR1* does not appear to be involved, as antisense suppression of the gene does not negate the inhibitory response (Zhang and Forde, 1998).

NR-deficient *Nicotiana* plants supplied with high NO_3^- levels were unable to assimilate NO_3^- adequately and subsequently accumulated large amounts of NO_3^- in the shoot (Scheible et al., 1997b). However, even though these plants were severely lacking in organic nitrogen, a nutritional status that might be expected to induce greater root growth, it was demonstrated that root growth of these plants was decreased and the number of LR reduced compared to WT plants in the presence of uniformly high NO_3^- supply (Scheible et al., 1997b; Stitt and Feil, 1999). Alterations to activities of key enzymes involved in carbon and nitrogen metabolism were also induced by NO_3^-, raising the possibility that changes to carbon allocation may be involved in alterations to root formation (Scheible et al., 1997a). Addition of sucrose to the plant medium was shown to partially reverse the negative effects of NO_3^- on root growth (Stitt and Feil, 1999; Zhang et al., 1999), however it has been suggested that inhibition of root growth is unlikely to be simply due to a general decrease in sucrose allocation to roots, as the effects of NO_3^- are highly specific to LR and particular stages of LR formation. Alternative suggestions are that the effect of supplying sucrose may have been to influence NO_3^- assimilation, thereby reducing the internal levels, or possibly to affect the plant C:N ratio, which may be a factor in the signalling process of LR inhibition (Zhang and Forde, 2000).

The two opposing signalling pathways in response to NO_3^- apparently provide the plant with a mechanism to regulate growth in response to both the amount of N present internally and external sources that may be available (Zhang and Forde, 2000). Thus, a plant may invest energy into LR growth to acquire NO_3^- from a localised source, depending on whether N levels are growth-limiting and the plant has a need to gain further N. This helps to maximise the efficiency of plant resource allocation. Some plant species have been shown to respond to local zones of NO_4^+ and inorganic phosphate with proliferation of LR specifically into these nutrient-rich zones, similar to the response of *Arabidopsis* to NO_3^- (Robinson, 1994; Leyser and Fitter, 1998). It is possible that analogous pathways to the nitrate signalling system, independent of metabolism of the nutrient, may exist for these nutrients.

1.2. LATERAL ROOT FORMATION

For potential modification of growth of plant root systems, one appropriate target for genetic manipulation may be the alteration of lateral root initiation and growth. Lateral roots are a key characteristic of the architecture, or spatial configuration, of the root system, which is determined by the pattern of root branching (topology) and rate and orientation of root growth (Lynch, 1995). Clearly these characteristics affect the size and spread of a root system and hence the ability of a plant to efficiently explore and exploit the soil environment.

At the cellular level, lateral root (LR) formation involves the postembryonic initiation of a new organ, with the development of a new meristem from differentiated root cells. Key events in LR development involve the reactivation of specific pericycle cells, a systematic series of cell divisions and expansions to form an organised primordium, emergence of the primordium and the precisely regulated activation of an autonomous meristem to control subsequent growth of the lateral root (Laskowski et al., 1995; Malamy and Benfey, 1997a; 1997b).

The physiological sequence of events taking place early in lateral root initiation and development have been observed and described in detail in a range of species including *Arabidopsis*, radish, chicory and white clover (Blakely et al., 1982; Laskowski et al., 1995; Malamy and Benfey, 1997a; Mathesius et al., 1998; Vuylsteker et al., 1998). More recently, some of the genes involved in LR initiation and development have been identified and isolated (Rost and Bryant, 1996; Malamy and Benfey, 1997b).

1.2.1. Physiolcgical events of LR formation

Lateral root primordia (LRP) are initiated in the pericycle, the cell layer adjacent to the root stele (Fig. 1.1). Initiation of LRP occurs in pericycle cells in the differentiated zone of roots, rather than the actively dividing cells in the root apical meristem region (Malamy and Benfey, 1997b). The radial location of lateral root initiation is determined by the architecture of the vasculature, with LRP originating in pericycle cells that are in specific positions in relation to the xylem or phloem poles, depending on the plant species (Fig. 1.2). The number of xylem or phloem poles also varies among different species. In diarch species, including *Arabidopsis thaliana*, tobacco (*Nicotiana tabacum)* and radish (*Raphanus sativus*), lateral roots originate from pericycle cells located adjacent to either

of the two xylem poles (xylem-radius), so that lateral roots are formed in two files on opposite sides of the primary root. Xylem-radius pericycle cells are distinguishable in longitudinal sections by their shorter cell length, indicating that a greater number of divisions have occurred than for other pericycle cells (Casero et al., 1989; Laskowski et al., 1995). For example, xylem-radius pericycle cells are half as long as phloem-radius pericycle cells in *Arabidopsis* and one-third as long as phloem-radius pericycle cells in radish (Laskowski et al., 1995). Differences in cell division in the pericycle cells that are specifically stimulated to form LR may reflect alterations in cell cycle regulation. Unlike most other non-dividing cells, which are arrested in the G_1 phase, pericycle cells are arrested in the G_2 phase of the cell cycle (Blakely and Evans, 1979). Re-entry into the cell cycle is likely to be an important point of control governing the capacity of these cells to initiate LR primordia (Malamy and Benfey, 1997b).

Initiation of a lateral root primordium (LRP) begins with an ordered series of transverse (anticlinal) divisions in the xylem-radius pericycle cells (i.e. perpendicular to the root axis) (Fig.1.3). The transverse divisions are followed by radial expansion of these cells, which subsequently undergo periclinal divisions (parallel to root axis). Other pericycle cells adjacent to the specific xylem-radius pericycle cells also become involved in the formation of the LRP. In *Arabidopsis*, the number of original pericycle cells initially involved in LRP formation was estimated to be 11 by Laskowski et al. (1995)- 6 around the arc of the pericycle (transversely) and an average of 2.4 cells longitudinally. As a result of these cell divisions, a layered, domed structure is formed, extending outward from the primary root stele to the surface (Casero et al., 1989; Laskowski et al., 1995; Malamy and Benfey, 1997a). Primordia generally emerge through the primary root surface when about eight layers have been formed (Laskowski et al., 1995).

Changes in the cellular patterns and organisation of the LRP are observable around the 8-layer stage and the structure of the primordium develops similar characteristics to the primary root tip (Laskowski et al., 1995; Malamy and Benfey, 1997a). With the use of transgenic *Arabidopsis* marker lines expressing ß-glucuronidase (GUS) in specific cell types, cell layers in the primordium analogous to those in the primary root tip have been identified (Malamy and Benfey, 1997a). Periclinal divisions in the outermost layers are followed by longitudinal divisions in the central part of the primordium, behind the apex, that result in long, procambium-like cells. Further periclinal divisions near the apex result in formation of the root cap.

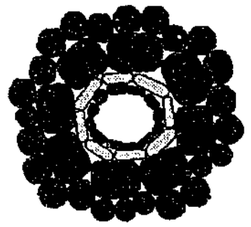


Figure 1.1. Schematic diagram of a cross-section of an *Arabidopsis* root, depicting cell layers. From centre: root stele (white); pericycle layer (green); endodermis (grey); cortex (blue); epidermis (brown).

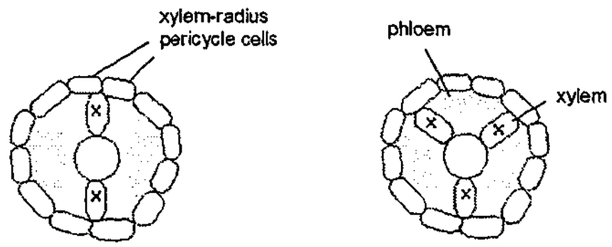


Figure 1.2. Schematic representation of cross section of root stele, showing two examples of different root vascular anatomy that may underlie lateral root initiation positions. (A) diarch root with two xylem bundles - xylem-radius cells are indicated; (B) triarch root. Depending on the species, LR may be initiated from pericycle cells adjacent to phloem poles (represented as shaded regions) or xylem poles (marked as x) or between the phloem and xylem poles. The relationship between xylem or phloem and position of LR initiation is presumed to facilitate the vascular connection of a new LR to the parent root (Atwell et al., 1997c).

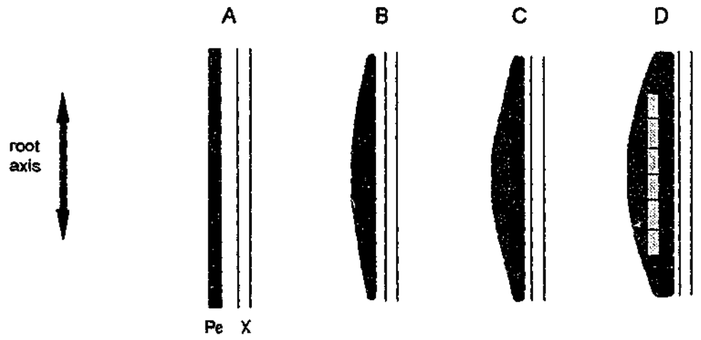


Figure 1.3. Schematic diagram of initial pericycle cell divisions in LRP initiation. (A) Longitudinal root section with file of pericycle cells (Pe, green) adjacent to xylem (X, white). (B) Anticlinal cell divisions (perpendicular to root axis), followed by radial expansion of specific pericycle cells. (C, D) Expanded cells undergo periclinal divisions (parallel to root axis) represented by brown and green cells. Subsequent cell divisions result in a domed layered structure growing outwards from the root stele.

The differentiation of an autonomous root apical meristem, capable of forming a root, has been shown to occur at around the 3-5 cell layer stage (Laskowski et al., 1995). This was demonstrated with short root segments excised from *Arabidopsis* seedlings that were cultured without auxin. On 0.5 mm long root segments, previously initiated primordia consisting of two cell layers were shown to be unable to continue formation of lateral roots, whereas most primordia of 5 cell layers or more were able to develop into lateral roots (Laskowski et al., 1995). However, Malamy and Benfey (1997a; b) noted evidence in *Arabidopsis* roots that the new meristem is not active until the primordium emerges from the parent root. During emergence, there is no increase in the number of cells in the outer layer of the primordium - emergence involves expansion of cells at the base of primordium, rather than cell division (Malamy and Benfey, 1997a). This is consistent with earlier studies in which the mitotic index in LRP was shown to be almost zero prior to LR emergence (MacLeod, 1972). After emergence through the parent root epidermis, the number of cells near the primordium root tip begins to increase, indicating activity of a functional meristem.

Characterisation of the *Arabidopsis root meristemless1* and 2 (*rml1* and 2) mutants also indicated that establishment of a root meristem occurs after initiation of the root primordium (Cheng et al., 1995). *rml* mutants were able to produce embryonic roots, which arrested soon after germination. Lateral roots were initiated from the short primary roots but growth was also arrested at a similar point after emergence. Cessation of growth was shown to be due to limited or lack of cell division specifically in root apical cells, so that mutants are unable to form an active root meristem. The formation and emergence of LRP prior to growth arrest is consistent with the late activation of the meristem.

1.2.2. The role of auxin in lateral root initiation

The primary role of auxin in lateral root formation has been clearly established, in physiological studies involving both exogenous and endogenous auxin and, more recently, following the characterisation of mutants with altered LR formation. Stimulation of LR initiation in response to application of exogenous auxin has been demonstrated consistently in roots of intact seedlings and plants, and in root cultures, for a multitude of species. Examples include classical experiments conducted with pea roots by Thimann (1936) and Torrey (1950), as well as more recent reports involving a range of species

(Wightman and Thimann, 1980; Blakely et al., 1988; Laskowski et al., 1995; Pelosi et al., 1995; Vuylsteker et al., 1998).

1.2.2.1. Role of endogenous auxin in LR development

Movement of endogenous auxin in roots occurs in two directions. From the site of synthesis in the shoot, auxin is transported acropetally to the root tip via the root stele (Torrey, 1976), the main transport stream of auxin in the root. Basipetal movement away from the root tip, via epidermal cells, has also been identified (Meuwly and Pilet, 1991), although this is likely to occur only in regions nearer to the root tip and it is not certain whether this auxin originates from the shoot or root apex, or both (Reed et al., 1998)

The involvement of an auxin gradient from shoot to root in LR development has been well established (Torrey, 1976; 1986; Wightman and Thimann, 1980). Further evidence that LR development is dependent upon acropetal auxin transport has been demonstrated by application of the auxin-transport inhibitor naphthylphthalamic acid (NPA) in specific positions along *Arabidopsis* roots (Reed et al., 1998). NPA treatment at the root-shoot junction reduced the flow of endogenous auxin (indole acetic acid, IAA) into the root, decreasing the number and density of LR formed compared to untreated roots. NPA application at sites along the root caused significant reductions in the density of LR formed, at the site at which it came into contact with root tissue and in regions apical to the point of application. NPA application in the apical half of the root did not affect LR formation in the upper (basal) half. Furthermore, application of IAA, apical to NPA, partially restored LR development in the apical regions of the roots. These results concur with previous observations of reduced LR formation in response to removal of aerial tissues (Wightman and Thimann, 1980) and in Transport Inhibitor Response (TIR) mutants of *Arabidopsis*, in which polar auxin transport is reduced (Ruegger et al., 1997).

A number of mutants with altered LR formation have been characterised which further establish the involvement of auxin and polar auxin transport in normal LR initiation. LR production is significantly repressed in the *axr1, axr4* and *aux1* auxin-resistant mutants of *Arabidopsis* (Hobbie and Estelle, 1995; Timpte et al., 1995). Similarly, a lack of response to auxin in the *Arabidopsis* mutant *altered lateral root formation4* (*alf4*) corresponds to a lack of lateral root production (Celenza et al., 1995). Conversely, extensive proliferation of lateral roots in the allelic *Arabidopsis* mutants *superroot* (*sur*), *rooty* (*rty*) and *altered lateral root formation1* (*alf1*) mutants (Boerjan et

al., 1995; Celenza et al., 1995; King et al., 1995) are likely to be due to the elevated IAA levels in these plants. Transformants of petunia and other species overexpressing microbial auxin biosynthetic genes also produce more LR than nontransgenic controls (Hamill, 1993).

The involvement of auxin in lateral root development was investigated using the promoter of an auxin-responsive gene from soybean, *GH3*, fused to the GUS reporter gene (Larkin et al., 1996). Expression of the construct in roots of transgenic white clover was identified in a group of cells located in the outer cortex, opposite sites of primordia initiation. Emerging LR were shown to grow towards and through these localised areas of GUS activity, suggesting the presence of an increasing auxin gradient from the root stele to the outer cortex (Larkin et al., 1996) and implying that asymmetry of auxin levels may be a mechanism by which stimulation of LR initiation is controlled and limited to specific pericycle cells.

In *GH3*-transformed white clover roots, it was also observed that the intensity of GUS staining increased with distance from the root tip (Larkin et al., 1996). This may indicate reduced root apical dominance and the presence of optimal concentrations of auxin (corresponding to the higher position along the auxin gradient). Further work with the same transgenic plants demonstrated that *GH3*-GUS expression in the cortex is preceded by expression in the earliest dividing pericycle cells (Mathesius et al., 1998). Pericycle cells which subsequently differentiate into primordia are located in a zone of high auxin prior to and during division of cells, verifying the involvement of localised regions of elevated auxin levels in the initiation of lateral root primordia.

The requirement for high auxin levels during primordium initiation is not maintained during subsequent stages of LR formation. Observations of expression patterns of the *GH3*-GUS gene construct noted above indicated reduced expression in primordia during meristem differentiation and emergence of the primordia (Mathesius et al., 1998). In fact, it has been demonstrated that continued treatment with high levels of exogenous auxin following stimulation of primordia initiation inhibits further development and conversion of primordia into lateral roots (MacIsaac et al., 1989; Pelosi et al., 1995).

1.2.2.2. Effects of exogenous auxin on LR formation

In a process analogous to that of endogenous auxin, exogenously applied auxin is transported acropetally to root tips, via phloem tissue (reviewed in Torrey, 1976) and stimulates increased initiation of LR. Exogenous supply of IAA to roots of *Arabidopsis*

and radish induces the activation of many more pericycle cells along the root than usual, extending the lateral meristem-initiating region to the whole length of the primary root and reducing the spacing interval between the lateral roots formed (Hinchee and Rost, 1992; Laskowski et al., 1995). In radish roots treated with 90 µM IAA, Laskowski et al. (1995) observed that all xylem-radius pericycle cells divided, forming two continuous files of pericycle-derived cells, with subsequent formation of many closely spaced lateral roots.

It is evident that many pericycle cells (in the correct location in relation to the xylem) along the length of a root have the capability to form LR meristems but may usually be restricted by limitations set by endogenous auxin levels. Evidently, treatment with exogenous auxin can stimulate LRP formation in these normally unresponsive cells. Constitutive expression of the *cdc2a* (cell division cycle) gene, a key gene in the control of cell cycle progression, was demonstrated in pericycle cells and meristematic tissue of *Arabidopsis* (Martinez et al., 1992), and has been suggested to be an indication of the competence of these cells to divide (Hemerly et al., 1993; John et al., 1993). In pea roots, levels of $p34^{cdc2}$, the 34kDa protein encoded by *cdc2*, were shown to increase when cells were stimulated to divide by auxin treatment (John et al., 1993) and the frequency of primordium formation was correlated with the level of $p34^{cdc2}$ protein induced. Thus, stimulation of LRP by auxin may be induced by the reactivation of differentiated pericycle cells, which then recommence cell cycle progression (Blakely et al., 1982; Malamy and Benfey, 1997b). The mechanism by which auxin triggers some pericycle cells to divide and not others is unclear but may be related to differences in auxin perception or transport.

Vera et al. (1994) demonstrated molecular evidence that pericycle cells of tobacco roots undergo developmental reprogramming during LR initiation, by analysis of expression of the *parA* gene in roots. *parA* is an auxin-regulated gene identified as a marker for cells undergoing cellular reprogramming. Expression of the gene was shown to be associated with protoplasts regaining the competence to divide, after isolation from leaf mesophyll cells (Takahashi et al., 1989). In roots of tobacco plants treated with auxin, expression of the *parA* gene was observed from 1-2 hours after auxin treatment, indicating reactivation of cell activity at an early stage of LR initiation. In comparison, the first pericycle cell divisions of primordium development occur between 5 and 10 hours after auxin treatment in radish roots (Laskowski et al., 1995). *parA* continued to accumulate for at least a further 48 hours, in initial cells and surrounding pericycle cells, during subsequent stages of primordium formation.

1.2.3. Auxin-cytokinin interactions in LR formation

Evidence of a role for cytokinin in LR production has also been demonstrated, as may be expected given the essential role of cytokinin in cell division (John et al., 1993; Zhang et al., 1996). The main sites of endogenous cytokinin synthesis are root tips, from which cytokinin is transported in a basipetal direction (Bollmark et al., 1988; Letham, 1994). A cytokinin gradient is thus established in the opposite direction to the auxin gradient, with the highest concentrations in the root tip decreasing with distance from the tip, then rising again in the basal region of the root

Evidence of negative control of LR initiation by one or more root tip factors, quite possibly a cytokinin, was demonstrated by a greatly increased frequency of LR formation in response to removal of the root tip (Wightman and Thimann, 1980; Biddington and Dearman, 1982). In support of these earlier studies, stimulation of lateral root formation has also been observed as an effect of ablation of root cap cells (Tsugeki and Fedoroff, 1999). Root cap cells of *Arabidopsis* were genetically ablated by root cap-specific expression of a diptheria toxin gene, DT-A, which inhibits protein synthesis. DT-A transgenic plants had morphologically normal shoots but roots were slow-growing and agravitropic, with increased numbers of secondary and tertiary lateral roots.

Classic studies involving tobacco tissue cultures demonstrated that the relative ratio in which the two phytohormones were supplied was the major determinant of whether undifferentiated tobacco stem pith tissue differentiated into shoot, callus or root tissues (Skoog and Miller, 1957). A relatively high auxin:cytokinin ratio led to root production, whereas a relatively high cytokinin:auxin ratio stimulated shoot production. Analyses of transformed plants overexpressing microbial genes for auxin or cytokinin biosynthesis have generally supported the studies involving exogenous application of these hormones (Hamill, 1993). Thus, with certain levels of auxin and cytokinin necessary to promote lateral root differentiation, LRP are most likely to be stimulated in regions of the root where the balance between the two phytohormones is appropriate (reviewed in John et al., 1993).

The effects of exogenous cytokinin on LR formation may differ depending on the concentration (John et al., 1993). In the presence of optimal levels of auxin, high exogenous cytokinin concentrations (more than 10^{-6}M) were found to be inhibitory to LRP formation and LR emergence in pea seedling roots, whereas cytokinin at lower concentrations (10^{-7}M) was stimulatory to LRP initiation (Wightman and Thimann, 1980). These respective inhibitory and stimulatory effects of high and low cytokinin treatments

have also been noted in other species including lettuce (MacIsaac et al., 1989) and eucalypt (Pelosi et al., 1995).

John et al. (1993) demonstrated that high cytokinin levels repress accumulation of $p34^{cdc2}$, the cell cycle protein encoded by *cdc2* and a key factor in the control of cell cycle progression. Reduced p34 protein levels, resulting in arrested cell division, were correlated with an inhibition of LRP induction in pea roots. However, the presence of low concentrations of cytokinin was shown to be stimulatory to, and necessary for, root cell division. Utilising quiescent tobacco pith tissue, which requires exogenous auxin and cytokinin for cell division, Zhang et al. (1996) found that the addition of auxin induced high levels of $p34^{cdc2}$-like proteins, but that these protein kinases were not activated without the presence of cytokinin. Cytokinin was shown to control tyrosine dephosphorylation and activation of the $p34^{cdc2}$ protein kinases and was necessary for the transition of cells from the G_2 cell cycle phase to mitosis.

1.2.4. Identification of genes expressed during LR initiation and development

1.2.4.1. Genes isolated via mutants

Recently, a number of genes have been identified that are involved in lateral or adventitious root initiation and development (Rost and Bryant, 1996; Scheres et al., 1996; Malamy and Benfey, 1997b).

Although not exclusively affecting roots, genes of the *AUX/IAA* family of early auxin-induced genes have been shown to have a central role in auxin signalling and are therefore relevant to root initiation and development (Guilfoyle et al., 1998; Rouse et al., 1998). Proteins encoded by members of the *Arabidopsis AUX/IAA* gene family interact with members of a family of transcription factors called auxin response factors (ARFs), which bind to auxin response elements in the promoters of many auxin inducible genes (Guilfoyle et al., 1998). Mutations in an *AUX/IAA* gene, *IAA17* (identical to *AXR3*) appeared to induce a hypersensitive response in *Arabidopsis* seedlings, with increased auxin responses including stimulation of adventitious rooting, reduced root elongation and a lack of gravitropism in roots and hypocotyls (Leyser et al., 1996). *axr3* point mutations were found to occur in domains that are highly conserved in AUX/IAA proteins and it was suggested that these mutations may affect interaction of IAA17/AXR3 with ARFs and/or increase stability of the protein (Rouse et al., 1998).

Another member of the *AUX/IAA* family, *SHY2/IAA3* (short hypocotyl) from *Arabidopsis* was demonstrated to regulate several auxin responses in roots (Tian and Reed, 1999), with mutations in *SHY2/IAA3* affecting auxin-dependent root growth, particularly LR formation, root elongation and gravitropism. SHY2/IAA3 appeared to have both positive and negative regulatory effects on auxin responses. LR formation was increased in loss-of-function *shy2/iaa3* mutants compared to WT, suggesting negative regulation by the gene. However, the short root phenotype of loss-of-function mutants could be rescued by low concentrations of auxin, at concentrations which inhibit root elongation in WT plants - these mutants may therefore have a reduced responsiveness to auxin and SHY2/IAA3 may also be interpreted as a positive regulator of auxin response. It was suggested that SHY2/IAA3 activity may involve differential expression or feedback control mechanisms, or alternatively may affect auxin transport (Tian and Reed, 1999).

RML (ROOT MERISTEMLESS) genes from *Arabidopsis* (as referred to in §1.2.1) have been shown to specifically affect root meristem formation and are thought to represent a key checkpoint for the establishment of root meristems. Activation of cell division in root apices of *rml* mutants following germination is defective and roots are therefore unable to establish a functional meristem. *RML1* has subsequently been cloned and found to encode the first enzyme in the pathway of glutathione biosynthesis (GSH) (Vernoux et al., 2000). Treatment of mutant seedlings with GSH reversed the mutant phenotypes. Furthermore, it was shown that adequate levels of GSH are essential for transition of cells from G_1-phase to S-phase. The findings suggest that a GSH-dependent pathway is necessary for normal post-embryonic cell division and meristem formation in roots. Interestingly, *RML1* was also shown to be allelic to the *Arabidopsis CADMIUM SENSITIVE2* gene (Vernoux et al., 2000).

LRP1, a gene expressed specifically in LRP and also adventitious root primordia (ARP), was isolated from *Arabidopsis* by insertional mutagenesis with a gene trap transposon, carrying a promoterless GUS reporter gene (Smith and Federoff, 1995). The gene was initially identified by expression of GUS in LRP and was subsequently cloned and sequenced. Utilising *in situ* hybridisations to *LRP1* cDNA probes, *LRP1* was shown to be expressed very early in LRP formation, until just prior to emergence of the LR from the parent root. Expression was not detected in root apical meristems. Evidence from DNA and RNA gel blots suggested *LRP1* to be part of a small gene family and as transposon insertion at *LRP1* did not result in a discernible mutant phenotype, it was suggested that some members of the gene family may have overlapping functions.

cdc2 genes and cyclin genes, in combination, are necessary for the control of cell cycle progression in plants, as well as many other eukaryotic systems (John et al., 1993). *cdc2* genes encode cyclin-dependent protein kinases which catalyse plant cell division. Cyclins, encoded by *cyc* genes, activate and direct the substrate specificity of protein kinases. As a consequence of the role of *cdc2* protein kinases in cell division (Hemerly et al., 1993; John et al., 1993), the regulation of *cdc2* and cyclin gene expression and protein activity are key factors affecting LR formation.

Although a key component of competence for cell division, expression of *cdc2a* is not confined to dividing cells (Martinez et al., 1992; Hemerly et al., 1993), suggesting that the $p34^{cdc2}$ protein is not the overall controlling factor in cell cycle progression. It is likely that the key role of the p34 protein kinase in regulation of cell division may be dependent on the cyclin proteins with which it interacts. In contrast to *cdc2*, expression of a cyclin gene from *Arabidopsis*, *cyc1At*, was shown to be specific to actively dividing cells, such as those of root apical meristems and LRP, and was not detected in quiescent pericycle cells (Ferreira et al., 1994b). Accordingly, the possibility that cyclin may be a key factor in triggering LRP initiation in pericycle cells was initially considered (Doerner et al., 1996). However, expression of *cyc1At* in non-dividing pericycle cells, under control of the *Arabidopsis cdc2a* gene promoter, was found to increase the rate of LR elongation but did not alter the frequency of LR initiation (Doerner et al., 1996). In these *cdc2aAt::cyc1At* transformants, growth of LR were accelerated due to stimulation of cell division in meristems, suggesting that *cyc1At* may be a limiting factor for growth of root meristems. Furthermore, as the frequency of LRP initiation was not altered by *cyc1At* expression in non-dividing pericycle cells, this indicated that control points other than *cyc1* are involved in triggering initiation of LR apical meristems.

Other cyclin genes expressed in roots, including the root-specific cyclin *2bAt* gene, are also highly correlated with cell division activity (Ferreira et al., 1994a). Hochholdinger and Feix (1998) investigated the expression of cyclins as potential molecular markers for initiation of crown root primordia in maize. Cyclin genes were specifically expressed in primordia regions of WT plants but were not expressed in crown root deficient mutants. In contrast, *cdc2* gene products were present in both mutant and WT tissues.

1.2.4.2. Genes isolated via differential expression approaches

Four cDNA clones, representing genes expressed during auxin-induced lateral root formation, were isolated from an *Arabidopsis* root culture cDNA library (Neuteboom et al., 1999). The gene sequences appeared to encode extracellular proteins, possibly involved in alteration of cell wall characteristics and structure during LR formation. Further characterisation revealed two very similar genes, *AIR1A* and *AIR1B*, which encode proteins that are likely to weaken plasmamembrane-cell wall connections. Another gene, *AIR3*, has been shown to encode a protease which may play a role in digesting cell wall proteins (Neuteboom et al., 1999). *AIR* genes are apparently exclusively expressed in cells of the outer layers of the parent root, at positions from which LR subsequently emerge. It is thought that *AIR* genes may be activated by signals sent from underlying auxin-activated pericycle or cortical cells, inducing a weakening of cells and/or cellular connections in cells expressing the *AIR* genes (Veth-Tello et al., 2000). Furthermore, although cell division in pericycle cells is not a prerequisite for auxin-induced *AIR* expression, expression is reported to be severely diminished in *Arabidopsis alf*4-1 mutants, in which the response of pericycle cells to auxin is deficient (Celenza et al., 1995).

Another gene identified as being involved in lateral root formation is *HR7*, a gene preferentially expressed in hairy roots of *Hyoscyamus niger* (Mikami et al., 1999). Expression conferred by the *HR7* promoter was shown to be specifically localised to LR primordia and the base of LR. Transgenic lines overexpressing *HR7* were shown to have greatly increased root system branching, with the degree of phenotypic effects correlated with the level of expression detected. The findings appear to indicate a substantive role for the gene in LR initiation. Some homology of the *HR7* protein to metallocarboxypeptidase inhibitors was identified and it was suggested that *HR7* may interact with other proteins, possibly in signal transduction or as a peptidase inhibitor.

RSI-1 (Root System Inducible-1), an auxin-inducible gene predominantly expressed in roots and activated in early LRP, was isolated from tomato (*Lycopersicon esculentum*) (Taylor and Scheuring, 1994). *RSI-1* was identified in a search for genes involved in LR initiation, activated at a similar time to the initiation of LR by auxin treatment. A cDNA library was constructed from roots treated with auxin for 36 hours, and differentially screened with probes derived from untreated and auxin-treated roots. 36 hours of auxin treatment was found to be sufficient to induce initiation of approximately 50% of the number of LR that would eventually form. Following

identification and cloning of *RSI-1*, root expression patterns in tomato were examined by transformation with *RSI-1* promoter-GUS fusions. Expression of the reporter gene was detected at a very early stage of LRP initiation, in pericycle cells which had just undergone the first periclinal division. High level *RSI-1* expression was continued during emergence of the LR but was diminished in more mature meristems - in LR that had reached approximately 3-4 mm in length and in parent roots.

1.2.5. Comparison of adventitious and lateral root formation

Functions of adventitious roots (AR) are generally considered to be equivalent to that of the primary root system, e.g. with respect to basic functions of nutrient and water acquisition and plant anchorage. However, it has been demonstrated that adventitious roots of some species may have alternate physiological and physical roles to underground roots, with differing roles in the uptake of nutrients and water (Kovar and Kuchenbuch, 1994). Complementary functions of adventitious and seminal roots may be needed to maximise plant growth and adaptability in certain conditions and/or developmental stages.

AR may arise from preformed or induced primordia. The series of anatomical events occurring during *de novo* AR formation appear to be analogous to those involved in LR formation. As with LR, AR formation involves dedifferentiation of initial cells and cell divisions to form a primordium structure, followed by formation of a new meristem and subsequent root extension (Blakesley, 1994). Adventitious roots differentiate from cells close to vascular tissues, commonly from phloem parenchyma or inner cortical parenchyma cells (Esau, 1977; Lovell and White, 1986). Although an exact equivalent of a pericycle cell layer is not present in tobacco stems, expression of a root-specific *HRGPnt3*-GUS gene fusion construct in transgenic plants demonstrated AR to be initiated in cells surrounding the vascular system in tobacco stems, i.e. tissue analogous to pericycle cells of roots (Vera et al., 1994). However, unlike LR, initiation of AR was not limited to specifically positioned cells, with AR primordia apparently initiated in any of the cells around the perimeter of vascular tissue.

Auxin has a comparable role in AR and LR formation. As with LR formation, AR requirements for auxin differ according to developmental stage. Relatively high endogenous auxin levels have been reported to be present during the early initiation stage, but are decreased as further cell divisions take place (Blakesley, 1994).

Furthermore, initiation of adventitious roots is also induced by exogenous auxin but further growth can be inhibited when exogenous auxin is present at later stages (Blakesley et al., 1991). This is similar to the differential effects of auxin on LR initiation and LR emergence observed in eucalypt seedlings (Pelosi et al., 1995).

AR initiation mutants

In contrast to cells competent to initiate AR, which form meristemsin response to auxin, non-competent cells often differentiate in an unorganised manner, forming callus (Lovell and White, 1986). Difficulty in obtaining adventitious rooting is of commercial significance in some clonal micropropagation programs, particularly in woody species important in horticulture or forestry (Davies et al., 1994; Ritchie, 1994; Häggman and Aronen, 2000). A lack of rooting and/or excessive callus formation may prevent useful clones from being propagated on their own rootstock.

Few mutants specifically defective with respect to AR have been described (Schiefelbein and Benfey, 1991). Among these are nodal root mutants identified in clover (White et al., 1998) and maize (Hetz et al., 1996), as well as the *rac* tobacco mutant, which is unable to form AR from stem tissues in response to auxin (Lund et al., 1996; 1997). A number of genes have also been identified that are specifically expressed in both LRP and ARP, e.g. *HRGPnt3* from tobacco and *LRP1* from *Arabidopsis* are expressed in early LRP development and also in ARP (Vera et al., 1994; Smith and Federoff, 1995).

Mortal mutants of white clover are spontaneous mutants with defective initiation of nodal AR, when grown between 10 and 25°C. The mutation specifically affects nodal root formation, as LR formation and shoot morphology of *Mortal* plants are normal. The *Mortal* defect in development of nodal root primordium was found to be inherited as a monogenic, dominant trait (White et al., 1998).

Rooting-incompetent *rac* tobacco mutants are deficient in AR formation, with no AR formed in response to auxin at concentrations optimal for AR in WT plants (Lund et al., 1996). Some cells in stem cuttings of *rac* mutants were found to form callus but none differentiated into AR, indicating that the *rac* mutation blocked AR formation prior to the commencement of organised cell division. To further investigate the molecular basis of AR initiation, effects of the *rac* mutation on auxin-regulated gene expression were studied (Lund et al., 1997). It was suggested that there may be several different auxin signal transduction pathways to AR formation, one of which is blocked by the *rac* mutation. Alternatively, *rac* may repress increases in auxin sensitivity that are required

for signal transduction pathways leading to AR initiation, whereas lower auxin sensitivity may be sufficient for unorganised cell division.

1.3. *AGROBACTERIUM RHIZOGENES*

One potential approach to modification of root growth and/or architecture involves the use of the soil bacterium *Agrobacterium rhizogenes*. *Agrobacterium* strains are generally prominent as plant pathogens, with the common characteristic of invading crown, stems or roots of wounded plants and subsequently inducing neoplastic growth in the form of tumours or adventitious roots. The two most studied species are *A. tumefaciens* and *A. rhizogenes*. Pathogenic strains of *A. tumefaciens* generally cause crown gall disease, which is typically manifested by undifferentiated cell growth forming tumours, or galls, at the base of the plant stem. In contrast, infection of dicotyledenous plants by *A. rhizogenes* typically induces some callus formation together with a proliferation of adventitious roots at the site of infection. Often termed hairy roots, due to a hair-like appearance, these roots are highly branched and plagiotropic and have the important features of growing in culture independently of exogenously supplied hormones. Furthermore, in many species it is possible to readily regenerate hairy roots into whole, transformed plants (Hamill and Lidgett, 1997).

The mechanisms by which agrobacteria identify and are chemotactically attracted to appropriate host plants, infect these plants and initiate differentiation of new roots have been extensively studied and reviewed (Zambryski et al., 1989; Winans, 1992; Baron and Zambryski, 1995; Kado, 1998). Many of the bacterial and plant genes involved and the roles they may play in these processes have also been elucidated. The understanding of the mechanisms have allowed the natural genetic engineering properties of the bacterium to be harnessed to genetically transform many plants, including initially recalcitrant monocot species, with a vast range of genes from many sources. While earlier work tended to focus on *A. tumefaciens*, due to its negative effects on growth, studies into particular aspects of *A. rhizogenes* mechanisms and genetics have subsequently increased.

Elucidation of the root-inducing properties of *A. rhizogenes* and characterisation of genes involved are relevant to further understanding of the processes of root induction. Furthermore, *A. rhizogenes* and the particular genes responsible for root induction may have potential applications for generating useful modifications to root

growth or architecture of transgenic plants (see §1.4.) (Hamill and Chandler, 1994; Chriqui et al., 1996; van der Salm et al., 1996a, b; Häggman and Aronen, 2000).

1.3.1. Prevalence and host species of *Agrobacterium*

Agrobacterium strains have been identified in soils worldwide (Kersters and De Ley, 1984), proliferating in the rhizosphere of plants, although to date the presence of *A. rhizogenes* has not been reported in Australian soils. De Cleene and De Ley (1981) carried out an extensive survey of 218 plant species for susceptibility to induction of hairy root by *A. rhizogenes*. Subsequent studies have identified at least 460 dicot and monocot species from over 100 families that have been successfully transformed with *A. rhizogenes* (Porter, 1991). However, although a wide range of plant species are identified as host plants when inoculated with an *A. rhizogenes* strain, under natural conditions the known host range appears to be limited to perennial species - apple (*Malus domestica*) and two species of *Spiraea* (De Cleene and De Ley, 1981).

1.3.2. *Agrobacterium* Ri and Ti virulence plasmids and T-DNA

The molecular mechanisms for infection by *A. tumefaciens* and *A. rhizogenes* are similar and involve the transfer of sections of a bacterial plasmid, known as the Ti-plasmid (tumour inducing) in *A. tumefaciens* and Ri-plasmid (root inducing) in *A. rhizogenes*. Various classes of Ri and Ti plasmids have been identified but common features are the large size (200 kb to greater than 800 kb) and the regions necessary for both tumorigenesis and rhizogenesis - the transferred DNA (T-DNA) and virulence (*vir*) regions (Gelvin, 1990).

The transfer of T-DNA is initiated and mediated by the *vir* genes, which are encoded on the Ri- or Ti-plasmid but are not themselves transferred (reviewed by Zupan and Zambryski, 1995; Kado, 1998). The T-DNA is flanked by two 25 bp repeat sequences, called left and right borders, which are *cis*-elements required for T-DNA processing and transfer. DNA between these border sequences is transferred to plant cells and integrated into the plant genome and subsequently transcribed and regulated by plant factors. Thus, this process represents a natural example of horizontal, or interkingdom, transfer of DNA.

The advantages for agrobacteria in achieving the genetic colonization of plants are gained from the introduction of genes encoding a distinctive class of metabolites, termed opines. Opines are generally low molecular weight amino acid or sugar derivatives, utilisable by agrobacteria as a carbon and/or nitrogen source (Dessaux et al., 1992). More than twenty different opines have been identified, comprising several structural classes. *Agrobacterium* strains carry genes encoding synthesis of a particular opine on the T-DNA, so that, following integration of the T-DNA into the plant, the transformed tumour or root tissues subsequently formed produce that particular type of opine. Correspondingly, the bacteria carry the genes for catabolism of this same opine on a nontransferred section of plasmid (reviewed Zambryski et al., 1989). Thus, by inducing a multiplication of transformed plant cells, in the form of tumours or roots, which secrete these novel compounds, the bacteria establish a soil environment conducive to their specific nutritional needs. This mediation of the interaction between *Agrobacterium* and the plant host by opines has been described as the opine concept (Tempé et al., 1979).

Although the general structure of Ti and Ri plasmids and molecular mechanisms of transfer are similar, the strategies by which each controls cell growth and induces neoplastic growth are very different. Ti T-DNA includes genes encoding for enzymes involved in auxin and cytokinin biosynthesis (*tms1*, *tms2* and *tmr*) (reviewed by Nilsson and Olsson, 1997). The consequent overproduction of auxin and cytokinin alter the hormonal balance in the plant cells, resulting in unorganised cell division and tumour production. As the plant cells are genetically transformed with the hormone biosynthesis genes, the continuous presence of bacteria is not required for maintenance of the disease (reviewed by Gelvin, 1990). Hormones are secreted by *A. tumefaciens*-transformed cells into neighbouring cells, so that the tumours produced will be chimeras of untransformed and transformed cells.

In contrast to the uncontrolled cell growth of crown gall disease, the physiological basis of the hairy root disease is a more precise and refined process, evident in the organized cell growth and differentiation of functional organs. Thus, hairy roots often have the capacity to regenerate into whole plants that contain and express T-DNA genes. The Ri T-DNA is stably integrated into the plant genome of regenerated plants and is inherited in a Mendelian and dominant fashion (Tepfer, 1984). Plants regenerated from hairy roots show distinctive alterations in phenotype, termed T (transformed) phenotype, including wrinkled leaves, reduced apical dominance in roots and stems, high growth rate of roots in culture and plagiotropic roots (Tepfer, 1984). Other common

effects across different species are reduced internode lengths, altered leaf and flower morphology, male sterility or reduced fertility and reduced seed production. Unlike crown gall tumours, which can be chimaeric tissues, hairy roots arise from a single transformed cell and consist only of transformed cells (Chilton et al., 1982; Bercetche et al., 1987).

1.3.3. Agropine Ri plasmid TL-DNA

Ri plasmids are classified by the type of opine produced by transformed tissues. The T-DNA of agropine plasmids, the most extensively studied type, is comprised of two T-DNA regions - TL-DNA (left) and TR-DNA (right) (Fig 1.4). The TR-DNA genes include homologues (*aux1* and *aux2*) to the auxin synthesis genes of the *A. tumefaciens* Ti plasmid as well as opine synthesis genes (reviewed by Nilsson et al., 1997). In contrast, mannopine and cucumopine Ri plasmids both have a single T-DNA region - these regions do not include any auxin biosynthesis genes but share strong homology with regions of agropine TL-DNA (Cardarelli et al., 1985; Filetici et al., 1987; Brevet and Tempé, 1988; Hansen et al., 1991). Furthermore, as the TR-DNA is also not always present in hairy roots induced by agropine strains (Jouanin et al., 1987), it is apparent that hairy root formation can be induced by TL-DNA alone in some cases and is not necessarily dependent on *aux* gene-encoded auxin synthesis. Rather, transformation with Ri T-DNA confers on cells a capacity to respond to auxin, whether provided by the plant, by *aux* genes or by exogenous supply, thereby stimulating differentiation of roots (Cardarelli et al., 1987b; Spanò et al., 1988).

Polarity of root induction from carrot discs by *aux*$^-$ agropine strains, or by mannopine and cucumopine strains was also demonstrated, with roots formed on apical surfaces but not basal surfaces (Cardarelli et al., 1985; Ryder et al., 1985). This is due to auxin depletion on the basal side, as a result of a polar auxin flow towards the root apex. However, *aux*$^+$ strains induced roots in equal abundance on both apical and basal surfaces and it was shown that either the presence of *aux* genes or an exogenous supply of auxin in strains without *aux* genes are sufficient to enable TL-DNA-mediated root induction (Cardarelli et al., 1985; 1987b). *Aux* genes may be considered to extend the virulence of agropine strains in environments of insufficient auxin to promote adventitious root formation, whereas mannopine and cucumopine strains do not have this capacity. Thus, although not essential for hairy root production, genes located on the TR-DNA can play a role in enhancing or enabling induction of hairy root formation

(Cardarelli et al., 1987b; Capone et al., 1989) and in some cases have also been shown to produce different phenotypic alterations in transformed roots and plants (McInnes et al., 1991; Amselem and Tepfer, 1992).

1.3.4. TL-DNA genes capable of inducing hairy roots

Eighteen open reading frames (ORFs) were identified by sequence analysis of the Ri TL-DNA of *A. rhizogenes* strain A4 (Slightom et al., 1986). By transposon mutagenesis of the TL-DNA and subsequent infections of kalanchoe leaves with strains mutant for each single *rol* gene, White et al (1985) identified four loci responsible for root induction and growth and these were termed root locus A-D (*rol*A-D), corresponding to ORF10, 11, 12 and 15 respectively (Fig. 1.4).

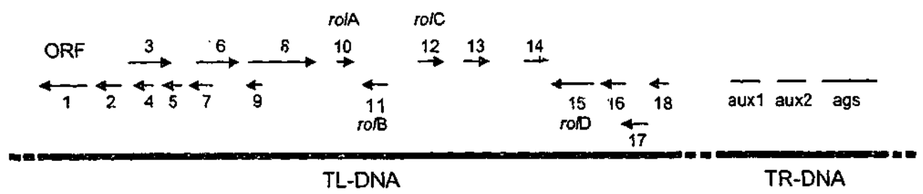


Figure 1.4. Schematic diagram of structure of agropine Ri T-DNA, with open reading frames (ORF) represented as numbered arrows. Direction of arrow indicates direction of transcription; arrow sizes are approximate representations of relative ORF length. TL-DNA (left) - *rol* genes A, B, C and D are ORFS 10, 11, 12 and 15, respectively. TR-DNA (right) contains auxin synthesis genes (aux1 and aux2) and agropine synthesis gene (ags).

Different roles for each *rol* gene in the hairy root phenotype were identified by the root growth characteristics of each mutant. The roots of *rol*A mutants grew straight and out from the plant surface instead of curling, *rol*B mutants were avirulent, indicating the key role of this locus in root induction, *rol*C mutants showed attenuated root growth and *rol*D mutants induced increased formation of callus and also reduced root growth.

The roles of the *rol* genes A, B and C in root induction were further confirmed and characterised using gain-of-function approaches (Cardarelli et al., 1987b; Spena et al., 1987; Capone et al., 1989). Infections of kalanchoe and tobacco leaves and carrot discs with recombinant *Agrobacterium* demonstrated that expression of the *rol*A, B and C genes in combination was generally sufficient to induce prolific growth of roots displaying the characteristic hairy root phenotype.

The independent capacity of the *rol* genes to induce root formation, to varying degrees, has also been established. Of the single *rol* gene constructs, *rol*B has been shown to elicit the strongest response and have a primary role in induction of adventitious root formation (Spena et al., 1987; Capone et al., 1989). Both *rol*A and *rol*B were able to induce AR from transformed tobacco leaf discs placed on media without phytohormones (Spena et al., 1987; Vilaine et al., 1987). However, the *rol*B gene stimulated earlier and more prolific root growth than the *rol*A gene, when each gene was under control of its own promoter (Spena et al., 1987). In carrot discs, *rol*B expression was able to induce formation of phenotypically altered roots, when coinoculated with ORF13+14 or the TR-DNA *aux* genes (Cardarelli et al., 1987b; Capone et al., 1989). Both the *rol*A and *rol*C genes, expressed separately, were not observed to induce root formation on carrot or kalanchoe leaf discs, even in the presence of the Ti-plasmid auxin synthesis genes or ORFs13+14 (carrot) (Spena et al., 1987; Capone et al., 1989).

*rol*C was demonstrated to stimulate root formation in tobacco leaves when expressed under control of the CaMV35S promoter (Spena et al., 1987). In contrast, over-expression of the *rol*B gene in tobacco and kalanchoe using the CaMV35S promoter (35S-B) appeared to be less effective for root induction than when under control of the native promoter, although the combination of 35S-B with *rol*C generated a greater response than 35S-B alone in kalanchoe leaves (Spena et al., 1987).

1.3.5. Increased sensitivity to auxin of TL-DNA- and *rol* gene-transformed tissues

The increased capacity of Ri T-DNA-transformed tissues to form adventitious roots has been extensively described in the literature and is indicative of auxin-related effects. It appears that this involves an enhanced sensitivity and capacity to respond to auxin (Cardarelli et al., 1987b; Spanò et al., 1988; van der Salm et al., 1996a) as major increases in auxin levels have generally not been measured in T-DNA or single *rol* gene-transformed plants (Spanò et al., 1988; Nilsson et al., 1993a, b; Delbarre et al., 1994; Fladung et al., 1997).

TL-DNA and *rol* gene-induced alterations to auxin sensitivity were demonstrated in tobacco leaf discs (Spanò et al., 1988). Explants containing the whole TL-DNA or the three genes *rol*A+B+C were much more responsive to IAA than untransformed tissues, in terms of their capacity to form roots. It was suggested that normal physiological levels of auxin may be sufficient to trigger spontaneous root differentiation in *rol* gene and

TL-DNA transformed tobacco leaves, as a result of an increased sensitivity of the transformed cells to the hormone. Transformation with *rol*B alone has been demonstrated to be sufficient to confer an increased capacity to respond to auxin in kalanchoe and carrot discs, whereas no roots were formed in *rol*A-, *rol*C-transformed or untransformed explants in response to auxin (Spena et al., 1987; Capone et al., 1989). Similarly, intact shoots of *rol*B-transformed apple rootstock M26 (*Malus* x *domestica* Borkh.) were able to initiate adventitious roots without exogenous auxin treatment, in contrast to untransformed shoots (Welander et al., 1998). Stem disc segments of *rol*B transgenic apple did not produce roots unless supplied with auxin, presumably due to low endogenous auxin production. When treated with auxin however, *rol*B-transformed stem discs were much more sensitive than untransformed stems, with root induction being stimulated at lower concentrations.

In addition to enhanced rhizogenesis, increased sensitivity to auxin caused by the presence of TL-DNA genes is also apparent in other auxin-induced responses. These include auxin-induced modification of transmembrane electrical potential difference (E_m) in protoplasts, proton excretion from root tips and root elongation - considered to be short- medium- and long-term responses to auxin respectively (Shen et al., 1988; 1990). In TL-DNA-transformed roots of *Catharanthus trichophyllus* and *Lotus corniculatus*, these auxin responses were susceptible to alteration by lower concentrations of naphthalene acetic acid (NAA) than in untransformed roots, indicating an elevated sensitivity to auxin in the transformed roots (Shen et al., 1988; 1990). However, roots transformed by TR-DNA only were not different from normal roots, indicating that modifications in auxin sensitivity are due to expression of TL-DNA genes. Measurements involving tobacco leaf protoplasts showed protoplasts from *rol*A+B+C transformed plants to be 1000 times more sensitive to auxin than normal protoplasts, in terms of NAA-induced modifications to the transmembrane E_m (Maurel et al., 1991). The presence of single *rol* genes conferred increases in sensitivity, by factors of 30-1000 for *rol*A, 3000-10,000 for *rol*B and 10 for *rol*C.

The increased sensitivity to auxin of protoplasts from *A. rhizogenes* transformed tobacco plants has been suggested to be due to the presence of increased numbers of auxin receptor sites at the plasma membrane (Barbier-Brygoo et al., 1990; 1991). This was demonstrated by modifying the number of functional receptors using antibodies raised against a maize auxin binding protein (ZmER-abp), which also recognise and inactivate an immunologically related protein on tobacco protoplasts. Treatment with these anti-ZmER-abp antibodies thus reduces the amount of functional auxin-binding

protein and subsequently causes inhibition of the transmembrane electrical response. Protoplasts transformed with *A. rhizogenes* were shown to require 10-fold more anti-ZmER-abp than normal protoplasts to induce a 50% inhibition of the maximal auxin-induced transmembrane E_m response, indicating the presence of a higher number of functional auxin receptors. Conversely, protoplasts from auxin-resistant mutants required 10-fold less anti-ZmER-abp to achieve the same response (Barbier-Brygoo et al., 1991). A similar assay was carried out with protoplasts from *rol*B transgenic plants, which were found to require 100 to 1000-fold more anti-ZmER-abp antibodies than untransformed protoplasts, to achieve comparable reductions in auxin sensitivity (Maurel et al., 1994).

1.3.6. Growth and developmental alterations induced by single *rol* genes

Expression of single *rol* genes in transgenic plants induces distinct morphological alterations, which have been characterised in a variety of species. The precise functions of the *rol* genes have not been completely elucidated. However, examination of the phenotypes and physiology of transformed plants, along with studies of expression patterns, promoter regulatory elements and deregulated and tissue specific expression, have gradually added to information on effects of the *rol* genes on growth and development (Chriqui et al., 1996; Nilsson and Olsson, 1997).

Consistent and stable production of particular phenotypic, physiological and biochemical characteristics resulting from *rol* gene expression have been considered as having potential for commercial, agricultural and horticultural applications in some species (van der Salm et al., 1996a; Häggman and Aronen, 2000). Tissue-specific expression conferred by promoters, or certain promoter elements, of *rol* genes have also been utilised, for ectopic expression of other genes (e.g. Graham et al., 1997; Almon et al., 1997).

1.3.7. *rol*A

1.3.7.1. Effects of *rol*A expression on phenotype

Expression of *rol*A was identified as being responsible for the distinctive leaf wrinkling observed in plants transformed with Ri T-DNA (Sinkar et al., 1988). *rol*A transgenic tobacco plants display the wrinkled leaf phenotype as well as stunted growth due to reduced internode distances, condensed inflorescence, larger flower size and reduced

length-to-width ratio of leaves (Schmülling et al., 1988; Sinkar et al., 1988). Similar alterations are observed in other species - *rol*A-transformed *Arabidopsis* have a stunted phenotype due to inhibited growth of the hypocotyl, internodes and petioles of cotyledons and leaves. Leaves have reduced length-to-width ratio and are curled due to epinastic growth (Dehio and Schell, 1993). *rol*A transgenic apple plants also have shortened stem and internode lengths and show some reduction in leaf area (Holefors et al., 1998).

35S-*rol*A plants display similar but even more pronounced phenotypic abnormalities, with severely wrinkled leaves that are dark green in colour and have reduced length-to-width ratios. 35S-*rol*A tobacco plants are very stunted, with internode lengths and plant height reduced to about half that of *rol*A transgenics. Plants are also late flowering with greatly reduced flower numbers (Dehio et al., 1993). Growth of 35S-*rol*A transformed tobacco roots were shown to be greater than normal, with greater biomass accumulation in tobacco root cultures grown without plant hormones and enhanced root growth in seedlings compared to non-transgenic controls, although no overall differences in root:shoot weight ratios were observed (Ben-Hayyim et al., 1996).

1.3.7.2. *rol*A expression patterns

Promoter analysis of *rol*A identified three regulatory domains which control the gene expression at different levels in stem, leaf and root of transformed tobacco plants (Carneiro and Vilaine, 1993). Utilising a *rol*A-GUS chimaeric gene, *rol*A promoter activity was analysed and expression found to be localised in phloem cells of tobacco leaves and stems, in a similar pattern to *rol*B and *rol*C promoters (Schmülling et al., 1989). In roots, however, *rol*A-GUS expression was not detectable in emerging lateral roots, unlike *rol*B-GUS and *rol*C-GUS expression.

Vilaine et al. (1998) also studied expression and localisation of a *rol*A-GUS fusion gene product in tobacco as a step to understanding its function. The *rol*A-GUS fusion protein was found to be localised to the plasmalemma, indicating that RolA is unlikely to be directly involved in regulation of gene expression, as would be expected if it were a DNA-binding protein, but raising the possibility of an involvement in signal transduction.

1.3.7.3. Possible primary and secondary functions of *rol*A

Molecular mechanisms explaining the effects of *rol*A on plant morphology are not fully understood. However, *rol*A expression has been found to have secondary effects on several plant growth regulators, in particular giberellic acid (GA). Analysis of other major plant hormones in *rol*A plants (Dehio et al., 1993) indicated alterations in levels of IAA, abscisic acid (ABA), cytokinins and the ethylene precursor (aminocyclopropane carboxylate, ACC), in some tissues.

GA is known to have a role in stem elongation (Atwell et al., 1999c) and in apical shoots of *rol*A tobacco plants, GA levels were 22% lower than normal levels (Schmülling et al., 1993). Furthermore, treatment of normal SR1 tobacco plants with inhibitors of GA synthesis induced a phenotype similar to *rol*A of stunted growth with shortened plant height and reduced internode elongation and dark-green, wrinkled leaves (Dehio et al., 1993). Exogenous application of GA to apical shoot meristem of *rol*A plants partially restored a normal phenotype, reducing the severity of leaf wrinkling and inducing earlier flowering and stem lengths more comparable to WT (Schmülling et al., 1993). However, growth was not fully restored, suggesting that the decrease in GA was not the only cause of the dwarfed plant phenotype. Another factor may be the ethylene precursor, ACC, which was found to be increased 5-fold in the stems of 35S-*rol*A plants compared to non-transgenic controls (Dehio et al., 1993). As excess ethlylene inhibits stem elongation (Romano et al., 1993), this may contribute to the shortened stem length characteristic of *rol*A plants.

The phenotype of *rol*A and Ri T-DNA transformed plants have also been shown to be affected by levels of polyamines and their conjugates, with polyamine levels decreasing as the degree of phenotypic alterations increases (Martin-Tanguy et al., 1990). Treatment of normal tobacco roots with the polyamine biosynthesis inhibitor DFMO caused a reduction in polyamine titres and resulted in accelerated root growth and branching, a phenotype similar to that of *rol*A-transformed roots. These observations were confirmed in 35S-*rol*A and DFMO-treated tobacco plants (Ben-Hayyim et al., 1994; 1996). These plants displayed similar developmental changes, including stimulation of root biomass accumulation and inhibition of shoot height, accompanied by reductions in putrescine levels. The addition of GA treatment partially reversed the negative effects on stem growth and putrescine levels (Ben-Hayyim et al., 1996) and it was suggested that *rol*A and GA may both act via alterations in metabolism of polyamines and polyamine conjugates.

1.3.8. *rol*B

1.3.8.1. Effects of *rol*B expression on phenotype

As well as an increased propensity to form adventitious roots, phenotypic effects of *rol*B in tobacco and tomato transformants include reduced apical dominance and internode length, alterations to leaf morphology, larger flower size and heterostyly (Schmülling et al., 1988; van Altvorst et al., 1992). Expression of the *rol*B gene under control of the CaMV35S promoter produces a distinctive leaf necrosis, round-edged leaves and heterostyly (Schmülling et al., 1988; Nilsson et al., 1993a). It was reported that apical dominance and adventitious root formation were not noticeably increased in these plants (Nilsson et al., 1993a).

1.3.8.2. *rol*B expression patterns and promoter studies

Expression of a *rol*B-GUS chimaeric gene indicated that *rol*B expression is localised in the phloem of leaves and stems and the root cap, cell-division regions and lateral root primordia in roots (Schmülling et al., 1989; Maurel et al., 1990). The expression pattern of *rol*B in roots is different from that of *rol*A or *rol*C, with much lower *rol*B-GUS activity in the vascular tissue of roots and leaves. Altamura et al. (1994) demonstrated that the presence of *rol*B in tobacco thin cell layers enhanced both flower and root differentiation, suggesting that *rol*B may promote not just formation of new roots but affect meristem formation in general.

The *rol*B promoter is strongly and specifically activated by auxin, as demonstrated by treatment of *rol*B and *rol*B-GUS transformed tobacco mesophyll protoplasts with NAA (Maurel et al., 1990; 1994; Capone et al., 1991). Up to 70- to 80-fold increases in GUS activity in *rol*B-GUS protoplasts were measured in response to NAA, whereas ABA, GA and cytokinin treatments did not induce similar changes. The pattern of GUS activity was also substantially modified by NAA treatment, with high levels of activity induced in some tissues previously without expression, such as root cortex cells, and reduced GUS levels in other tissues (Maurel et al., 1990). In *rol*B-transformed protoplasts 300-fold increases in *rol*B mRNA accumulated in response to auxin (Maurel et al., 1994). However, increased *rol*B activity was not observed until 5-6 hrs after NAA treatment (Maurel et al., 1990; 1994) whereas known auxin-induced genes are much more rapidly activated once auxin is added (Abel and Theologis, 1996). Thus

the induction of the *rol*B promoter is unlikely to occur via a direct action of auxin but may be mediated by other factors.

Analysis of the *rol*B promoter identified regulatory regions responsible for the level and tissue specificity of expression in tobacco (Capone et al., 1991; 1994). Five domains were identified, with deletion of any one of these domains resulting in up to 2- to 4-fold reductions in activity of *rol*B-GUS chimaeric genes (Capone et al., 1994). Each of the domains was shown to control expression in a different population of cells in the root apex. Furthermore, the same four domains were found to be essential for *rol*B expression in root meristematic cells and for the capacity of leaf mesophyll protoplasts to respond to auxin, confirming the association between these processes.

Elements of the molecular regulatory mechanisms involved in *rol*B expression have been elucidated. A transcription factor, NtBBF1, which binds to a specific sequence in a regulatory domain of the *rol*B 5' upstream sequence, was isolated from tobacco (De Paolis et al., 1996). The target DNA sequence was identified in domain B of the *rol*B promoter, which is necessary for expression of the gene in root meristematic cells and for the induction of *rol*B by auxin (Capone et al., 1991; 1994). NtBBF1 is a member of the Dof-family, a family of transcription factors with a conserved zinc finger domain that has been found in a wide range of plant species (references in Baumann et al., 1999). The NtBBF1 recognition sequence was shown to mediate the specificity and auxin-inducible expression of *rol*B (Baumann et al., 1999). Another nuclear factor from *N. tabacum* has also been identified, binding to a *rol*B promoter sequence within the region defined as domain A (Filetici et al., 1997).

1.3.8.3. Evidence that *rol*B is unlikely to increase auxin levels in plants

When expressed in *E. coli,* the RolB protein was demonstrated to have glucosidase activity, hydrolysing a compound similar to an auxin conjugate, (indole-3-acetyl)-ß-D-glucoside (IAGluc) (Estruch et al., 1991a). Consequently, it was suggested that *rol*B expression could possibly lead to increases in the intracellular pool of auxin, by releasing free auxin from inactive conjugated auxin compounds. However, this hypothesis was not supported by data from *in vivo* experiments with *rol*B-transformed plants or cells. Intracellular concentrations and metabolism of IAA and NAA and conjugates in *rol*B transgenic tobacco plants (Nilsson et al., 1993a) or protoplasts (Delbarre et al., 1994)

were not different from the wild-type. Furthermore, the RolB protein was not found to recognize IAGluc as a substrate for hydrolysis *in vivo* (Nilsson et al., 1993a).

A comparison of phenotypes of *rol*B-expressing tobacco plants and plants overexpressing IAA (Nilsson et al., 1993a) also contradicts a model involving an increased auxin pool in *rol*B transgenics. Dwarfism and very strong apical dominance were striking alterations in IAA-overproducing plants but were not observed in *rol*B-transgenic plants. Conversely, plants in which *rol*B was overexpressed (35S-*rol*B) displayed a distinctive leaf necrosis and lack of increased adventitious root formation compared to WT plants, effects not seen in IAA-overproducing plants (Nilsson et al., 1993a).

1.3.8.4. *rol*B-induced increased sensitivity to auxin

The alterations in growth and development observed in *rol*B transgenic plants are evidently not due to a direct role of the RolB protein in biosynthesis or metabolism of endogenous auxin. It is considered more likely that *rol*B effects are due to increased sensitivity of transformed cells to auxin, possibly involving changes in auxin binding and/or reception or transduction of the hormone signal (Costantino et al., 1994).

The RolB protein is localized to the plasma membrane and has been shown to have tyrosine phosphatase activity, with a suggested involvement in auxin-mediated signal transduction (Filippini et al., 1996). Correspondingly, *rol*B protoplasts have been shown to be 3000 to 10,000 times more sensitive to auxin than normal protoplasts, in terms of the transmembrane electrical response to auxin (Maurel et al., 1991; 1994). The increase in sensitivity to auxin was concomitant with auxin-induced accumulation of *rol*B mRNA, although particularly high levels of *rol*B mRNA were not required to achieve markedly greater sensitivity to auxin (Maurel et al., 1994). As noted previously (§1.3.5), modifications in transmembrane electrical responses by anti-ZmER-abp1 antibodies were observed to be different in *rol*B protoplasts compared to untransformed protoplasts (Maurel et al., 1994), also suggesting that increased sensitivity of *rol*B protoplasts to auxin relates to alterations in auxin signal perception or transduction.

Increased auxin binding to plant cell membranes was also demonstrated in *rol*B-transformed tobacco cells in culture (Filippini et al., 1994). Two different classes of auxin-binding proteins were identified in tobacco cells (both transformed and untransformed), one of which was immunologically related to RolB, as it was inhibited by

antibodies raised against RolB. The other type, not recognized by anti-RolB antibodies, was immunologically related to a maize ABP. In *rol*B-transformed cells, the overall auxin binding capacity was greater than that of untransformed cells. This was shown to be due to an increase in the component of auxin-binding activity recognized by anti-RolB antibodies, whereas the ABP-related activity remained unchanged compared to untransformed cells.

1.3.9. *rol*C

1.3.9.1. Effects of *rol*C expression on phenotype

Distinctive *rol*C phenotypic effects are well characterised in transgenic tobacco and other species including potato (*Solanum tuberosum*), *Atropa belladonna*, petunia and a deciduous tree species, hybrid aspen (*Populus tremula* x *tremuloides*). Plants expressing *rol*C are generally shorter with reduced apical dominance, shortened internodes and altered leaf morphology. Pollen production is often reduced and plants flower early and produce smaller flowers (Schmülling et al., 1988; Spena et al., 1989; Fladung, 1990; McInnes et al., 1991; Scorza et al., 1994; Nilsson et al., 1996b; Bell et al., 1999). Flowering in carrot plants was stimulated by *rol*C to the extent of inducing annual flowering in this biennial species (Limani et al., 1998).

35S-*rol*C plants generally demonstrate a more exaggerated dwarfed and bushy phenotype with reduced apical dominance, have very small flowers and are usually male sterile due to severely deficient pollen production. Leaves are often smaller than those of controls, as well as lanceolate and pale-green due to reduced chlorophyll levels (Schmülling et al., 1988; Fladung, 1990; Kurioka et al., 1992; Fladung et al., 1997; Winefield et al., 1999).

Although *rol*C, under control of the endogenous promoter, was not able to induce root formation on tobacco or kalanchoe leaf discs (Spena et al., 1987), root production in transgenic plants expressing *rol*C has been shown to be increased (Lambert and Tepfer, 1990; Martin-Tanguy et al., 1993; Tepfer et al., 1994). Increased root dry weight was measured in *rol*C transgenic alfalfa compared to WT plants (Frugis et al., 1995). In transgenic tobacco root cultures, *rol*C roots were more highly branched than *rol*A or *rol*B roots and grew better in phytohormone-free medium (Schmülling et al., 1988). Palazón et al. (1998b) also noted a much greater capacity than normal for root formation

in 35S-*rol*C and *rol*A+B+C transgenic tobacco root cultures, and root biomasses of *in vitro* grown plants regenerated from the transformed roots were correspondingly greater than that of untransformed controls (Palazón et al., 1998b).

1.3.9.2. *rol*C expression patterns and promoter elements

Analysis of *rol*C-GUS chimaeric gene expression has shown that the *rol*C promoter directs expression to phloem cells of leaves and stems (Schmülling et al., 1989; Sugaya et al., 1989; Guivarc'h et al., 1996). In tobacco roots, *rol*C-GUS chimaeric gene expression was reported to be phloem-specific in both main and lateral roots and unlike *rol*B-GUS was not present in lateral root primordia (Schmülling et al., 1989). In roots of *rol*C-GUS transgenic hybrid aspen, the *rol*C expression pattern was notably different to that reported in annual species (Nilsson et al., 1997). Expression of both the *rol*C and *rol*B promoters were most pronounced in pericycle cells, in particular the pericycle cells from which lateral roots are initiated. Strong expression continued during development of the lateral root primordia, which may indicate a role in growth of new meristems.

Activation of the *rol*C promoter by sucrose in phloem tissue was demonstrated in tobacco seedlings transgenic for *rol*C-GUS chimaeric gene fusions (Yokoyama et al., 1994). GUS activity and expression were increased in the phloem of roots, cotyledons and hypocotyls in response to exogenous sucrose and, to a much lesser extent, by fructose and glucose. Deletion analysis of the promoter indicated the presence of a *cis*-acting sucrose-response region. The *rol*C promoter is also activated to some degree by auxin, as demonstrated in *rol*C-GUS transformed protoplasts treated with NAA (Maurel et al., 1990). A moderate increase in GUS activity was measured in response to NAA and some alteration in the tissue-specific expression of *rol*C-GUS was also observed, although these did not occur to the same extent as similarly treated *rol*B-GUS protoplasts.

Promoter analysis has also identified *cis*-elements regulating expression levels and cell-specificity of the *rol*C promoter (Sugaya and Uchimiya, 1992). The proximal -153 bp upstream sequence is apparently required for phloem-specific expression and the -120 to +23 region is necessary for expression in seed embyros. Leach and Aoyagi (1991) found that root specificity of the *rol*C promoter was increased by deleting upstream regions and retaining the proximal -417 bp region, although *rol*C activity was correspondingly decreased.

1.3.9.3. Evidence that *rol*C does not have primary function in cytokinin metabolism

The RolC protein, when expressed in *E. coli*, exhibits ß-glucosidase activity and can hydrolyse inactive cytokinin conjugates *in vitro* to release free cytokinins (Estruch et al., 1991b). However, although some *rol*C phenotypic modifications, such as reduced plant height and apical dominance, are reminiscent of effects caused by increased levels of cytokinin, other effects are inconsistent with a direct role for *rol*C in increasing cytokinin levels. In plants transformed with a cytokinin synthesis gene, *ipt*, and plants to which cytokinin is exogenously applied, phenotypic alterations generally include increased chlorophyll synthesis and inhibition of rooting, which are the reverse of *rol*C-induced phenotypic alterations (Hamill, 1993; Faiss et al., 1996). In hybrid tobacco plants expressing both the *rol*C and *ipt* genes, mixed phenotypic alterations were observed, suggesting that the *rol*C phenotype is mediated by a signalling pathway different to that of cytokinin overproduction (Faiss et al., 1996).

In measurements of cytokinins in 35S-*rol*C transformants of several species, major differences between overall cytokinin levels of controls and *rol*C transgenics have generally not been detected (Schmülling et al., 1993; Nilsson et al., 1993b; Faiss et al., 1996; Fladung et al., 1997). Variations in reported results were also likely to result from the different cytokinin types measured and the analysis of differing tissue types. For example, cytokinin levels were somewhat increased in shoot apices, which are strong sink tissues (Nilsson et al., 1993b), whereas in other studies utilising fully expanded source leaves, *rol*C did not appear to influence cytokinin biosynthesis. To further analyse effects of *rol*C activity on cytokinins and cytokinin conjugates, Faiss et al. (1996) produced transgenic tobacco plants in which *rol*C was expressed under control of a tetracycline-inducible promoter. Upon induction of *rol*C expression by tetracycline feeding, *rol*C phenotypic effects were observed to develop but no alterations in the endogenous cytokinin balance were measured (Faiss et al., 1996). Moreover, although an 8-fold increase in the cytokinin zeatin riboside was measured in shoot apices of 35S-*rol*C transgenic aspen, increased levels of the conjugated cytokinin were also detected, indicating that the RolC protein was not acting to hydrolyse these compounds (Nilsson et al., 1996b). Hybrid plants expressing both the *rol*C and *ipt* genes were also found to accumulate high levels of conjugated cytokinins (Faiss et al., 1996). Furthermore, it was noted that while the RolC protein is localized in the cytoplasm (Estruch et al., 1991c), cytokinin conjugates are compartmentalized in vacuoles and would therefore not be available for hydrolysis *in planta* (Faiss et al., 1996).

From the accumulated evidence, it can therefore be concluded that phenotypic alterations in *rol*C transgenic plants are not likely to be due to the release of free cytokinins from inactive cytokinin conjugates by the RolC gene product. Alterations to cytokinin metabolism are considered more likely to be a secondary, rather than primary, effect of *rol*C (Nilsson and Olsson, 1997).

1.3.9.4. Secondary effects of *rol*C on other hormones

Apart from cytokinin, *rol*C has also been demonstrated to cause secondary effects on the metabolism of, and sensitivity to, other hormones, evidently contributing to the pleiotropic morphological and developmental alterations of *rol*C transgenic plants. Reduced GA levels have been associated with inhibition of stem elongation (Atwell et al., 1999c), analogous to the dwarfed phenotype of *rol*C-expressing plants. In fact, the concentration of GA in apical shoots of 35S-*rol*C tobacco and 35S-*rol*C potato were found to be reduced by 28-60% from normal levels, depending on *rol*C expression levels (Schmülling et al., 1993). Shortened stem lengths of 35S-*rol*C transgenic aspen were similarly associated with reduced ratios of GA to ABA in the stems (Fladung et al., 1997).

Normal stem elongation in *rol*C tobacco was restored by exogenous application of GA to the shoot meristem (Schmülling and Röhrig, 1995), suggesting that the lowered GA levels are the main cause of the dwarfism, as a consequence of *rol*C expression. However, as other *rol*C characteristics such as early flowering, reduced leaf pigment and male sterility were not reversed by application of GA (Schmülling et al., 1993), this indicated that the alteration to GA metabolism is most likely a secondary effect of *rol*C activity.

Altered levels of ABA in 35S-*rol*C transgenic hybrid aspen were hypothesised to be responsible for the characteristic early release from dormancy in these transgenic trees (Fladung et al., 1997). ABA levels have been found to be reduced by up to 50% compared to controls, in leaves and buds of 35S-*rol*C hybrid aspen and in 35S-*rol*C tobacco leaves (Schmülling et al., 1993; Fladung et al., 1997). Modifications to ethylene content have also been reported - in transformed root cultures of *Catharanthus roseus*, ethylene content was negatively correlated with the level of *rol*C gene product (Palazón et al., 1998b). Inhibition of ethylene production was also observed in *rol*C transgenic tobacco plants (Martin-Tanguy et al., 1993).

Alterations in free or conjugated auxin levels in *rol*C-transgenic plants have not generally been observed. In 35S-*rol*C tobacco, only very small differences in free IAA levels were noted compared to nontransgenic controls, although levels of IAA conjugates were considerably more reduced in leaves (Nilsson et al., 1993b). However, no differences in IAA metabolism, transport and turnover were observed and reduced levels of IAA conjugates were presumed to be a secondary *rol*C effect. In two separate studies of 35S-*rol*C transformed transgenic aspen, apparently conflicting results of IAA levels were reported, although respective measurements were made in plants of different ages (Nilsson et al., 1996b; Fladung et al., 1997).

1.3.9.5. Possible mechanisms for effects of *rol*C

One mechanism proposed for the activity of *rolC* is that, rather than directly interacting with hormones, *rol*C may be more likely to affect sucrose transport and/or metabolism (Nilsson et al., 1997). The predominant expression of *rol*C in the phloem, through which sucrose is transported, and the regulation of the *rol*C promoter by sucrose (Yokoyama et al., 1994) are compatible with this hypothesis. Higher water turgor, transpiration rates and fresh weight-to-dry weight ratios of *rol*C plants (Nilsson et al., 1993b) compared to WT plants may also be evidence of increased sugar in leaves and suggest that *rol*C activity may relate to the creation of a sink for assimilates and promotion of sugar unloading in leaves.

In accordance with this evidence, Nilsson and Olsson (1997) suggested a possible model for the functions of *rol*B and *rol*C in hairy root induction. Plant cells in which suitable levels of auxin and sucrose are present will be competent to initiate AR; these cells are therefore ideal targets for *Agrobacterium rhizogenes* infection. Correspondingly, the *rol*C and *rol*B promoters are regulated by sucrose and auxin, respectively (the *rol*C promoter is also influenced by auxin), so that concurrent expression of both genes will most likely occur in cells in which levels of these two factors are high, such as phloem cells (Uggla et al., 1996).

Thus, following transfer and integration of Ri T-DNA into the genome of plant cells, increased sensitivity to auxin as a result of *rol*B expression may induce root initiation. A supply of sucrose is also needed by the new root meristem to continue growth and this may be facilitated by the expression of *rol*C, by increasing sucrose levels in the phloem cells in which it is expressed by promotion of sucrose unloading. As

phloem parenchyma cells can serve as initial cells for adventitious roots (Esau, 1977; Lovell and White, 1986), the combined expression of *rol*B and *rol*C in conjunction with other *rol* genes may thus control development of these cells, leading to the formation of hairy roots. As noted by Nilsson and Olsson (1997), expression of these genes in the phloem also concurs with previous observations that for *A. rhizogenes* infection and induction of hairy roots, a wound deep enough for the bacteria to reach the phloem region was required.

In an alternative hypothesis, Faiss et al. (1996) proposed that the substrate for RolC ß-glucosidase activity may be oligosaccharins, a subclass of oligosaccharides. Oligosaccharides are essentially short chains of sugar residues, some of which are able to exert regulatory effects on plant development when at low concentrations. Naturally occurring oligosaccharins are most likely generated by fragmentation of cell-wall polysaccharides (Fry et al., 1993). Effects of various oligosaccharin types include eliciting plant disease resistance responses, inhibition or promotion of growth and some anti-auxin effects (Fry et al., 1993; Bellincampi et al., 1996). Some oligosaccharins have been found to serve as substrates for specific enzymes. For example, *Rhizobium nodA* and *nodB* gene products modify a class of lipo-oligosaccharides, which, in modified form, are the signal molecules for nodule production in leguminous plants (Dénarié et al., 1996). Expression of these *nod* genes in tobacco plants was shown to induce distinct phenotypic alterations, suggesting the natural presence *in vivo* of the oligosaccharin substrate and therefore its relevance in plant developmental processes (Schmidt et al., 1993).

Plant wounding is one cause of cell wall degradation and thus may be a source of oligosaccharins. Plant wounding is also a factor in *Agrobacterium rhizogenes* infection, at which time expression of the *rol*C gene is required. Furthermore, as noted by Faiss et al. (1996), oligosaccharins of the lipo-oligosaccharide class have been shown to sustain cell growth without auxin or cytokinin (Röhrig et al., 1995), which could explain the short-term ability of protoplasts expressing *rol*C plants to divide and form callus independently of auxin and cytokinin (Walden et al., 1993). Roots transgenic for *rol*C have also been shown to be able to grow prolifically *in vitro* in phytohormone-free medium, in contrast to untransformed roots and roots transgenic for other *rol* genes (Schmülling et al., 1988). Thus, based on these observations, a role for oligosaccharins as a substrate for *rol*C may be possible.

1.3.10. *rol*D

Transgenic plants expressing *rol*D have been shown to flower strikingly early (Mauro et al., 1996). In root induction assays with an *A. rhizogenes rol*D transposon insertion mutant, root initiation was demonstrated to still occur but with subsequently retarded growth, indicating that *rol*D is not necessary for root initiation but is a factor in root growth (White et al., 1985). Strong expression of *rol*D in roots of transformed plants has been noted (Leach and Aoyagi, 1991; Elmayan and Tepfer, 1995). Analysis of expression of *rol*D-GUS chimaeric genes showed *rol*D expression to be much stronger in roots than in stems and leaves, particularly in mature plants and in the root elongation zones and vascular tissues (Elmayan and Tepfer, 1995).

Expression of *rol*D-GUS in tobacco was noted to be developmentally regulated in all organs, being strongest during elongation and expansion (differentiation) phases in both immature and mature plants (Trovato et al., 1997). For example, in roots and stems, expression was noted in vascular tissue of elongation zones but no GUS staining was present in apical regions. Similarly, no expression was noted during meristematic growth of leaves or flower petals but strong staining was observed during ensuing developmental expansions. Following continued high levels of expression in mature tissues, expression of *rol*D was reduced in ageing tissues and subsequently switched off at the onset of senescence.

It seems likely that regulatory elements in the *rol*D promoter may control development during different developmental phases and in particular tissues, in a similar manner to the other *rol* genes (e.g. Sugaya and Uchimiya, 1992; Carneiro and Vilaine, 1993; Capone et al., 1994). Leach and Aoyagi (1991) noted that a truncated version of the *rol*D promoter (373bp of 5' flanking sequence) conferred root specific expression, whereas a longer promoter segment did not. Analysis of the *rol*D promoter sequence identified several motifs also present in other root-specific genes, including the tobacco *HRGPnt3* gene, and the *rol*B promoter domain conferring root-specificity (Elmayan and Tepfer, 1995).

1.3.11. Other TL-DNA ORFs

ORF13 and ORF14 from TL-DNA of *A. rhizogenes* agropine type strains have been demonstrated to have promotive effects on hairy root formation. Induction of root formation from carrot discs by *rol* genes was shown to require coinoculation with an

Agrobacterium strain carrying either the TR-DNA auxin synthesis (*aux*) genes or ORF13+14 (Capone et al., 1989). Transformed carrot roots induced by *rol*A+B+C and ORF13+14 were found to contain both inserts and had a similar phenotype to hairy roots. The requirement for the presence of ORF13 and 14 in the same cell as the *rol* genes suggests that the ORF13 and 14 gene products are not diffusible and therefore are not likely to be auxin. However, ORF13 and 14 may play a key role in hairy root induction by allowing the *rol*A, B and C genes to induce root differentiation under limited auxin conditions. Synergistic effects of ORF13 and ORF14 on adventitious root formation were also observed in tobacco leaf segments. While tobacco leaves inoculated with *Agrobacterium* strains carrying *rol*B alone produced a moderate number of roots, significantly more roots were induced by *rol*B in conjunction with one of *rol*C, ORF14 or ORF13 (Aoki and Syōno, 1999).

In an investigation of other TL-DNA ORFs, apart from the *rol* genes, none had the capacity to induce roots independently, when expressed in tobacco under control of the CaMV35S promoter (Lemcke and Schmülling, 1998). Morphological alterations were caused by three of the ORFs under control of this promoter - ORF3, 8 and 13. Expression of ORF3 caused tissue necrosis of tips of upper leaves and on bracts and sepals. Internode lengths were also altered, with shorter lower internodes and longer upper ones. Phenotypic effects induced in ORF13 plants have been shown to include dwarfed stature with altered leaf initiation and variable internode lengths, irregular flower shapes and inhibition of root elongation (Hansen et al., 1993; Lemcke and Schmülling, 1998).

Effects of TL-DNA ORF8 on root induction from carrots (Capone et al., 1989) resembled that of ORF13+14, in enabling root induction by the *rol*A+B+C genes without the presence of *aux* genes. The ORF8 protein was previously shown to have homology to tryptophan 2-monooxygenase (t2m) proteins (Levesque et al., 1988). t2m is the catalyst for the production of indole-3-acetamide (IAM), the first in the two-step process of auxin synthesis in *A. tumefaciens*, *A. rhizogenes* and *Pseudomonas savastanoi*. t2m is encoded in these species by *tms1*, *aux1* and *iaaM*, respectively. Recently, further analysis of the ORF8 protein has provided additional insight into the potential role of this ORF in *A. rhizogenes*-induced root formation (Lemcke et al., 2000). The N-terminal domain of ORF8 was shown to have some homology to the RolB protein (26%) and a low degree of homology to t2m proteins (36%) was located in the C-terminal end. Although this homology was low compared with other known t2m proteins, evidence of t2m activity of ORF8 was demonstrated (Lemcke et al., 2000). This activity of ORF8 may

have an important role in the effectiveness of root induction by *A. rhizogenes*; for example, in plant species in which endogenous auxin levels are not sufficient to trigger root initiation (Cardarelli et al., 1987a; Capone et al., 1989). The role of ORF8 in increasing IAM levels may help to elevate endogenous auxin levels in certain hosts, which are able to convert IAM to IAA by endogenous production of an IAM hydrolase. Thus, the presence of ORF8 in Ri T-DNA may facilitate the activity of *rol*B in root induction, following *A. rhizogenes* infection. Significant auxiliary roles in root induction may also account for the high degree of conservation between different Ri plasmids of the TL-DNA regions including ORF8 and ORFs 13 and 14 (Filetici et al., 1987).

Seedlings of ORF3 and ORF8 tobacco transformants demonstrated altered sensitivity to both auxin alone and to auxin/cytokinin treatments (Lemcke and Schmülling, 1998). ORF3 seedlings displayed reduced formation of callus in response to treatment with auxin/cytokinin. It was suggested that the ORF3 gene product may have a role in suppressing tissue dedifferentiation, thus favouring root differentiation triggered by *rol* genes. ORF8 seedlings are apparently more resistant to auxin, as they were capable of growing with auxin concentrations that were inhibitory to other genotypes. Interestingly, as transformants containing the homologous *iaaM* gene were not similarly resistant, this trait may be encoded in the N-terminal end of the ORF8 protein, which has homology to the RolB protein (Lemcke et al., 2000).

Possible negative regulatory roles, perhaps in moderating the effects of *rol* genes (Lemcke et al., 2000) were suggested for these ORFs. For example, ORFs 3 and 8 appear to increase resistance to auxin and auxin/cytokinin in combination, which may alleviate toxic effects of high levels or sensitivity to phytohormone induced by *rol* genes. Similarly, expression effects of ORF13 seemed to be related to restrictions on cell division and elongation of roots and leaves, which may help to counterbalance the effects of *rol* genes.

In summary, *A. rhizogenes rol* genes are generally responsible for the root-inducing capacity of *A. rhizogenes*; these genes are capable of independently stimulating adventitious root induction as well as a variety of phenotypic effects in other plant organs. Other TL-DNA genes have possible roles in modulating and optimising effects of the *rol* genes. *rol* gene mechanisms are likely to involve influencing the local balance of auxin and cytokinin within roots, and possibly other physiological processes such as sugar metabolism, subsequently resulting in stimulation of root initiation in transformed tissues.

1.4. BIOTECHNOLOGICAL APPLICATIONS OF TRANSFORMATION WITH *A. RHIZOGENES*

Although infection with *A. tumefaciens* does not necessarily cause the death of the plant, the crown gall formation can severely stunt growth and cause tissue necrosis and therefore decrease productivity in agricultural industries. On the other hand, the main symptom of infection with *A. rhizogenes* root-inducing bacteria is the formation of a mass of adventitious roots on the stem of the plant. In some circumstances this may be regarded as a negative effect, such as during dense culturing of plants for horticultural purposes. In nature, however, it is possible that *A. rhizogenes* infection may be advantageous in some cases - for example, by providing the plant with an improved ability to obtain nutrients or water. In fact, possible *A. rhizogenes*-induced positive effects on root growth, which may be beneficial in enhancing plant growth, persistence and productivity, have been considered in terms of agronomic and horticultural applications for some time (Strobel and Nachmias, 1985; Lambert and Tepfer, 1991). For example, Strobel and Nachmias (1985) observed significant promotion of root number and root mass of almond (*Prunus amygdalus*) and olive (*Olea europaea*) bare root stock inoculated with *A. rhizogenes*, with accompanying positive effects on growth of upper plant parts.

Stimulatory effects on plant growth induced by the presence of *Agrobacterium* may group these species with a general class of asymbiotic rhizosphere bacteria termed *plant growth promoting rhizobacteria* (PGPR). These are defined as naturally occurring soil bacteria able to colonize roots and stimulate plant growth. Bacteria of many different genera have been demonstrated to have asymbiotic plant growth-promoting activity, including *Agrobacterium* spp. and most commonly *Pseudomonas* spp. (Chanway, 1997). The use of PGPR for inoculation of roots of greenhouse and field crops has potential as a useful approach to improving productivity; in particular, inoculation of tree species is an area of particular interest and may have potential applications for reforestation programs (Chanway, 1997). *Agrobacterium radiobacter* has been reported to increase growth of tree species, *Pinus sylvetris* (pine) and *Fagus sylvatica* (beech), under greenhouse conditions (Leyval and Berthelin, 1989; 1993). However, under field conditions, successful promotion of plant productivity has not been widely documented; plant growth promotion effects observed in greenhouse or controlled environment trials are apparently often not reproduced in field experiments (Alabouvette and Steinberg,

1995; Chanway, 1997). This is likely to be at least partly due to limitations on the ability of the introduced microorganisms to survive in new soil environments.

Improvement of root initiation capacity of rootstocks and cuttings is an important consideration in propagation of horticultural and agronomically significant species (Kovar and Kuchenbuch, 1994; Häggman and Aronen, 2000). The use of *A. rhizogenes* and/or Ri T-DNA genes in inducing root formation in difficult-to-root woody species is one particular area of interest (Häggman and Aronen, 2000), and has been investigated in numerous species including apple (Lambert and Tepfer, 1992), walnut (*Juglans regia*) (Falasca et al., 1999), eucalypt (Machado et al., 1997), *Pinus nigra* (Mihaljevic et al., 1996), *Pinus contorta* (Yibrah et al., 1996), aspen (*Populus tremula*) (Tzfira et al., 1998) and elm (*Ulmus* spp.) (Rinallo et al., 1999). Induction of adventitious rooting on cuttings of recalcitrant species can facilitate micropropagation of desirable clones and also reduce difficulties and costs associated with tissue culture propagation techniques and methods such as grafting. Stimulation of more vigorous root systems earlier in development are also likely to be advantageous for plant survival rates, particularly during transplantation or outplanting, and may also reduce the need for intensive watering regimes.

Possible applications for the phenotypic effects of *A. rhizogenes* Ri T-DNA gene expression have also been indicated in horticulturally important species (Davies et al., 1994), such as geranium (Pellegrineschi et al., 1994) and *Begonia* (Kiyokawa et al., 1996) and rose (van der Salm et al., 1996b; 1998). Transformation of lemon-scented geranium with *A. rhizogenes* was shown to induce ornamentally desirable characteristics of increased lateral branching and reduced stature, as well as more branched root systems and inhibition of flowering (Pellegrineschi et al., 1994). Interestingly, essential oil production and fragrance intensity were also increased, modifications relevant to the commercial uses of this plant as an ornamental species and as a crop for production of geraniol oil for the perfume industry.

Root cultures transformed with *A. rhizogenes* grow rapidly and are genetically stable, and have therefore been well established as a system for the study and production of secondary metabolites (Hamill and Lidgett, 1997; Flores et al., 1999). Interest remains strong in using transformed root cultures grown on a large scale in bioreactors as a source of biologically important products (Doran, 1997). Transformed root cultures have also been cocultivated with organisms such as fungi, nematodes and insects - these have been utilised in studies of root pathogens (Flores et al., 1999) and may also have other potential applications. For example, cocultivation with mychorrizal

fungi may facilitate cultivation of the fungi for use in inoculating plants, to induce symbiotic interactions beneficial to plant growth (Flores et al., 1999).

1.4.1. Potential biotechnological applications for *rolC*

*rol*C has been a gene of particular interest in terms of commercial applications, including horticultural and forestry plant species. The ability to manipulate plant form and developmental characteristics are an advantage for horticultural crops, such as ornamental plants, garden annuals and fruit tree and vegetable species. Aspects of the *rol*C phenotype in transgenic plants have been recognised as having significant potential for commercial horticultural applications (Scorza et al., 1994; Bell et al., 1999; Giovannini et al., 1999; Winefield et al., 1999). For example, the characteristic dwarfed stature of *rol*C plants has application in the production of compact miniature versions of ornamental plants and tree species, such as *Petunia* (Winefield et al., 1999) and pear trees (*Pyrus communis L.*) (Bell et al., 1999). Earlier flowering, altered flower size and increases in flower number are also modifications of interest induced by *rol*C, enhancing traits often desired in ornamental plants (Scorza et al., 1994). Reductions in fertility, although clearly a disadvantage for crops propagated by seed, may have advantages in other cases where vegetative propagation is possible, for minimising the probability of crossbreeding with non-transgenic plants.

Increased production and accumulation of secondary metabolites in root cultures have been reported for several species transformed with *rol*C, with implications for the commercial production of useful compounds, such as in the pharmaceutical industry. *rol*C expression levels are correlated with nicotine production in transformed tobacco root cultures (Palazón et al., 1998b), alkaloids in root cultures of *Catharanthus roseus* (Palazón et al., 1998a) and also increased production of ginsenosides in ginseng root cultures (Bulgakov et al., 1998).

Potential agronomic benefits to potato were suggested as a result of *rol*C expression effects. 35S-*rol*C transgenic potato plants were found to have reduced glucose levels, which are correlated with increased resistance to fungal and bacterial pathogens (Fladung and Gieffers, 1993). However, productivity of the 35S-*rol*C potato plants was concomitantly limited, due to reduced tuber number and yield. As a strategy to overcome this disadvantage, Fladung et al. (1993) transformed plants with *rol*C under control of a promoter, *rbcS*, conferring expression only in photosynthetic tissues and

thereby generating plants in which glucose levels in tubers were reduced without alteration in tuber number or yield.

Transformation of tree species apple and hybrid aspen have been shown to cause characteristic *rol*C effects similar to that in tobacco and potato, highlighting the influence of *rol*C on basic plant processes. The phenotype of *rol*C transgenic hybrid aspen was stably maintained through a cycle of growth and dormancy and second period of growth (Nilsson et al., 1996b). Maintenance of transgene expression and phenotype is clearly an important factor for consideration in strategies for agronomic improvement of perennial species by genetic modification. Lambert and Tepfer (1991) investigated the possibility of improving apple tree propagation using either wild type *A. rhizogenes* strains (A4 and 8196) or the *rol*C gene controlled by CaMV35S promoter to increase root induction. Successful root induction was increased for the *rol*C-transformed microcuttings *in vitro*, compared to control and A4-inoculated cuttings. The resulting plants were chimaeric, with untransformed aerial parts and transformed root systems. This raised the possibility of inserting the *rol*C gene into the genome of a recalcitrant rootstock to improve rooting ability, onto which untransformed scions could be grafted. The success in overcoming rooting deficiency by inoculation of cuttings was dependent on the genotype of the apple line and the strain of bacterium used.

Phenotypic modifications of possible agronomic benefit were identified in alfalfa (*Medicago sativa*) transformed with *rol* genes (Frugis et al., 1995). In creeping-rooted strains of this important forage legume, shoot buds develop from laterally spreading, highly branched root systems. *rol*C-transformed alfalfa plants showed improvements in productivity, with significantly greater root mass production and correspondingly increased number of stems produced along the roots, in comparison to WT plants. In contrast, CaMV35S-*rol*C transformed alfalfa did not have improved root growth compared to WT plants, suggesting that expression levels of *rol*C may have been relevant to plant phenotype in this case. Although increased expression of *rol*C has been demonstrated to stimulate increased root growth in some species, in others the altered regulation of expression does not enhance growth and sometimes has other adverse effects (Fladung and Ballvora, 1992; Frugis et al., 1995; Palazón et al., 1998a). There may not necessarily be a simple correlation between *rol*C expression levels and effects on plant growth, as the influence of *rol*C on alterations to hormonal balance or sensitivity and also the effects of the environmental conditions in which the plants are grown are likely to be significant and complex.

ABBREVIATIONS

ABA	abscisic acid
AR	adventitious root (s)
ARP	adventitious root primordium/primordia
aux:cyt	auxin:cytokinin ratio
BAP	6-benzylaminopurine
C	carbon
Chl	chlorophyll
DW	dry weight
FW	fresh weight
GA	giberellic acid
GUS	ß-glucuronidase reporter gene
HF	hormone-free
IBA	indole-3-butyric acid,
IAA	indole-3-acetic acid
IPA	indole-3-propionic acid
ipt	isopentenyl transferase
Kan^R	kanamycin resistant
Kan^S	kanamycin sensitive
LR	lateral root (s)
LRP	lateral root primordium/primordia
N	nitrogen
n	sample number
NAA	α-naphthalene acetic acid
nptII	neomycin phosphotransferase II
PCR	polymerase chain reaction
Ri	root inducing
RMF	root mass fraction
r:s	root:shoot ratio
s.e.m	standard error of the mean
T- DNA	transfer DNA
Ti	tumour inducing
WT	wild-type

AIMS OF THIS STUDY

1. Investigation of the effects of *rol* genes on root initiation and root growth in *Nicotiana tabacum* transformed with individual or combined *rol* genes.

The main objectives of this section were:
(a) to investigate potential effects of *rol* genes on lateral root formation, by an analysis of lateral root primordia (LRP) initiation in response to exogenously applied auxin, in primary roots of *rol* gene transgenic tobacco seedlings.

(b) analysis of the effects of *rol* genes, particularly *rol*C, on growth of transgenic tobacco plants, by quantification of root and shoot biomasses and root:shoot balance of plants grown in soil or cultured *in vitro*.

(c) to model the effects of restricting *rol*C gene expression to the root system on relative root and shoot growth, by grafting of transformed rootstocks with untransformed scions or by genetic regulation using a root-specific promoter fragment.

2. Transformation of *Trifolium repens* (white clover) with *rol*C.

The objectives of this section were to generate transgenic white clover lines expressing the *rol*C gene and characterise the effects of the gene on a range of phenotypic traits and shoot and root growth parameters. In view of the importance of white clover as a pasture crop in Australia and in many regions of the world, potential agronomic advantages of alterations induced by *rol*C are considered.

3. Survey for the presence of *A. rhizogenes* in local soils.

As the presence of free-living *A. rhizogenes* in Australian soils has not been reported to date, the aim of this preliminary survey was to explore the possibility that *A. rhizogenes* may exist in the local environment. This may be relevant to the potential release of transgenic crop plants containing *rol* genes. Soil samples from a range of cultivated and natural plant environments, from locations around Melbourne and regional Victoria, were included in the study.

CHAPTER 2

MATERIALS AND METHODS

General molecular biology, plant tissue culture and bacterial experimental procedures are described in this chapter, with methods specific to particular experiments detailed in the relevant chapters.

2.1. GENERAL MOLECULAR BIOLOGY PROCEDURES

In general, standard molecular biology methods were used, with modifications as noted. Single glass-distilled (dH_2O) and ultrahigh quality (UHQ) water were used for molecular biology procedures. All solutions used were at concentrations and pH as listed in §2.1.2 (Solutions and Reagents) unless otherwise stated. Abbreviations of solution and reagent names are listed in §2.1.2. Centrifuge speeds are expressed in K = 1000 rpm.

2.1.1. DNA AND RNA METHODS

Extraction of genomic DNA from plant tissues

This method was modified from the protocol of Pich and Schubert (1993). Tissue was harvested and immediately snap-frozen with liquid nitrogen, stored at -70°C. Without thawing, tissue was ground to a fine powder using a ceramic mortar and pestle. The powder was added to 1.5 ml extraction buffer (500 mM NaCl; 50 mM Tris-HCl pH 8.0; 50 mM EDTA, 1% (v/v) ß-mercaptoethanol added immediately before use). The mixture was kept on ice and 0.65 ml ice cold 20% PVP added (final concentration 6%). 0.4 ml of 10% (w/v) SDS was then added and the mixture incubated at 65°C for 10 min with occasional mixing. 0.1 volume of 5 M KAc was added and the mixture incubated on ice for 30 min before centrifugation at 13 K, 10 min at 4°C. The supernatant was transferred to a new tube and mixed with 0.6 volume isopropanol by gentle inversion, placed on ice for 10 min, then centrifuged again (13 K, 10 min, 4°C). The supernatant was discarded thoroughly and the pellet dissolved in 500 µl 1xTE (pH 8.0). The DNA was treated with RNase A (10 µl of 1 mg ml^{-1} solution) and incubated 37°C, 10-15 min. The DNA solution was then extracted 1-2 times with an equal volume of phenol:$CHCl_3$:IAA (25:24:1). After

centrifugation at 13 K, 5-10 min, the aqueous phase was transferred and the DNA precipitated as per standard methods (see *E. coli* plasmid preparation).

Extraction of total RNA from plant tissues

RNA was extracted using the method described in Hamill and Lidgett (1997). Tissue was harvested, immediately snap-frozen with liquid nitrogen and stored at -70°C. Without thawing, tissue was ground to a fine powder using a ceramic mortar and pestle. The powder was added to extraction buffer consisting of 750 µl phenol and 750 µl TLES and vortexed. 750 µl $CHCl_3$:isoamyl alcohol was then added and the mixture divided into two eppendorf tubes and centrifuged at 10 K, 5 min. The supernatant was placed in a new tube, an equal volume of 4 M LiCl added and the mixture placed in ice at 4°C overnight. Following this, the tubes were centrifuged (10 K, 30 min, 4°C), the supernatant discarded and pellets resuspended in 250 µl water. To precipitate RNA, 0.1 volume NaAc and 2 volumes 100% ethanol were added and the solution placed at -70°C for 1 h or -20°C overnight. The RNA was then spun down by centrifuging at 10 K, 30 min and the RNA pellet resuspended in 70 -100 µl water.

Restriction enzyme digests

Restriction enzyme digests were generally carried out in 20-30 µl volume, utilising 5-10 units of enzyme used per microgram of DNA and 1 mg ml^{-1} BSA. In some less readily digestible samples, 2 mM spermidine was also added.

Southern blot hybridisation

DNA was separated on a 0.8% agarose gel. The gel was treated with 0.25 M HCl (for 15 min), denaturation buffer (1.5 M NaCl, 0.5 M NaOH) (30 min) and neutralisation buffer (1.5 M NaCl, 1 M Tris pH8) (30 min). A capillary blot transfer was set up, using 20xSSC as the blotting buffer and allowing transfer to the nylon membrane (Hybond-N+) to proceed overnight. The DNA was then fixed to the membrane by treatment with 0.4 M NaOH and rinsed briefly in 2xSSPE.

Prehybridisation: Membranes were incubated in prehybridisation solution (5xSSC; 0.5% (w/v) SDS; 5xDenhardt's solution (see below); 0.4 mg ml^{-1} denatured herring sperm DNA) for 1-3 hr at 65°C. Denatured, radioactively labelled probe was then added and allowed to hybridise overnight at 60-65°C.

Post-hybridisation treatment: Membranes were washed depending on the stringency required:

Low stringency: 3xSSC; 0.5% (w/v) SDS, room temperature for 10 min.

Medium stringency: 1xSSC; 0.5% (w/v) SDS, 60°C for 10 min.

High stringency: 0.1xSSC; 0.5% (w/v) SDS, 60°C for 10 min or longer, depending on the level of background radioactivity on membrane.

After washing, membranes were wrapped in plastic and placed with X-ray film in cassette with intensifying screen.

Northern blot hybridisation

A formaldehyde denaturing gel was used (1.5% agarose; 1xMOPS; 5.4% formaldehyde, 0.4 µg ml^{-1} EtBr). Electrophoresis running buffer was 1xMOPS. Gels were usually run for 16-20 h at 20 volts. After electrophoresis was complete, the formaldehyde was removed by rinsing gel twice in 10xSSC for 20 min. The blot was then set up in the same way as a Southern blot (see above), with 10xSSC as the buffer. Transfer was allowed to take place over a period of 2-3 days. The nylon membrane was then removed and placed face up for 5 min on blotting paper soaked in 0.05 M NaOH to fix RNA to the membrane. The membrane was then rinsed briefly in 2xSSPE.

Hybridisation was carried out in a hybridisation tube using ExpressHyb solution (Clontech). Prehybridisation was carried out with 5 ml ExpressHyb solution at 65°C for 30 min. This solution was then replaced with 4 ml fresh ExpressHyb containing the denatured radioactively labelled probe and the hybridisation allowed to proceed for 60 min at 65°C. Membranes were then washed as described in ExpressHyb manufacturers instructions: three washes in Wash Solution 1 (2xSSC; 0.05% (w/v) SDS) for 10-12 min each, two washes in Wash Solution 2 (0.1xSSC; 0.1% (w/v) SDS) for 20 min each. The membrane was then wrapped in plastic and exposed to X-ray film.

***Agrobacterium* colony blots**

Colony blots were carried out by direct transfer and fixation of bacteria to Hybond N+ nylon membrane, for hybridisation and probing of bacterial DNA. As *Agrobacterium* colonies generally exude a viscous, polysaccharide slime, the most controlled way to

transfer bacteria was to dab small amounts of bacterial colonies onto relevant positions on the membrane using sterile toothpicks. Membranes were treated as for Southern blots with alkaline denaturation solution and neutralising solution; DNA was then fixed with 0.4 M NaOH. Probing was carried out as for Southern blots.

Genomic DNA isolation from *Agrobacterium* strains

10 ml MG broths inoculated with *Agrobacterium* were incubated with shaking at 25°C for 16 h. 5 ml of cells were centrifuged at 5K, 5 min and the pelleted cells resuspended in 900 µl lysis buffer (125 µg ml^{-1} Proteinase K; 8.3 mg ml^{-1} lysozyme; 1.25% (v/v) sarkosyl; in TE buffer pH 8.0) and incubated at 37°C for one hour. After centrifugation at 15 K, 15 min, the supernatant was removed to a new tube and then centrifuged again at 15 K, 15 min. This repeat spin is to help remove as much of the polysaccharide slime as possible. The supernatant was again removed to a clean tube, an equal volume of isopropanol added and the mixture placed on ice for 30 min. The precipitated chromosomal DNA was very compact and viscous and could be removed by scooping out with a sterile disposable pipette tip. It was then rinsed with 70% ethanol, allowed to air dry and resuspended in water.

Probe preparation

Plasmid preparation from *E.coli*

30-100 ml bacterial cultures in LB were grown overnight and centrifuged at 5 K, 10 min. Pellet resuspended in 2.5 ml cold buffer (25 mM Tris-HCl pH8; 10 mM EDTA). 5 ml freshly prepared lysis solution (0.2M NaOH/1%(w/v) SDS) were added and mixture placed on ice for 5 min before addition of 3.75 ml cold 5 M/3 M KAc and another 10 min on ice. Mixture was centrifuged 15K, 15 min, the supernatant transferred to a new tube and 40 µl RNase (10 mg ml^{-1}) added with an incubation at 37°C for 30 min. This was followed by addition of 20 µl Proteinase K (10 mg ml^{-1}) and incubation at 37°C for 30 min. 0.5 volume of isopropanol was added and the mixture centrifuged 10 K, 15 min. The resulting pellet was resuspended in 500 µl TE. This DNA solution was extracted with phenol and $CHCl_3$:IAA and the DNA precipitated by a standard procedure: 0.1 volume NaAc and 2 volumes 100% EtOH were added, the mixture placed ~30 min at-20°C then centrifuged 13 K, 30 min. The DNA pellet was washed in 70% EtOH, dried and resuspended in water (or 1xTE).

Recovery of DNA from agarose gels

(a) Phenol freeze purification

EtBr-stained DNA was cut from the agarose gel and crushed in an eppendorf tube (and in some cases was also pushed through a small syringe). 0.5 ml phenol was added and the mixture vortexed, before placing at -20°C for several hours or at -70°C for 30 min. The mixture was then centrifuged at 13 K, 30 min and the top layer transferred to a new tube. To the lower layer, 200 µl 1xTE was added and this was centrifuged at 13 K, 15 min. The top layer was then combined with the previous aqueous layer in the new tube. This solution was extracted with an equal volume of $CHCl_3$:IAA (24:1) and centrifuged 13 K, 10 min. The top aqueous layer was removed and the DNA precipitated as per standard method (see plasmid prep procedure) and resuspended in 10 µl water.

(b) Gel-spin purification (alternative procedure)

The Gel-Spin™ DNA Recovery kit, from Worthington Biochemical Corporation, allows isolation and purification of DNA from agarose gels without use of chemical or enzymatic reagents to depolymerise the agarose. Agarose slices were placed in supplied filter cup in microcentrifuge tube and centrifuged 11K, 5 min. Salt solution (0.1 M KCl) was added and the tube centrifuged again. DNA was then precipitated with addition of 100% ethanol, as per standard procedures.

^{32}P-labelling of probes for DNA and RNA hybridisations

Probes were labelled using the Gigaprime Labelling Kit (Bresatec), as per manufacturer's instructions. 50-100 ng of DNA was labelled for each probe. DNA was first denatured by boiling for 5 min. [α-^{32}P]-dATP, mixed nucleotides, buffer and DNA polymerase were added and incubated with DNA at 37°C for 15-20 min. The radioactively labelled DNA was then separated from unincorporated radioactive nucleotides by passing the mixture through a Sephadex G-50 drip column and collecting the relevant fractions. The labelled probe was denatured before adding to hybridisation mix by boiling for 2 min and immediately placing on ice.

Removal of radioactive probe

DNA membranes were stripped by placing in 0.4 M NaOH at 45°C for 20 min, then rinsed in a neutralising solution (0.1xSSC; 0.1% (w/v) SDS; 0.2 M Tris-HCl pH 7.5) for 20 min. From RNA membranes and some DNA membranes, probes were removed by

placing membranes for 10 min or longer in 10% (w/v) SDS solution heated to 90-100°C, with gentle rotation.

Phosphorimaging

A phosphorimager was used to quantify the relative signal strengths of Northern blot hybridisations. A phosphor screen was exposed to the hybridised membranes, then scanned with Molecular Dynamics Storm phosphorimage scanner. Scans were analysed with ImageQuant software. Following hybridisation(s) with the relevant *rol* gene probes, Northern blots were stripped and probed with ubiquitin to correct for differences in RNA loading levels. Blots were scanned again with the phosphorimager and *rol* gene expression relative to loading levels was calculated.

2.1.2. MOLECULAR BIOLOGY SOLUTIONS AND REAGENTS

Abbreviations used and additional details of composition, concentrations or pH

BSA bovine serum albumin

$CHCl_3$ chloroform

CrO_3 chromium trioxide: 3% (w/v) in water

Denhardts solution (50x): BSA fraction V 10 g l^{-1}; Ficoll Type 400 10 g l^{-1}; PVP 10 g l^{-1}

dH_20 single glass distilled water

EDTA ethylenediamine tetraacetic acid

EtBr ethidium bromide 10 mg ml^{-1}

EtOH ethanol

IAA isoamyl alcohol

KAc potassium acetate: 3 M potassium/5 M acetate; pH 4.8

LiCl lithium chloride 4 M

MOPS 3-(morpholino) propane sulfonic acid: 200mM MOPS, 90mM NaAc, 10mM Na_2EDTA: pH 7

NaAc sodium acetate 3 M

NaOH sodium hydroxide: 0.4M DNA blot fixation; 0.05M RNA blot fixation

phenol pH 7.5

PVP polyvinyl pyrrolidone 20% (w/v) soluble in water

TLES 100 mM Tris pH 8.0; 100 mM LiCl; 10 mM EDTA; 1% (w/v) SDS.

SDS sodium dodecyl sulphate 10% (w/v) in water

SSC (20x): 3 M sodium chloride; 0.3 M trisodium citrate; pH 7.0

SSPE (20x): 3 M sodium chloride; 0.2 M $NaH_2PO_4.H_20$; 0.02 M EDTA; pH7.7

TE TrisEDTA: 10mM Tris HCl; 1mM EDTA; pH 7.5

2.2. GENERAL TISSUE CULTURE AND PLANT CULTIVATION METHODS

All tissue culture procedures were carried out under sterile conditions in a laminar air flow cabinet. Ultrahigh quality (UHQ) water was used for all tissue culture procedures, solutions and media.

2.2.1. MEDIA AND SOLUTIONS

Solid Murashige and Skoog (M+S) medium

M+S medium powder 4.43 g l^{-1}; agar 8 g l^{-1}; pH 6.0. Unless otherwise specified, M+S medium was supplemented with sucrose at 30 gl^{-1} for general plant cultivation.

Solid half-strength M+S medium for *in vitro* culture of white clover clones

MS medium powder 2.215 g l^{-1}; sugar 1.5% (w/v); agar 8 g l^{-1}; pH 6.0

B5 liquid medium

B5 powder 3.875 g l^{-1}; sugar 30 g l^{-1}

White clover transformation media:

Regeneration medium RM73

NAA (Naphthaleneacetic acid) 0.5 μM; TDZ (Thidiazuron) 5 μM; added to M+S 4.43 g l^{-1}; sucrose 20 g l^{-1}; agar 8 g l^{-1}

Selection medium

Cefotaxime 250 μg ml^{-1} and kanamycin 25 μg ml^{-1} are added to RM73

Root induction medium

IBA 1.2 μM; M+S 2.215 g l^{-1}; sucrose 15 g l^{-1}; agar 8 g l^{-1}

***Tobaccc transformation:* Shoot regeneration medium MSR1**

IAA 2 mg l^{-1}; BAP 0.5 mg l^{-1} added to M+S medium 4.43 g l^{-1} with sucrose 30 g l^{-1}.

Preparation of media supplemented with antibiotics and plant growth hormones

Solutions of antibiotics and plant hormones were made by dissolving powder in relevant solvent. Solutions were then filter-sterilised in laminar flow cabinet through 0.45μm pore filters (Millipore). Exogenously applied hormone or antibiotic supplements were added in appropriate concentrations to sterile liquefied medium (cooled to around 60°C) under aseptic conditions, prior to pouring plates.

Modified Hoagland's (hydroponic) nutrient solution

Stock solutions:

Macro I (100x): KH_2PO_4 13.6 g l^{-1}; KNO_3 50.5 g l^{-1}; $MgSO_4.7H_20$ 49.3 gl^{-1}

Macro II (100x): $Ca(N0_3)_2.4H_20$ 118 g l^{-1}

Micro (1000x): Boric acid 2.86 g l^{-1}; Manganese chloride 1.81 g l^{-1}; Zinc sulphate 0.22 gl^{-1}; Copper sulphate 0.08 g l^{-1}; Molybdic acid 0.02 g

Iron (100x): $FeSO_4.7H_20$ 0.5 g l^{-1} ; Na_2EDTA 0.7 g l^{-1}

Per litre of ¼ strength Hoagland's:

Macro I 2.5 ml; *Macro II* 2.5ml; *Micro* 250 μl; *Iron* 2.5 ml

2.2.2. TISSUE CULTURE AND PLANT CULTIVATION PROCEDURES

Surface sterilisation of tobacco seed for *in vitro* germination

A dilute solution of a commercial bleach (Domestos, consisting of 5% (w/v) available chlorine) was used. Tobacco seed were placed in 10% (v/v) solution of Domestos and gently mixed for 30 min then thoroughly rinsed 5 times with sterile UHQ water.

Kanamycin selection of tobacco transformants

Tobacco seed were sown and germinated on M+S medium supplemented with 30 g l^{-1} sucrose and 75 μg ml^{-1} kanamycin. Kanamycin-resistant seedlings were usually identifiable by 10 days after sowing.

Transfer of *in vitro*-grown plants to soil

Tobacco plantlets were outplanted into 1 litre volume pots, in 800 ml of an equal ratio of soil mixed with perlite and fertilised with 5 g l^{-1} of Osmocote, a slow-release complete fertiliser suitable for pot-grown plants. To maintain a humid environment until established

in the soil, plants were covered with plastic for one week, gradually decreasing humidity by provision of air vents.

Manual crosses between tobacco plants

Anthers from the maternal plant were removed while the flowers were immature i.e. before they dehisced. The flowers were sliced open while still closed and the anthers removed. Flowers were kept under paper bags to mature, to avoid accidental cross pollination. When the flowers were mature and the stigma appeared sticky, dehisced anthers were taken from the paternal parent and the pollen smeared over the stigma of open flowers of the maternal parent. The flowers were kept covered as the seed pods developed. When the seed pods were completely dry and brown they were removed from the plant.

Tissue harvest for dry weight measurements

Tissues were collected in paper bags and placed in oven at 50°C for 7 days to ensure complete dehydration.

Greenhouse light and temperature conditions

Temperature: 25°C ± 2°C. Lighting: 16 h photoperiod (commencing 6:00 am) with fluorescent lighting (Sylvania Gro-Lux, Thorn, UK); waveband ~360-720 nm; photon flux density of 150 μmol m^{-2} s^{-1}.

***In vitro* culture light and temperature conditions**

Temperature: 22°C ± 2°C. Lighting: fluorescent lights (Sylvania Gro-Lux, Thorn, UK); waveband ~360-720 nm; photon flux density of 60 μmol m^{-2} s^{-1} under a 16 h photoperiod.

2.3. BACTERIAL CULTURE MEDIA

For solid media, 15 g l^{-1} agar was added before autoclave sterilisation.

1xLB tryptone 10 g l^{-1}; yeast extract 5 g l^{-1}; sodium chloride 10 g l^{-1}; pH 7.5

1xYMB yeast extract 0.4 g l^{-1}; mannitol 10 g l^{-1}; K_2HPO_4 0.5 gl^{-1}; $MgSO_4$ 0.2 g l^{-1}, NaCl 0.1 g l^{-1}; pH 7.0

1xTY tryptone 5 g l^{-1}; yeast extract 3 g l^{-1}; pH 7.0

1xMG mannitol 5 g l^{-1}; L-glutamic acid 1 g l^{-1}; KH_2PO_4 0.25 g l^{-1}; NaCl 0.1 g l^{-1}; $MgSO_4$ 0.1 g l^{-1}; biotin 1 µg l^{-1}; tryptone 5 g l^{-1}; yeast extract 2.5 g l^{-1}; pH 7.0

Agrobacterium selection medium 2E

For 500 ml of medium 2E: NH_4NO_3 (0.08g); erythritol ($C_4H_{10}O_4$) (1.525g); KH_2PO_4 (0.27g); K_2HPO_4 (0.52g); $MgSO_4.7H_2O$ (0.125g); sodium taurocholate ($C_{26}H_{44}NNaO_7S$) (0.145g); yeast extract (0.05g); malachite green (2.5 ml of 0.1% (w/v) solution); agar (7.5g). After autoclaving and cooling to ~50°C, add filter-sterilised cycloheximide (0.5 ml of 2% solution) and sodium selenite (Na_2SeO_3) (3.3 ml of 1% solution).

2.4. SOURCES (brand/company)

Plant growth hormones: IBA, IAA, NAA, Thidiazuron (TDZ) *Sigma*
Antibiotics: Kanamycin sulphate, Ampicillin *Sigma*
Cefotaxime *Roussel*
M+S medium *Sigma*
B5 medium *Sigma*
Agar *Difco*
Sodium selenite *Sigma*
Sodium taurocholate *Sigma*
Micropore tape *3M*
Osmocote slow-release complete fertiliser *Scotts*
Domestos (bleach) *Lever & Rexona*
Restriction enzymes *Promega*
RNase (DNase-free) *Promega*
RNA standard size markers *Promega*
Gel-Spin DNA purification kit *Worthington Biochemical Corporation/ScimaR*
Hybond N+ nylon membrane *Amersham*
ExpressHyb *Clontech/Integrated Sciences*
[α-^{32}P]-dATP radioactive isotope *Geneworks* (formerly *Bresatec*)
Gigaprime DNA labelling kit *Geneworks* (formerly *Bresatec*)
Sephadex G-50 *Pharmacia*
Medical X-ray film *Fuji*

CHAPTER 3

EFFECTS OF *rol* GENES ON ROOT INITIATION AND GROWTH IN TRANSGENIC *NICOTIANA TABACUM*

3.1. INTRODUCTION

The processes of adventitious root (AR) and lateral root (LR) formation are analogous with respect to the basic sequence of developmental events, with both involving dedifferentiation of initial cells and cell divisions to form a primordium, followed by activation of a new meristem and subsequent root emergence (Blakesley et al., 1991; Malamy and Benfey, 1997a). Moreover, the role of auxin is similar in both AR and LR formation, with comparable requirements for auxin at key developmental stages and similar responses to exogenously supplied auxin at high or low concentrations, depending on the stage of development. Considering the effects of *A. rhizogenes rol* genes on auxin sensitivity in terms of AR induction (Spanò et al., 1988; Capone et al., 1989), it is therefore conceivable that the *rol* genes may also alter LR initiation by influencing the sensitivity of transformed root cells to auxin. The *rol* genes may thus have potential applications for modifying root growth and possibly the root-shoot balance of plants.

Specific effects on LR formation by *rol* genes have not been investigated to the same extent as AR formation, although expression of *rol* genes in some transgenic plants has been reported to cause increases in the size and branching of root systems. In particular, larger root systems have been reported in plants expressing *rolC*, including tobacco and alfalfa (Martin-Tanguy et al., 1993; Tepfer et al., 1994; Frugis et al., 1995; Palazón et al., 1998a). However, very few studies have quantified these effects in intact plants.

A detailed analysis of LR branching in *Arabidopsis* plants transformed with Ri T-DNA demonstrated that much higher numbers of lateral roots were produced than in plants not containing Ri T-DNA, resulting in root dry weights three times greater than non-transgenic controls (Hamill and Chandler, 1994). *In vitro* root cultures of tobacco roots transformed with single *rol* genes have also been reported as being more branched than untransformed roots (Schmülling et al., 1989). The use of individual *rol* genes, rather than the whole Ri T-DNA, may have advantages for obtaining modifications to rooting; for example, negative effects in the upper plant may be less severe with single *rol* genes, as the range of phenotypic effects is narrower (Schmülling et al., 1988).

This chapter describes experiments aimed at investigating the effects of *rol* genes on root initiation and root growth in tobacco (*Nicotiana tabacum* var SR1) lines transformed with individual or combined *rol* genes. The analyses of *rol* gene transgenic tobacco lines undertaken in this study may add to information on the influence of *rol* genes on root initiation and root growth. Two main aspects were investigated:

(a) *LR and AR initiation in response to varying levels of auxin.* Possible alterations to auxin sensitivity in terms of root initiation were assessed in *rol* gene transformed tissues. The frequency of lateral root primordia (LRP) initiation was determined in primary roots of young, intact seedlings. LRP induction was also analysed in seedlings of a tobacco line transformed with *A. rhizogenes* Ri plasmid T-DNA. The effects of the presence of all the *rol* genes as well as other T-DNA ORFs on LRP formation were thus compared to the effects of the individual *rol* genes. AR induction was examined on leaf discs from mature tobacco plants transformed with various *rol* genes, as a comparison with the LRP analysis and also with other reports in the literature of AR formation from *rol* gene transformed leaves. Formation of AR in response to different exogenous auxin concentrations was also investigated.

(b) *Growth analyses.* An increased capacity for root branching as a result of *rol* gene expression may in turn lead to effects on overall plant growth. Although this has been alluded to by others, quantitative data regarding these effects have rarely been reported. In the study undertaken here, plants of transgenic tobacco lines containing certain *rol* genes were analysed to assess whether expression of the *rol* genes would lead to corresponding effects on the growth of the root system. Root and shoot growth and root:shoot balance were quantified in plants of selected transgenic tobacco lines grown *in vitro*, with a particular focus on transformants containing *rol*C. Growth measurements were also made of tobacco plants grown in soil, including lines in which *rol*C was expressed individually or expressed in conjunction with *rol*B. In addition, an investigation into the effects of restricting *rol* gene expression to the root system of transgenic plants was undertaken, as a comparison with the effects observed using native or constitutive promoters which allow *rol* gene expression in aerial tissues. Root and shoot growth were assessed when expression of *rol*C was limited to the root system, genetically or by grafting of transformed rootstocks with untransformed scions.

3.2. MATERIALS AND METHODS

3.2.1. Plant material

***rol* gene transformants**

Transgenic lines of *N. tabacum* var. SR1 containing *rol* genes were kindly supplied by Dr A. Spena. A transgenic line for each individual *rol* gene or *rol* gene combination, as listed below, was supplied as a representative example of each genotype, demonstrating typical phenotypic characteristics caused by expression of the individual *rol* genes (Spena et al., 1987; Schmülling et al., 1988).

Transformed *N. tabacum* SR1 lines containing the individual *rol*A, *rol*B or *rol*C genes, controlled by their endogenous promoters, are designated Nt*rol*A, Nt*rol*B and Nt*rol*C respectively. Transformants expressing *rol*C or *rol*B under control of the CaMV35S promoter are designated Nt35SC and Nt35SB respectively. Nt35SB+C transformants contain both *rol*B and *rol*C expressed in combination, with *rol*B driven by the CaMV35S promoter and *rol*C by its native promoter. Transgenic control plants were of a SR1 tobacco line transformed with the GUS reporter gene under control of the domain A (-90 to +8 region) of the CaMV35S promoter (Benfey et al., 1989) (Figure 3.1). The domain A promoter element of 35S is here termed A/35S and the control line is designated A/35S-GUS.

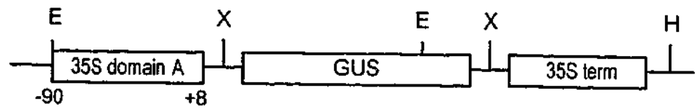


Fig. 3.1. Structure of construct transformed into control line A/35S-GUS, containing domain A of the CaMV35S promoter ligated to the GUS coding sequence, with the CaMV35SS terminator sequence (term) (construct not drawn to scale). Restriction enzyme site abbreviations: E, *Eco*RI; H, *Hind*III; X, *Xba*I

The transformation vectors used to produce the control and *rol* gene lines also carried the neomycin phosphotransferase II (*nptII*) gene, which encodes kanamycin resistance to enable selection of transgenic tissue *in vitro*. Seed of all plants, including controls, were germinated *in vitro* on kanamycin selection medium, and selected kanamycin-resistant (Kan^R) transformants were subsequently used as the maternal parent line in crosses with the SR1 line. In the F_1 generation, Kan^R seedlings were observed to

segregate as expected in 1:1 ratios with kanamycin-sensitive (Kan^S) seedlings. Kan^R *rol* gene transformants and Kan^R A/35S-GUS controls were selected for analyses.

9402 Ri T-DNA transformants

An SR1 transformant root line transformed with T-DNA from *A. rhizogenes* strain LBA 9402 was generated following standard laboratory procedures (Hamill and Lidgett, 1997) and portions of transformed roots were subsequently cultured on shoot regeneration medium MSR1 (see §2.2.1 Media and Solutions). Plants were cultured in soil when about 4-5 cm in height and possessed a typical T-phenotype (Tepfer, 1984), consisting of slightly wrinkled, lanceolate leaves, shorter stature and essentially no pollen, leading to male sterility. Being male sterile these plants were used as the maternal parent in crossings with the SR1 parent line. F_1 seed resulting from this cross were germinated and assessed for LRP formation, with an anticipated segregation into equal proportions of transformed and untransformed plants, assuming a single copy of Ri T-DNA in the T_0 line. For identification of transformed plants in the progeny, phenotypic analysis of mature plants was undertaken and also analysis of a subgroup of plants by Southern hybridisation to ascertain the presence or absence of T-DNA genes. Plants of unknown transformation status were designated as 9402/SR1.

Plants expressing *rol*C under control of the CaMV35S domain A promoter

Tobacco lines putatively transformed with *rol*C under control of A/35S promoter (Fig. 3.2) are designated as A/35S-C lines, with a corresponding number for independent transformant lines, i.e. A/35S-C1 etc[1]. After crossing to SR1 as paternal parents, F_1 seedlings containing transgenes were selected by resistance to kanamycin, as described above for other *rol* gene transformants.

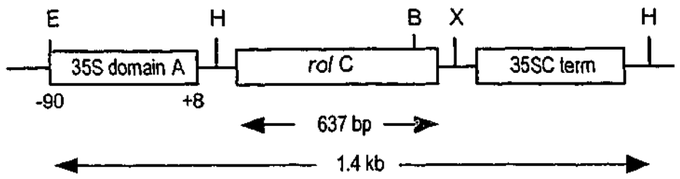


Fig. 3.2. Structure of construct used to generate A/35S-C lines, containing domain A of the 35S promoter ligated to the *rol*C coding sequence, with the 35SS terminator region (term) (construct not drawn to scale). Restriction enzyme site abbreviations: E, *Eco*RI; H, *Hind*III; B, *Bam*HI; X, *Xba*I.

[1] Transformations were initiated by a former research assistant in the laboratory, P. Webb.

3.2.2. Analysis of lateral root primordium formation

A preliminary investigation involving a range of auxins at various concentrations and treatment times was undertaken, prior to commencing this study, to determine the most suitable auxin for further analyses. Roots of 11-day-old seedlings of *N. tabacum* var SR1 were treated with the auxins indole-3-butyric acid (IBA), indole-3-acetic acid (IAA), indole-3-propionic acid (IPA) and naphthalene acetic acid (NAA), at a range of concentrations ranging from 10^{-8}M to 10^{-3}M IBA, increasing in 10-fold increments (see Chapter 2 for preparation of culture media). Of these auxins, IBA was found to be quite consistent in stimulating LRP formation, and induced relatively high levels of LRP, at 10^{-4}M and 10^{-5}M. IBA was consequently chosen as a suitable auxin for use in further experiments. IBA has also been used effectively in other studies of LR induction (Pelosi et al., 1995; Biondi et al., 1997). It has been shown that IBA is more stable than IAA, as it is metabolised more slowly and is less susceptible to oxidation and non-enzymatic degradation (which may also explain its effectiveness as a rooting agent for plant cuttings) (Normanly, 1997).

In response to exposure to IBA (10^{-5}M) over a range of treatment times, the majority of LRP were visible in SR1 tobacco roots treated for 48 hours, with no further increases in LRP measured after 72 hours. This is comparable to the timeframe of LRP formation in auxin-treated seedling roots of eucalyptus (Pelosi et al., 1995), radish (Blakely et al., 1988) and tomato (Taylor and Scheuring, 1994). It was also observed that with the lower levels of IBA, 10^{-9}M to 10^{-7}M, the numbers of LRP formed were invariably similar to the numbers in formed in untreated roots (hormone-free), typically 2-3 LRP/cm. Therefore, in this study, the range of IBA concentrations tested were mostly limited to the higher, effective concentrations, ranging from 10^{-6}M to 10^{-3}M.

Seedlings were grown for 11 days on vertically oriented plates containing solid Murashige and Skoog (M+S) medium with 3% sucrose (w/v) (see §2.2.1) and were then transferred to MS medium containing indole-3-butyric acid (IBA). Seedlings not treated with IBA were placed on hormone-free (HF) M+S medium for the same period of time. After 72 hours treatment with IBA, the seedlings were placed in a 1% (w/v) solution of chromium trioxide (CrO_3) for 16-20 hours and then rinsed several times with distilled water to remove the CrO_3. Treatment with CrO_3 causes the tissue of the roots surrounding the vascular system to become semi-transparent (Pelosi et al., 1995), allowing primordia to be clearly visible as dense dome-like structures and counted when examined under a stereomicroscope placed above a light source (see Fig. 3.7). The length of the roots was also measured, to take account of slight variation between individuals, and measurements were then standardised as the number of LRP formed per centimeter of root tissue.

As the transformant lines could not all be assayed at the same time, it was necessary to maintain the experimental conditions and set-up of each assay as consistently as possible. Each assay included a separate set of controls to provide further standardisation for measurements made at different times. For this reason, some slight variations in control LRP numbers are apparent between assays but patterns of LRP formation are consistent and the controls are always matched to the associated transformant line assayed at the same time.

For LRP analysis of 9402/SR1 roots, the first stage of the procedure was carried out as for other LRP experiments. Each seedling was numbered for subsequent identification. After treatment with IBA, rootswere separated from the seedling shoot and placed in CrO_3 for tissue clearing. LRP formed in roots were counted, as for previous experiments. Treatments were hormone-free (HF) and IBA concentrations 10^{-6}M, 10^{-5}M and 10^{-4}M; sample sizes for 9402/SR1 were around 30 seedlings for each treatment. The untreated shoots were cultured on M+S medium, in correspondingly numbered sections of divided plates. Almost all plantlets rapidly developed new root systems and were transferred to soil when roots had grown to a sufficient size.

3.2.3. Analysis of adventitious root formation on leaf discs

Transformed plant lines utilised were N*tro*lA, N*tro*lB, N*tro*lC, Nt35SC and Nt35SB+C. Control leaves were taken from the plants of the same A/35S-GUS line as used in the LRP experiments. Leaves from 9-week-old greenhouse-grown plants were surface sterilised in a solution of 10% (v/v) Domestos for 20 min with gentle shaking and then rinsed with sterile UHQ water several times to ensure no bleach remained. Discs 15mm in diameter were cut from these leaves with a sterilised metal cutter, ensuring that each disc contained a similar number of leaf veins. Discs were placed in Petri dishes (90 mm diameter x 20 mm) and sealed with parafilm. Sample sizes were 12-15 leaf discs for each treatment, with 4-5 leaf discs per Petri dish.

Leaf discs were cultured on solid M+S medium with 30g l^{-1} sucrose, either hormone-free or containing IBA, at concentrations increasing in 10-fold increments from 10^{-8}M to 10^{-3}M increments (see §2.2.1 for preparation of culture media). The number of adventitious roots (AR) produced were counted after 17 days treatment with IBA. With longer time periods, leaf discs with the greatest responses to IBA treatment had produced so many roots that it was difficult to count them with accuracy. It was found that leaf discs cut from almost fully expanded leaves were more suitable for these treatments than younger leaves, which continued to grow so that leaf pieces increased greatly in size over the 17-day time period, causing difficulties in accurately estimating the number of roots on a leaf area basis.

3.2.4. Analysis of root and shoot growth of *rol* gene transgenic lines grown in soil

Seeds were germinated on M+S medium supplemented with 30 g l^{-1} sucrose and 100 mg ml^{-1} kanamycin. After 12 days, Kan^R transformants were selected and transplanted individually into 50 ml M+S medium in 250 ml volume culture jars. Plants were grown for 4 weeks *in vitro* to allow them to reach an appropriate size then transplanted into soil in 1 litre capacity plastic pots containing an equal ratio of a standard seed-raising soil and perlite, supplemented with 5 g l^{-1} Osmocote slow-release complete plant fertiliser (see §2.2.2). When transplanted, plants were acclimatised under plastic wrap for one week, with a gradual decrease in humidity during this time, by provision of air vents. Plants were arranged randomly in the greenhouse, to ensure minimal bias from any environmental variations that may occur in different areas of the greenhouse.

3.2.5. Dry weight measurements of soil-grown plants

For the growth analyses of plants grown in soil, plants were harvested on the day on which the first flower opened, or at other time points as specified. Flowering times typically ranged between 12 and 14 weeks after sowing, depending on genotype. The shoot and roots of plants were separated, the roots carefully washed clean and all the plant tissue placed in a drying oven at 50°C for 7 days to ensure complete dryness before weighing. Root and shoot dry weights and relative root and shoot proportions were compared in soil-grown tobacco plants. Relative root and shoot growth are measured by the root dry weight (DW) as a percentage of the total plant DW and referred to as the root mass fraction (RMF).

3.2.6. Grafting of transgenic and SR1 tobacco plants

Shoots of SR1 tobacco were grafted onto rootstocks of N*tro*lC, Nt35SC and Nt35SB+C plants. These grafted plants are denoted N*tro*lC/SR1, Nt35SC/SR1 and Nt35SB-C/SR1, respectively. Reciprocal grafts were also made, with transformed shoots grafted onto SR1 roots, designated SR1/N*tro*lC, SR1/Nt35SC and SR1/Nt35SB+C. As a control, SR1 shoots were self-grafted onto SR1 rootstocks and are denoted SR1/SR1.

Grafting was undertaken 6 weeks after germination, which represented the earliest stage at which successful grafting could be reproducibly achieved. A plant of appropriate genotype was taken and the shoot removed, leaving a few centimetres of stem above the roots. A V-shaped wedge was cut out of this stem about 1 cm in depth,

exposing the vascular tissues of the stem. The relevant scion was trimmed to produce a pointed wedge, which fit into the stem section of the corresponding root system, placing the vascular tissue of the two parts together. The stem sections were bound tightly together with waterproof tape to reduce moisture loss from the wounds. The grafted plant was enclosed in a plastic bag for one week to maintain a humid environment then gradually acclimatised over a period of 4 days to the external environment.

Once the graft had successfully taken and the plant was established (Fig. 3.3A), the plant was trimmed to leave the stem and the first three leaves above the graft, with a minimal root system (Fig. 3.3B). After replanting, the three older leaves were removed as soon as the plant had begun to produce new leaves, which could support plant growth (Fig. 3.3C, D). This ensured, as far as possible, that all further growth was essentially a product of the interaction between the new combination of shoot and root systems- all new leaf and stem material above the graft was thus produced utilising the resources of the new root system which formed after grafting, and vice versa. In a similar manner to the previous growth measurements, plants were harvested when the first flower had opened, as a means of standardising the developmental stage at which measurements were made. In measuring root and shoot weights, all new leaves and shoots were harvested, leaving only the "old" stem section. Roots were cut off close to the stem and carefully washed to remove soil and debris before drying at 50°C for 7 days to measure dry weights.

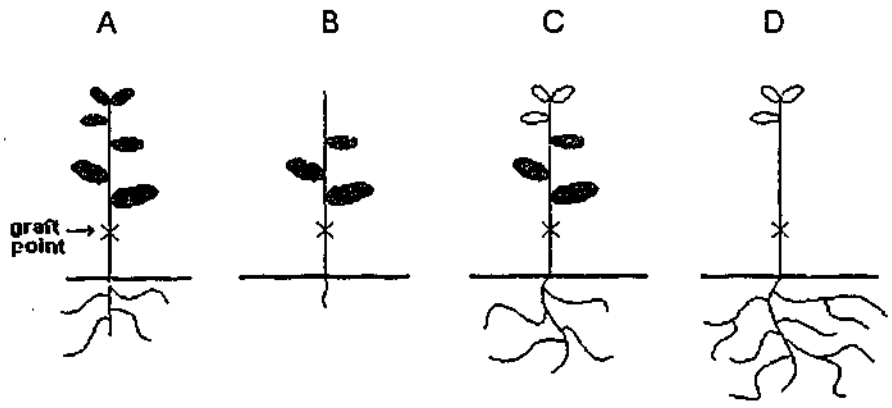


Figure 3.3. Schematic diagram of the experimental procedure for grafted plants. After the grafted plant was established (A), only three leaves and a very minimal root system were retained (B). Once three new leaves (coloured white) had been formed (C), the old leaves (coloured grey) were removed (D).

3.2.7. *In vitro* growth analyses

Seedlings of selected plant lines were initially germinated on medium containing kanamycin to select for the transformed genotypes (see §2.2). After 12 days, individual Kan^R seedlings were transplanted to medium lacking kanamycin in glass jars of 250 ml capacity, with 50 ml volume of M+S medium with or without 3% sucrose (w/v) as a carbon source. 7-9 plants were assessed for each line and medium type, with growth measurements taken after 7.5 weeks. Roots and shoots were separated and washed clean of agar, placed in the drying oven for seven days and then weighed.

3.2.8. Plant greenhouse and *in vitro* culture conditions

In vitro culture and PC2 greenhouse light and temperature conditions as stated in §2.2.2.

3.2.9. Statistical tests

In the analysis of LRP formation, the difference between the mean number of LRP/cm in each *rol* gene transformant line and the corresponding A/35S-GUS control line, for each IBA concentration were tested for statistical significance using a Student's (independent) t-test. For growth analyses, the statistical significance of differences in growth (mean biomass) between the relevant *rol* gene transformant line and A/35S-GUS control line were also tested using an independent t-test.

Significance levels of $p<0.05$ (significant); $p<0.01$ (highly significant) and $p<0.001$ (very highly significant) are specified, and are represented by *, ** and *** respectively.

3.3. ANALYSIS OF EFFECTS OF *rol*C ON GROWTH OF TOBACCO PLANTS *IN VITRO*

3.3.1. INTRODUCTION

The objective of experiments undertaken in this section was to quantify growth alterations caused by *rol*C or *rol*B expression in tobacco plants grown *in vitro*, in terms of root and shoot biomass and determine any changes in the relative balance between these two components.

The supply of resources (mineral and organic) in an *in vitro* culture medium is clearly of key importance to plant growth and the presence or absence of an exogenous carbon (C) source can greatly influence plant growth, including biomass, photosynthesis and uptake of water and nutrients (Kozai et al., 1997). Conventional culture media commonly include sucrose, as a C source. This generally establishes photomixotrophic conditions, with plants gaining C from both photosynthesis and utilisation of the artificial C source. Plants cultured *in vitro* without an artificially supplied carbon source must grow photoautotrophically. For this analysis, plants were grown *in vitro* with either high nutrient and carbon source provision (M+S medium with 3% sucrose) or on high nutrient medium without sucrose (M+S medium), to investigate whether the presence of specific *rol* genes would result in any differential accumulation of biomass between shoots and roots when plants were either photoautotrophic or photomixotrophic.

3.3.2. RESULTS

3.3.2.1. Root and shoot biomass

The plant lines analysed for growth *in vitro* were N*tro*lC, Nt35SC, N*tro*lB, A/35S-C8[2] and A/35S-GUS. After several weeks of growth, the biomasses of all plants provided with sucrose were considerably higher than that of plants grown without sucrose (Table 3.1A,B). Root and shoot dry weights of A/35S-GUS control plants grown on 3% sucrose medium were increased by factors of approximately 2 and 1.5 respectively, compared to growth on medium containing no sucrose. The promotion of growth by the presence of 3% sucrose was even greater for N*tro*lC and Nt35SC plants, particularly with regard to root dry weights, which were 7 to 8-fold larger when grown on medium containing 3% sucrose compared to growth on medium lacking sucrose.

[2] As will be detailed in §3.5, A/35S-C8 is a transformant line with root-specific expression of *rol*C.

When supplied with 3% sucrose, total plant dry weights for all transformant lines, particularly N*tro*lC, were significantly greater than that of A/35S-GUS controls. Notably, root masses of N*tro*lC and 35SC plants were the most substantially increased compared to controls ($p<0.001$). As the shoot masses of N*tro*lC and Nt35SC plants grown with 3% sucrose were considerably higher than that of control plants ($p<0.001$), this indicates that *rol*C phenotypic alterations did not have a limiting effect on *in vitro* shoot growth under these conditions and at this age (7.5 weeks). This is in contrast to plants grown in soil (see §3.5), where shoots of *rol*C transformants were much smaller than controls at the time at which they were measured. Without provision of sucrose *in vitro*, the shoot weights of controls, N*tro*lC and Nt35SC plants were more comparable ($p>0.05$).

Table 3.1. Root and shoot dry weights (DW) and root mass fractions (RMFs) of tobacco plants grown *in vitro*. Plants were grown for 7.5 weeks on (A) M+S medium containing 3% sucrose (w/v) or (B) on M+S medium without sucrose. n = 7-9 for each line and each treatment. Statistical differences were tested between the mean root or shoot DW for each line and the corresponding A/35S-GUS control DW. Statistically significant differences between means indicated as * $p<0.05$; ** $p<0.01$; *** $p<0.001$. RMF is the root DW as a percentage of the total plant DW.

(A) **No sucrose**	root DW (mg)	shoot DW (mg)	total DW (mg)	RMF (%)
A/35S-GUS	1.90 ± 0.55	15.44 ± 2.3	17.34 ± 2.78	11.1 ± 1.3
N*tro*lC	2.89 ± 0.42	22.22 ± 2.67	25.11 ± 28.26	11.7 ± 1.7
Nt35SC	3.25 ± 0.59	18.75 ± 3.62	22.0 ± 3.88	17.2 ± 2.9
A/35S-C8	2.56 ± 0.50	17.44 ± 0.99	20.0 ± 1.35	12.3 ± 1.7
N*tro*lB	1.89 ± 0.45	15.89 ± 1.98	17.78 ± 2.34	11.2 ± 1.6

(B) **3% sucrose**	root DW (mg)	shoot DW (mg)	total DW (mg)	RMF (%)
A/35S-GUS	3.92 ± 0.80	24.50 ± 4.03	28.42 ± 4.68	13.0 ± 1.7
N*tro*lC	23.78 ± 3.73***	93.78 ± 7.26***	117.6 ± 10.85 ***	20.5 ± 1.1 ***
Nt35SC	22.78 ± 3.71***	49.67 ± 8.07*	72.44 ± 10.56 **	31.9 ± 3 ***
A/35S-C8	8.88 ± 1.54*	70.63 ± 8.05***	79.5 ± 9.15 ***	11.1 ± 1.1
N*tro*lB	8.11 ± 1.22*	58.33 ± 15.71	66.44 ± 16.33	14.0 ± 1.7

3.3.2.2. Root mass fractions of *in vitro*-grown tobacco

When grown on medium containing 3% sucrose, the RMFs of Nt35SC and N*trolC* plants were significantly higher than for A/35S-GUS controls ($p<0.001$) (Fig. 3.4; Table 3.1 B). Nt35SC plants clearly produced a much higher relative root mass than all other lines, including *rolC* ($p<0.001$). Plants of the N*trolB* and A/35S-C8 lines were not different from controls with respect to this parameter. Even though actual root masses were larger in these plants, the relative proportions of roots were not increased as the corresponding shoot masses were also increased.

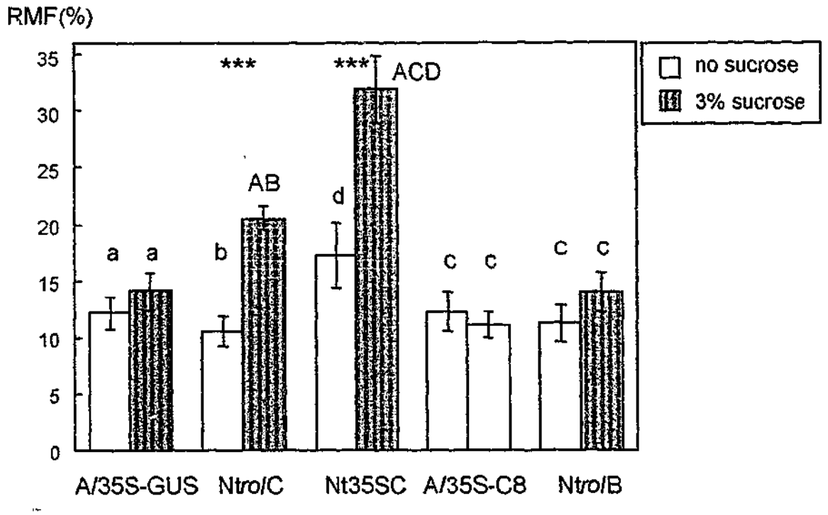


Figure 3.4. Root dry weight as a percentage of the total plant dry weight (RMF) of tobacco plants grown *in vitro* for 7.5 weeks. Plants were grown on M+S medium with 3% (w/v) sucrose (solid bars) or M+S medium without sucrose (striped bars). Vertical lines represent standard error of the mean. Sample sizes were 7-9 for each treatment.

Statistical analysis was carried out between each test sample and the control when grown with 3%; and between samples and the control grown with no sucrose. Within each genotype, the t-test was applied for comparison between plants grown with and without sucrose. Statistically significant differences: *** $p<0.001$.

Bars with lower case letters are not significantly different from each other ($p>0.05$). Bars with upper case letters are significantly different to bars with the corresponding lower case letter. i.e. Nt35SC (3%) significantly different from Nt35SC (no sucrose) and A/35S-GUS (3%); N*trolC* (3%) significantly different from N*trolC* (no sucrose) and A/35S-GUS (3%). In addition, the difference between RMFs of Nt35SC (3%) and N*trolC* (3%)was very highly significant. A/35S-C8 and N*trolB* transformants did not produce significantly different RMFs compared to controls or when grown under the alternative conditions.

Although comparative values clearly imply a tendency to greater root growth in 35SC plants, no significant differences from control plants were evident in any of the lines grown without sucrose. Due to fairly large variations between individual plants, the somewhat higher average proportion of roots in Nt35SC plants (17.2%) compared to A/35S-GUS plants (11.1%) is not statistically significant ($p>0.05$).

In comparing relative root and shoot growth with and without sucrose for each individual transformant line, differences were only evident in N*trolC* and Nt35SC plants. Growth of the root systems were increased to a much larger extent than that of shoots when cultured in medium including 3% sucrose, and RMFs of N*trolC* and Nt35SC plants were correspondingly much higher than for controls ($p<0.001$).

3.3.2.3. Northern blot hybridisation

Relative *rolC* expression levels were quantified in plants grown with and without sucrose by Northern blot hybridisation, comparing the density of *rolC* hybridisation signals relative to that of ubiquitin hybridisation signals (Fig. 3.5).

rolC transcript levels appeared to be higher in leaves of N*trolC* transgenic plants grown with 3% sucrose than comparable plants grown without sucrose supply. This is consistent with the reported activation of the *rolC* promoter in response to sucrose (Yokoyama et al., 1994). *rolC* expression levels were also slightly higher in roots of *rolC* plants grown with 3% sucrose than without sucrose, although this differencewas not as great as in leaves.

In Nt35SC plants, *rolC* transcript levels were slightly lower in leaves grown with sucrose than without sucrose. From this Northern blot, it is not possible to compare *rolC* expression levels in Nt35SC roots grown with and without sucrose, as hybridisation signals were very faint. This is most likely due to the low RNA levels present, apparent from the amount of RNA on the agarose gel and also when the blot was probed with ubiquitin DNA (Figures 3.5B,C). *rolC* transcripts are clearly evident in other Northern blots of the Nt35SC transformant line (see §3.4.1 and 3.5.2, Figures 3.6, 3.23 and 3.24).

No real differences were apparent between Nt35SC and N*trolC* plants in terms of *rolC* expression levels, similar again to the results of other Northern blot analyses with 11-day-old seedlings and with soil-grown plants (Figures 3.7, 3.23 and 3.24). This confirms that this Nt35SC tobacco line does not express *rolC* at a substantially higher level than the N*trolC* transformant line utilised in these experiments, which contain *rolC* under control of the native promoter.

No *rol*C transcripts were detected in A/35S-C8 leaves and roots. However, as low levels of *rol*C transcript were detected in roots by a separate Northern blot (see Fig. 3.23), the lack of hybridisation on this blot is most likely to be due to the low loading levels of A/35S-C8 root RNA on the gel (Fig. 3.5C). Levels of RNA loaded on the gel for leaf tissue of A/35S-C8 appeared to be sufficient to detect *rol*C transcripts if present.

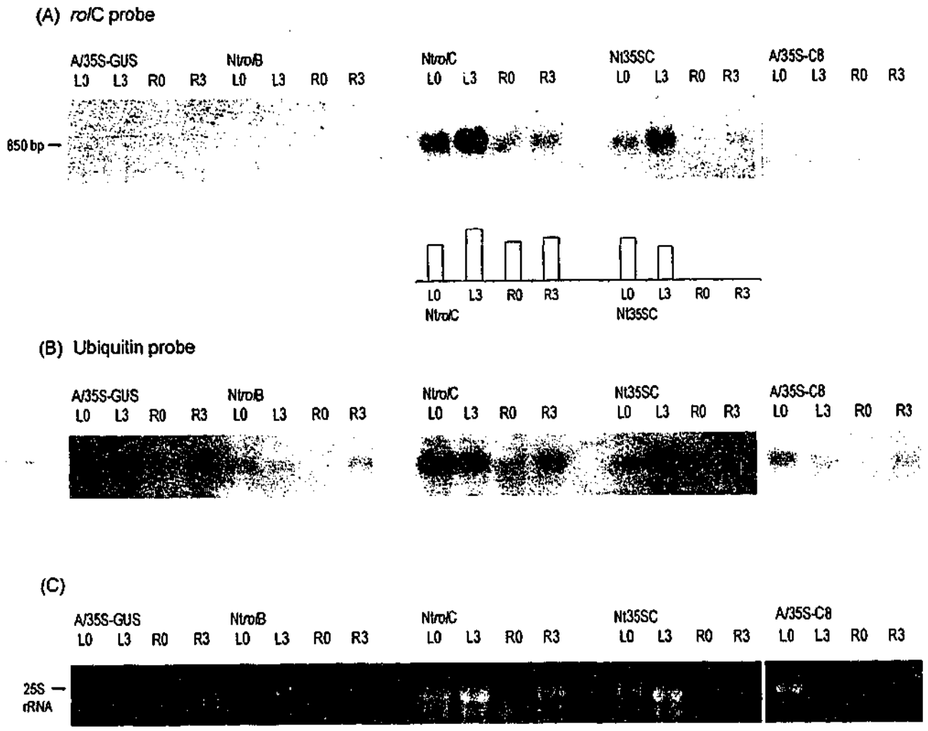


Figure 3.5. Northern blot analysis of total RNA from tobacco transgenic lines, grown *in vitro* with 3% sucrose or without sucrose supplement. The blot was probed with the *rolC* coding sequence from *A. rhizogenes* A4 (A), then stripped and rehybridised with the ubiquitin probe (B). A lack of hybridisation to the ubiquitin probe in some samples most likely indicates a low RNA loading level; this is also evident from loading levels on the agarose gel electrophoresis (C) prior to blotting. L0 = leaf, no sucrose; L3 = leaf, 3% sucrose; R0 = root, no sucrose; R3 = root, 3% sucrose.

3.3.3. DISCUSSION

3.3.3.1. Growth under photoautotrophic and photomixotrophic conditions

From the results of this study, it was apparent that although tobacco plants were able to grow photoautotrophically on sucrose-free medium, growth was clearly reduced in comparison to plants grown with a sucrose supply. Evidently, plants were able to fix carbon in the sucrose-free conditions but the process of photosynthesis may have been compromised, and growth subsequently limited by the decreased levels of photosynthate available. Conventional *in vitro* conditions used do not generally favour photosynthesis (Kozai et al., 1997; Pospísilová et al., 1997); in fact, net photosynthetic rate is often observed to be increased when plants are transferred from *in vitro* to *ex vitro* conditions (Van Huylenbroeck and Debergh, 1996; Pospísilová et al., 1998; Fila et al., 1998). This has been suggested to be due to lower concentrations of photosynthetic enzymes, such as Rubisco, in *in vitro* grown plants (Desjardins et al., 1995).

In contrast, the provision of high sucrose levels to tobacco plants as an artificial energy source appears to have more than compensated for reduced photosynthetic activity, with substantially larger root and shoot biomasses produced by all plants, compared to growth on medium lacking sucrose. These observations are consistent with previous experimental evidence of greater biomass produced by plants grown in medium containing sucrose. For example, fresh and dry weights of melon shoots were greater when grown in medium containing 3% sucrose than with 1% or no sucrose (Adelberg et al., 1999). Tichá et al. (1998) observed that tobacco plantlets grown with 3% sucrose had higher carbohydrate content, dry matter accumulation and total leaf area than plants grown photoautotrophically.

3.3.3.2. Effects of *rol*C on relative biomass allocation to roots

With the provision of a readily accessible carbon supply, optimal partitioning models predict that the need for allocation of resources to shoot growth may accordingly be diminished and a correspondingly increased level of resources may be made available for partitioning to roots. Differences in partitioning in response to sucrose provision have been noted in other reports with various species. For example, grapevine plantlets demonstrated greater partitioning to roots and lower partitioning to leaves when provided with 2.5% and 3.75% sucrose, compared to growth with low levels of sucrose (0.625%) (Fila et al., 1998).

It has been observed in studies with numerous plant species that high sucrose levels provided in the growth medium can cause reductions in photosynthesis and reduced investment into components of photosynthesis, such as chlorophyll (Langford and Wainwright, 1987; Van Huylenbroeck and Debergh, 1996; de la Vina et al., 1999). This lower net photosynthesis in response to higher sucrose levels may be due to feedback inhibition of Rubisco (Desjardins et al., 1995; de la Vina et al., 1999). Photosynthesis genes and relevant enzymes are generally downregulated by abundant supplies of sugars/carbohydrates (Koch, 1996).

However, although large differences in absolute biomass were evident between tobacco plants grown photoautotrophically or photomixotrophically in the present study, partitioning between root and shoot was altered to varying degrees in the different *rol* gene and control plant lines. For A/35S-GUS control plants, partitioning of resources to root and shoot systems were comparable when grown both with and without sucrose, and the RMFs remained similar in both environments. In medium lacking sucrose, the RMFs of plants containing the *rol*C or *rol*B genes were all comparable although the data suggested a tendency to an elevated RMF in Nt35SC plants. Growth in medium containing 3% sucrose produced a significantly higher relative allocation of biomass to the root systems in N*trol*C and Nt35SC plants than in photoautotrophically grown plants.

As biomass partitioning in A/35S-GUS controls was apparently unaffected by the presence or absence of sucrose in the medium, the effectiveness of photomixotrophic conditions on increasing partitioning to roots in N*trol*C and Nt35SC plants relate to the expression of *rol*C. While there may be a somewhat reduced investment of biomass in photosynthetic tissue (shoots) for plants grown *in vitro* on medium containing 3% sucrose, this did not seem to result in significantly increased RMF in control plants. However, the increased capacity for root growth induced by expression of *rol*C appears to have been effective in augmenting root system growth, resulting in a substantial enhancement of root biomass relative to shoot biomass in N*trol*C and Nt35SC plants. This stronger root growth also resulted in the production of correspondingly larger shoots, particularly marked in N*trol*C plants.

Furthermore, the *rol*C promoter has been reported to be induced by sucrose in tobacco (Yokoyama et al., 1994) and also in hybrid aspen (Nilsson et al., 1996a). Stimulation of expression of *rol*C in transformed plants by the presence of sucrose in the growth medium may be a factor in the increased root growth compared to *rol*C plants grown without sucrose provision. However, any effect resulting from this is probably quite moderate, as Nt35SC plants produced much greater relative root growth on both media and a larger increase in growth with 3% sucrose compared to growth without sucrose. The CaMV35S promoter controlling expression of *rol*C in these plants is not

similarly activated by sucrose, generally conferring strong, constitutive expression (Holtorf et al., 1995; Nilsson et al., 1996a).

3.3.3.3. *rol*B and A/35S-C plants

An increased propensity for LR initiation in response to auxin has been demonstrated in both transgenic N*trol*B and A/35S-C8 tobacco roots (Figures 3.10 and 3.27). As both N*trol*B and A/35S-C8 plants produced more root mass than A/35S-GUS controls when grown with 3% sucrose *in vitro*, this suggests that an enhanced capacity for LRP formation may have affected overall root system growth, in these conditions.

The augmented root growth in *rol*B and A/35S-C8 plants grown *in vitro* were correlated with increased shoot growth, so that the relative root and shoot proportions of the whole plant were maintained. This suggests that targeted expression of *rol*C to roots in this A/35S-C8 line may have the potential to produce larger plants, without detrimental effects on shoot growth. Shoot growth was also promoted in *rol*C and 35SC plants, however increases in root growth were much greater and therefore altered the overall balance between root and shoot. The contrasts between root growth effects in A/35S-C8 plants and *rol*C or 35SC plants may be a reflection of the relatively low levels of *rol*C expression in roots of the A/35S-C8 transformants.

3.4. LATERAL AND ADVENTITIOUS ROOT INDUCTION IN TRANSGENIC *N. TABACUM* EXPRESSING *rol* GENES

It is apparent that *rol*C gene expression is able to stimulate the growth of root systems of transgenic tobacco plants grown *in vitro*, when expressed under control of either the endogenous promoter or the 35SC promoter. This is consistent with observations that roots transgenic for *rol*C, but not roots transgenic for other individual *rol* genes, could grow well axenically *in vitro* in nutrient medium containing sucrose but lacking phytohormones (Schmülling et al., 1988). To further investigate the influence of *rol* genes, particularly *rol*C, on root growth, an examination of the effects of expression of *rol* genes on the initiation of LRP and AR in response to auxin was undertaken.

3.4.1. RESULTS

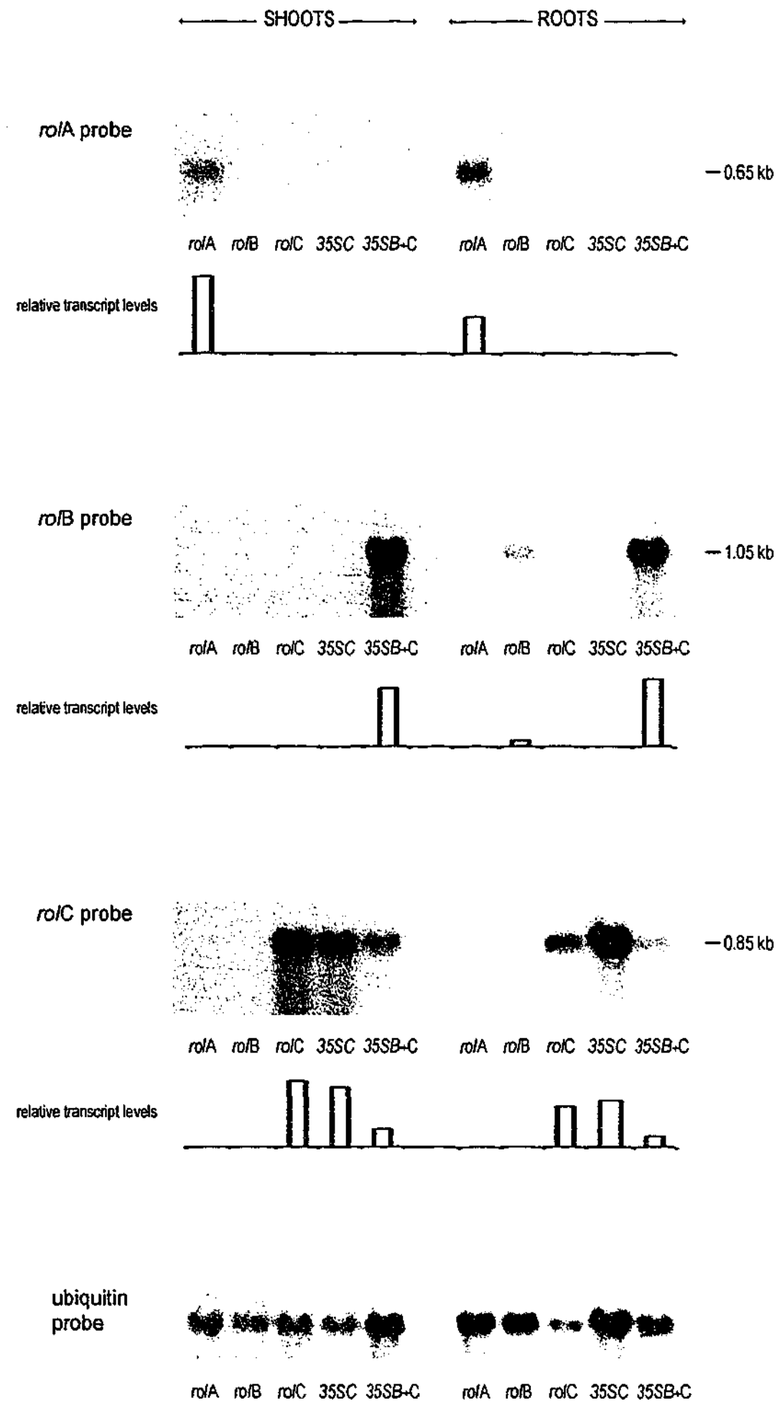
3.4.1.1. Confirmation of expression of *rol* genes in tobacco seedlings

Northern blot analysis was carried out to verify that the *rol* genes were expressed in seedling roots during the period in which LRP formation was occurring. The results (Fig. 3.6) confirm that in each transgenic line, the corresponding *rol* gene is expressed at the 48-hour time point, by which time the majority of LRP have formed in tobacco SR1 seedlings (see §3.2.2). As anticipated, no expression of *rol*A, *rol*B or *rol*C was evident in auxin-treated roots of the control line, A/35S-GUS.

Figure 3.6. *(Facing page)* Northern blot hybridisation of *rol* gene expression in roots and cotyledons of 11-day-old transgenic tobacco seedlings. Total RNA was extracted from seedlings grown for 11 days and then treated with 10^{-4}M IBA for 48 hours, in an identical procedure to that used for setting up roots for LRP measurement. The Northern blot was probed with each of the *rol*A, *rol*B and *rol*C and ubiquitin genes, separately, and was stripped between each probing. (NB for picture clarity the names of transgenic lines are abbreviated on the figure labels to exclude the Nt prefix).

Transcript levels of each *rol* gene were quantified relative to the ubiquitin transcript levels – these relative levels are represented by bar graphs under each blot figure. Expression of *rol*A and *rol*C in the relevant transgenic lines were somewhat higher in cotyledons than roots. Unexpectedly, *rol*B expression in Nt*rol*B cotyledons was not detected in this Northern, even though plants of this line displayed typical *rol*B-induced alterations to shoot phenotype. It is possible that transcript levels were too low to be detected in this particular case.

As may be expected, expression of *rol*B under control of the constitutive CaMV35S promoter in Nt35SB+C seedlings was substantially higher than that of *rol*B under the native promoter. It is interesting to note, however, that *rol*C transcript levels in both roots and cotyledons of Nt*rol*C and Nt35SC seedlings were fairly equivalent, although it was also expected that expression of *rol*C under control of the CaMV35S promoter would result in higher expression. Also, *rol*C transcript levels in Nt35SB+C, in which *rol*C is under regulatory control of the native *rol*C promoter were much lower than in the Nt*rol*C seedlings.



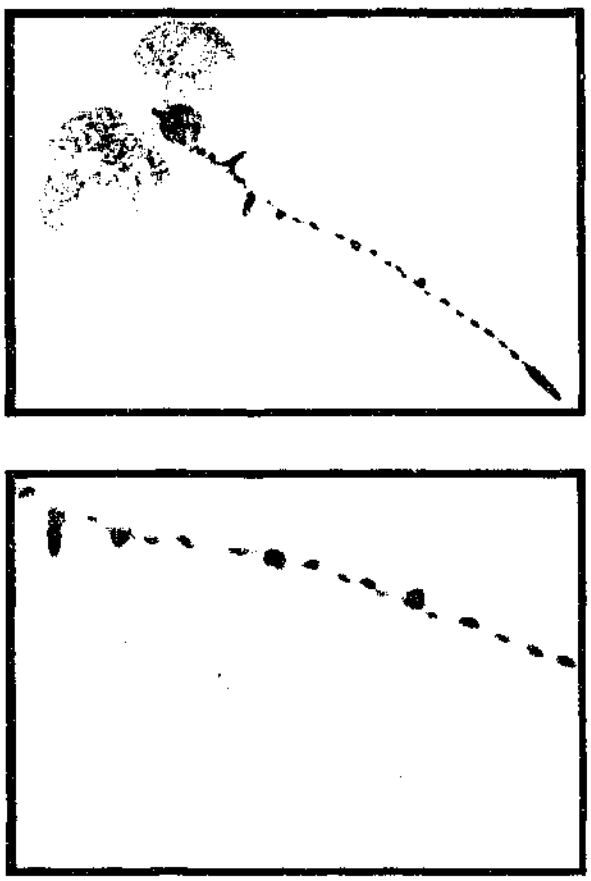


Figure 3.8. LRP formation in roots of 11-day-old SR1 tobacco seedlings treated with 10^{-6}M IBA, followed by treatment with CrO_3 to visualise primordia. Whole seedling (top panel) and closer view of auxin-treated and untreated roots (lower panel). In roots treated with 10^{-4}M or 10^{-5}M IBA, many LRP at various stages of development are typically observed to be crowded along the roots. Consistent with observations of others (Laskowski et al., 1995; Pelosi et al., 1996), LRP can be seen to originate from the edge of the vascular stele, from the pericycle layer.

3.4.1.2. LRP initiation in A/35S-GUS control seedlings

Numbers of LRP formed in roots of one set of A/35S-GUS control seedlings, in response to various concentrations of IBA, are represented in Figure 3.7. This is a representative set of A/35S-GUS roots, as separate groups were used for assays carried out at different times. Variations in LRP numbers between control sets were minor and the pattern of LRP formation remained uniform. When roots were placed on hormone-free (HF) medium, an average of 3.7 LRP/cm were formed and equivalent numbers were initiated at the lower concentrations of 10^{-9}M, 10^{-8}M and 10^{-7}M IBA. Markedly increased numbers of LRP formed in response to higher concentrations of IBA - 10^{-6}M IBA induced a two-fold increase in the number of LRP and 10^{-5}M IBA induced four times as many LRP compared to HF treatments. Within the range of IBA concentrations tested, treatment with 10^{-4}M IBA stimulated the maximum number of LRP in the control roots (24.2 LRP/cm), which appear densely packed in the vascular tissue of the root (Fig. 3.8). At the highest concentration of IBA tested, 10^{-3}M, the incidence of LRP was very low and equivalent to that of untreated roots.

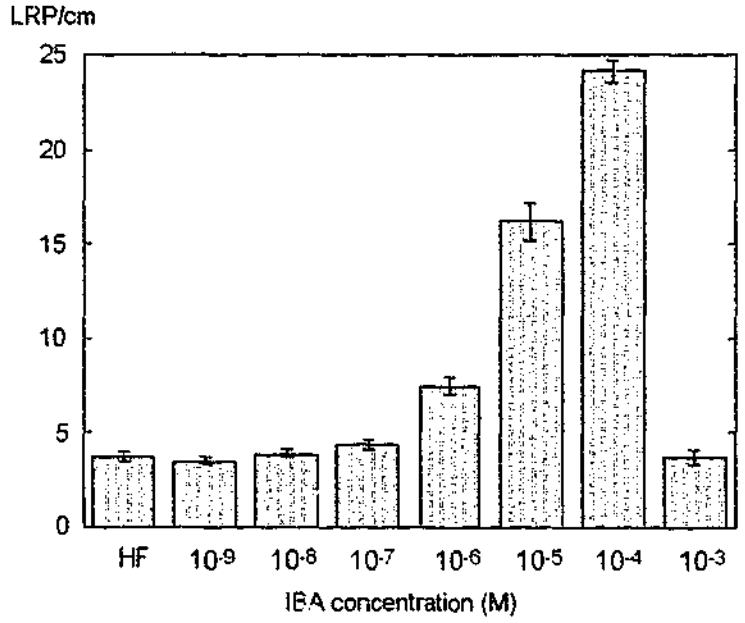


Figure 3.7. Number of LRP/cm in roots of 11-day-old A/35S-GUS seedlings, treated with IBA at a range of concentrations or without hormone treatment (HF). Bars represent mean number of LRP/cm of 8-12 seedling roots ± s.e.m.

The A/35S-GUS transformants were utilised as controls, as these plants contain a kanamycin resistance marker and could therefore be exposed to the same kanamycin selection regime as the *roi* gene transformed lines. This ensured that any disparities potentially arising as a result of kanamycin treatment or initial transformation procedures would be minimised. LRP formation in the kanamycin-selected A/35S-GUS transgenic

roots was compared with untransformed SR1 tobacco seedlings, which were not exposed to kanamycin treatment. No significant differences in the pattern and numbers of LRP were apparent between the two lines.

3.4.1.3. LRP formation in *rol* gene-transformed lines

In all *rol* gene transformed lines, the numbers of LRP formed at the lower concentrations of IBA, 10^{-8}M and 10^{-7}M, were not notably different from the numbers formed in A/35S-GUS seedling roots. Similarly, LRP initiation in response to 10^{-3}M IBA was found to be as low as in untreated roots in all transformants. Any variations in LRP formation from the levels observed in A/35S-GUS controls occurred in response to 10^{-6}M, 10^{-5}M or 10^{-4}M IBA. Data from these treatments are shown in Figures 3.10 - 3.13.

LRP formation in roots of N*trol*A seedlings in response to IBA followed a very similar pattern to that of the A/35S-GUS controls, with no differences observed between the numbers of LRP formed with any of the IBA treatments (Fig. 3.9). In N*trol*B roots, up to 1.5-fold more LRP were induced by IBA at 10^{-6}M and also 10^{-5}M, compared to similarly treated control roots (Fig. 3.10A). The other IBA concentrations tested did not induce significant alterations in LRP initiation. Nt35SB seedlings showed the same pattern of LRP responses as N*trol*B, with significant differences from normal roots produced in response to 10^{-5}M and 10^{-6}M IBA but not other concentrations (Fig. 3.10B).

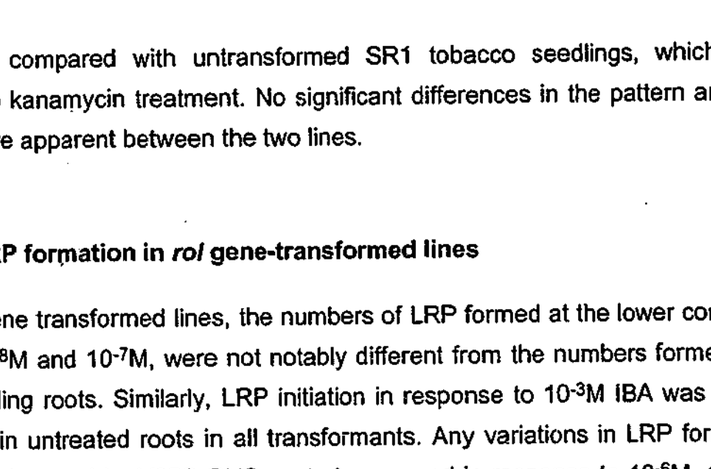
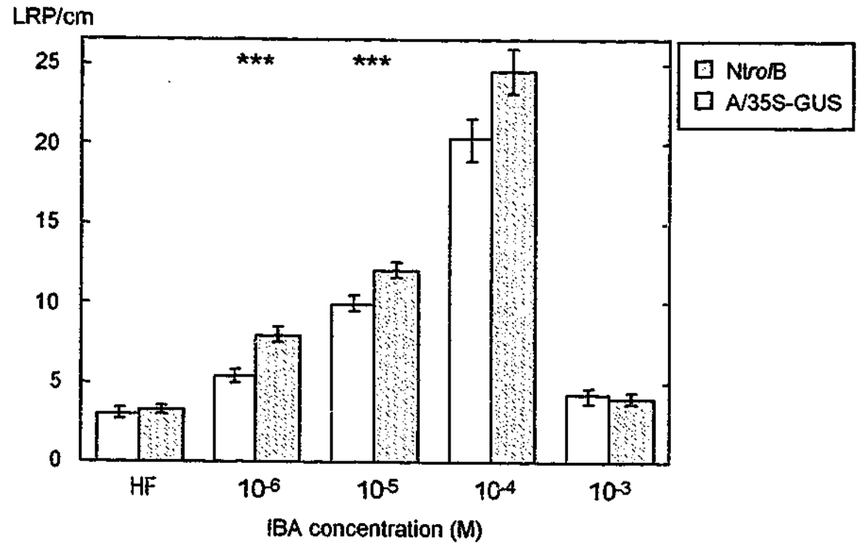


Figure 3.9. Number of LRP/cm in roots of 11-day-old N*trol*A and A/35S-GUS seedlings, treated with IBA at a range of concentrations or without hormone treatment (HF). Bars represent mean number of LRP/cm of 8-12 seedling roots ± s.e.m. No significant differences in LRP/cm were observed between N*trol*A and A/35S-GUS roots, in response to any of the IBA concentrations ($p>0.05$).

(A)



(B)

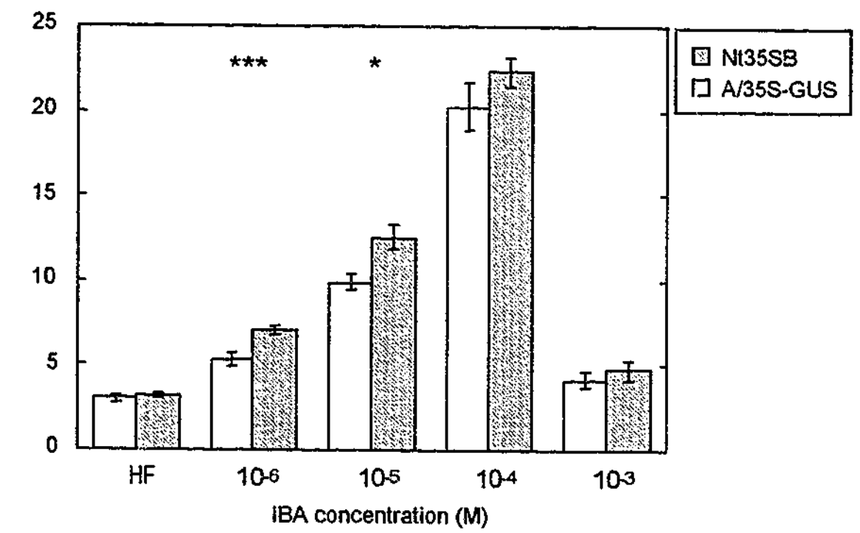
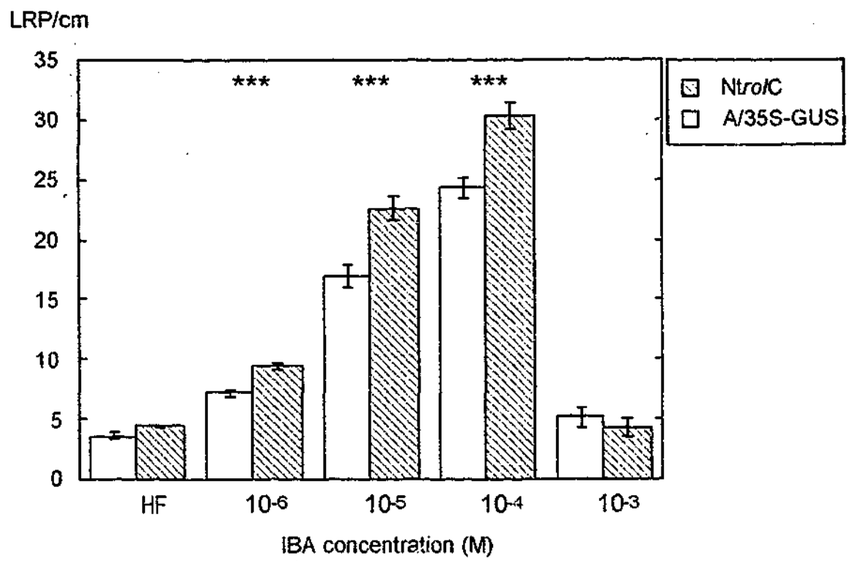


Figure 3.10. Number of LRP/cm in roots of 11-day-old seedlings treated with IBA at a range of concentrations or without hormone treatment (HF). (A) N*trol*B and A/35S-GUS; (B) Nt35SB and A/35S-GUS. Bars represent mean number of LRP/cm of 8-12 seedling roots ± s.e.m. Statistically significant differences: * $p<0.05$; *** $p<0.001$.

(A)



(B)

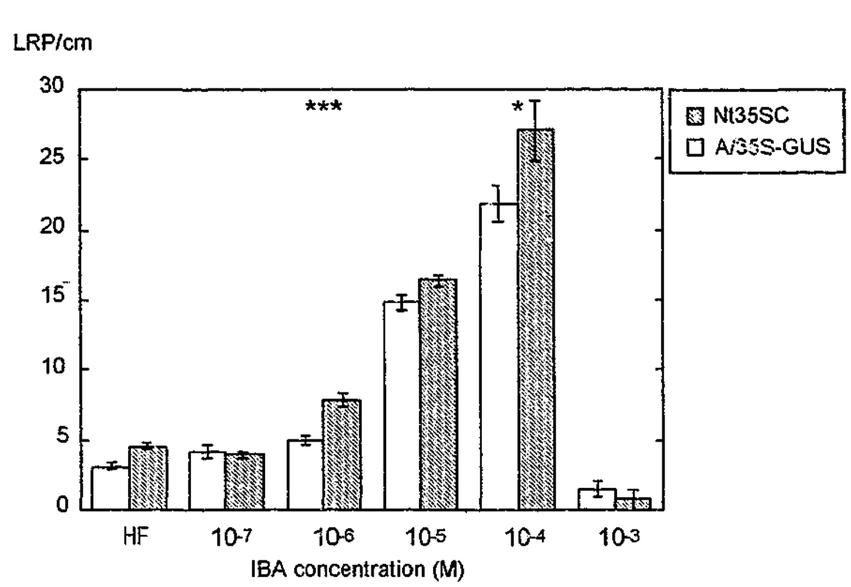


Figure 3.11. Number of LRP/cm in roots of 11-day-old seedlings treated with IBA at a range of concentrations or without hormone treatment (HF). (A) N*trolC* and A/35S-GUS; (B) Nt35SC and A/35S-GUS. Bars represent mean number of LRP/cm of 8-12 seedling roots ± s.e.m. Statistically significant differences: * p <0.05; ** p <0.01; *** p <0.001.

In N*trolC* transformants, highly significant increases in LRP formation compared to control seedlings were observed in roots treated with 10^{-6}M, 10^{-5}M and 10^{-4}M IBA ($p<0.001$). The highest numbers of LRP were elicited by 10^{-4}M IBA, with 25% more LRP induced in N*trolC* roots than controls ($p<0.001$) (Fig. 3.11A).

In Nt35SC roots, significantly more LRP were formed than in controls in response to 10^{-6}M IBA ($p<0.001$) and 10^{-4}M IBA ($p<0.05$); no statistically significant differences were apparent in samples with 10^{-5}M IBA (Fig. 3.11B).

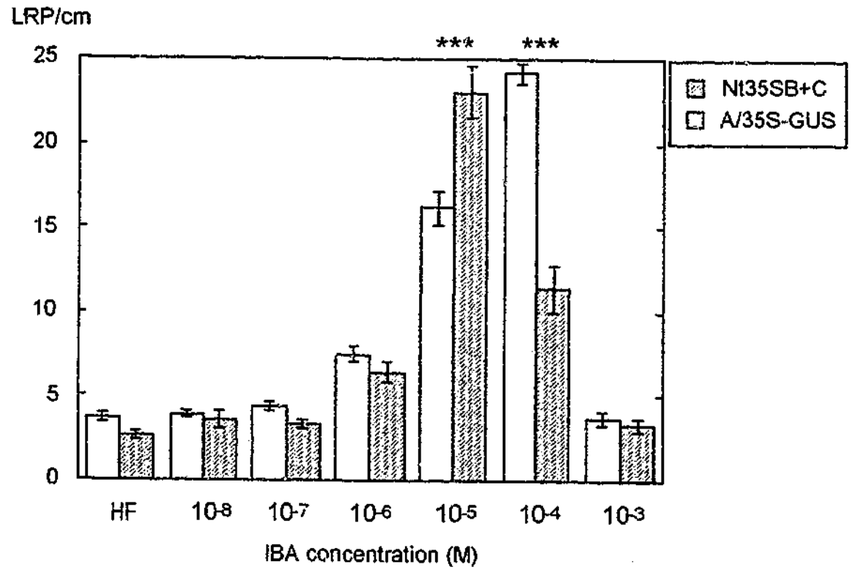


Figure 3.12. Number of LRP/cm formed in roots of 11-day-old 35SB+C and A/35S-GUS seedlings, treated with IBA at a range of concentrations or without hormone treatment (HF). Bars represent mean number of LRP/cm of 8-12 seedling roots ± s.e.m. Statistically significant differences: *** p <0.001.

In Nt35SB+C seedling roots, the pattern of LRP formation in response to IBA differed from that of controls and other transformants (Fig. 3.12). The maximal LRP responses to auxin occurred at 10^{-5}M IBA, rather than 10^{-4}M IBA, as in the control and other*rol* gene lines. In fact, a significantly lower level of LRP was induced in Nt35SB+C roots by 10^{-4}M IBA ($p<0.001$), compared to controls. This suggests a suppression of LRP formation,

similar to that normally caused by a higher auxin concentration (10^{-3}M), although to a lesser extent.

These results suggest that the combined expression of the *rol*B and *rol*C genes may have a synergistic effect on the sensitivity of these transformants to auxin, in terms of LRP induction, augmenting the effects caused by each gene when expressed separately. Interestingly, however, treatment with 10^{-6}M IBA did not induce additional LRP compared to A/35S-GUS, unlike roots expressing *rol*C and *rol*B individually.

3.4.1.4. LRP formation in response to IBA in 9402/SR1 roots

As the Ri T-DNA introduced into the transformed plants was from a wild-type *Agrobacterium* Ri plasmid, a convenient selection marker such as an antibiotic resistance gene was not available for selection of transformants at germination or seedling stage. For identification of T-DNA transformed plants, phenotypic descriptions were made of all mature plants recovered from excised seedling shoots, as it was considered that alterations in phenotype would be likely to provide a reliable indication of the presence of T-DNA. As the plants matured, many developed distinct phenotypic effects, commonly including wrinkled leaves and very shortened internode lengths. Some plants showed intermediate effects. These phenotypes were consistent with the T' and T phenotypes, respectively, of tobacco plants transformed with Ri T-DNA as described by Tepfer (1984).

A number of plants were chosen to test more conclusively for the presence of the introduced T-DNA. Plants were selected that showed phenotypic alterations as well as plants of normal wild-type appearance. A Southern blot of genomic DNA from these plants was probed with the *rol*C coding sequence and also reprobed separately with the *rol*A sequence, to detect any major rearrangements or deletion in this region of T-DNA.

Results of these hybridisations (Fig. 3.13) indicate that some plants appearing phenotypically altered were not necessarily transformed with either the *rol*C or the *rol*A genes (e.g. Fig. 3.13, plants 41, 62). Thus, the phenotype of the plants used in this experiment do not appear to have been a reliable indicator of whether the plants were T-DNA transformed (9402) or untransformed (SR1) and other approaches would be required to determine this conclusively. However, even without precise identification of genotype, some inferences can be made from an assessment of the overall trends of the combined 9402/SR1 LRP data compared to the A/35S-GUS data.

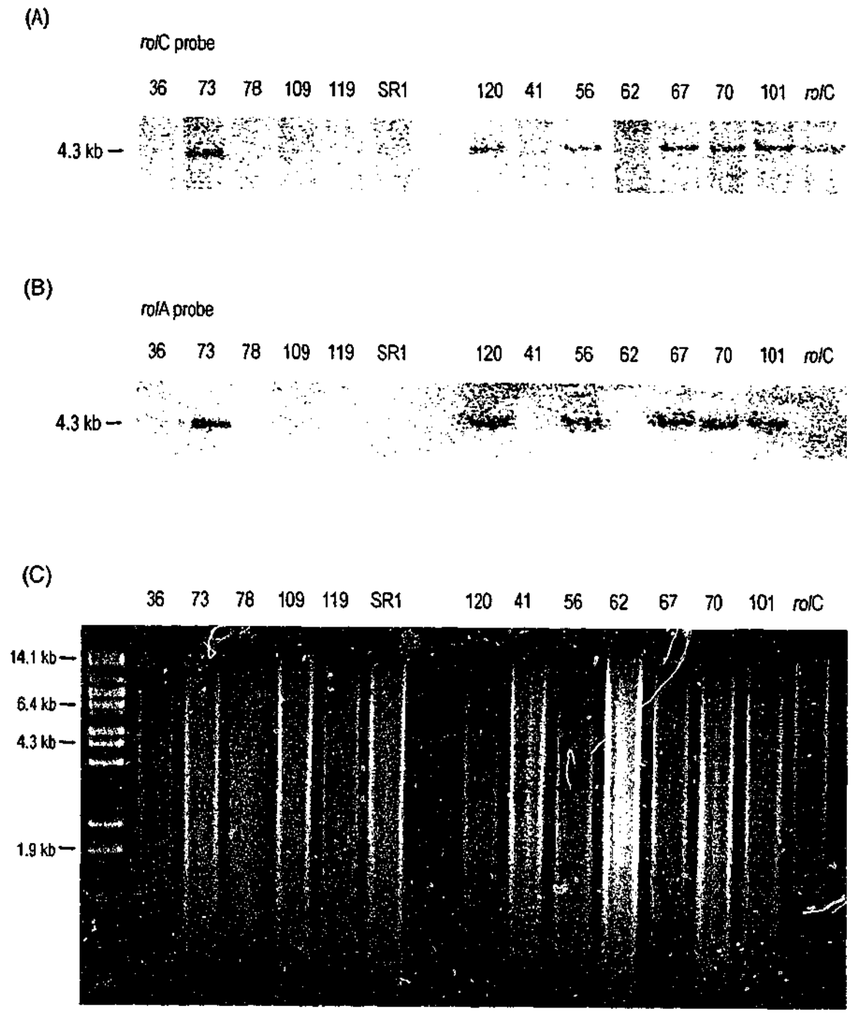


Figure 3.13. Southern blot analysis of genomic DNA of selected putative 9402 Ri-transformed tobacco plants. Numbers above lanes correspond to plant identification numbers. Blot was probed with *rol*C coding sequence (A); blot was then stripped and rehybridised with *rol*A coding sequence (B). Controls were *rol*C transgenic plants (last lane) and SR1 plants (lane 6). (C) Agarose gel of DNA prior to blotting, digested with *Hind*III and *Eco*RI; approximately 10 µg DNA loaded per lane. Left lane, DNA size markers.

A comparison of mean number of LRP/cm (Fig. 3.14) indicates that 10^{-6}M IBA treatment induced significantly more LRP in 9402/SR1 roots than in A/35S-GUS roots ($p<0.001$). Differences were also observed at 10^{-5}M IBA (slightly more LRP) and 10^{-4}M (slightly less LRP), although these were not statistically significant ($p>0.05$). There was no obvious clustering of the 9402/SR1 data into two distinct groups, which, if present, may have reflected differences in number of LRP in Ri-transformed roots compared to untransformed roots. However, highly disparate LRP levels would not necessarily be expected, given that large differences have not been observed between any of the *rol*-transformed lines and untransformed controls. Data may instead be expected to fall into two overlapping groups, in which case it is less likely that the mean of combined data from 9402 and SR1 roots will be significantly different from controls.

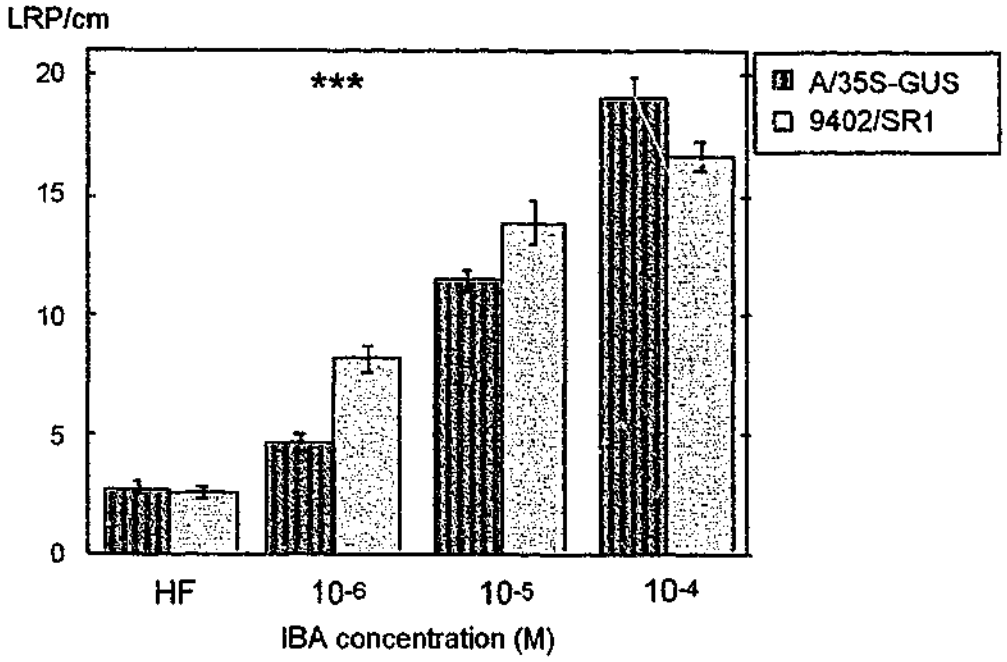


Figure 3.14. Number of LRP/cm in roots of 11-day-old 9402/SR1 and A/35S-GUS roots, treated with IBA at a range of concentrations or without hormone treatment (HF). Bars represent mean number of LRP/cm ± s.e.m. n = 29-31 for 9402/SR1; n = 8-10 for A/35S-GUS. Statistically significant differences: *** $p < 0.001$.

The large variation in LRP numbers produced in 9402/SR1 roots was also notable (Fig. 3.15). The boxplot represents the range of data and medians for each of the IBA treatments for the collective 9402/SR1 and A/35S-GUS data. The LRP data for 9402/SR1 roots are clearly spread over a much larger range than the A/35S-GUS controls and may indicate that two, or more, separate and somewhat different groups of data have been combined. This suggestion supports the hypothesis that the presence of

9402 T-DNA has some effect on LRP initiation in response to auxin, thereby producing LRP at different levels to untransformed SR1 roots.

Overall, the results of these measurements are consistent with the observations noted above of increased sensitivity to auxin induced by the expression of *rol*B and *rol*C individually or together. As mentioned above, any differences between two possible sets of 9402/SR1 data do not seem to be large and it appears likely that the effects on LRP formation caused by presence of T-DNA are not greater than that caused by the *rol*C and *rol*B genes.

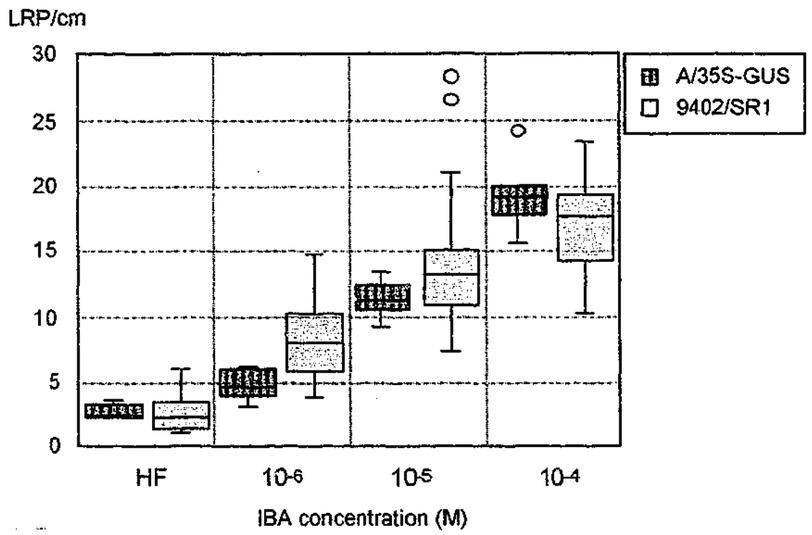


Figure 3.15. Boxplot of combined 9402/SR1 LRP data compared to A/35S-GUS data, in response to different IBA concentrations or without IBA treatment (HF). Vertical lines indicate the full range of data (LRP/cm), boxes represent the middle 50% of the data and horizontal lines in boxes indicate the median. Circles represent outliers.

3.4.1.5. Effects of *rol* gene expression on adventitious root formation from tobacco leaves

Leaf discs of tobacco containing various *rol* genes were tested for their capacity to produce adventitious roots in response to treatment with different auxin levels (Table 3.2). A/35S-GUS leaf discs placed on hormone-free medium or medium containing

10^{-8}M IBA or 10^{-7}M IBA did not produce any roots after 17 days. With IBA treatment at 10^{-6}M, a low frequency of root formation was evident, with an average of about one root per leaf disc. Much greater stimulation of root formation occurred with treatments of 10^{-5}M and 10^{-4}M IBA, with the highest numbers of roots formed in response to 10^{-4}M IBA (an average of 13.3 roots per leaf segment) (Fig. 3.16).

Table 3.2. Mean number of adventitious roots produced per leaf disc of *N. tabacum rol* gene transformants. Leaf discs were cultured for 17 days on M+S medium, either without auxin or containing IBA at concentrations as specified.

IBA concentration	A/35S-GUS control	Nt*rol*A	Nt*rol*B	Nt*rol*C	Nt35SC	Nt35SB+C
Hormone Free	0	1.5	1	0	0	0
10^{-8}M	0	0.4	1.5	0	0	0.4
10^{-7}M	0	0.25	4.2	0.25	0	1.0
10^{-6}M	1.1	2.6	30	2.7	1.1	2.6
10^{-5}M	5.9	20	>80	16	7.9	15
10^{-4}M	13.3	23.7	40	31.8	20	20

A conspicuous aspect of Nt*rol*A transformants was the spontaneous production of small numbers of AR on hormone-free medium and also in response to very low IBA treatments (10^{-8}M and 10^{-7}M); this did not occur in controls or Nt*rol*C and Nt35SC leaves. With other IBA concentrations, the number of roots produced on Nt*rol*A leaves were 2- to 3-fold greater than controls. The largest difference between Nt*rol*A leaves and controls occurred on medium containing 10^{-5}M IBA, with a three-fold higher number of roots produced on average from Nt*rol*A leaf discs.

Nt*rol*B leaf discs also demonstrated a capacity for spontaneous AR formation, producing an average of one or two AR per leaf disc when placed on hormone-free medium or 10^{-8}M IBA. On medium containing higher levels of auxin, the AR formation response in Nt*rol*B leaves was by far the most pronounced of all the *rol* gene transformants. With 10^{-6}M IBA, an almost 30-fold increase in AR formation was

observed compared to controls. In response to 10^{-5}M IBA, a huge proliferation of new roots were formed (too numerous to count), although many of the roots were very short and stunted with a generally unhealthy appearance and colour (a typical appearance of roots inhibited by auxin) (Fig. 3.16). Also notable was the fact that 10^{-4}M IBA was clearly not the optimal concentration for AR stimulation in Nt*rol*B leaves, as less AR were produced with 10^{-4}M IBA than with 10^{-5}M IBA. This is in contrast to other *rol* gene transformants and the controls, for which the greater number of AR were invariably stimulated by 10^{-4}M IBA. These results are therefore consistent with an increased sensitivity of Nt*rol*B leaf tissues to the effects of auxin on induction of AR.

In Nt*rol*C and Nt35SC leaves, very few or no AR were produced in response to the lower IBA concentrations (Table 3.2). The maximum numbers of AR in these leaf discs were induced by 10^{-4}M IBA treatment, with Nt*rol*C and Nt35SC leaves displaying heightened AR responses at this auxin level, compared to controls. Overall, auxin-induced AR formation was more pronounced in Nt*rol*C leaves than Nt35SC leaves, producing two- to three-fold more roots than controls at the higher IBA concentrations.

A synergistic activity of *rol*B and *rol*C, apparent in terms of LRP initiation in response to auxin, was not evident with respect to induction of AR. The presence of *rol*B, in addition to *rol*C, was observed to result in the formation of some roots in Nt35SB+C leaves in response to 10^{-8}M IBA, which did not occur in tobacco leaf discs of Nt*rol*C transformants. However, although there was some increase in the overall number of AR formed in Nt35SB+C leaves, compared to controls, the numbers of AR induced from Nt35SB+C leaf discs were generally comparable to that of leaves transformed with *rol*C alone and were less than the number of AR on Nt*rol*B discs.

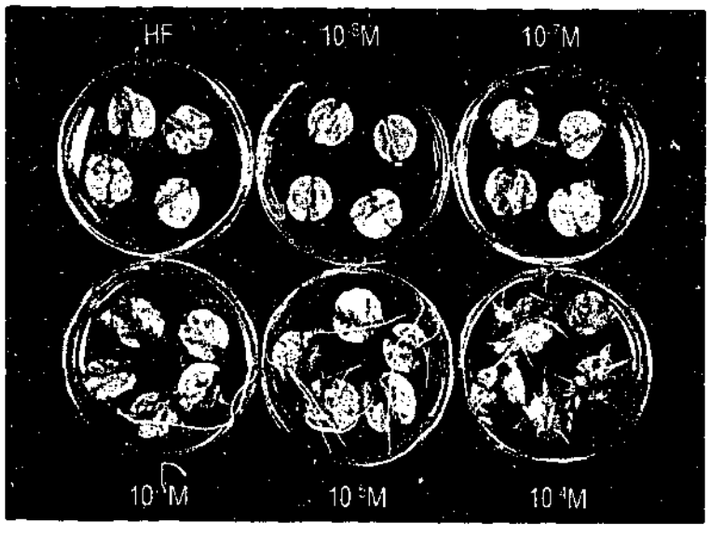
3.4.2. DISCUSSION

The effects of *rol* genes on the sensitivity of transgenic tobacco roots to auxin, interms of induction of LRP, were examined. The observations may present further insights into the involvement of *rol* genes in the promotion of root proliferation in transformed plants.

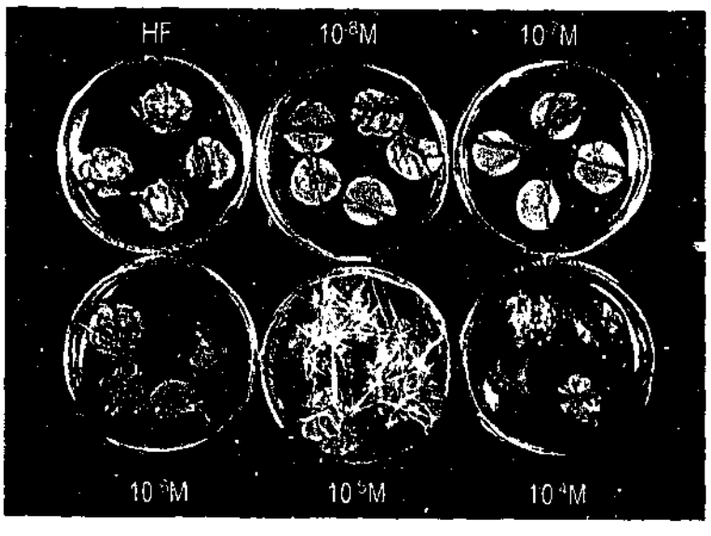
3.4.2.1. LRP induction in normal roots

The observation that LRP formation in A/35S-GUS roots was similar to that of SR1 indicated that the process of selection of transformants by kanamycin resistance did not adversely affect subsequent LRP formation. In untreated roots (hormone-free, HF) of tobacco seedlings, LRP are initiated at low frequency, with averages of 3-4 LRP/cm

(A) *rol*C leaf discs different IBA concentrations



(B) *rol*B leaf discs different IBA concentrations



(C) Leaf discs from different lines treated with 10^{-5}M IBA

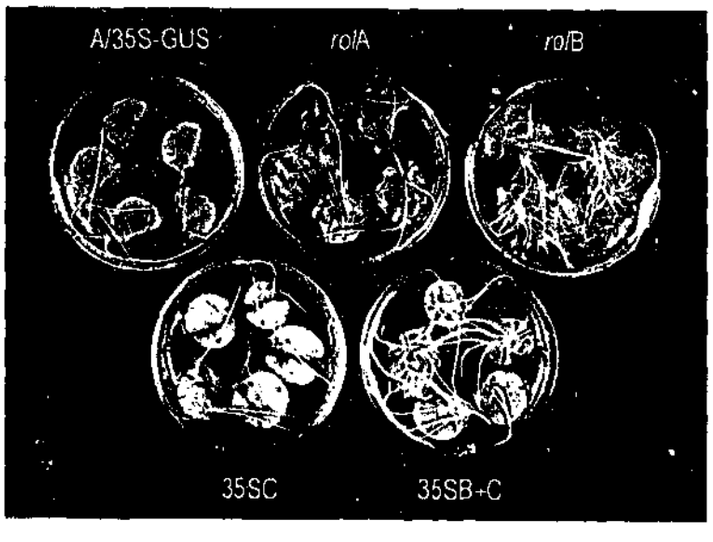


Figure 3.16. Adventitious root formation from leaf discs of tobacco *rol* gene transgenic lines. (A, B) *rol*C and *rol*B leaf discs respectively, after 17 days treatment with different IBA concentrations as indicated. (C) Leaf discs of different *rol* gene transgenic lines after 17 days treatment with 10^{-5}M IBA.

consistently produced in both control and *rol*-transformed seedlings. Low concentrations of IBA, 10^{-8}M - 10^{-7}M, also do not induce formation of LRP. As levels of endogenous auxins in root tissues are generally in the order of 10^{-7}M (Torrey, 1986), the low concentrations of exogenous auxin are probably close to physiological levels and therefore would not be expected to stimulate greatly increased numbers of LR.

In contrast, IBA at the higher concentrations of 10^{-4}M, 10^{-5}M and 10^{-6}M induces substantially more LRP than are formed in untreated roots. Although high concentrations of exogenous auxin, such as 10^{-6}M and above, probably represent artificial hormone conditions that would not be likely to occur *in vivo*, the augmented responses facilitate analyses of LRP formation. In an assay such as that undertaken here, small differences in LRP frequency between individual roots at physiological auxin levels may exist but are difficult to quantify. With higher auxin concentrations however, differences between roots become more evident as the overall LRP frequency is increased.

The pattern of LR frequency in response to IBA treatment in tobacco seedlings was very similar to that reported in excised radish roots, in response to increasing IAA concentrations (Blakely et al., 1988). IBA treatment of excised eucalyptus seedling roots also induced generally similar LRP initiation responses, although in that case the most effective IBA concentration was 10^{-5}M (rather than 10^{-4}M)(Pelosi et al., 1995). Reported observations of LR initiation in response to auxin treatment in other species are varied, although this also likely to be a reflection of the different experimental systems and species used. Untransformed *Hyoscyamus muticus* roots in cultured *in vitro* produced significantly more LR in response to IBA at 0.5, 1 and 2.5 μM than were formed in untreated roots, as would be expected (Biondi et al., 1997). However, it is interesting to note that in Ri T-DNA-transformed roots of *H. muticus*, the number of LR formed without any auxin treatment was already high and that the lower IBA concentrations did not cause any difference in LR initiation. This contrasts markedly with the low frequency of LRP formation on HF medium in the *rol* gene-transformed and putative 9402 Ri-transformed tobacco roots in this work. In addition, LR formation in the transformed *H. muticus* roots was inhibited by IBA at 2.5×10^{-6}M, a level much lower than the IBA concentration which repressed LRP in both normal and *rol*-transformed tobacco seedlings. These inconsistencies may reflect the different root types used - intact seedling roots in this work, compared to *in vitro* root cultures of *H. muticus*. The disparity between auxin effects on roots cultured *in vitro* and roots of intact plants was demonstrated by Vuylsteker et al. (1998). In roots of intact chicory plantlets, 10^{-6}M NAA induced an average of 0.8 LR meristems and 10^{-5}M NAA induced 2.3 LR meristems. This pattern was reversed in excised roots cultured *in vitro*, with 10^{-6}M NAA inducing more new LR than 10^{-5}M NAA.

3.4.2.2. Toxic effects of auxin at very high concentrations

Treatment of roots with 10^{-3}M IBA is apparently inhibitory to LRP formation to a large extent, with the very low numbers of LRP formed at this concentration similar to that of untreated roots. This was consistently observed in all the *rol* gene-transformed and untransformed lines of *N. tabacum* SR1 tested. As growth and elongation of the roots are also inhibited by the high concentration of IBA, this indicates that excessive IBA is probably toxic to all root functions and not just differentiation of LRP. Similar effects are mentioned in other reports, although the threshold concentration for toxicity differs depending on the growth system and type of auxin used. For example, in transformed roots of *H. muticus*, NAA at $2.5x10^{-6}$M was inhibitory to LRP formation and root elongation (Biondi et al., 1997). Pea roots treated with various auxins at 10^{-3}M showed root tip necrosis and markedly reduced LRP formation and LR emergence and length (Wightman and Thimann, 1980). Similarly, in roots of eucalyptus seedlings, LRP formation was repressed by treatment with 10^{-3}M IBA (Pelosi et al., 1995).

3.4.2.3. Suggested model for auxin-mediated stimulation of lateral root initiation

Root cells that are normally triggered to initiate primordia may have a greater capability to perceive auxin than cells which are not stimulated by endogenous levels of auxin. Postulated mechanisms for this include increased density and/or efficiency of auxin receptors or influx carriers (Malamy and Benfey, 1997b).

The *Arabidopsis ALF1* gene (Celenza et al., 1995) plays an important role in LR formation, possibly by negative regulation of internal auxin levels. *alf1* mutants produce greater numbers of LR than controls and this effect was shown to be further increased by addition of exogenous auxin. Evidently, by raising auxin to higher levels than normal, additional pericycle cells are stimulated to initiate LRP formation, indicating that these pericycle cells have retained the capacity to respond to auxin. Conversely, *alf4* mutants are defective in LR formation and also unresponsive to exogenous auxin, indicating a role for the *ALF4* gene in allowing pericycle cells to perceive or respond to auxin.

Increases in auxin levels in root cells, as a result of exogenous applications, will affect the auxin:cytokinin balance. This is relevant to the initiation of root formation, as root differentiation is induced by high auxin levels relative to cytokinin levels, as demonstrated by classical studies of Skoog and Miller (1957) and others (reviewed in (Hamill, 1993). Thus, in normal roots, exogenous auxin supplements may increase the auxin:cytokinin ratio to a level that overrides the limits imposed by the endogenous auxin

gradient. This may subsequently elicit primordium-initiating responses from pericycle cells that are inherently responsive to auxin signalling, although not stimulated by the normal auxin:cytokinin ratio. It is conceivable that there may be a "threshold" auxin:cytokinin ratio which, if exceeded, stimulates extra root pericycle cells to differentiate into LR initials and thereby increases the frequency of LRP initiation significantly above normal levels. With the supply of low exogenous concentrations of IBA (10^{-7}M, 10^{-8}M), the overall auxin levels will be raised to some degree but the small increase in the overall auxin:cytokinin ratio *in vivo* may be insufficient to exceed this threshold level. In the present study, exogenous IBA concentrations of 10^{-6}M were sufficient to induce significant increases in LRP compared to untreated roots, whereas 10^{-7}M IBA (and below) did not stimulate more LRP than were formed in untreated roots.

Exogenous auxin has also been demonstrated to have some effect on cytokinin levels, with auxin treatment shown to promote cytokinin catabolism and bring about a reduction in cytokinin levels, in *ipt* transgenic tobacco plants (Zhang et al., 1996). Eklöf et al. (1997) similarly demonstrated the mutually interactive effects of auxin and cytokinin, with overproduction of auxin in transgenic tobacco plants resulting in reduced levels and rates of synthesis of cytokinin, and reduced auxin levels in cytokinin-overproducing plants. This interaction between auxin and cytokinin may contribute to the effect of exogenous auxin treatment on lateral root differentiation, with higher auxin levels stimulating extra pericycle cells to undergo division, as well as possibly reducing relative cytokinin levels, so that ratios of auxin:cytokinin are further increased in favour of root formation.

3.4.2.4. Effects of *rol* genes on LRP and AR formation

(i) Individual *rol* genes

The responses of the different tobacco transformants to auxin treatment, in terms of LRP and AR formation, generally reflect the root growth characteristics described for whole plants and explants. Roots of Nt*rol*A transformants did not produce LRP in numbers significantly different from control roots. Correspondingly, reported phenotypes of *rol*A transgenic plants are not usually associated with notably increased root growth. In fact, in *rol*A-transformed tobacco and apple, decreased root growth was observed (Carneiro and Vilaine, 1993; Ben-Hayyim et al., 1996; Holefors et al., 1998). Furthermore, GUS reporter gene expression conferred by the *rol*A promoter was not detected in emerging lateral roots, in contrast to expression patterns conferred by the *rol*B and *rol*C promoters (Schmülling et al., 1989).

However, N*trol*A tobacco leaf discs produced some adventitious roots on hormone-free medium and also with low IBA concentrations, 10^{-8}M and 10^{-7}M IBA, in contrast to N*trol*C, Nt35SC and control leaves. These results concur with other reports of root induction from *rol*A tobacco leaves (Spena et al., 1987; Carneiro and Vilaine, 1993). The contrast in root induction effects in leaves and roots may reflect the differential expression of *rol*A under the control of promoter regulatory elements, with different levels of *rol*A transcripts accumulating in leaf, stem and root tissues types (Carneiro and Vilaine, 1993). This may also explain why *rol*A tobacco leaf mesophyll protoplasts appeared to be more sensitive to auxin than untransformed and *rol*C protoplasts, in terms of transmembrane potential difference (Maurel et al., 1991), yet enhanced auxin-sensitivity in terms of an increased stimulation of root cells to initiate LRP was not apparent in N*trol*A roots in this study.

Both *rol*B and *rol*C were shown to have an influence on root induction but with different effects on the processes of lateral and adventitious root formation. The expression of either *rol*C or *rol*B conferred a somewhat greater propensity for initiation of LRP in response to auxin, compared to WT roots. Adventitious root growth in response to exogenous auxin treatment was much greater from *rol*B-transformed leaf discs than any other line tested. This corresponds with previously reported observations in experiments with transformed kalanchoe and tobacco, in which *rol*B alone was able to induce roots on leaves of kalanchoe and, of all the individual *rol* genes, induced the strongest root response from tobacco leaf discs (Spena et al., 1987). Moreover, the phenotype of *rol*B transgenic tobacco plants includes an increased tendency to form adventitious roots from stems (Schmülling et al., 1988). Also consistent with an apparently greater sensitivity to IBA, in terms of capacity to form AR, the repressive effects of excess auxin were evident in the *rol*B leaf discs at a lower concentration than usual (10^{-4}M). This normally optimal IBA concentration may be perceived as being higher by the *rol*B-transformed cells. Testing of higher IBA concentrations would probably identify levels that are inhibitory to AR formation in these other lines.

In this study, N*trol*C leaf discs produced moderately increased numbers of AR compared to control leaves. In other reports, *rol*C was not able to induce roots from leaf discs of tobacco or kalanchoe (Spena et al., 1987). However, in analyses of root growth in whole plants, *rol*C transformants can have measurably larger root mass and increased root:shoot ratios (Frugis et al., 1995). This could be due to a greater propensity for lateral root branching that increases the overall mass of roots, as was suggested by the results of the LRP analysis in this study. This also corresponds with observations that *rol*C roots in *in vitro* culture branch more frequently than roots transformed with other *rol* genes (Schmülling et al., 1988).

(ii) Effects of combined expression of *rol*B and *rol*C

Sensitivity to auxin, in terms of LRP initiation, was greater in the Nt35SB+C line, compared to the *rol*B and *rol*C lines, most likely as a result of the combined expression of the two genes. Synergistic effects of *rol*B and *rol*C were suggested by the induction of maximal LRP numbers in response to a lower IBA concentration (10^{-5}M) than in the other lines (10^{-4}M). Furthermore, 10^{-4}M IBA was somewhat inhibitory to LRP formation for the Nt35SB+C line, in contrast to the N*trol*B and N*trol*C lines. An enhanced sensitivity to auxin may have raised the perceived auxin:cytokinin ratio in Nt35SB+C roots treated with 10^{-4}M IBA, to a level at which auxin is inhibitory, rather than promotive, to LRP initiation. This inhibitory effect was only apparent in other lines when treated with 10^{-3}M IBA.

In a study by Schmülling et al. (1988), visual assessment of root growth and branching of *rol*B+*rol*C and 35SB+C transformed tobacco root cultures revealed an increase in growth compared to roots transformed with either of the genes separately. In contrast, in *rol*B+*rol*C transformed alfalfa plants grown in soil, root mass was not increased as much as in plants transformed with *rol*B or *rol*C separately (Frugis et al., 1995). The alfalfa cultivar used in the study was a "creeping-rooted" type that spreads laterally, with frequently branching roots from which shoots are produced. A lower root mass could be due to a lower frequency of LR initiation or other reasons, such as reduced root elongation.

The synergistic activity of *rol*B and *rol*C in enhancing LRP formation in tobacco was not observed to the same extent in the induction of AR from leaf discs. The combined presence of *rol*B and *rol*C resulted in root induction that was generally comparable to that of leaves transformed with *rol*C alone and was considerably less than for *rol*B transformed leaves. A similar analysis was carried out by Spena et al. (1987), with tobacco leaves transformed with the same constructs but untreated with auxin. In contrast to the present study, it was found that 35SB+C leaf discs produced notably more roots than both *rol*C and 35SB leaf discs. However, the 35SB+C leaf discs of Spena et al. (1987) also produced fewer roots than *rol*B leaves, as was observed in the current study. In other investigations of *rol*B and *rol*C in combination, Aoki and Syōno (1999) found that tobacco leaf discs transformed with *rol*B+*rol*C produced more roots than *rol*B leaves. Conversely, Capone et al. (1989) observed that on carrot discs, fewer roots were induced by *rol*B and *rol*C together, compared to the independent effects of *rol*B.

Disparities in the details of separate studies may be due to differences in varieties or species, culture conditions or relative expression levels of the relevant genes in transgenic lines. In the Nt35SB+C line used in this study, *rol*B expression under

control of 35S promoter was very strong and *rol*C transcript levels were much lower than in *rol*C and 35SC transformants (Fig. 3.6). Thus, in the current analysis, it is not clear whether reduced AR induction from Nt35SB+C leaves is due to a mitigation of expression effects by the presence of both genes or is a result of different *rol*B and *rol*C expression levels in this particular line, compared to the single *rol* gene lines.

In the analyses of Spena et al. (1987) and Aoki and Syōno (1999), it is also noted that comparisons of root formation from 35SB+C or *rol*B+C tobacco discs were made in leaves untreated with exogenous auxin. By contrast, auxin treatment of leaves in the current study appeared not to promote synergistic effects from *rol*C and *rol*B. Carrot discs also required auxin (or *aux* genes or ORFs 13+14) for induction of root formation (Capone et al., 1989). It is therefore possible that the joint activity of *rol*C and *rol*B may be adversely affected by an over-supply of auxin, which could account for the apparently contradictory observations in these different experiments. Conceivably, the effects of an exogenous auxin supply, in addition to the enhancement of auxin-sensitivity by the presence of *rol*C and *rol*B, may be too great to be optimal for adventitious root induction. On carrot discs, Capone et al. (1989) demonstrated that a much greater abundance of roots were induced by *rol*B and *rol*C in conjunction with *rol*A and other ORFs - this may relate to a suggested role for some TL-DNA ORFs in negatively regulating *rol* gene-enhanced auxin responses and thus helping to optimise conditions for root induction (Lemcke and Schmülling, 1998).

(iii) 35S-*rol*C and 35S-*rol*B lines

As shown by Northern analysis, *rol*C expression levels in the Nt35SC transformant lines were not higher than in *rol*C transformants, even though the 35S promoter may have been expected to confer higher expression. Consistent with this, root induction responses in Nt35SC leaves and roots were not greater than that of *rol*C transformants. Thus, these results do not indicate whether sensitivity to auxin, in terms of LR and AR initiation, can be correlated with *rol*C expression levels.

In other studies, *rol*C-transformed alfalfa was shown to have increased root growth compared to WT plants, whereas root growth in 35S-*rol*C transformed alfalfa was not improved (Frugis et al., 1995). Also, in root cultures of *Catharanthus roseus*, roots expressing higher levels of *rol*C gene product were found to have lower growth rates than roots with low expression (Palazón et al., 1998a). These reports contrast, however, with some other reported observations of root growth in 35S-*rol*C and *rol*C transformants. While *rol*C tobacco leaf discs were not able to spontaneously produce roots, an abundance of roots were formed on 35S-*rol*C leaf discs cultured on medium

lacking auxin (Spena et al., 1987). A qualitative assessment of root growth and branching of transformed tobacco roots cultured axenically also revealed increased growth in 35S-*rol*C roots compared to *rol*C-transformed roots (Schmülling et al., 1988).

Although LRP initiation response of Nt35SB transformed seedlings to auxin was not greater than that of Nt*rol*B seedlings, *rol*B transcript levels were not ascertained in the Nt35SB line relative to the Nt*rol*B transformant line. Therefore, the effects of higher *rol*B expression levels on LRP initiation in tobacco seedlings cannot be determined from this study. Evidence from other studies suggests that overly elevated expression levels of *rol*B can be less conducive to root initiation than normal expression levels. Spena et al. (1987) observed that root production on 35SB discs was less than for *rol*B discs and 35SB tobacco plants did not exhibit the increased AR production and reduced apical dominance observed in *rol*B plants (Schmülling et al., 1988; Nilsson et al., 1993a). This is consistent with increased auxin sensitivity of tissues due to expression of *rol*B, with elevated levels of auxin becoming toxic to LRP formation and LR growth.

3.4.2.5. Different pathways for auxin-mediated processes

Variations between *rol* gene-induced effects on AR formation from leaves and LRP formation in roots may be a result of the particular effects of auxin on each process. For example, synergistic effects of 35S-*rol*B and *rol*C were apparent in enhancement of LRP initiation but not AR initiation. Conversely, Nt*rol*A transformants produced slightly increased numbers of AR compared to controls but LRP initiation was not altered by the presence of *rol*A; in Nt*rol*C transformants, the reverse pattern was observed.

While it is likely that precise transcript levels of *rol* genes influence the capacity of a transgenic tissue to produce root primordia in response to exogenous auxin supply, different receptors and signal transduction pathways are also likely to be involved in the control of various auxin-mediated processes (Guilfoyle et al., 1998). Variations in distribution of receptors in particular tissues and cell types may contribute to observable differences in auxin responses. For example, Maurel et al. (1991) demonstrated that the sensitivity of protoplasts to auxin in terms of electrical response was increased in *rol* transformants but that cell division, although also mediated by auxin, was not correspondingly modified. Similarly, *rol*B clearly increases sensitivity to auxin in relation to some traits, including changes to transmembrane E_m and leaf necrosis (Schmülling et al., 1988; Maurel et al., 1994), however other auxin-related traits are not affected. For example, it was demonstrated that sensitivity of *rol*B tobacco seedlings to inhibition of

germination by auxin, and auxin requirements for division of *rol*B callus cells are not different from WT tissues (Schmülling et al., 1993).

Differential effects of auxin on root developmental processes have also been noted with respect to lateral root initiation and root elongation. *Arabidopsis alf4-1* mutants were shown to be sensitive to inhibition of root elongation by auxin but remained insensitive to auxin-induction of LR. Roots of *alf4-1* seedlings did not initiate LR, even when treated with IAA at concentrations normally stimulating LR formation (Celenza et al., 1995). In double mutants of the *Arabidopsis* auxin-resistance mutations *axr4* and *aux1* (Hobbie and Estelle, 1995), the effects of each mutation on reducing the frequency of LR formation appeared to be additive, possibly indicating that the two genes act in separate pathways. However, with respect to root elongation, resistance to auxin was not more affected in the double mutant than in the *aux1* mutant alone, suggesting epistatic interactions of two genes in the same pathway. The contradictory responses may be due to differences in the auxin mechanisms invoived in these developmental processes. As previously noted (Chapter 1), it has been demonstrated that auxin requirements during LRP initiation differ from that of LR elongation (Pelosi et al., 1995).

3.4.2.6. LRP formation in 9402/SR1 roots in response to IBA

The phenotypic characteristics of many of the putatively Ri-transformed plants were quite marked, being characteristic of well-described effects for plants regenerated from transformed roots (Tepfer, 1984). Southern blot analysis showed, however, that the phenotypic alterations did not appear to necessarily correlate with the presence of the T-DNA in these plants. The reason for the distinctively modified phenotype of plants that did not contain *rol*C and *rol*A is not certain. It is unlikely that the altered phenotype of the plants is due to transformation with the *rol*B gene alone, as *rol*B is flanked by the *rol*A and *rol*C genes in the Ri T-DNA. In addition, *rol*A is the *rol* gene responsible for the distinctive leaf wrinkling of T-DNA transformed plants (Sinkar et al., 1988; Carneiro and Vilaine, 1993), which was utilised as a clear phenotypic marker. Similarly, although the presence or absence of TR-DNA genes and other TL-DNA ORFs were not specifically determined, these are also unlikely to be responsible for the phenotypic alterations. The abnormal phenotype of plants regenerated from hairy roots has been correlated with the presence of TL-DNA, rather than TR-DNA (Taylor et al., 1985). Likewise, of fifteen different transgenic tobacco plant lines harboring single non-*rol* ORFs of TL-DNA, only two - ORF3 and ORF13 - have shown altered morphologies when compared to wild-type (Lemcke and Schmülling, 1998). Leaf wrinkling was not evident as a feature of ORF3

plants. The phenotype of ORF13 transgenic plants have some similarities to Ri-transformed plants, including dwarfed size and variable internode length (Lemcke and Schmülling, 1998). An uneven leaf morphology has been observed but distinctly wrinkled leaves were only noted with overexpression of the gene in 35S-ORF13 plants (Hansen et al., 1993). These plants exhibited small, asymmetric and wrinkled leaves. However, other differences in 35S-ORF13 plants were noted that were not seen in the putative 9402 Ri-transformants, including slower growth and reduced apical dominance, with production of offshoots from the base of plants.

The 9402/SR1 LRP data suggest that, in comparison to the effects of the single *rol*C and *rol*B genes, the presence of the Ri T-DNA containing all T-DNA genes does not further magnify LRP initiation in response to auxin. As discussed above, the increased stimulation of LRP observed in roots transgenic for *rol*C and *rol*B, compared to *rol*A, is consistent with the general influence of these genes on root initiation. Therefore, although T-DNA transformants may contain all three *rol* genes and other Ri T-DNA ORFs, this may not necessarily have more effect on the induction of LRP than *rol*B+*rol*C. The *rol* genes were originally identified and defined by their primary role in the induction of roots resulting from plant infection by *A. rhizogenes* (White et al., 1985). The functions of all other T-DNA genes have not been established, although the capacity for independent initiation of root growth appears to be limited to the *rol*A, B and C genes. It has been shown that none of ORFs 2-9 and 13-18 from TL-DNA of *A. rhizogenes* strain HR1, under control of the 35S promoter, are able to induce AR formation on tobacco leaf discs (Lemcke and Schmülling, 1998).

With plant phenotype found to be an ambiguous indicator of transformation status, other feasible methods for identification of genotype were considered. These included typing each individual plant by molecular techniques such as Southern hybridisation, as already carried out with some plants (Fig. 3.13) or PCR, using appropriate primers for a relevant T-DNA marker such as the *rol*C gene or the intergenic region between the *rol*C and *rol*B coding sequences. Preliminary experiments showed that this was feasible, however as the LRP data obtained strongly suggested that the presence of Ri T-DNA did not have markedly greater effects on LRP formation than the single *rol* genes, it was considered unlikely that identification of individual plant genotypes would provide information of additional consequence and this aspect was not investigated further in the present study.

3.4.2.7. A suggested model for the effects of *rol* genes on LRP formation

The results of the LRP experiments described above indicated that *rol*C and *rol*B expression, particularly in combination, increased frequencies of LRP formation in response to certain auxin concentrations. While current understanding of *rol*C and *rol*B gene functions remains uncertain, in terms of their capacity to alter phytohormone levels *in vivo* (Nilsson and Olsson, 1997), it has been demonstrated that *rol*B- and *rol*C-transformed cells may have an enhanced sensitivity to auxin compared to normal cells and thereby perceive an effectively higher auxin concentration. For *rol*B transformed cells, this could be due to the presence of an increased number of transmembrane auxin receptors (Barbier-Brygoo et al., 1991), thereby enabling *rol*B transformed cells to bind more auxin than normal cells. A likely mechanism for *rol*C transformed cells has not yet been proposed.

Incorporating known effects of auxin and cytokinin on root initiation, and observations made in this analysis with tobacco seedling roots, a possible model for the effects of *rol* genes on LRP formation is outlined below and in Fig. 3.17.

This model is based on a hypothesised threshold auxin:cytokinin ratio for triggering of LRP initiation (§3.4.2.3) and an increased sensitivity to auxin of some *rol* gene transformants, with respect to LRP initiation. Thus, the *perceived* auxin:cytokinin ratio may consequently be higher in these *rol* gene transformant lines than in control lines. Exogenous application of auxin increases the auxin:cytokinin (aux:cyt) ratio to which pericycle cells in roots are exposed. When the perceived aux:cyt ratio reaches a certain threshold level, LRP initiation is triggered. Numbers of LRP increase in proportion to increasing auxin levels, as normally unresponsive pericycle cells (in terms of LRP initiation) are stimulated to begin the cell division process.

According to this model, in *rol*B and *rol*C transformed lines in which the perceived aux:cyt ratio may be higher than in WT roots, the frequency of LRP formation is increased to a greater extent than in WT roots treated with the same concentration of exogenous auxin. This was the effect observed in roots of N*trol*C, N*trol*B, Nt35SC and Nt35SB transformants, at IBA concentrations of 10^{-6}M, 10^{-5}M and 10^{-4}M.

A further aspect of this proposed model is the presence of a second aux:cyt threshold. This threshold occurs at a high relative auxin concentration, at which auxin is inhibitory, rather than promotive, to LRP initiation and root growth. Therefore, in transformed cells perceiving a higher aux:cyt ratio, this second threshold may be reached at a lower exogenous auxin concentration than normal cells. This effect was observed in Nt35SB+C roots treated with 10^{-4}M IBA, in which frequencies of LRP formation and primary root elongation were substantially reduced by a level of auxin

normally optimal for LRP induction (10^{-4}M IBA). In controls and other *rol* gene transformant lines, these effects are induced by 10^{-3}M IBA.

This model may also account for the fact that in N*trol*B and N*trol*C transformed lines, which are postulated to have a higher sensitivity to auxin, increased LRP frequency is not observed in response to auxin concentrations of 10^{-7}M and lower. At these lower concentrations of exogenous IBA, the perceived auxin:cytokinin ratio, though perhaps greater than in WT cells, may not be greater than the threshold level required for LRP induction and therefore not in the range at which significantly more LRP are initiated.

Increased frequencies of LRP initiation were not observed in *rol* gene transformed roots of seedlings in the absence of auxin treatment, which is clearly more analogous to a normal plant environment than the presence of high levels of exogenous auxin. However, differences in sensitivity between roots at physiological auxin levels may exist which, although not easily measurable or significant in terms of increase LRP formation in individual roots, may nevertheless be biologically significant. If expression of the *rol*B and *rol*C genes cause slightly increased numbers of LRP to be initiated in each transformed root due to an increased sensitivity to endogenous auxin, the compounding effect of these differences could result in substantial effects on the size and growth of the root system as a whole. With slight increases in the number of LR formed from primary roots, which each in turn produce more LR and so on, the overall root system may potentially become much more highly branched than WT root systems. As observed in the previously cited example of Ri-transformed *Arabidopsis* (Hamill and Chandler, 1994), a much higher number of lateral roots produced by Ri T-DNA transformed plants cultured *in vitro* was shown to result in a greatly increased root mass. Although the number of primary LR did not significantly differ between the two plant types, transformed plants subsequently produced progressively greater numbers of secondary, tertiary and quaternary LR roots. By 27 days after germination, the total number of LR tips was almost three times higher for transformed plants than for normal plants.

Figure 3.17. *(Facing page)* A suggested model for the effects of exogenous auxin on LRP initiation in normal tobacco roots and in transgenic tobacco lines expressing *rol* genes.

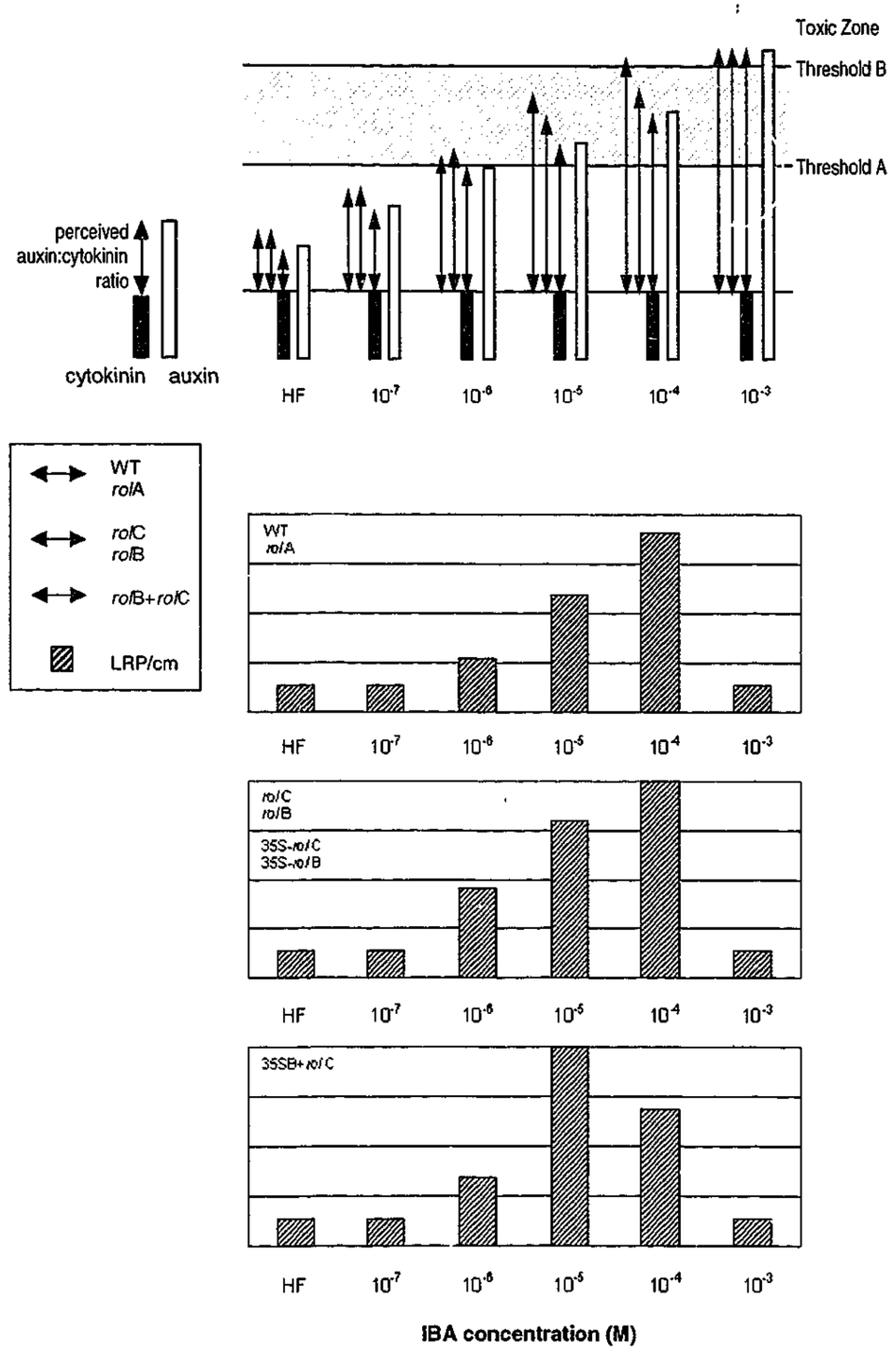
Threshold line A represents the aux:cyt ratio at which LRP formation is significantly increased. Threshold line B represents the aux:cyt ratio at which the auxin level becomes toxic or inhibitory to LRP formation.

Arrowed bars represent the auxin:cytokinin (aux:cyt) ratio. These are coloured to represent the perceived aux:cyt ratio of different genotypes. Blue bars represent WT or control roots, and N*trol*A transformants, in which LRP formation does not significantly differ from WT. Red bars represent N*trol*C transformants and genotypes observed to have similar LRP patterns i.e. N*trol*B, Nt35SC and Nt35SB. Green bars represent Nt35SB+C roots.

In WT roots, the aux:cyt ratio is perceived as normal; therefore, blue bars are same height as the arrowed bar. In "red" and "green" genotypes, the aux:cyt ratio is perceived to be higher than normal, possibly due to a higher sensitivity to auxin. Thus, the red and green bars are depicted as extending further than the arrowed bar representing the actual aux:cyt ratio.

As the concentration of exogenous auxin is increased, the relative aux:cyt ratio also increases and at some point, certain threshold aux:cyt ratios are reached. In this model, the aux:cyt ratio in Nt35SB+C roots reaches this second threshold at a lower concentration of exogenous auxin (10^{-4}M) than the other genotypes, due to an enhanced perception of auxin.

The corresponding bar charts represent relative LRP formation patterns. On HF medium and with 10^{-7}M IBA treatment, low levels of LRP are formed. With 10^{-6}M IBA, this number is significantly increased, as the aux:cyt ratio reaches Threshold line A, although the increase is greater in N*trol*C (and other "red" genotypes) and Nt35SB+C roots. Similarly, with 10^{-5}M IBA, large numbers of LRP are formed, particularly in Nt35SB+C roots. With 10^{-4}M IBA, LRP numbers in WT and N*trol*C roots are increased again; in Nt35SB+C roots, however, a toxic aux:cyt level (threshold line B) is reached at 10^{-4}M IBA and the number of LRP consequently drops. This threshold is reached by all other genotypes when treated with 10^{-3}M IBA.



3.4.2.7. Possible alternative mechanisms for *rol*C-induced stimulation of LRP initiation

Although strong evidence for the effects of *rol*B in increasing sensitivity to auxin has been reported (Maurel et al., 1991; 1994), a similarly strong link between *rol*C and auxin sensitivity has not been established. A moderate increase in sensitivity, in terms of transmembrane E_m response, was detected in *rol*C transgenic leaf mesophyll protoplasts (Maurel et al., 1991). The *rol*C promoter was also shown to be activated to some extent by auxin, in transgenic mesophyll protoplasts and also in leaves and roots of transgenic tobacco plants (Maurel et al., 1990). However, the results of the LRP analysis undertaken in the present study suggest an effect of *rol*C on LR formation generally equivalent to that of *rol*B, with an increased responsiveness to exogenously applied auxin. This is in accordance with other reports of increased branching of *rol*C-transformed roots (Schmülling et al., 1988; Tepfer et al., 1994; Palazón et al., 1998b).

A model was proposed by Nilsson and Olsson (1997) for the functions of *rol*B and *rol*C in AR formation, which may be relevant to observations made here. It was suggested that *rol*B may stimulate root primordia formation by inducing increased sensitivity to auxin, and the expression of *rol*C in phloem cells may promote sucrose unloading, thereby increasing sucrose supply levels. As phloem cells can serve as initials for AR, the expression of these genes in phloem cells of aerial tissues may thus promote formation of AR. The regulation of the *rol*C and *rol*B promoters by sucrose and auxin respectively (Maurel et al., 1990; 1994; Yokoyama et al., 1994), further correlate with the concept of phloem cells as a prime target for *A. rhizogenes*.

However, as *rol*C is only expressed in phloem cells in tobacco roots (Schmülling et al., 1989; Guivarc'h et al., 1996) it is not immediately apparent how increases in sucrose unloading, induced by *rol*C in phloem cells, could lead to stimulatory effects on xylem-radius pericycle cells competent to initiate lateral root primordia. The effects of *rol*B on increased formation of LRP are ostensibly more understandable, as this gene is expressed in pericycle cells in tobacco roots (Altamura et al., 1991) and thus may be capable of stimulating LRP initiation by enhancing their auxin-sensitivity. Interestingly, in roots of the perennial species hybrid aspen, both *rol*C and *rol*B are expressed in pericycle cells (Nilsson et al., 1997), in contrast to roots of tobacco and rice, in which *rol*C expression is strictly phloem-specific (Matsuki et al., 1989; Guivarc'h et al., 1996).
In addition, Nilsson and Olsson (1997) also refer to evidence of a role for *rol*C in sucrose unloading in phloem cells and note that cell division activity is stimulated by increased sucrose concentration. This may suggest that increased sucrose unloading in this region could contribute to *rol*C-induced stimulation of LRP initiation in pericycle cells.

A proposal put forward by Faiss et al. (1996) that oligosaccharins may be a substrate for *rol*C, could also potentially have some relevance to the enhanced root-initiating effects of *rol*C. A prominent example of developmental effects elicited by a group of oligosaccharins is the role of lipo-oligosaccharides (LCOs) in nodule formation in legumes. Although derived from cell division initiated in different cell types, similarities between nodule formation and LR formation have been observed. In fact, it has been suggested that mechanisms of nodule development may have been adapted from that of LR development (Mathesius et al., 2000). In particular, analogous changes in expression patterns of the auxin-induced gene *GH3*, indicating developmental alterations in auxin balance, were observed during formation of both nodule and LR primordia (Mathesius et al., 1998). Furthermore, a number of other similar plant cell responses triggered by both initiation of LR and rhizobium infection have been noted in addition to *GH3* expression - chalcone synthase gene expression, accumulation of a specific flavonoid, formononetin (Mathesius et al., 2000) and expression of *ENOD40* and *ENOD12A* genes during both nodule and LR development (Yang et al., 1993; Bauer et al., 1996).

Evidence of the association between nodulation and LR formation was observed in cortical cells during LR and nodule initiation in mature (differentiated) root tissues of white clover (Mathesius et al., 2000). Nodule formation is generally lacking in mature root zones, compared to the more acropetal region just behind the root tip (zone of developing root hairs) where nodulation preferentially occurs. However, nodules that do form in the mature root zone are usually associated with LR, initiation occurring at the sites of emerging LR. It was demonstrated that cortex cells overlying sites of LR initiation have similar responses to rhizobium-inoculated cells and thus, when stimulated by LR initiation, can apparently be "hijacked" for nodule initiation. This is consistent with observations that the general inability to form nodules in mature root zones is likely to be due to a lack of signalling between infected root hairs and underlying cortical cells in this zone (Mathesius et al., 2000).

The process of nodulation involves modification of a class of LCOs to an active form by the expression products of *nod* genes, which trigger nodule formation (Dénarié et al., 1996). LCOs have been shown to induce a temporary inhibition of auxin flow in white clover roots, leading to a subsequent transient accumulation of auxin and stimulating initiation of root nodules at this site (Mathesius et al., 1998). It is conceivable that an analogous situation may occur with *rol*C and LRP initiation. For example, one scenario may be that *rol*C may modify a certain class of oligosaccharin (as suggested by Faiss et al., (1996)), which could then cause local alterations in auxin or cytokinin balance, possibly by influencing hormone gradients, transport or signalling. Alterations to auxin-cytokinin balance may consequently result in stimulation of lateral root initiation.

3.5. ANALYSIS OF SOIL-GROWN TRANSGENIC TOBACCO LINES

3.5.1. INTRODUCTION

The cumulative effect of increases in lateral root branching in plants containing *rol* genes may lead to measurable changes to overall root system growth in older plants. Plant growth in soil was analysed for several transgenic *N. tabacum* lines containing *rol*C under regulatory control of different promoters. As analysis of LRP formation in various *rol* gene transformant lines demonstrated the 35SB+C combination to have most effect on increasing capacity for LRP initiation in response to auxin, this transgenic line was included in analysis of root system growth in whole plants. Also analysed were *rol*C, 35SC and the A/35S-GUS control line as used for the LRP analysis.

Preliminary analysis of the transgenic tobacco lines analysed in this experiment showed them to display some variability in rates of growth and development, even when grown under identical conditions. This is presumably a result of *rol* gene expression, as the parental SR1 is a highly inbred line derived from doubled haploid plant material (Maliga et al., 1973) and has a high degree of uniformity. Thus, a particular stage of development was required at which plants could be compared with one another. One clearly identifiable stage, the commencement of flowering, was designated as the time at which root and shoot biomasses of the plants would be assessed.

Plant ontogeny is also an important consideration in undertaking root and shoot growth comparisons, as growth rates and patterns change over the course of a plant's development (Wilson, 1988; Gedroc et al., 1996). Therefore, to take into account ontogenetic variation and the possibility that different effects may be apparent at earlier stages, relative root and shoot growth were compared at periodic time points during development, as well as measurement of mature plants at a single developmental timepoint, the opening of the first flower.

Root-specific expression of *rol*C

In addition to the plant lines mentioned above, experiments were designed to express *rol*C specifically in the roots of plants and assess these plants for any alterations in growth patterns. Generation of increased root growth in plants, by expression of *rol*C specifically in roots, could conceivably have potential benefits for improving plant growth and productivity in some environments. Increased early root growth may help plants to become established more rapidly, which may be an advantage for survival, particularly if later conditions subsequently become unfavourable, e.g. restricted water or nutrient

availability. It is possible that plants with *rol*C root systems may also have greater potential than normal plants for increased overall growth in response to limitations in these resources. However, *rol*C also induces prominent modifications to shoot phenotype, some of which may be considered detrimental to growth and productivity, depending on the plant species and objectives. Features such as dwarfed plant stature, reduced fertility, lower chlorophyll levels and lower biomass (the latter shown in §3.5.2.1) may not be considered desirable or advantageous for many crop species, although some of these characteristics have particular applications in horticultural and ornamental species (as will be discussed in Chapter 4).

Tissue-specific expression of introduced genes is a key consideration for many transgenic plant and crop development objectives (e.g. Fladung et al., 1993; Graham et al., 1997; Gittins et al., 2000), with the isolation of many potentially beneficial genes, including pest and virus resistance and particular nutritive factors. Specific targeting of *rol*C expression to roots may potentially result in increased root growth without inducing negative effects in aerial parts. To assess the effects of directing *rol*C expression to the root system of tobacco plants, two experimental approaches were considered: (1) transformation of plants with *rol*C under control of a root-specific promoter; (2) grafting of *rol*C-transformed tobacco rootstocks and untransformed shoots. The effects of limiting expression of *rol*C to the root system were subsequently investigated by analyses of root and shoot growth and the balance between the two systems.

_rol_C expression directed by CaMV35S domain A promoter

One potentially suitable 5' regulatory sequence for the regulation of root-specific expression of *rol*C is the domain A regulatory element (-90 to +8 region) from the CaMV35S promoter. By fusion with the GUS reporter gene, domain A has been shown to confer expression predominantly in root tissue of tobacco plants (Benfey et al., 1989). In seedlings, expression was conferred primarily in the root tip and lateral roots and was also evident in the region below the apical meristem. In seven-week-old plants, expression remained strongest in roots, in the root cortex, meristematic region and in pericycle cell regions involved with lateral root initiation but was not evident in vascular tissue. Very low level expression was also detected at some stages of development in the vascular tissue of young leaves and upper stem by Benfey et al. (1989), although this was not observed in histochemical staining of similar domain A-*GUS* transformant leaves by Elmayan and Tepfer (1995).

With predominantly root-specific expression, consistent throughout development (Benfey et al., 1989), the CaMV35S domain A was considered an appropriate promoter for targeting expression of *rol*C to the root system and potentially inducing increased

root growth, without the accompanying morphological effects of *rol*C on the upper parts of the plant.

Grafted tobacco plants

Reports of grafts between *rol*C-transformed plants and normal plants have generally indicated that the RolC gene product is not graft-transmissible. *rol*C transgenic shoots or rootstocks of potato and tobacco did not alter the growth or phenotype of the respective SR1 rootstock or shoots to which they were grafted (Fladung, 1990; Estruch et al., 1991b). In grafted rose plants consisting of *rol*ABC rootstocks and WT scions, no evidence of transgenic phenotype was found in shoots with the exception of an accelerated bud release (Van der Salm et al., 1998). No detailed examinations of r:s relationships were undertaken in these studies

With regard to certain phenotypic and physiological alterations, the RolC gene product has been shown to exhibit cell-autonomous behaviour. In terms of inducing rhizogenesis, the RolC gene product is unlikely to be transported or diffusible as *rol*C-induced roots are always transformed (Schmülling et al., 1988) and root induction only occurs from transformed cells. Cell-autonomy of *rol*C expression was also demonstrated with regard to leaf pigmentation. In chimaeric leaves, regions consisting of cells expressing *rol*C were clearly delineated by the pale-green colour (resulting from reduced chlorophyll levels), whereas cells not expressing *rol*C were darker green and remained unaffected by the presence of *rol*C in the adjacent sectors (Spena et al., 1989; Schmülling and Schell, 1993; Oono et al., 1993b). Furthermore, plants expressing *rol*C regulated by its native promoter do not show pale green leaves, as expression is directed to vascular tissue (Sugaya et al., 1989; Guivarc'h et al., 1996). This confirms the requirement for *rol*C to be specifically expressed in the mesophyll cells, for the pale-green phenotype to be manifested.

However, though the *rol*C gene product itself may be localised to one cell, it has been suggested that some physiological effects of its activity may be transportable or diffusible to a different location (Guivarc'h et al., 1996). For example, effects of *rol*C in anthers are apparently not limited to transformed tissues, as chimaeric *rol*C tobacco plants were found to be male sterile, even though the meristem layer from which anthers are derived were not transformed (Schmülling and Schell, 1993). Also, although early flowering and altered flower morphology are apparent in *rol*C-transformed plants, expression of the gene was not detected in tobacco shoot apical meristems entering the prefloral phase (Guivarc'h et al., 1996).

3.5.2. RESULTS

3.5.2.1. Root and shoot growth of plants measured at time of flowering

In this experiment, plants were harvested on the day on which their first flower opened, so that plants were all assessed at a comparable developmental stage. The average root, shoot and total plant dry weights of the four plant lines were measured (Table3.3). Growth of both root and shoot were highest in control (A/35S-GUS) plants, which produced more than double the biomass of N*trol*C and Nt35SC plants. From visual comparisons, growth and development of A/35S-GUS plants appeared to be identical to that of untransformed SR1 plants. Plants expressing *rol*C tended to flower earlier (approximately 10 weeks from sowing seed) than A/35S-GUS plants (approximately 12 weeks from sowing seed).

Table 3.3. Average dry weights of soil-grown A/35S-GUS, N*trol*C, Nt35SC and Nt35SB+C tobacco plants at time of flowering ± standard error of the mean. n = 5-7.

	Total plant dry weight (g)	Shoot dry weight (g)	Root dry weight (g)
A/35S-GUS	7.456 ± 1.010	7.210 ± 0.992	0.246 ± 0.027
N*trol*C	3.091 ± 0.265	2.960 ± 0.255	0.134 ± 0.011
Nt35SC	2.786 ± 0.372	2.668 ± 0.357	0.118 ± 0.016
Nt35SB+C	5.593 ± 0.130	5.352 ± 0.133	0.241 ± 0.003

The relative proportions of root and shoot of plants of the N*trol*C, Nt35SC and Nt35SB+C transformant lines were compared (Fig. 3.18), with the root dry weight represented as a percentage of the total plant dry weight (root mass fraction, RMF). The data show that, although the total dry weight (DW) of N*trol*C plants was significantly reduced relative to controls, the average RMF of N*trol*C plants was higher than that of control A/35S-GUS plants ($p<0.05$). The difference was moderate but indicates that the expression of *rol*C resulted in some increase in relative root growth, in tobacco plants grown under the conditions described. Although the RMFs of Nt35SC and Nt35SB+C plants were very similar to that of N*trol*C, population variances were slightly higher and the Nt35SC and Nt35SB+C RMFs were not statistically different from A/35S-GUS

controls ($p>0.05$). However, this suggests that some growth effects may have been induced in these Nt35SC and Nt35SB+C plants, which may have been clearer with a larger sample size to reduce the degree of variation.

The similarity in relative root and shoot proportions between the three transgenic lines suggests that, at this developmental stage, neither the presence of *rol*C under control of the CaMV35S promoter, nor the combined presence of *rol*C and *rol*B in these particular lines induced greater effects on root system growth than were caused by *rol*C alone.

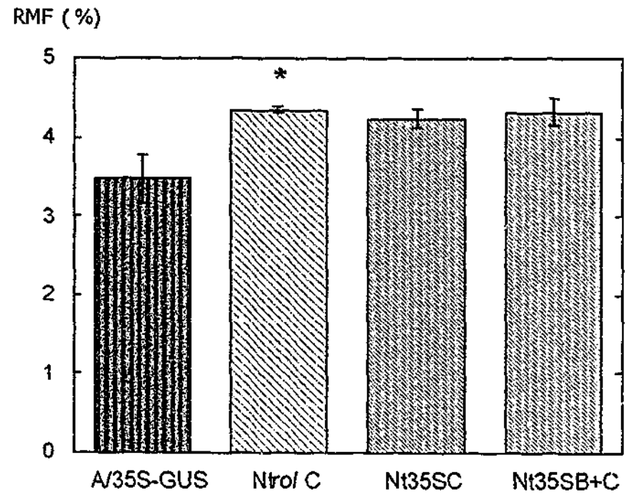


Figure 3.18. Root dry weight as a percentage of the total plant dry weight (RMF) of A/35S-GUS, N*trol*C, Nt35SC and Nt35SB+C tobacco plants. Plants were grown in soil with high-nutrient conditions and harvested at time of opening of the first flower on each plant. Vertical lines represent standard error of the mean, n = 5-7. Statistically significant differences: * $p<0.05$.

3.5.2.2. Root and shoot growth of non-grafted plants measured at weekly time points

To assess root and shoot growth at different plant stages, plants from the N*trol*C, Nt35SC and A/35S-GUS lines[1] were harvested after 7, 8, 9 or 10 weeks growth (see Fig. 3.20). 4-6 plants of each genotype were chosen randomly and harvested at each time point. Analysis of plants prior to 7 weeks growth was not practicable as plants were very

[1] 35SB+C plants were also initially included in the experiment, however growth of these plants was compromised by insect infection of several individuals and the plants were excluded from the analysis.

small, consisting of only a few leaves, and the amount of root tissue was too low to obtain accurate dry weight measurements. Conversely, after 10 weeks, some plants were beginning to flower.

At the 7-week timepoint, the total biomasses of N*trol*C and Nt35SC plants were larger than that of control plants (Table 3.4A). However, over the course of subsequent weeks, this pattern was reversed, with the average biomasses of control plants equaling, then exceeding those of Nt35SC plants by week 8 and those of N*trol*C plants between weeks 9 and 10. This comparative relationship between Nt35SC, N*trol*C and control plants at week 10 is consistent with that of plants harvested at the time of flowering (Table 3.3). Furthermore, measurements of root biomass (Table 3.4B) show that the comparative root masses of N*trol*C and control plants paralleled the pattern of total biomass, with N*trol*C root growth more advanced at the early stages (weeks 7 and 8) but surpassed by growth of control plant roots by week 10. This may be related to the slightly earlier flowering of N*trol*C plants, as relative biomass allocation to roots is generally reduced as plants begin to flower (Atwell et al., 1999b). Conversely, earlier flowering of N*trol*C compared to WT plants may be brought about by faster root growth early in development.

Table 3.4. Average dry weights of A/35S-GUS, N*trol*C and Nt35SC plants at 7, 8, 9 and 10 weeks ± standard error of the mean. (A) Total plant dry weights (B) Root dry weights. n =5-6.

(A)	Total plant dry weight (g)			
	week 7	week 8	week 9	week 10
A/35S-GUS	0.057 ± 0.007	0.278 ± 0.026	0.746 ± 0.107	1.781 ± 0.135
N*trol*C	0.079 ± 0.010	0.337 ± 0.042	0.739 ± 0.167	1.200 ± 0.130
Nt35SC	0.077 ± 0.014	0.251 ± 0.051	0.421 ± 0.048	1.042 ± 0.097

(B)	Root dry weight (g)			
	week 7	week 8	week 9	week 10
A/35S-GUS	0.005 ± 0.001	0.015 ± 0.001	0.040 ± 0.006	0.075 ± 0.006
N*trol*C	0.006 ± 0.001	0.023 ± 0.003	0.042 ± 0.012	0.049 ± 0.005
Nt35SC	0.006 ± 0.001	0.011 ± 0.001	0.021 ± 0.002	0.046 ± 0.004

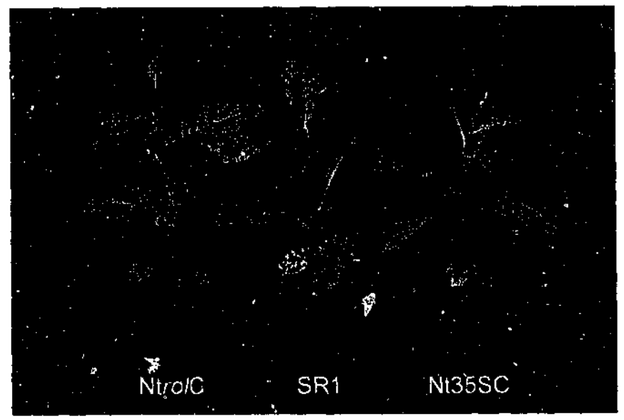


Figure 3.20. Transgenic N*trol*C and Nt35SC tobacco and SR1 tobacco plants grown in soil, 8 weeks after sowing seed. Although the overall sizes of plants are not markedly different at this stage, reduced internode length is evident in plants expressing the *rol*C gene, compared to SR1 plants.

3.5.2.3. Relative root growth of non-grafted plants measured at weekly time points

The RMFs of plants were highest at seven weeks, with a gradual decrease in the relative root tissue proportion observed in all three lines over the following three week time period (Fig. 3.19). At week 7, absolute root dry weights were very low and somewhat variable and overall there were no statistically significant differences between the relative root weights of the three plant lines. At 8 weeks of age, the root weight of N*trolC* transformants was approximately 7% of the total plant dry weight, which is significantly greater than that of the control plants (5.4% of total plant dry weight) ($p<0.001$). This suggests that expression of the *rolC* gene in these plants had some effect on increasing the relative biomass distribution in favour of root growth, at this stage of development. A measurable increase in the proportional size of the root system of N*trolC* plants was not observed at the other time points at which measurements were made. The relative root to shoot weights at weeks 9 and 10 were very similar for all plants.

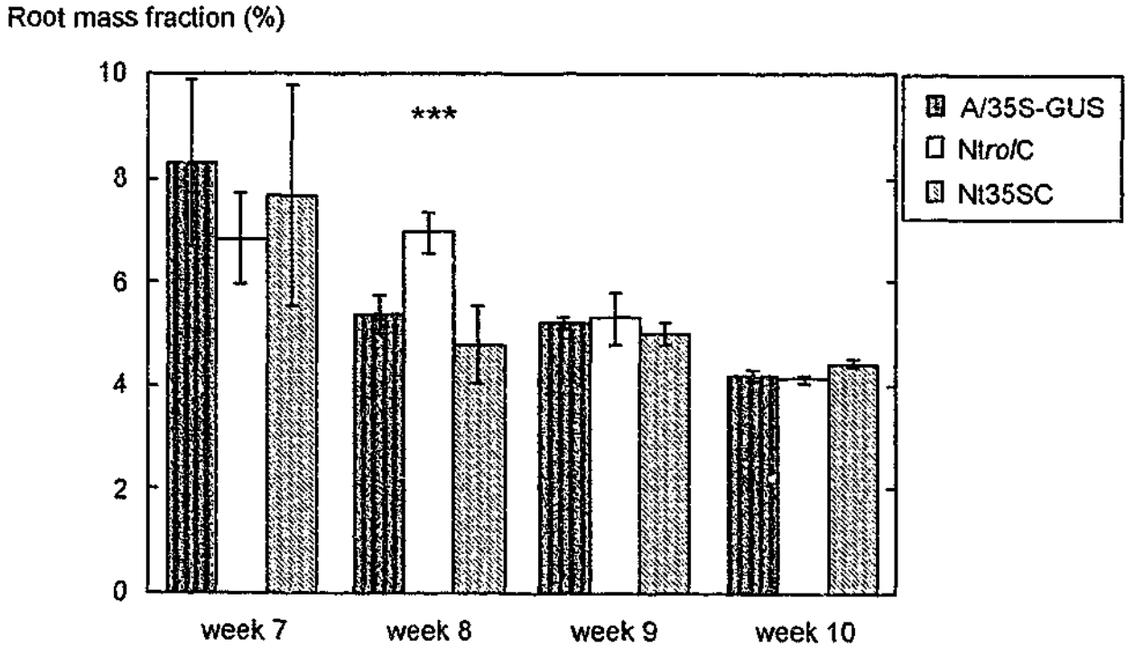


Figure 3.19. Root mass fraction (%) of A/35S-GUS, N*trolC* and Nt35SC tobacco plants at 7, 8, 9 and 10 weeks. Plants were grown in soil under high-nutrient conditions. Vertical lines represent standard error of the mean, n= 5-6. Statistically significant difference *** $p<0.001$, between N*trolC* and A/35S-GUS at 8 weeks.

The similarity of RMFs of the three plant lines at week 10 (Fig. 3.19) is in contrast to the significant difference apparent between N*trolC* and control plants in the previous experiment, which were harvested at time of flowering (Fig. 3.18). This may be because the N*trolC* plants tend to flower earlier than normal and these plants were therefore closer to flowering at week 10 than control plants. As noted, floral buds were evident on some N*trolC* plants at 10 weeks but did not appear on A/35S-GUS plants until later, at approximately 12 weeks. It is likely that biomass allocation to roots would have been naturally lower in the flowering N*trolC* plants than in A/35S-GUS plants at 10 weeks. Differences between relative root growth of N*trolC* plants and controls may therefore not have been evident when all plants were harvested at week 10. Consistent with this hypothesis, the RMFs of A/35S-GUS control plants at week 10 (Fig. 3.19) are somewhat higher (4%) than those calculated at the time of flowering (3.5%) (Fig. 3.18). Although measured in different experiments, precluding direct comparisons, this probably reflects changes in RMF as plants reach the stage of flowering. When each plant was harvested only after flowering, the ontogenetic stages of *rolC* and control plants would have been more comparable than at 10 weeks, and the enhanced root growth of N*trolC* plants compared to controls was evident as an increased RMF.

Although the results for week 7 are variable, the tendency is clearly also towards a generally higher proportion of roots than at later stages. It is possible that there may be some difference between N*trolC* and control plants at week 7 but that this was obscured by the variation in measurements. The primary reason for the large variations in RMFs is likely to be the very small size of the plants at this early stage of growth and therefore the much larger margin of error associated with relative measurements of this scale. With root and shoot biomasses of less than 0.01 g and 0.1 g respectively, even slight variations in growth rate and development are likely to have a disproportionate effect on plant mass and on root:shoot ratios in particular. As a result, there is a high degree of discrepancy between individual plants. By 8 weeks, root and total plant masses had generally tripled and more consistency between samples is observed at this stage and later time points.

Conclusion

Expression of *rolC* in transgenic tobacco plants appears to influence root growth and may have the potential to alter root-shoot balance of transgenic plants. From these measurements, however, it was not clear whether the effects of *rolC* on root growth are separable from effects of the gene on shoot growth. The overall growth of N*trolC* transformants was reduced compared to WT plants; in particular, *rolC*-induced negative effects on shoot growth as reported in other studies - lower biomass, reduced stature

and shortened internodes, and smaller leaves. This may at least partly account for the higher r:s ratio of N*rol*C plants compared to WT plants.

Expression of *rol*C specifically or predominantly in the root system may provide a means of distinguishing between effects on the root system independently of negative effects on shoots. If manipulation of root systems of crop plants is ultimately attempted via the use of *rol* genes, such an approach may be necessary.

3.5.2.4 Analysis of grafted plants

The combination of transgenic rootstocks with SR1 scions (N*rol*C/SR1, Nt35SC/SR1 and Nt35SB+C/SR1) were more amenable to grafting than the reciprocal combinations (SR1/Nt35SC and SR1/Nt35SB+C), with many more graft unions of the former type persisting. Reasons for this are not clear but may be related to the altered shoot form characteristics of *rol*C transformants, such as smaller stems, which may have reduced the efficiency of the procedure.

Grafted plants with transgenic rootstocks and SR1 shoots, at 4 weeks after grafting, are shown in Figure 3.21. Plants grew healthily with new stem and leaf growth produced after grafting; the phenotype of the shoots remained normal as compared to untransformed plants. Conversely, in the reciprocally grafted plants SR1/Nt35SC (Fig. 3.21), the *rol*C phenotype was clearly maintained in the transformed scion with untransformed SR1 rootstock[4].

It is apparent from the phenotype of N*rol*C/SR1, Nt35SC/SR1 and Nt35SB+C/SR1 grafted plants that the *rol*C gene product is not graft transmissible - given the distinctive effects induced by *rol*C, any alterations to WT phenotype shoot phenotypes would be expected to be clearly visible. Thus, resulting plants were regarded as being transformed by *rol*C only in the root system, thereby establishing a set of plants for the analysis of the effects of specific *rol*C expression on root and shoot growth.

3.5.2.5. Root and shoot dry weight measurements of grafted plants

The grafting method used here is quite an injurious process, involving cutting and damaging plants during a period of active shoot growth and development. Growth of SR1/SR1 control plants was the most affected by the grafting process, with root and shoot masses reduced by about half compared to non-grafted plants, although relative

[4] Of the reciprocal grafts joining SR1 rootstocks and *rol* gene-transformed scions, a limited number were successfully established. From these plants, it was possible to make general observations of the effects of this graft on plant phenotype. However, comparative growth could not be validly assessed due to the small sample size.

root:shoot ratios remained similar to that of non-grafted plants. The three other lines were not detrimentally affected in terms of plant growth; in fact, Nt35SC/SR1 and Nt35SB+C/SR1 grafted plants produced greater amounts of both root and shoot tissues than intact Nt35SC and Nt35SB+C plants.

When harvested at flowering, the N*trol*C/SR1, Nt35SC/SR1 and SR1/SR1 grafted lines were comparable to one another in the amount of total biomass produced. In comparison, the overall growth of Nt35SB+C/SR1 plants was substantially higher than the other grafted lines, with more than double the dry weight produced in both root and shoot systems (Table 3.5).

Relative root and shoot growths (expressed as RMF) were also compared for the grafted plants at the time of flowering (Fig 3.22). The root dry weight of SR1/SR1 grafted controls comprised an average of 4.7%. of the total plant dry weight. For the transformed grafted plants Nt35SC/SR1 and N*trol*C/SR1, the root dry weights were higher, at 5.4% and 6.2% of the total plant dry weight, respectively. These averages are higher than for controls, although statistically are not significantly different ($p>0.05$), due to fairly high variation between samples. However, these results do suggest a tendency to relatively increased root growth in plants expressing *rol*C in the root system.

A highly significant increase in relative root dry weight was observed for Nt35SB+C/SR1 grafted plants ($p<0.001$), with the root dry weight constituting 7.7% of the total plant dry weight at flowering. Thus, while root and shoot biomass of Nt35SB+C/SR1 plants were both considerably larger than in other plants, relative root growth appears to have been enhanced to an even greater degree than shoot growth.

Table 3.5. Average total plant dry weight, root dry weight and shoot dry weight of grafted plants ± standard error of the mean. Plants were harvested at eleven weeks. n = 5-9. Statistically significant differences: *** $p<0.001$.

Graft type (root/shoot)	Total plant dry wt (g)	Shoot dry wt (g)	Root dry wt (g)
SR1/SR1	3.558 ± 0.381	3.391 ± 0.361	0.167 ± 0.022
N*trol*C /SR1	2.663 ± 0.876	2.514 ± 0.854	0.148 ± 0.023
Nt35SC /SR1	4.315 ± 1.420	4.153 ± 1.407	0.162 ± 0.050
Nt35SB+C/SR1	10.567 ± 2.059 ***	9.786 ± 1.922 ***	0.781 ± 0.140 ***

Ntrol*C* rootstock
WT scion

WT rootstock
Ntrol*C* scion

Figure 3.21. Grafted tobacco plants, 4 weeks after grafting. Arrows indicate graft point (bound with waterproof tape). The phenotype of grafted scions do not appear to be affected by the genotype of the rootstock.

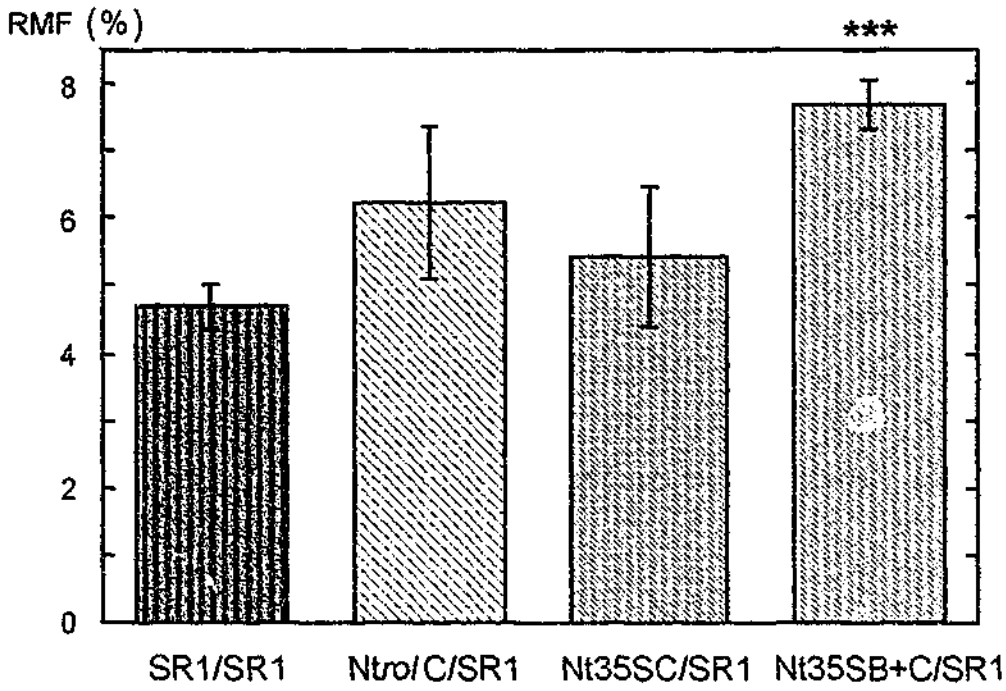


Figure 3.22. Root mass fraction (RMF) of grafted plants. Plants were grown in soil under high-nutrient conditions and harvested at the time of opening of the first flower. Vertical lines represent standard error of the mean, n = 5-9. Statistically significant differences: *** $p < 0.001$.

3.5.2.6. Analysis of A/35S-*rol*C plants

Eight A/35S-C lines analysed. Putative A/35S-C heterozygous transformant progeny (R_1) were produced from crosses between R_0 regenerants and SR1 plants and selected by resistance to kanamycin. Phenotypic analysis of kanamycin-resistant R_1 plants showed that most plants were generally normal and that any changes that were observed were minor compared to the effects on shoot phenotype induced by *rol*C under control of the native promoter. In terms of characteristics commonly altered by *rol*C, namely plant height, internode length and shoot branching, no differences from control plants were evident. The most noticeable feature of most of the putative A/35S-C transformants was a higher tendency for bud drop than normal when plants first commenced flowering, and also some very slight leaf wrinkling. However, rates of bud drop were reduced to normal levels at the second flowering phase. Furthermore, in second-generation kanamycin-resistant (R_2) plants, produced from crosses between R_1 and SR1 plants, differences in leaf phenotypes and flower production were no longer apparent.

The segregation ratio of kanamycin-resistant (Kan^R) to kanamycin-sensitive (Kan^S) R_2 progeny was somewhat variable for different transformant lines. Ratios were

predicted to be 1:1 for the R_2 generation, the progeny of heterozygous Kan^R R_1 transformants backcrossed to an SR1 parent. Deviation from this ratio was observed for A/35S-C8 progeny, with a much higher proportion of Kan^R seedlings than expected (Table 3.6); conversely, a relatively low number of A/35S-C4 and A/35S-C2 R_2 progeny were Kan^R. All A/35S-C6 R_2 seedlings were Kan^S, indicating the likely absence of the Kan^R coding sequence and thus possibly the remainder of the *rol*C construct. Further χ^2 analysis of A/35S-C8 R_2 progeny indicates that the segregation ratio of Kan^R and Kan^S seedlings is 3:1 ($p>0.05$), a ratio consistent with a possible presence in the population of two independently segregating copies of the Kan^R gene.

Table 3.6. Number of kanamycin-resistant and kanamycin-sensitive R_2 progeny of A/35S-C tobacco lines. Seedlings were germinated and grown on M+S medium supplemented with 75 mg ml^{-1} kanamycin sulphate and scored 12 days after sowing. Expected Kan^R:Kan^S segregation ratio for a single transgene copy is 1:1. Segregation ratios were tested by χ^2 analysis; a χ^2 value less than 3.84 indicates that the observed ratio is consistent with a 1:1 ratio. * $p<0.05$.

genotype	Kan^R	Kan^S	χ^2 (1:1)
A/35S-GUS	20	18	0.11
A/35S-C1	39	40	0.01
A/35S-C2	20	50	12.86 *
A/35S-C3	46	41	8.95 *
A/35S-C4	6	17	5.26 *
A/35S-C5	17	15	0.11
A/35S-C6	0	31	31 *
A/35S-C8	49	12	22.44 *

3.5.2.7. Molecular analysis

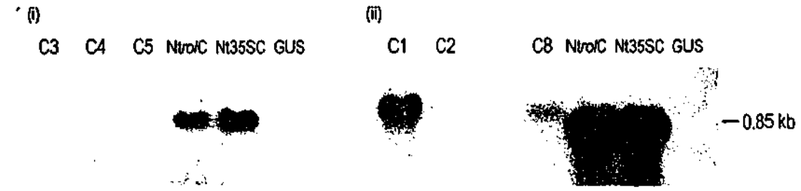
Expression of *rol*C in the A/35S-C lines producing Kan^R seedlings was analysed by Northern blot hybridisation. Some lines were tested further by Southern blot hybridisation to check for possible rearrangements or deletions of the transgene. Plant tissue for Northern and Southern blot analyses were collected from kanamycin-resistant R_2 plants.

Northern blot analysis

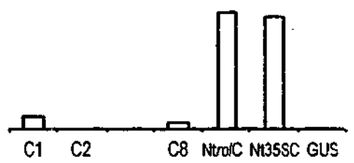
Expression of the *rol*C gene was analysed in root and shoot of plants from six of the A/35S-C lines. Northern blots were probed with the *rol*C coding sequence (Figures 3.23 and 3.24). Root-specific *rol*C expression was detected in roots of A/35S-C8 plants at a low level and no expression was detected in leaves of these transformants. In A/35S-C1 plants, *rol*C transcripts were detected in both leaves and roots, with comparatively high expression levels apparent in both tissues. No evidence of expression was observed in roots or leaves of A/35S-C2, C3, C4 or C5 plants.

The apparent lack of expression in most of these lines may be due to an absence of the transgene in the genome or possibly because of very low *rol*C transcript levels, below the limits of detection of Northern blot hybridisation. Although use of reverse transcriptase PCR (RT-PCR) may have provided this information, further investigation of these lines was not undertaken, as only tobacco lines with *rol*C expression detectable by Northern hybridisation were sought for this analysis. Expression could also be prevented by transgene rearrangements or gene silencing as a result of integration position. Similarly, expression of *rol*C in leaves of A/35S-C1 plants may be caused by the position of integration, which may have placed the construct near an enhancer element directing leaf expression.

(A) *rol*C probe



(B)



(C) Ubiquitin probe

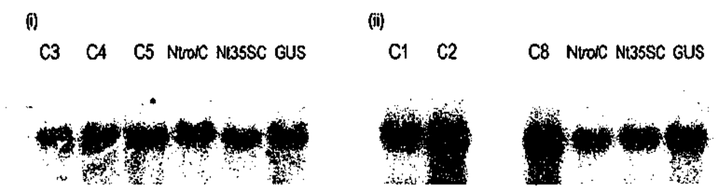
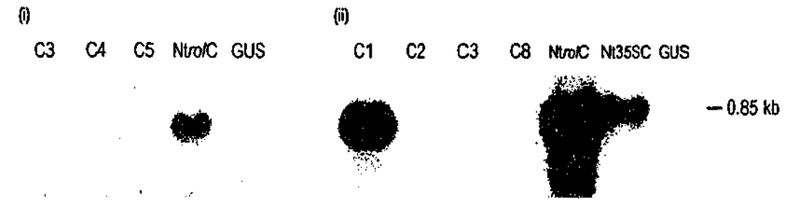
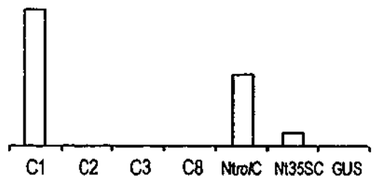


Figure 3.23. Northern blot hybridisations of root total RNA from putative A/35S-C tobacco transformants. Approximately 20 µg of total RNA loaded per lane. Two separate Northern blots (i) and (ii) were carried out with the various lines, shown with the corresponding ubiquitin blots on the left and right of the figure, respectively. (A) Hybridisation to *rol*C probe – (i) N*trol*C control hybridised to the *rol*C probe, (ii) signals correspond to the A/35S-C1, A/35S-C8, N*trol*C and Nt35SC lines; (B) *rol*C expression levels relative to ubiquitin expression levels; (C) Hybridisation to the ubiquitin probe, indicating relative RNA loading levels.

(A) *rol*C probe



(B)



(C) Ubiquitin probe

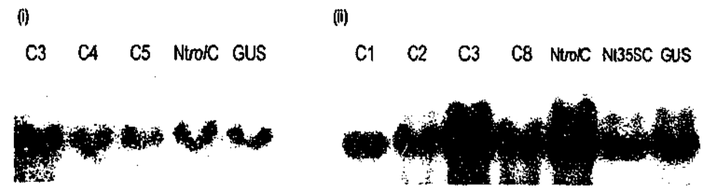


Figure 3.24. Northern blot hybridisations of leaf total RNA from putative A/35S-C tobacco transformants. Approximately 20 µg of total RNA loaded per lane. Two separate Northern blots (i) and (ii) were carried out with the various lines, shown with the corresponding ubiquitin blots on left and right of the figure, respectively. (A) Hybridisation to *rol*C probe - (i) only the N*trol*C control hybridised to the *rol*C probe, (ii) signals correspond to the A/35S-C1, N*trol*C and Nt35SC lines; (B) *rol*C expression levels relative to ubiquitin expression levels; (C) Hybridisation to the ubiquitin probe, indicating relative RNA loading levels

Southern blot analysis

Four of the putative transformant lines were selected for confirmation of transformation with the A/35S-*rol*C construct - these were the two lines in which *rol*C expression was apparent (A/35S-C1 and C8) and two lines in which expression was not detected (A/35S-C2 and C3). The presence of the transgene was detected for lines A/35S-C1 and A/35S-C8, however no hybridisation was evident for A/35S-C2 and A/35S-C3 (Fig. 3.25). This implies that the lack of *rol*C RNA transcripts in these lines was due to absence of the transgene, rather than rearrangements or gene silencing due to position effects.

Surprisingly, the initial selection of A/35S-C3 and C5 seedlings by kanamycin resistance displayed an equal proportion of Kan^R and Kan^S genotypes, even though the presence and expression of the rest of the *rol*C transgene construct was not subsequently indicated. It is possible that the high number of Kan^R seedlings could be due to the separate transfer of the *nptII* sequence conferring kanamycin-resistance, independently of the A/35S-*rol*C construct. This has been noted as a potential characteristic of the pBin19 binary vector, which was used in making this construct, as the *nptII* sequence is located close to the T-DNA right border (RB) element inthis vector (Frisch et al., 1995). The initiation of polar T-DNA transfer from the RB (Zambryski et al., 1989) may thus result in a more frequent transfer and integration of *nptII* without the remainder of the construct.

The A/35S-C2 and A/35S-C4 lines also produced Kan^R seedlings, despite an apparent absence of *rol*C transcript. In these lines, however, Kan^R seedlings were in low proportion to Kan^S seedlings, and a simple genetic explanation for the kanamycin resistance is not apparent. It is possible that T_0 plants were chimaeric for the binary vector T-DNA constructs.

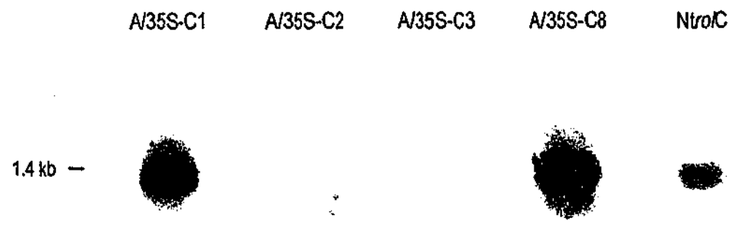


Figure 3.25. Southern blot hybridisation of A/35SC tobacco lines and N*trol*C control line, probed with the *rol*C coding sequence from *A. rhizogenes* A4. Genomic DNA was extracted from leaves of plants and digested with *Eco*RI and *Hin*dIII; approximately 10 µg DNA loaded per lane.

3.5.2.8. Root growth analyses of A/35S-C8 transformants

Further quantitative analysis of the potential effects of root-specific *rol*C expression was focused on A/35S-C8 transformants, as this was the only A/35S-C line in which *rol*C expression was confirmed to be restricted to the roots. The effects of root-localised expression of *rol*C on lateral and adventitious root initiation and root and shoot growth were assessed.

LRP formation in response to auxin in A/35S-C8 seedlings

The procedure used was identical to that used for the lateral root primordia (LRP) analysis of *rol* gene transformant lines (§3.2.2). Seedlings were treated with concentrations of 10^{-4}M, 10^{-5}M or 10^{-6}M IBA or placed on auxin-free medium. Consistent with all other transgenic lines tested, the untreated roots showed no differences in LRP numbers from untreated roots of control seedlings (A/35S-GUS). In a similar pattern to the control and also N*trol*B and N*trol*C transgenic lines, the number of LRP that were produced increased with the IBA concentration, with the peak number of LRP formed in response to 10^{-4}M IBA (Fig. 3.26). No differences between A/35S-C8 roots and controls were observed with 10^{-6}M or 10^{-5}M IBA treatments but 10^{-4}M IBA induced a very highly significant increase ($p<0.001$) in LRP formation in the A/35S-C8 seedlings. An average of 35.21 LRP/cm were formed in A/35S-C8 roots compared to 25.13 LRP/cm in control roots.

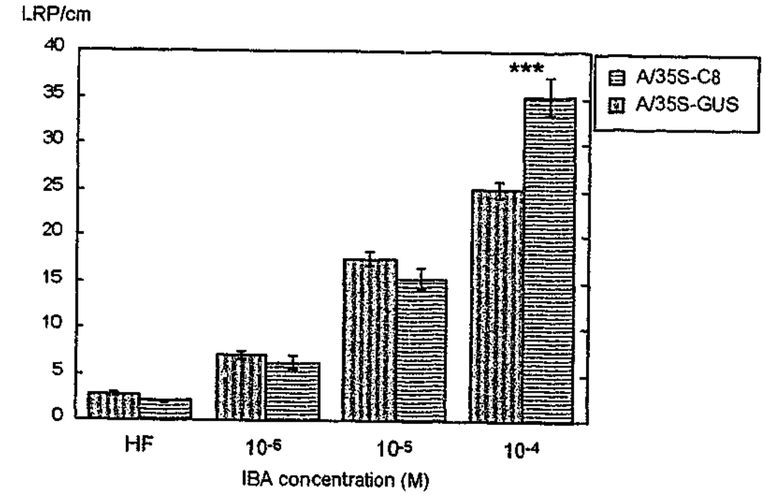


Figure 3.26. Mean number of LRP formed in roots of 11-day-old A/35S-C8 seedlings, with or without IBA treatment. Seedlings were treated with IBA for 72 hours. Vertical lines represent standard error of the mean; n = 7-11. Statistically significant differences between control and A/35S-C8 at each IBA concentration *** $p<0.001$.

Adventitious root formation from A/35S-C8 leaves in response to auxin treatment

Adventitious root (AR) production in leaves of A/35S-C8 transformants was compared with that of controls. Expression of *rol*C was not detected in A/35S-C8 leaves of this line and the results of this assay are consistent with the absence of *rol*C-induced effects on AR formation and the root-specificity of *rol*C expression. A/35S-C8 leaf discs show a similar pattern of AR formation to the A/35S-GUS control leaves, in response to IBA treatment - no significant differences between the two were apparent at any of the concentrations tested (Table 3.7). On hormone-free medium and 10^{-7}M IBA, no roots were produced; with 10^{-6}M IBA, one or two roots at most were usually formed. With the range of concentrations tested, the greatest numbers of roots were produced in response to 10^{-4}M IBA.

Table 3.7. Mean number of adventitious roots per leaf disc of *N. tabacum* transformants containing *rol*C under control of the A/35S promoter. Leaf discs were cultured for 17 days on medium containing IBA. n = 12-15. These leaf discs were cultured using an identical procedure to the previous leaf disc AR analysis (§3.2.3). No statistically significant differences were apparent between control and A/35S-C8 leaves at any of the auxin concentrations tested ($p>0.05$).

IBA concentration	Number of roots per leaf disc	
	A/35S-GUS control	A/35S-C8
Hormone Free	0	0
10^{-7}M	0	0
10^{-6}M	1.3 ± 0.6	1.5 ± 0.7
10^{-5}M	6.4 ± 1.9	7.9 ± 1.7
10^{-4}M	12.1 ± 1.9	16.8 ± 1.9

3.5.2.9. Root and shoot dry weight measurements of A/35S-C transformants.

Root and shoot dry weights of A/35S-C8 plants grown in soil were measured at the time of flowering for each individual plant, similar to the previous experiments with Ntrol*C* transformed plants (§3.5.2.1). The flowering time of A/35S-C8 plants was quite varied, with the earliest plants flowering around 11 weeks after sowing and the last plant at around 14.5 weeks. In contrast, all but one of the A/35S-GUS plants flowered within

eight days of each other, with flowering occurring between 11 and 12 weeks after sowing. Reasons for this difference between A/35S-C8 and A/35S-GUS are not clear, as no evidence of transgene expression was apparent in A/35SC8 shoots. Furthermore, alterations to flowering induced by *rol*C normally involve promotion of early, rather than late, flowering times (present study; Schmülling et al., 1988; Guivarc'h et al., 1996).

The average total-plant dry weights (Table 3.8) of A/35S-C8 plants were comparable to those of A/35S-GUS plants. This is in contrast to the dry weights of Ntrol*C* and Nt35SC plants (Table 3.3), which were much lower overall than control plants due to considerably less shoot growth. As A/35S-C8 plants have a normal shoot phenotype, shoot biomass is evidently not affected by the presence of the *rol*C gene. With regard to relative root growth, the average RMF of A/35S-C8 plant was also very similar to that of A/35S-GUS controls (Fig. 3.27). The root dry weights of the A/35S-C8 plants were a relatively small proportion, slightly less than 4%, of the total plant dry weight at flowering, which were similar to the RMFs of the Ntrol*C*, Nt35SC and Nt35SB+C (non-grafted) plants previously measured (Fig. 3.18).

Table 3.8. Average total plant dry weights of A/35S-C8 and control tobacco plants ± s.e.m. n = 12-14. Plants were grown in soil and harvested at opening of the first flower.

	Total plant dry weight (g)
A/35S-GUS	6.902 ± 0.356
A/35S-C8	7.074 ± 0.752

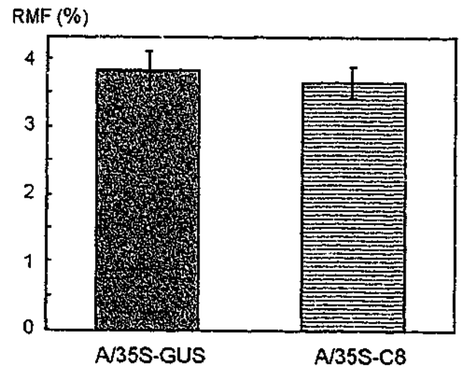


Figure 3.27. Root mass fractions (RMF) of A/35S-C8 and A/35S-GUS plants. Plants were grown in soil with high-nutrient conditions and harvested at opening of first flower. n = 12-14. There is no significant difference between the mean RMFs (%) of the two lines ($p>0.05$).

3.5.3. DISCUSSION

3.5.3.1. Root specificity of *rol*C expression in grafted plants and A/35S-C transformants

The phenotypes of grafted tobacco plants suggested that the *rol*C gene product was not transmitted through the graft union. The presence of *rol*C-transformed roots did not alter the phenotype of WT scions and correspondingly, in the reciprocal grafts, distinct *rol*C shoot phenotypic characteristics were not modified by the untransformed roots. These observations concur with those of Fladung (1990) involving reciprocal grafts with *rol*C-transformed potato and tomato plants, in which shoot phenotype was independent from rootstocks, thereby demonstrating the non-diffusible nature of the gene product. In experiments with *rol*ABC rootstocks of rose (*Rosa hybrida*) grafted with WT scions (van der Salm et al., 1996a), many of the typical pleiotropic effects induced by *rol* genes in shoots were not present, although increased axillary budding was observed.

It was assumed that WT roots of grafted plants were not directly modified by the RolC protein produced in transformed scion tissues - given the evidence of the non-diffusible nature of *rol*C in the reverse grafts, this seems a reasonable inference to make. Conversely, it seems reasonable to assume that *rol*C expression was maintained in *rol*C rootstocks grafted to SR1 shoots.

The shoot phenotype of A/35S-C8 plants also remained normal. Northern blot analysis indicated that most of the A/35S-C lines studied here did not express *rol*C in the roots and, of all the lines analysed, line A/35S-C8 was the only one to show expression limited to root tissue. Clearly, additional independent transformant lines would be necessary for a more conclusive assessment of alterations to shoot morphology, however the A/35S-C8 line demonstrates that the A/35S promoter may enable *rol*C to be specifically expressed in roots, without accompanying negative effects on upper parts of the plant.

3.5.3.2. Root and shoot growth of intact (non-grafted) plants grown in soil

Analysis of growth of whole plants grown in soil showed that, at maturity, the average total mass of A/35S-GUS control plants was much greater than that of *rol*C and 35SC tobacco transformants (Table 3.2). This corresponds with the generally observed smaller stature of *rol*C-transformed plants, which has been reported for a wide range of species (e.g. Schmülling et al., 1988; Fladung et al., 1993; Nilsson et al., 1996b; Winefield et al., 1999). However, actual quantifications of plant masses of *rol*C transgenics are not commonly reported in the literature. In one study, total shoot masses of *rol*C transformed tobacco plants after seed-set were less than half that of WT plants(Scorza et al., 1994).

Comparisons of root-shoot balances showed that relative allocation to the root system was higher in N*rol*C plants than in WT plants, particularly in the early stages of growth. Differences between root growth of control and N*rol*C transformants, although not large, were statistically significant at the earlier timepoints and may possibly be a consequence of an increased propensity for lateral root initiation, as observed in N*rol*C seedling roots (§3.4.1.3). Although no exogenous auxin was applied to soil-grown plants, *rol*C-transformed root cells may have an increased sensitivity to endogenous auxin that could lead to a more frequent stimulation of lateral root primordia than in controls, as discussed in §3.4.2.7. Even a small increase in the probability of each root branching more often would conceivably have a measurable effect on growth (e.g. Hamill and Chandler, 1994). Furthermore, changes in branching frequency and pattern affect the architecture (i.e. spatial arrangement) of a root system, and thus can have potentially significant effects on the function and growth of the root system (Lynch, 1995).

Ontogenetic changes in biomass allocation

The early stages of plant development after germination are a key period for root growth. In general, herbaceous plant species have higher r:s ratios during early stages of development, that decrease as the plant matures (Atwell et al., 1999b; Poorter and Nagel, 2000). This may be due to a need for direction of more resources to root growth during earlier stages as the plants become established in the soil, thus ensuring nutrient and water supply and in turn supporting increasing shoot size (Friend et al., 1994). Ontogenetic changes in plant biomass allocation were also highlighted by observations of Gedroc et al. (1996) in growth experiments with two annual species. Greater active partitioning to roots in the earlier developmental stages of the plants was observed, with the relative rate of root growth versus shoot growth decreasing in later stages of development.

Correspondingly, in the experiments undertaken here, RMFs of tobacco plants decreased over the time period from 7 to 10 weeks after sowing, in accordance with the normal ontogenic development of plants (Atwell et al., 1999b). The more active growth of the root system in earlier growth phases may also have contributed to the greater root biomass of N*rol*C plants compared to controls at these stages, as the relative partitioning of plant resources towards root growth may have further promoted *rol*C-induced initiation of lateral roots. However, presupposing that *rol*C expression remained

consistent throughout growth, the capacity for *rol*C to stimulate root initiation and development may have been reduced as the plant matured, being counteracted by a relative increase in partitioning to shoots.

Shifts in r:s ratios may relate to the fact that *rol*C transgenic tobacco plants flower earlier than normal (Scorza et al., 1994; this study), as an ontogenetic decrease in partitioning to roots associated with the onset of flowering may occur earlier in *rol*C plants. In fact, it could be speculated that earlier increases in relative root biomass (and possibly increased nutrient acquisition) as a result of *rol*C expression may be a causal factor in the earlier flowering in *rol*C transgenic plants.

Plant growth *in vitro* and in soil

In vitro plant growth conditions are clearly different to those of an *ex vitro* environment, in particular factors such as humidity and CO_2 levels and the root zone environment (Kozai et al., 1997). With the fundamental role of these factors in plant growth and plant processes, differences between the growth patterns of plants in soil and *in vitro* are likely.

The RMFs of all tobacco plants grown *in vitro* were higher than that of soil-grown plants harvested at a comparable age (7-8 weeks). Differences were particularly apparent in N*trolC* transgenics, with root proportions of N*trolC* and Nt35SC plants greater than 20% of the total dry weight when grown in the presence of sucrose. It is also noteworthy that when grown *in vitro*, dry weights of N*trolC* plants were not reduced compared to controls as they were in soil; in fact, both N*trolC* shoot and root systems exceeded that of A/35S-GUS controls several-fold when grown on medium containing sucrose.

3.5.3.3. Root and shoot growth of grafted plants and comparisons with non-grafted plants

Root-specific expression of *rol*C and *rol*B in grafted plants stimulated marked increases in root growth, resulting in a higher RMF than in SR1/SR1 grafted plants. The augmented root growth of Nt35SB+C/SR1 plants corresponds with the finding that auxin-induced LRP initiation was the most enhanced in roots of Nt35SB+C seedlings. A greater shoot mass may arise as a result of increased root growth, asthe size of a plant root system is likely to have an effect on the size of the shoot system it can support. Superior growth of roots in Nt35SB+C transformed plants may allow the plant to more efficiently gather nutrients and water from the soil and transfer them to the upper parts of the plant, leading to a correspondingly greater increase in shoot growth.

To further assess the consequences of root-specific expression of *rol*C on root system growth, it is pertinent to compare growth of plants expressing *rol*C exclusively in roots (grafted plants), with that of intact (non-grafted) plants in which *rol*C is expressed in both root and shoot. However, the large differences between the biomasses of grafted SR1/SR1 and intact SR1 plants indicate that considerable alteration to normal growth can occur as a result of the grafting process. Accordingly, actual biomasses of the grafted and non-grafted plants cannot be directly equated, and it is more valid to compare the root and shoot proportions of the respective plants.

In general, roots comprised a higher proportion of the total DW of grafted plants possessing transgenic roots, than the intact transgenic plants. This was most notable in Nt35SB+C/SR1 grafted plants compared to whole Nt35SB+C plants, while the mean RMFs of Nt35SC/SR1 and N*trolC*/SR1 plants were also somewhat higher than mean RMFs of intact plants (§3.5.2.2). Root-specific expression of *rol*C and *rol*B thus produced the greatest increase in relative root growth, when compared to the expression effects of the genes in both root and shoot. However, although LRP analysis indicated that the combined expression of *rol*B and *rol*C may considerably influence the capacity of tobacco roots for lateral root initiation, expression of the two genes in non-grafted plants did not have a significant effect on root growth or r:s ratio. These results suggest that relative root growth of non-grafted Nt35SB+C plants (and, to a lesser extent, Nt35SC and N*trolC* plants) may have been compromised/moderated by effects of expression of the *rol*B and *rol*C genes in shoots.

For example, it is possible that lower chlorophyll levels often found in leaves of *rol*C transformed plants (Fladung, 1990; Chapter 4, this study) may affect the r:s balance of transformed plants. With a correspondingly reduced photosynthetic capability (Fladung, 1990), the capacity of *rol*C-transformed plants to assimilate normal amounts of carbon into photosynthate is altered, which may consequently affect normal growth patterns. According to optimal partitioning models, factors restricting photosynthesis generally cause a relatively elevated distribution of biomass to shoots (Ericsson, 1995). Correspondingly, the fraction of available photosynthate allocated to the root would be reduced (Minchin et al., 1994). Therefore, although *rol*C expression in transgenic plants may have had some promotive influence on relative root growth, the effects may be comparatively diminished by reduced partitioning of dry weight to roots. In contrast, chlorophyll levels in the SR1 shoots of grafted plants are not reduced and it would be expected that normal levels of photosynthate are produced, with an appropriate proportional allocation to roots.

However, while this *rol*C effect may account for the moderate differences between root-specific and whole plant expression of *rol*C and 35S-*rol*C, the magnitude

of the increase in RMF in grafted Nt35SB+C/SR1 plants compared to intact Nt35SB+C plants suggest that expression of *rol*B may have had an additional compounding effect on the r:s balance in intact plants. Expression of *rol*B under control of the CaMV35S promoter in shoots has been shown to result in increased formation of leaves and shoots (i.e. reduced apical dominance) (Schmülling et al., 1988). *rol*B expression has also been associated with a general promotion of apical meristem formation(Altamura et al., 1994), with an apparent involvement in IAA signal transduction (Filippini et al., 1996). It is possible that the stimulation of active meristem formation and leaf growth may favour allocation of resources to shoots and thereby result in a reduced relative allocation of resources to roots in intact Nt35SB+C plants grown in soil, compared to grafted Nt35SB+C/SR1 plants.

Thus, while the *rol*B and *rol*C genes in combination can evidently induce significant increases in root growth when expressed specifically in roots, it seems likely that this effect may be mitigated by the concurrent expression of the genes in the aerial tissues. This suggests again the value of obtaining appropriate root-specific expression of these genes for agricultural applications, in terms of achieving useful alterations in root growth.

3.5.3.4. Growth conditions

As the plant growth environment is fundamental to plant growth responses and r:s balance (Wilson, 1988; Ericsson, 1995), environmental factors must be taken into account in comparing plant growth. In this study, care was taken to standardise as many of these factors as possible to allow comparable measurements of growth to be made. External conditions of light, temperature and nutrition for all plants were kept as uniform as possible.

The specific environmental conditions of these tobacco growth experiments included provision of high nutrient levels and ample supply of water, to ensure non-limiting conditions. According to the root-shoot equilibrium models of Brouwer and Thornley, substantiated by the results of many studies (reviewed in Wilson, 1988; Ericsson, 1995; Poorter and Nagel, 2000), plants would be expected to allocate relatively less biomass to roots when grown under high nutrient conditions with ample water, compared to growth in low nutrient and low water conditions. Thus, investment in maximal root growth was probably not necessary for optimum growth in the current experiments. Although some augmentation of relative root growth was apparent in transgenic lines expressing *rol*C, it is possible that an increased capacity for root

branching may be more fully utilised in low nutrient conditions. Further investigation of growth of *rol*C transformants under different nutrient and water regimes would be relevant to obtaining a clearer picture of the extent of the potential for *rol*C to alter root growth.

As the root and shoot system of a plant are fundamentally interrelated, the use of root:shoot balance (r:s or RMF) as a measurement of growth may blur observation of effects on root growth in itself. Increased root system growth is often accompanied by increased shoot growth and therefore may not necessarily result in significantly higher overall r:s ratios. In the present study, this was observed for N*trol*B and A/35S-C8 transgenics cultured *in vitro* with 3% sucrose. In addition, as the mass of root systems often constitute a small fraction of the total plant weight, relatively small increases in root growth may not have much noticeable impact on the overall r:s ratio. However, the ratio of root to shoot biomass is a comparable trait that is relatively straightforward to determine and is commonly used as a measurement for growth analyses (Wilson, 1988; Ericsson, 1995; Poorter and Nagel, 2000).

3.5.3.5. Alterations to root growth in A/35S-C8 tobacco

Expression of *rol*C under control of the A/35S promoter appears to have some influence on LRP induction, with a significant increase in LRP compared to controls in A/35S-C8 roots treated with 10^{-4}M IBA. This suggests a degree of enhanced sensitivity to auxin caused by root-specific activity of *rol*C and is consistent with observations of *rol*C and 35SC genotypes (§3.4.1.3).

No notable distinctions between the numbers of adventitious roots formed on control and A/35S-C8 leaf discs were recorded. As expression of *rol*C was apparent only in roots of the A/35S-C8 tobacco plants, significant modifications to adventitious root formation from leaf tissue were not likely. This presumption is corroborated by the results of the AR assay. This contrasts with events observed in N*trol*C and Nt35SC leaves, in which *rol*C was expressed, promoting moderate but significant increases in AR differentiation.

The somewhat increased capacity for LRP formation of A/35S-C8 roots in response to auxin was not subsequently evident as an increase in the overall root mass of whole plants at flowering. It is possible that larger differences existed earlier in development, as observed in N*trol*C and Nt35SC transformants, however if present, these differences did not lead to higher overall dry weights of plants or earlier flowering. A substantial difference in root mass of A/35S-C8 plants would probably not be

anticipated, given that differences between RMFs of N*rol*C or Nt35SC plants and control plants are not large, even with the much higher *rol*C expression levels conferred by these stronger promoters. As demonstrated in the Northern hybridisation (Fig. 3.23), low levels of *rol*C expression are conferred by the A/35S promoter in roots of these transformants, particularly compared to the expression from the *rol*C promoter or the CaMV35S promoter, and any resulting modifications would be likely to be correspondingly moderate. As noted by Benfey et al. (1989), substantially higher GUS expression levels were conferred in tobacco roots by the CaMV35S promoter (both domains A and B) than by domain A alone. For example, GUS enzyme activities, quantified in roots of 15 day old seedlings, were almost 10-fold higher with the GUS gene controlled by domains A+B, compared to the activity driven by domain A.

3.5.3.6. A/35S-*rol*C expression levels and patterns

Expression of *rol*C was detected in both roots and leaves of A/35S-C1 plants but the very high level of expression exhibited in leaf tissue is somewhat anomalous. Although CaMV35S domain A was observed to direct some expression to tobacco leaf and stem tissues (Benfey et al., 1989), histochemical staining and quantification of GUS enzyme activity indicated this expression to be at very low levels. Contrary to observations of Benfey et al. (1989) in mature plants, Elmayan and Tepfer (1995) did not observe any histochemical staining resulting from 35S domain A expression in leaves of mature tobacco plants and did not detect a difference between leaf GUS activity in 35S domain A and control plants. It is quite likely that the position at which the *rol*C construct integrated into the A/35S-C1 genome was influenced by a *cis*-element causing enhanced expression in leaves; alternatively, interactions between *trans*-factors and *cis*-elements of the transgenic promoter may alter expression patterns. Benfey et al. (1989) similarly noted enhanced GUS expression in seedling cotyledons of one particular domain A-GUS line, in addition to the normal strong root expression.

Transgene expression levels may also vary depending on the independent transformation event. Benfey et al. (1989) observed variations between independent transgenic plants in the intensity and extent of expression conferred by CaMV35S domain A. Possible reasons suggested for this were differences in copy number or position effects due to integration at different sites. Lindroth et al. (1999) compared the GUS expression conferred by three root-specific promoters, in four plant species. Differences in expression patterns and levels were evident between the four species, indicating the possible variability in expression conferred by a promoter due to factors

such as plant species or individual transformant. In apple plants transformed with the GUS gene controlled by the *rbcS* tissue specific promoter from tomato, GUS expression was found to vary between different transgenic lines (Gittins et al., 2000). Expression levels were either positively or negatively affected by the presence of multiple transgene inserts, and were probably influenced by other factors, such as the position of integration.

Although the relatively low level of *rol*C expression conferred by A/35S promoter in the A/35S-C8 line is not necessarily abnormal for this promoter, it is possible that further transformations may produce plants with higher levels of root-specific *rol*C expression and consequently greater increases in root initiation and root mass. A larger number of transformant lines would be required to establish a more detailed estimation of the range of *rol*C expression levels possible from this construct. In addition, as discussed previously in §3.5.3.4, it is possible that cultivation of plants in low-nutrient conditions may favour a more extensive promotion of root initiation in *rol*C-transformed roots. Ontogeny of the A/35S-C plants may also have influenced biomass allocation to roots. As observed in the N*rol*C transgenic tobacco, allocation to roots, and hence r:s ratio, may be relatively higher earlier in development of these transformants.

3.5.3.7. Possible alternative promoters for root-specific expression of *rol* genes

In addition to CaMV35S domain A, a number of other potentially appropriate root-specific promoter and *cis*-element sequences have been reported. These include promoters of two genes isolated from tobacco, *HRGPnt3* (hydroxyproline-rich glycoprotein) and *TobRB7*, and elements of both the *rol*C and *rol*D promoters which direct expression predominantly to roots.

Expression of the *HRGPnt3* gene is highly localised and is restricted to pericycle cells involved in LR initiation; specifically, pericycle cells which subsequently become part of the new root tip (Keller and Lamb, 1989). Expression has also been detected in AR initial cells (Vera et al., 1994). HRGPs are a class of structural proteins in plant cell walls and the function of the *HRGPnt3* gene product may be to reinforce the cell wall of lateral root tips as they emerge through the main root. Strong expression of the gene occurs in the pericycle cells during early lateral root initiation but is greatly diminished once the LR begins to emerge through the cortex of the parent root. Curiously, Lindroth et al. (1999) found this pattern of *HRGPnt3*-directed GUS expression only in tomato roots and not in tobacco roots, even though the gene was originally isolated from tobacco. These

differences could have been due to position effects or possibly an absence of other *cis*-elements in the construct that are required for expression in tobacco.

TobRB7 is expressed early in root meristems and immature central cylinder regions, with expression occurring from as early as two days post-germination (Yamamoto et al., 1991). In contrast to *HRGPnt3*, root-specific expression of *TobRB7* in lateral roots was observed to begin at the initiation stage and continue throughout LR development.

Deletion analysis of the *rol*C and *rol*D promoters identified regions which direct expression primarily to roots (Leach and Aoyagi, 1991). By analysis of GUS activities resulting from *rol*C-GUS gene fusions, a 417 bp section of the *rol*C promoter (5' flanking sequence of *rol*C gene) was shown to direct expression predominantly to the roots in tobacco, with activity 5-fold higher in roots than in leaves. However, the level of GUS activity in both roots and leaves was considerably reduced (by approximately 25%) compared to the maximum level conferred by the 872 bp region 5' of the *rol*C gene.

The *rol*D promoter in intact form was shown to direct a high level of expression throughout young tobacco plants, with levels up to three times that of the CaMV35S promoter (Leach and Aoyagi, 1991). Expression of *rol*D in Ri T-DNA transformants was predominantly directed to roots and this root-specificity was further increased by truncation of the promoter to a 373 bp region, resulting in a root activity 20-fold higher than in leaves. Expression levels from the truncated *rol*D promoter were much higher than those conferred by the 35S domain A (Elmayan and Tepfer, 1995) and also the root-specific *rol*C promoter region (Leach and Aoyagi, 1991).

GUS expression conferred by other root-specific promoters, *RSI-1* (Root System Induced-1) and *sam-1* (S-adenosyl-L-methionine synthetase), were investigated in four different plant species (Lindroth et al., 1999). The *RSI-1* gene, an auxin-responsive gene isolated from *Lycopersicon esculentum* (Taylor and Scheuring, 1994) was expressed in this species specifically at the initiation of lateral root development. *sam-1*, isolated from *Arabidopsis*, was expressed in root vascular tissue (Peleman et al., 1989). Using promoter-*GUS* fusions, Lindroth et al. (1999) found GUS staining resulting from these promoters mainly localised in lateral root meristems of *Arabidopsis*, tobacco and tomato, with *sam-1* also expressed in roots of *Pinus contorta* and in vascular regions in *Arabidopsis* and tomato.

3.5.3.8. Root-specific promoters for potential use with *rol* genes - general conclusions

The results of the LRP analyses in §3.4 suggested that both *rol*C and *rol*B may increase the capacity of transformed root cells to initiate LR in response to auxin. As treatment with exogenous auxin generally increases the frequency of LR formation, by stimulating additional pericycle cells to differentiate, this indicates that these cells are competent to differentiate but may require higher than normal auxin concentrations to do so. It appears that root-specific expression of *rol*C, particularly in conjunction with *rol*B, may have some potential to produce stimulatory effects on root growth, without inducing adverse effects on upper plant growth and development. Therefore, to promote increased lateral root formation by *rol* gene expression, an effective promoter may be one that directs *rol*C+*rol*B expression to pericycle cells, increasing the auxin-sensitivity of these normally unresponsive cells and stimulating the LR differentiation process.

The localisation of expression in pericycle cells by domain A of the CaMV35S promoter (Benfey et al., 1989) raised the possibility that it may be a potentially suitable promoter for root-specific regulation of *rol*C+*rol*B. Preliminary experiments undertaken here, involving the *rol*C gene, are somewhat inconclusive but suggest an increased sensitivity to auxin in A/35S-*rol*C transgenics with respect to LRP initiation *in vitro*. In terms of overall r:s biomass ratios of plants grown in soil, divergences from controls were clearly not significant. However, when grown *in vitro* in medium with or without sucrose, A/35S-C8 plants were larger than controls. Low *rol*C expression levels in the A/35S-C8 line may have limited the effectiveness of the transgene, and a greater number of transformant lines would clearly give a better indication of the potential influence of this 5' regulatory sequence when fused to *rol* genes.

Placement of *rol*C+*rol*B under transcriptional control of some other suitable promoters or regulatory elements, such as the *TobRB7* promoter, would also be a relevant approach for further expansion of this study of root-specific expression of *rol*C. Preliminary work towards this objective has been carried out, with PCR amplification and subsequent subcloning of the *TobRB7* promoter into a vector carrying *rol*C. Further work remains to confirm the efficacy of the *rol*C construct, which can then potentially be transformed into tobacco and also species of agronomic interest, such as white clover (*Trifolium repens*) (see Chapter 4).

Clearly, careful experimental analysis would be needed to assess whether any of these promoter elements fused to *rol* genes may lead to root-specific expression in appropriate cell types and, consequently, to altered r:s relationships.

3.6. CONCLUSIONS AND FUTURE DIRECTIONS

Although the results of the present study are not conclusive, it appears that the *rol*C and *rol*B genes have some capacity to increase the relative root biomass of transgenic tobacco plants. Both genes, separately and particularly in combination, increase the sensitivity of tissues to exogenously supplied auxin with respect to initiation of LRP.

Consistent with an increased capacity to initiate lateral roots, expression of *rol*C and *rol*B resulted in increases in overall mass of both roots and shoots in plants grown *in vitro*, when an ample supply of reduced carbon was made available. Grafting experiments involving a WT scion with transgenic rootstocks also suggest that root-specific or predominant expression of these genes, if obtained at appropriate levels and possibly in appropriate cell types, may have the capacity to increase both the r:s ratio and the overall mass of plants. This may be due to an enhanced capacity to take up nutrients and/or water from the soil. The present study did not investigate the effects of altering nutrient or water supplies to *rol* gene transgenic plants. A comparative study would thus be needed to evaluate the effects of altering these parameters, before any conclusions could be drawn with respect to the potential agricultural applications of introducing *rol* genes intro crop plants, with a view to altering root system growth.

A model for *rol* gene action was devised in an endeavour to account for observed effects of *rol* gene expression upon auxin-induced LRP formation. Further experimentation is required to test the validity of the model, particularly with regard to the influence of the auxin:cytokinin ratio. Treatment of roots with cytokinin at concentrations greater than 10^{-7}M is known to be generally inhibitory to lateral root formation and root elongation, even in the presence of otherwise optimal levels of auxin (Wightman and Thimann, 1980; MacIsaac et al., 1989; Pelosi et al., 1995). However, if N*trol*B and N*trol*C transformants have an increased capacity to perceive auxin, with respect to LRP formation, the model predicts that auxin:cytokinin ratios in these roots may not be affected by exogenous cytokinin application to the same extent as normal roots. The levels of exogenous cytokinin at which lateral root differentiation is inhibited may therefore be higher for these transgenic roots than for normal roots. An analysis of LRP formation in roots treated with a range of cytokinin concentrations could thus add further information relating to the suggested model of altered auxin sensitivity in *rol*-transformants, with respect to lateral root initiation.

Stability of transgene integration and variations in expression levels are relevant considerations for the potential usefulness of transgenic plants. Expression levels of *rol*C in the Nt35SB+C line used here were fairly low and *rol*B transcript levels were high (Fig. 3.6). This combination may be favourable for the effects of *rol*B and *rol*C with

respect to promotion of LR induction and root system biomass. In contrast, AR production from Nt35SB+C leaves was not increased compared to *rol*C leaves. An optimal AR formation response may require higher or lower expression levels of *rol*C or *rol*B respectively; alternatively, it is possible that *rol*B and *rol*C may have had antagonistic effects on each other in terms of AR formation. Further investigations would be required to determine whether different expression levels may affect LR or root growth differently, as this may be a possible disadvantage of the use of *rol*B+*rol*C in combination. In terms of promoting greater root biomass in particular plants, both AR and LR formation may be relevant targets for improvement. For example, in species such as perennial clovers and alfalfa, persistence and productivity are dependent on efficient adventitious (nodal) root production, in addition to a requirement for adequate LR formation from AR roots (see Chapter 4).

The transgenic lines used in this study were selected as phenotypically representative examples of each line, as reported in the literature (Spena et al., 1987; Schmülling et al., 1988). Expression levels in each of the transgenic lines used were evidently appropriate to induce typical shoot phenotypes, although other independently transformed lines could conceivably have different *rol* gene expression levels.

Aspects such as differential expression of the *rol*B+*rol*C combination, by placement under control of alternative promoters, are relevant for future work. Expression of *rol*C under transcriptional control of domain A of the CaMV35S promoter was shown to have potential effects on root system growth, with increased initiation of LR in response to auxin. However, *rol*C expression in A/35S-C8 transformants did not result in significant promotion of root growth in soil, although it is possible that production of more transformant lines to obtain stronger *rol*C expression or expression of *rol*C under control of other root-specific promoters may produce different results. A more comprehensive study than the one undertaken here would be needed to properly evaluate the potential of this promoter fragment.

CHAPTER 4

CHARACTERISATION OF EFFECTS OF *rolC* EXPRESSION IN *TRIFOLIUM REPENS*

4.1. INTRODUCTION

Trifolium repens, white clover, is a long-lived perennial legume, extensively distributed worldwide and the principal forage legume utilised in pastures in most temperate regions (Frame et al., 1998). Although generally considered as a temperate region species, many white clover varieties have a high tolerance for more extreme climates of cold and heat and poor soil conditions (Duke, 1981). A large number of cultivars and ecotypes of white clover have been described and classified from locations worldwide, along with a considerable diversity of adaptations for survival in different environments (Williams, 1987b; Frame et al., 1998). In a 1997 world checklist, at least 319 different white clover cultivars and ecotypes were listed (Caradus and Woodfield, 1997), although not all are commercially utilised.

Many new white clover cultivars have been developed over sixty years of breeding in various countries (Woodfield and Caradus, 1994). In general, efficiency of breeding programs have been improved by international exchange of germplasm and better screening for superior genotypes. Examples of traits that have been enhanced or combined in new white clover cultivars by breeding include leaf sizes, plant height, stolon densities, forage yield, disease resistance and tolerance to climate stresses such as cold and drought (Duke, 1981; Woodfield and Caradus, 1994).

Adaptations to sub-optimal conditions, such as periodic cold or drought, clearly influence the suitability of cultivars to their particular environment. In addition, adaptability can depend on growth characteristics such as persistence, plant height and flowering time. For example, small-leaved cultivars generally have lower short-term yields but higher persistence and grazing tolerance (Williams, 1987b; Woodfield and Caradus, 1994; Frame et al., 1998). Small leaves and shorter internodes have also been associated with resistance to winter stress (Williams, 1987b; Frankow-Lindberg, 1999). Some growth attributes have been useful for the introduction of alternative cultivars to particular regions or climate types, which may not be conducive to local varieties; in one example, Duke (1981) refers to a winter-hardy *T. repens* cultivar introduced to Canada

from Russia, which subsequently produced higher yields than previously introduced cultivars. Mediterranean cultivars with heat and drought tolerance have been beneficial in Australia and New Zealand - for example, the widely used cultivar Haifa was developed in Australia from germplasm sourced from Israel (Williams, 1987b; Frame et al., 1998).

4.1.1. Overview of white clover structure and development

White clover form and development have been extensively reviewed by Thomas (1987) and Frame et al. (1998). The following overview of white clover form anddevelopment is referenced from these sources and others as cited.

In the early stages of vegetative growth, for approximately 4 weeks after germination, white clover plants remain in rosette form, with an upright stem. The primary root of seedlings develops into a tap root, from which an extensive system of lateral roots is formed. Subsequently, white clover acquires a stoloniferous growth habit, with stolons - elongated, horizontally growing stems - emerging from the primary seedling axis. Stolons consist of a series of internodes separated by nodes, from which leaves, adventitious roots and axillary buds are initiated (Fig. 4.1). Leaves are trifoliate with upright, extended petioles.

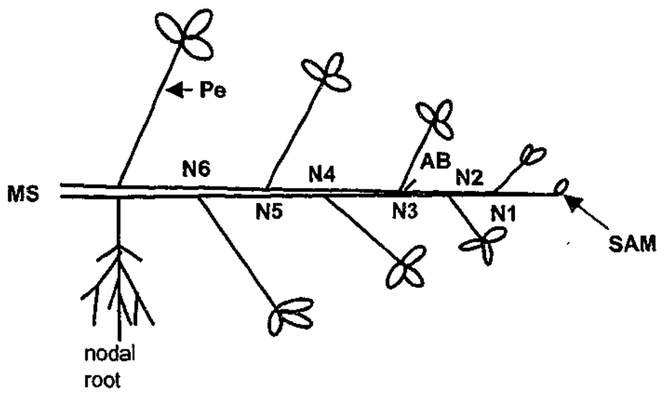


Figure 4.1. Schematic representation of white clover stolon structure, indicating the formation of nodes (N) along the main stolon (MS). Leaves are produced at nodes alternating from upper and lower surface of stolon. Nodal roots are produced from primordia initiated at the axillary bud, generally on the underside of the stolon. SAM, shoot apical meristem; P, petiole; AB, axillary bud.

As the stolons spread laterally from the centre of the plant, secondary stolons branch out, forming a network of stolons supported by adventitious (nodal) roots. This structure is the basis of the ability of white clover to persist and spread vegetatively in pastures. Axillary buds, formed at the leaf axil, either develop into laterally branching stolons or into inflorescences, depending on the prevailing climate. In dry, warm conditions and long days, flower production is favoured, whereas vegetative persistence by stolon growth is promoted in cooler temperatures.

Each stolon node typically produces a trifoliate leaf and two root primordia, one each above and below the axillary bud, on the upper and lower surfaces of the stolon. As development of the primordia into roots requires moist conditions, with a relative humidity of at least 90% (Stevenson and Laidlaw, 1985), only the lower primordia in contact with soil are usually located in a microenvironment conducive for further differentiation. The upper primordia generally remain inactive. Nodal roots can subsequently develop into fully functional root systems, with prolific lateral root branching, providing stolons with anchorage and the capacity to gain nutrition independently of the main axis. The primary axis does not usually persist for more than 2-3 years (Duke, 1981) and as new growth centres are formed along the rooted stolons, the connections between the stolons and the primary axis break down. As older stolons also eventually die, vegetative persistence of clover in pasture is dependent on continued lateral branching and extension of the stolon network, and production of nodal root systems to support individual stolons.

Nodal roots further assist in persistence of white clover by promoting the burial of stolons beneath the soil surface. Stolon burial, initially attributed to animal trampling and wormcasts, was also observed to occur in greenhouse-grown plants where these factors were not present (Cresswell et al., 1999). Stolon nodal roots were demonstrated to have an ability to contract and actively draw stolons down to or below the soil surface and this was suggested to be a developmental adaptation to promote clover persistence, by holding stolons more firmly to the soil and helping to resist uprooting by grazinganimals (Cresswell et al., 1999). In some environments, stolon burial may also have a role in protecting stolons from frost during winter (Grant et al., 1991). It was suggested by Cresswell et al. (1999) that there may be potential for enhancement of this characteristic by improved breeding or management strategies.

Production of seed can also be a factor in clover persistence. As noted above, white clover flowering and consequently seed production are promoted by hot and dry climate conditions, which are not conducive to vegetative growth. This developmental

feature may help maintain persistence of a clover stand in conditions in which there may not be enough water for individual plants to thrive. A certain proportion of seed produced in white clover pastures are hard seed with impermeable seed-coats, which can remain viable for many years in soil before germination. In non-mechanically harvested samples, hard seed can comprise as much as 97.5% of seed collected (Pederson and Brink, 2000) although this proportion is likely to be closer to 10% with mechanical harvesting, due to damage caused by the process (Frame et al., 1998). Production of hard seed may be an adaptation to ensure survival of a white clover stand in case of unsuccessful establishment of soft seed in adverse environmental conditions (Frame et al., 1998).

4.1.1. Agronomic applications of white clover

White clover is a highly nutritious animal feed and is commonly used as grazed pasture or harvested for hay and seed. In some warmer climates, white clover is grown in pastures as a winter annual crop (Duke, 1981; Frame et al., 1998). In Australia, white clover is generally grown in mixed pastures with grasses, commonly phalaris and perennial ryegrass (*Lolium perenne*), for dairy pasture (Kemp and Culvenor, 1994). The balance between the companion species is important for clover performance, which is more susceptible to adverse conditions and competitive disadvantage than the grass species (Mason, 1993). The white clover content of dairy pastures is also a key factor in milk production, due to its high nutritive value. In 1993, the average Australian dairy pasture clover content of 20% was calculated to be worth around $400m to the Australian dairy industry (Mason, 1993). It was estimated at the time that an increase in the white clover content of pasture from 20% to 50% would add more than $100m to that value, even if overall pasture biomass were not increased. Further potential gains were estimated to be as much as $450m for a 30% increase in pasture biomass produced entirely from additional white clover growth (Mason, 1993).

As a legume, white clover has a valuable and significant role in contributing to the nitrogen (N) economy of mixed pastures by fixation of atmospheric nitrogen (N_2). Nitrogen fixation by white clover proceeds from a specific symbiotic association with *Rhizobium leguminosarum* biovar *trifolii* (*Rl trifolii*) (Frame et al., 1998). N_2 fixation in grazed swards in New Zealand was estimated at an average of up to 400 kg N ha^{-1} annually (Crush, 1987). Similarly, analysis of the N accumulation by white clover and

pea (*Pisum sativum*) crops indicated that both crops generated net N gains in the field, due to N_2 fixation of 327 kg N ha^{-1} and 286 kg N ha^{-1} for clover and pea respectively (Kumar and Goh, 2000). The contribution of N to fields by white cloverand other legume crops have been found to potentially reduce or eliminate the need for additional N fertilizers for subsequent wheat crops, for at least 1-2 years (Holford and Crocker, 1997).

Further applications for white clover crops have also been described. An established white clover stand can function as a cover crop, providing soil stabilisation and reducing erosion and also enriching the N content of depleted soils (Duke, 1981; Kumar and Goh, 2000). Ground cover provided by legume species *T. repens* and *T. alexandrium* were found to be involved in reducing erosion rates, during land restoration of motorway embankments (Andrés and Jorba, 2000). White clover has also been found to be a possible alternative to herbicide treatments, for suppressing weed vegetation in tree plantations. In experiments with ash tree plantations, white clover showed some potential for weed suppression and did not appear to be as competitive with trees, not restricting restrict tree growth as much as other ground cover treatments tested (Willoughby, 1999).

4.1.2. Improvement of white clover crops

The importance of white clover as a pasture crop in many regions worldwide has made it the focus of much research to develop strategies for its improvement. In Australia, persistence of white clover in pasture is an objective of high priority (Mason, 1993; Lane et al., 1997). Many detailed analyses have been carried out on white clover persistence during drought and winter stresses and growth under different soil nutrition, temperature and moisture conditions. Also, effects of environmental conditions such as high CO_2 and ozone levels and management of grazing regimes and crop rotation to improve long-term viability have been extensively studied. As indicators of growth, parameters frequently measured include leaf size and rate of appearance, stolon extension rates and density, stolon branching, number of rooted nodes, internode length, shoot and root biomass production and partitioning.

The vegetative persistence of white clover has been shown to be inversely correlated with yield. A high yield of biomass is obtained from plants with large leaves and long petioles, while persistence is associated with a higher density of stolons and correspondingly small leaves (Williams, 1983; Evans et al., 1992). The better

persistence of small-leaved cultivars may also relate to their closer proximity to the ground and therefore not grazed as heavily (Evans et al., 1992). Cultivars tolerant to cold climate are also usually small-leaved and tend to predominate in soils of low fertility (Williams, 1983; 1987b). Cultivars of intermediate leaf sizes (e.g. cv. Grasslands Huia) can provide a balance between adequate persistence and good yield. Although there is apparently a tradeoff between productivity and persistence, improved root system growth is potentially of benefit to both attributes.

Improved root system growth and function are of particular importance in areas where soil conditions are not optimal and also where plants may experience seasonal stresses such as drought or flooding. In fact, soil and water limitations are considered to be key factors affecting white clover production (Mason, 1993). For example, restricted root growth resulting in low pasture yields has been a reported problem in sub-optimal soil conditions in Australia, such as in irrigated pastures in northern Victoria (Blaikie et al., 1988; Lane et al., 1997). Available water is restricted by shallow topsoil, the zone most accessible to the roots, and the dense subsoil is difficult for roots to penetrate. Although physical modification, such as breaking up of subsoil, and other improvements to soil management have been shown to improve yield (Blaikie et al., 1988), an ongoing need to promote rapid root proliferation of white clover has been indicated for Australian pastures (Blaikie and Mason, 1993), to achieve more productive shoot growth. An improved capacity for strong and rapid growth of the root system is likely to promote utilisation of water and nutrients and consequently support greater shoot yield and persistence, as well as strengthening anchorage during grazing (Blaikie and Mason, 1993; Lane et al., 1997; Caradus and Woodfield, 1998).

Blaikie and Mason (1993) demonstrated that good productivity of white clover pastures, in terms of shoot growth, is dependent on a prolific root system. In particular, large, highly branched root systems were associated with the most productive shoots. Caradus and Woodfield (1998) observed positive and high correlations between leaf size and taproot characteristics (i.e. number and diameter of vertically penetrating roots) in measurements with 11 different clover genotypes. Evidence of the role of the root system on white clover crop growth was also demonstrated by selection for plants with large root diameter and medium leaf size (Caradus and Woodfield, 1998). These selected lines were found to have better growth and survival in comparison to plants of the cultivar Huia, which have medium leaves and small taproots, and also in comparison with groups selected for small or large leaves. Furthermore, selection for increased ratio

of root weight dry weight (DW) to total plant DW was shown to result in improved growth and persistence in a dryland environment, compared to Huia plants.

Other important considerations for clover pastures include the nutritive quality of the forage, biomass productivity and clover content in the sward (Mason, 1993; Lane et al., 1997). Compatibility with companion grass species, with *Rhizobium* bacterial strains and with insect pollinators are also relevant aspects (Williams, 1983). Improvement of pest resistance is a crucial objective, particularly as it is likely that achieving increases in the clover content of pastures will correspondingly increase the need for improved disease and pest control (Mason, 1993). Pathogens and pests known to affect white clover include a wide range of viral and fungal diseases, nematodes and insects (Duke, 1981; Burgess and Gatehouse, 1997; Frame et al., 1998), which can have detrimental effects on growth, persistence and quality (Mason, 1993; Dudas et al., 1998). One of the most widespread viruses is white clover mosaic potexvirus (WC1MV) which has a world-wide occurrence and also attacks other clover and legume species. In investigating the impact of WC1MV on white clover in NZ pastures, Dudas et al. (1998) found that in mature pastures, an average of 69% of plants were infected with WC1MV. In field trials, significant negative effects on growth were measured in plants inoculated with the virus, which produced 36.5% less dry weight than uninfected plants over three harvests. This reduction in growth was correlated with an effect on stolon elongation, an important determinant of plant yield and competitiveness in pasture swards.

4.1.3. Strategies for genetic improvement of white clover

Improvement of clover and other forage legumes have been carried out by traditional breeding and selection methods since the 1930s (Woodfield and Caradus, 1994), generally through phenotypic recurrent selection (Williams, 1987b). Commonly utilised for cross-pollinated species such as clovers and alfalfa, the basis of this breeding method is a cyclical selection and intercrossing of individuals with the desired phenotype (Bowley, 1997). In each successive cycle, individuals are selected from an improved population so that over several cycles, the frequency of plants in the population with the desired traits can be increased substantially. Phenotypic recurrent selection is often combined with progeny testing, whereby performance characteristics of progeny are examined, before selecting parents for crossing (Bowley, 1997; Frame et al., 1998). This

helps to minimise possible effects, in choosing individuals for crossing, of phenotypic aberrations in the parent lines that may be caused by environmental factors.

A high degree of natural variation has been found between different clover populations for leaf size, stolon density and branching and root characteristics - taproot diameter and proportion of nodal to total root dry weight - which are relevant to breeding for improved persistence in dryland regions (Caradus and Woodfield, 1998; Lane et al., 2000). The potential for improvement of white clover crops by selection for these characteristics was demonstrated by Caradus and Woodfield (1998), with selection for increased taproot diameter with medium leaf size or selection for increased root weight ratio resulting in higher shoot yields in dry conditions.

Genetic improvement of white clover over the last six decades, in terms of yield and percentage content in the sward, has been estimated at a rate of 6% gain per decade (Woodfield and Caradus, 1994). This was reported to be a higher level than that determined for the forage crops alfalfa (*Medicago sativa*), perennial ryegrass (*Lolium perenne*) and Italian ryegrass (*Lolium multiflorum*). The increases in genetic improvement of white clover were suggested to be largely due to better screening for superior genotypes and utilisation of suitably adapted ecotypes in breeding programs. Inbreeding to reduce the number of recessive deleterious alleles is a further approach to genetically improving white clover.

Limitations to traditional breeding methods may include a lack of genetic variability for the particular traits desired, inadvertent adverse effects on other attributes or difficulty in uncoupling correlated traits, for example large leaves and large taproots. Genetic modification techniques have increased the possibilities for improvement of clover growth and productivity, including the introduction of useful genes from other plant species and genes conferring resistance to insects and viruses, as well as the ability to study aspects of development such as nodulation (Díaz et al., 1995; Burgess and Gatehouse, 1997; Dudas et al., 1998; Sharma et al., 1998).

Initial difficulties with genetic transformation of white clover were encountered due to lack of an *in vitro* regeneration procedure that would be suitable for a wide range of genotypes. As white clover is an outbreeding and genetically diverse species (Williams, 1987b), the ability of different genotypes to regenerate from tissue explants *in vitro* is variable (White and Voisey, 1994). An early transformation method, involving *Agrobacterium* inoculation of stolon internode segments, was limited to a particular rare genotype with a high regeneration capacity, WR8 (White and Greenwood, 1987). However this genotype was not considered to be desirable in terms of agronomic traits (Voisey et al., 1994).

Several successful regeneration methods have since been developed that are independent of genotype and have consequently facilitated the transformation of white clover (White and Voisey, 1994; Beattie and Garrett, 1995; Larkin et al., 1996). These methods involve *Agrobacterium*-mediated transfer of transgenes and regeneration from 3-day old cotyledonary stalks of germinated seed (Voisey et al., 1994) or cotyledonary tissue of imbibed seed (Beattie and Garrett, 1995; Larkin et al., 1996). Reported transformation frequencies of the different methods vary considerably, ranging between 1% and 50%.

Transformation of white clover with the gene coding for the coat protein of white clover mosaic potexvirus (WC1MV) was reported in 1998 (Dudas et al., 1998). Genes encoding enzymes that may disrupt metabolic activity in insects also have been shown to have potential effects on increasing insect resistance in white clover and other agronomically important species such as rice (Duan et al., 1996; Burgess and Gatehouse, 1997). These include *Bacillus thuringiensis* Bt endotoxin, protease inhibitors, α-amylase inhibitors and lectins.

Enhancement of forage quality is one important objective for white clover and other forage legumes that is highly relevant to the dairy, meat and wool industries. The availability of adequate sulfur-containing amino acids to sheep has a significant influence on wool production, however normal levels in forage legumes are relatively low and are degraded easily in the animal rumen. Sharma et al. (1998) increased the levels of sulfur amino acids accumulating in leaves of white clover, by stable expression of a sulfur-rich storage protein, δ-zein, from maize. Similarly, sulfur-rich sunflower seed albumin has been introduced into subterranean clover (*Trifolium subterraneum*) to improve nutritional quality (Khan et al., 1996).

In recent work involving the introduction into white clover of the cytokinin biosynthesis gene, isopentenyl transferase (*ipt*), under control of a senescence-related promoter, the induction of a delay in leaf senescence in *ipt* transgenic plants was demonstrated (Ludlow et al., 2000). A delay in plant senescence brought about by the developmentally regulated overproduction of cytokinin was suggested to have possible applications for extending the growth period of transgenic clover plants, subsequently resulting in increased crop biomass and seed production.

Diáz et al. (1989) introduced a pea lectin gene, *psl*, into clover to facilitate nodulation by a *Rhizobium leguminosarum* biovar (*Rl viciae*) that normally nodulates roots of pea, lentil and sweet pea but not white clover roots. Functional expression of the *psl* gene and targeting of the protein to root cells susceptible to infection (Díaz et al., 1995) allowed nodulation of white clover roots to occur following infection with *Rl viciae*.

Thus, a host plant-specificity barrier for nodulation was removed by the introduction of the *psI* gene.

4.1.4. Potential applications for *rol* genes in white clover improvement

As discussed in preceding chapters, transformation of plants with the *rol* genes from *Agrobacterium rhizogenes*, individually or in combination, have been found to induce phenotypic alterations in a range of plant species transformed with these genes. In particular, certain characteristics of the *rol*C phenotype correspond with traits which are considered to have potential commercial value for agricultural and horticultural crops, including a number of pasture, orchard and ornamental species (Lambert and Tepfer, 1991; Fladung et al., 1993; Frugis et al., 1995; Giovannini et al., 1999; Winefield et al., 1999). Traits of potential interest for white clover improvement include increased root branching and growth, reduced plant stature, reduced apical dominance and modifications to flowering and fertility, all of which may conceivably be advantageous to the persistence of plants in the field.

Several legume species have been characterised for effects of transformation with Ri T-DNA from wild-type *A. rhizogenes*. These include analysis of regenerants of bird's-foot trefoil (*Lotus corniculatus*) and adventitious roots of white clover, red clover (*Trifolium pratense*), alfalfa (*Medicago sativa*) and siratro (*Macroptilium atropurpureum*), a tropical forage legume (Beach and Gresshoff, 1988; Webb et al., 1990). However, there have been few reports of legumes transformed with specific Ri T-DNA genes; these include analysis of *rol* gene effects in *L. corniculatus* (Pozárková et al., 1995) and alfalfa (Frugis et al., 1995). These will be discussed at a later point in relation to the results of the experiments presented in this chapter.

The experimental objectives of the work presented in this chapter were to generate transgenic white clover plants expressing the *rol*C gene from *A. rhizogenes* and to determine the effects of the gene on plant phenotype and selected growth parameters. The cultivar from which transgenic plants were generated, Haifa, is a white clover variety utilised in Australian dairy pastures. Confirmation of transformation and expression were obtained, followed by quantification of a range of phenotypic traits frequently measured in growth analyses of white clover. The observed effects are discussed in the light of *rol*C effects common to other species and possible mechanisms for these alterations. The potential for *rol*C to improve agriculturally useful traits are discussed as well as aspects of interest for further investigation and improvement.

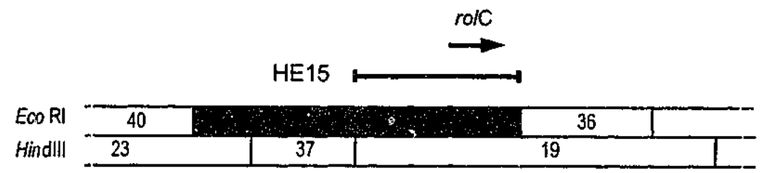
4.2. MATERIALS AND METHODS

4.2.1. WHITE CLOVER TRANSFORMATION

*rol*C construct and bacterial strains

The *rol*C sequence utilised for transformation of *T. repens* originated from *A. rhizogenes* strain A4 and was carried on fragment HE15, produced by a *Hin*dIII digest of A4 TL-DNA *Eco*RI fragment 15 (Fig. 4.2). The HE15 fragment placed in the vector pBin19 had previously been transferred into *A. tumefaciens* strain AGL0. The resulting strain, designated AGLO/HE15, was then used in the transformation (an overnight AGLO/HE15 culture incubated at 25°C). Transformation of white clover with strain AGLO/pBI121, carrying the pBI121 vector (Jefferson, 1987), was also attempted, to recover transgenic plants containing the GUS gene for use as controls. The presence of the GUS gene would not be expected to have an effect on phenotype.

(A)



(B)

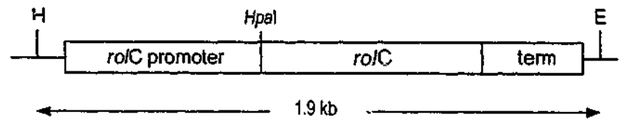


Figure 4.2. (A) Restriction map of section of Ri TL-DNA from *A. rhizogenes* A4 containing *rol*C (arrow): shaded section is *Eco*RI fragment 15, bracketed line represents fragment HE15 obtained by *Hin*dIII and *Eco*RI digests. (B) Schematic diagram of *rol*C gene and flanking regions on the HE15 fragment.

Transformation of *T. repens*

Trifolium repens cv Haifa was transformed with *rol*C using the method of Beattie and Garrett (1995). The transformation procedure involved *Agrobacterium*-mediated transfer of the gene in a binary vector and subsequent regeneration of whole plants from immature embryos via adventitious shoot organogenesis.

Seeds of *T. repens* cv Haifa were surface sterilised with a series of washes - 70% ethanol for 3 minutes, 1.5% hypochlorite for 40 minutes, 70% ethanol for 3 minutes then 6-8 rinses with sterile water. Seeds were then left in water to imbibe at 15°C for 15-17 hours. After this time, the seeds were dissected under microscope in sterile conditions. The seedcoat was removed to release the cotyledonary-stage embryo, which was then cut to separate the cotyledons from the developing hypocotyl, leaving a small portion of the cotyledon stalk. Around 300 cotyledons were dissected for each transformation procedure. The cotyledons were separated into individuals and collected in MG medium, before incubation with an overnight *A. tumefaciens* AGLO/HE15 culture for 40 minutes (log phase culture, approximately 10^9 cells). The excess liquid was then removed and the explants transferred to Regeneration Medium (RM73) (see §2.2.1) and left to cocultivate for a period of three days. After this time, the explants were rinsed several times in sterile water and transferred to selection medium (§2.2.1) containing cefotaxime and kanamycin. The explants were subcultured onto new selection medium every 3 weeks, with developing adventitious shoots excised and placed onto Root Induction Medium (§2.2.1). *In vitro* light and temperature conditions are specified in §2.2.2.

Transformant lines and controls

After regeneration, five independent transformants were obtained. These are designated as Trrol C1 (*Trifolium repens rol*C1) through to Trrol C5. Initial plans to obtain a greater number of transformants were hindered by excessive bacterial overgrowth, which was not sufficiently alleviated by antibiotic treatments and killed many explants. Consequently, the number of viable explants was markedly reduced, limiting the number of independent transformants that could be obtained.

The generation of white clover transformant lines expressing GUS gene for use as controls was not successful and as a result, untransformed wild-type (WT) clover lines derived from seed have been utilised as controls. Although generated via different processes, transgenic and WT lines were subsequently been treated identically and all plants cultivated in a similar growth environment. Both *in vitro*-grown and greenhouse plants of transformed and untransformed controls were maintained though many cycles of subculturing and repotting before evaluation of phenotypic traits and Southern and Northern blot analyses. Vegetative propagation involved repotting plants at regular intervals of 3-4 months, discarding older plant matter and retaining new growth, so that plants came to consist of completely fresh growth. Through this procedure, many individual clones of each transformant line were produced for experimental purposes.

DNA and RNA analyses of transformants

Genomic DNA and total RNA extractions, restriction enzyme digests and Southern and Northern blot analyses were carried out as described in §2.1.1. DNA and RNA for Southern and Northern analyses were extracted from the leaves of plants grown in soil in greenhouse conditions, approximately one year after transformation. The second Northern analysis was conducted approximately three years after transformation on soil-grown plants, which had been repotted at regular intervals during that time.

4.2.2. PHENOTYPIC MEASUREMENTS

Greenhouse temperature and light conditions

Temperature and lighting conditions are described in §2.2.2. Plants of different lines were placed in random positions in the greenhouse bay to minimise the effect of potential biases that may arise due to physical location.

Leaf protein content, chlorophyll content and leaf dry weight

The following data on characteristics of chlorophyll and protein content and internode length were obtained from intact soil-grown plants that had been vegetatively propagated for a period of approximately three years. Healthy young leaves of similar age were collected from soil-grown *rol*C-transformed and WT plants. Leaves were sourced from a number of different individual plants of each line. Each trifoliate leaf was separated into single leaflets, with one leaflet used for analysis of chlorophyll content, the second assayed for protein content and the third leaflet for measurement of dry weight. Leaflets corresponding to each particular leaf were labelled, so that possible correlations between these three characteristics could be checked.

Protein content

The protein contents of WT and transformant leaves were measured. Total water-soluble protein was extracted from leaves with 0.75 ml buffer (50 mM Tris-HCl pH 8.0; 10 mM ß-mercaptoethanol). Samples were centrifuged and the protein concentration measured using the method of Bradford (1976), using a dye reagent from Bio-Rad Protein Assay kit. Bovine serum albumin (BSA) was used as a protein standard.

Chlorophyll content

Chlorophyll levels of WT and *rol*C-transformed leaves were assayed by extraction with acetone in a method similar to MacLachlan and Zalik (1963). One leaflet was ground in 80% acetone, in a final volume of 0.75 ml. The extracts were centrifuged and 150 µl of supernatant added to 1350 µl of 80% acetone. Optical density of this solution was then measured at 645 nm and 663 nm and the chlorophyll concentrations calculated using the following equations (MacLachlan and Zalik, 1963).

$$\text{Chl } a = \frac{(12.3\ OD_{663} - 0.86\ OD_{645})\ V}{d \times 1000 \times W}$$

$$\text{Chl } b = \frac{(19.3\ OD_{645} - 3.6\ OD_{663})\ V}{d \times 1000 \times W}$$

Chl *a* = chlorophyll *a* concentration (mg g^{-1} fresh weight)
Chl *b* = chlorophyll *b* concentration (mg g^{-1} fresh weight)
OD = optical density, at wavelength indicated
V = volume of extract (ml)
d = length of light path (cm)
W = fresh weight of leaf (g)

Internode length

The internode length was measured as the distance between the second-and third-last nodes on stolons of *rol*C-transformed and WT plants (except for Tr*rol*C3 plants, which do not form stolons). This ensured that results would not be biased by selection of nodes for measurement.

New root and leaf production from stolon segments grown hydroponically

Quantitative measurements of root and shoot production were made in plants cultivated hydroponically from a stolon segment consisting of a single node with one leaf. Explants were taken from a number of individual plants of each transformant line. Each explant was placed in a cube of rockwool in small pots, with open bases to allow roots to grow through freely (Fig. 4.3). Pots were placed in individual containers, 11 cm in diameter, into which quarter-strength modified Hoaglands nutrient solution was added (see §2.2.1). The containers were covered with aluminium foil to minimise algal growth (Fig. 4.3). Plantlets were grown for five weeks, with Hoaglands solution replenished every three days. At five weeks, the numbers of leaves produced were counted and petiole length (height) and root and shoot dry weights were measured.

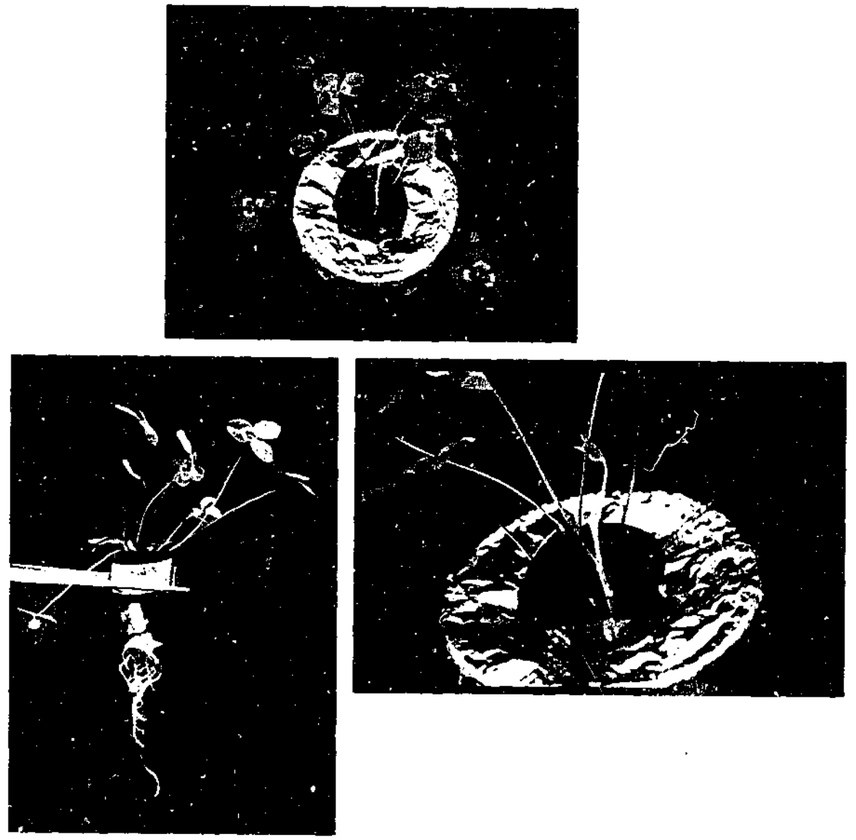


Figure 4.3. Hydroponic system for growth of plants from stolon segments. Stolon segments, each consisting of a single node and leaf with no nodal roots, were placed in rockwool cubes which were placed in pots standing in foil-covered containers (top, bottom right). These explants have been photographed after five weeks. The bottom of the pots were cut out, allowing roots to grow through freely (bottom left).

4.3. RESULTS

4.3.1 Confirmation of presence and expression of *rol*C in transgenic white clover lines

Southern blot hybridisation

To confirm the successful integration of the *rol*C gene into the putative transformants, Southern blot analysis was undertaken on genomic DNA extracted from leaves of greenhouse-grown plants and probed with the *rol*C coding sequence. Hybridisation bands of 1.9 kb were detected, corresponding to the expected size of the structurally intact *rol*C gene (Fig. 4.4A).

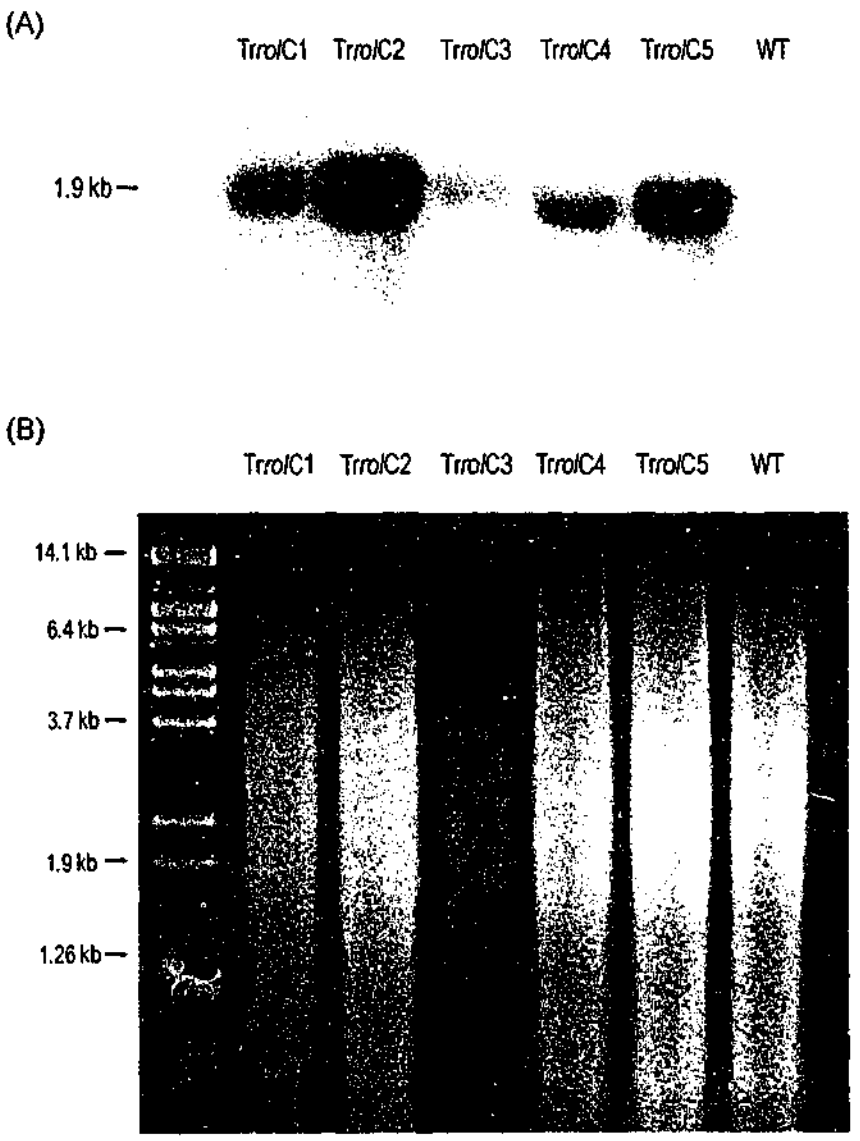


Figure 4.4. (A) Southern blot hybridisation of genomic DNA from leaves of Tr*rol*C transgenic and WT clover lines probed with *rol*C coding sequence from *A. rhizogenes*. DNA was digested with restriction enzymes *Hind*III and *Eco*RI. (B) Agarose gel of the digested DNA prior to blotting; approximately 10 μg DNA loaded per lane, with the exception of a reduced level of Tr*rol*C3 DNA.

With the exception of Tr*rol*C3, the amount of genomic DNA loaded onto the initial agarose gel (Fig. 4.4) was similar for all lines, so the intensity of the hybridising bands for these lines may serve as an indication of relative copy number. The weak signal for Tr*rol*C3 transformants is evidently due to the much smaller amount of DNA loaded on the agarose gel and copy number is difficult to estimate for this line from this Southern blot. Of the other lines, Tr*rol*C2 appears to have the highest number of integrated copies, followed by Tr*rol*C5 and Tr*rol*C1; in comparison, Tr*rol*C4 has a relatively weak signal, implying the presence of fewer integrated copies of the *rol*C gene.

For further estimation of the *rol*C copy number in each line, genomic DNA was digested with a single restriction enzyme, *Eco*RI, and probed with the *rol*C sequence (Fig. 4.5 A, B). With random integration of the gene into the genome, multiple copies will most likely be placed at different distances from *Eco*RI sites each time and therefore will be evident as hybridising bands of different sizes. The *rol*C construct does not contain internal *Eco*RI sites (Fig. 4.2); thus, each band probably represents one transgene copy. The hybridisation results suggest that the Tr*rol*C1 genome contains two *rol*C gene copies, Tr*rol*C3 and Tr*rol*C5 each contain three copies and the Tr*rol*C2 genome contains 4 copies.

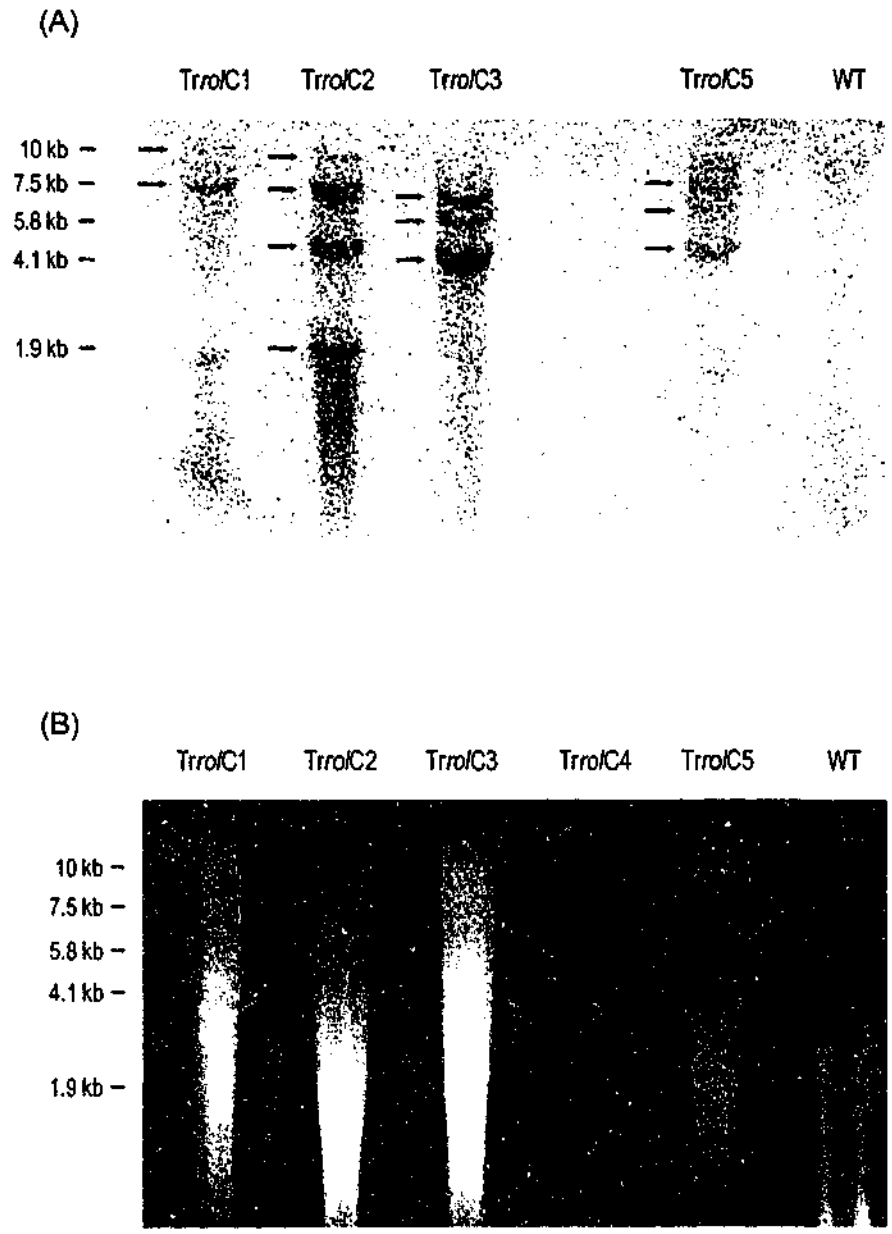


Figure 4.5. (A) Southern blot hybridisation of genomic DNA from leaves of T*rrol*C transgenic and WT clover lines to assess transgene copy number. DNA was digested with restriction enzyme *Eco*RI. Arrows indicate position of hybridisation bands. (B) Agarose gel electrophoresis of digested DNA prior to blotting; some unevenness in loadings is apparent.

The amount of T*rrol*C4 genomic DNA available for this second Southern blot was very limited and a detectable signal was not obtained. However, a lower T*rrol*C4 copy number, relative to the other *rol*C lines was suggested by the first Southern hybridisation. As T*rrol*C1 appeared to contain two copies of the transgene, it seems likely that T*rrol*C4 is transformed with a single *rol*C gene. As no evidence of expression of the gene in the T*rrol*C4 transformants has been observed, from either the Northern hybridisations or in phenotypic effects on the plants, determining the precise T*rrol*C4 copy number was of minimal value in terms of evaluating this particular transformant line. Possible future work on *rol*C inactivation in transformed clover may make obtaining this information a higher priority.

Northern blot hybridisation

Hybridisation with the *rol*C DNA probe shows that strong *rol*C transcript levels were detected in leaves of three of the independent lines - Tr*rol*C1, Tr*rol*C3, Tr*rol*C5. No expression of *rol*C was apparent in leaves of Tr*rol*C2 or Tr*rol*C4 plants (Fig. 4.6).

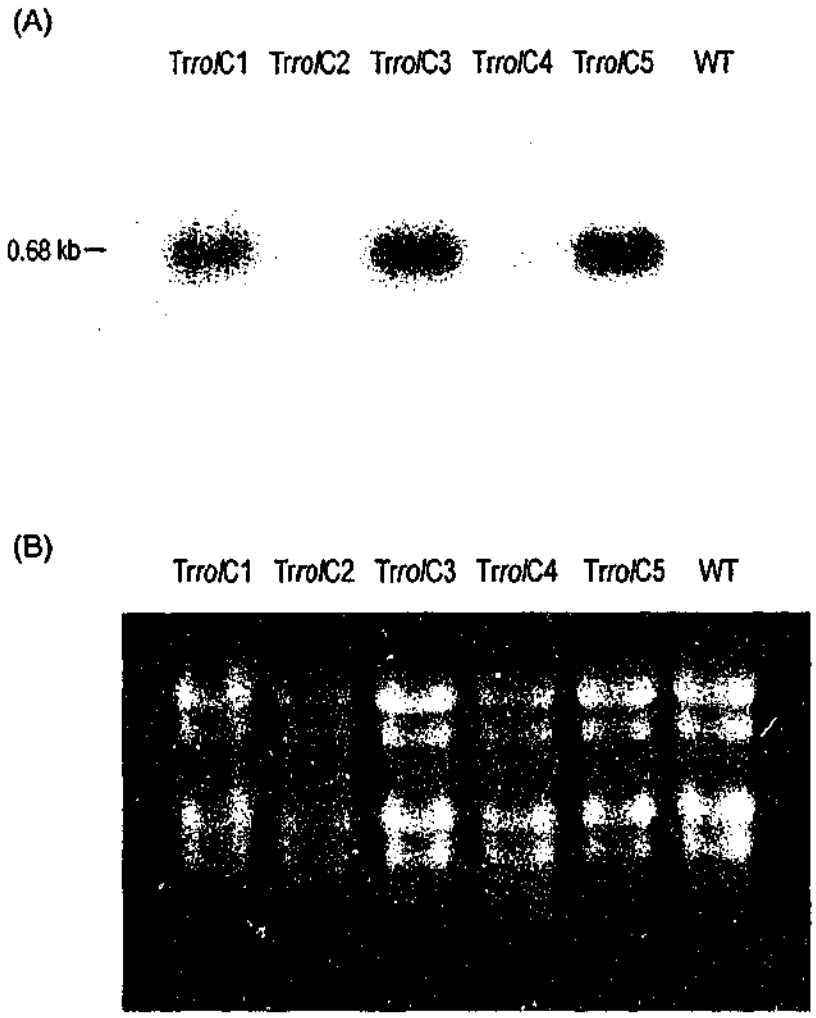


Figure 4.6. (A) Northern blot hybridisation of total RNA from leaves of Tr*rol*C transgenic and WT white clover lines, probed with *rol*C. (B) Agarose gel electrophoresis of clover leaf RNA. Approximately 20 μg of total RNA was loaded per lane.

Over the course of approximately 3 years, clover plants were maintained in a greenhouse and periodically repotted. The continued expression of the transgene after this period of time was assessed, by a second Northern blot of RNA extracted from plant material harvested at this later date. Expression was also examined separately in roots and leaves of transformant and WT plants - the expression of *rol*C in transgenic plants at this time was detected mainly in roots of Tr*rol*C3 and Tr*rol*C5 plants (Fig. 4.7), with very low transcript levels detected in leaves of Tr*rol*C3. These results raised the possibility

that a loss of expression in T*rol*C1 and T*rol*C5 leaves may have occurred over this period. However, as shoot phenotypic differences characteristic of *rol*C remained constantly evident in T*rol*C1 and T*rol*C5 plants, a more probable interpretation is that of continued expression of *rol*C in these plants but at a lower rate, leading to low transcript levels that may not have been detected by this Northern blot hybridisation. Slight phenotypic alterations observed in T*rol*C2 and T*rol*C4 plants may also be due to these transformants expressing *rol*C at low levels, below the limits of detection by Northern hybridisation; alternatively, these subtle differences may be due to variations in the genetic backgrounds of individual plants.

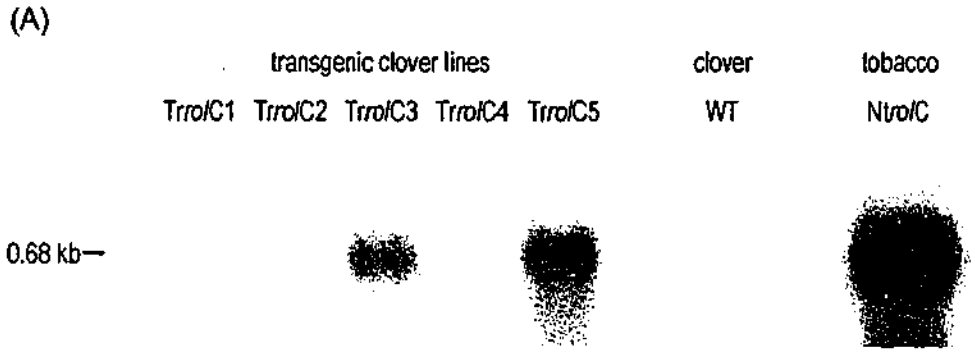


Figure 4.7. Expression of *rol*C in roots of transgenic white clover lines, after three years cultivation in greenhouse with regular repotting. (A) Northern blot hybridisation of total RNA from roots of T*rol*C transgenic lines, WT white clover line and *rol*C transgenic tobacco, probed with *rol*C. (B) Agarose gel electrophoresis of clover and tobacco RNA. Approximately 20 μg of total RNA was loaded per lane. Northern blot of RNA from shoots is not shown, due to low transcript levels.

4.3.2. Phenotypic analysis of Tr*rol*C white clover lines

A variety of distinct phenotypic characteristics were evident in several Tr*rol*C transformants, compared to non-transgenic controls, with specific modifications differing between the transformant lines. These alterations were found to be stable, persisting over three years and throughout many regular plant repottings. Phenotypic modifications of Tr*rol*C lines are summarised in Table 4.2, at the end of the Results section.

The following qualitative descriptions of phenotype, as well as data quantifying characteristics of internode length, chlorophyll and protein content, were obtained from intact soil-grown plants which have been vegetatively propagated over the course of three years, as described above. Measurements of root and shoot production were made in plants grown hydroponically from a stolon segment consisting of a single node and leaf.

4.3.2.1. Qualitative descriptions of phenotype

Leaves

Leaves of Tr*rol*C2 and Tr*rol*C4 plants appeared slightly smaller than WT leaves. Tr*rol*C3 and Tr*rol*C5 leaves were generally smaller than WT and also somewhat lanceolate, with a slightly wrinkled appearance (Fig. 4.8, 4.9). These Tr*rol*C3 and Tr*rol*C5 plants were also more compact than normal plants, with shorter leaf petioles than WT plants (Fig.4.10, 4.11). Tr*rol*C1 and Tr*rol*C5 plants were found to regularly produce leaves with four leaflets instead of the usual three (Fig. 4.8G), which rarely occurred, if at all, in other lines.

Stolons

By visual inspection, stolons of Tr*rol*C1, Tr*rol*C2 and Tr*rol*C4 plants appeared indistinguishable from WT stolons. A striking characteristic of Tr*rol*C3 plants was the near absence of stolon growth. The leaves of Tr*rol*C3 transformants instead tended to grow as a compact clump, with most leaves growing upwards from a central base (see Fig. 4.12). There did not appear to be any extension or lateral branching of stolons in these plants. Tr*rol*C5 plants were observed to have shorter stolons, with nodes and leaves produced much more closely together than in the WT and other transformant lines (Fig. 4.12).

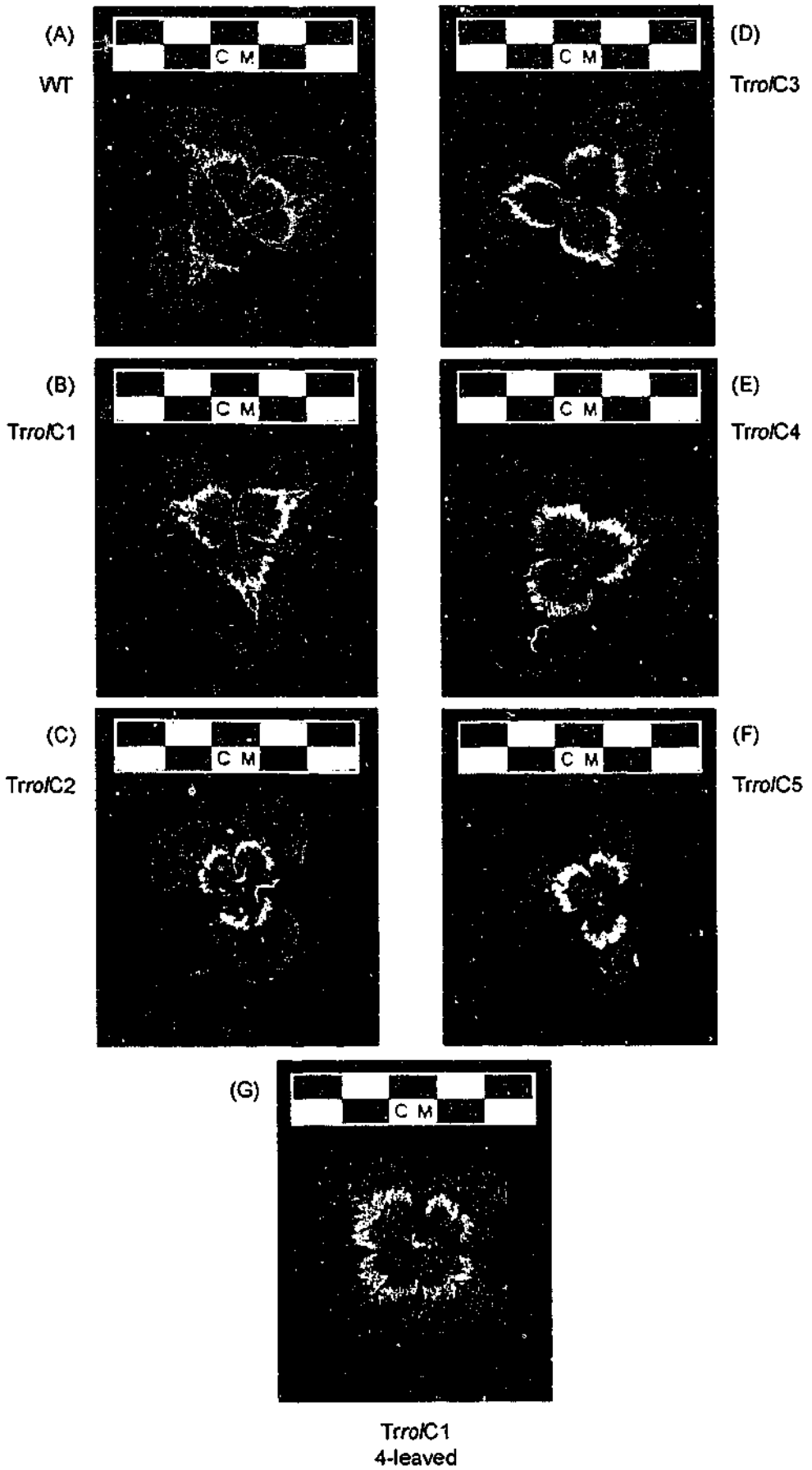
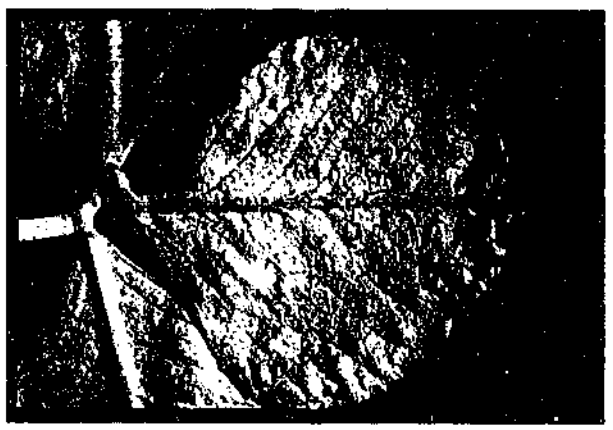


Figure 4.8. Single leaves of T*rrol*C and WT clover lines, including a leaf with four leaflets from the T*rrol*C1 line. T*rrol*C1, T*rrol*C3 and T*rrol*C5 leaves are smaller than WT. Leaves of T*rrol*C1, T*rrol*C3 and T*rrol*C5 are somewhat lanceolate. Some wrinkling is also apparent on the edges of the T*rrol*C3 and T*rrol*C5 leaves (see Figure 4.9 for closer view).

TrrolC3

TrrolC5



WT

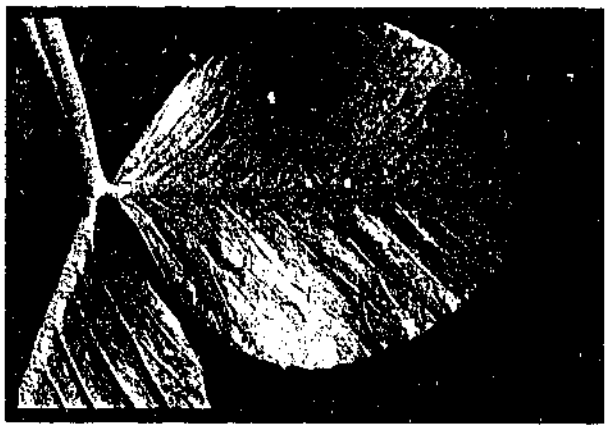
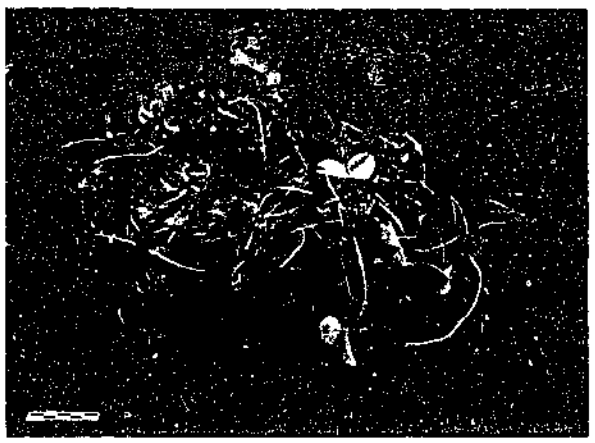


Figure 4.9. Close view of underside of leaflets from Tr*rol*C3, Tr*rol*C5 and WT plants, highlighting *rol*C-induced leaf wrinkling.



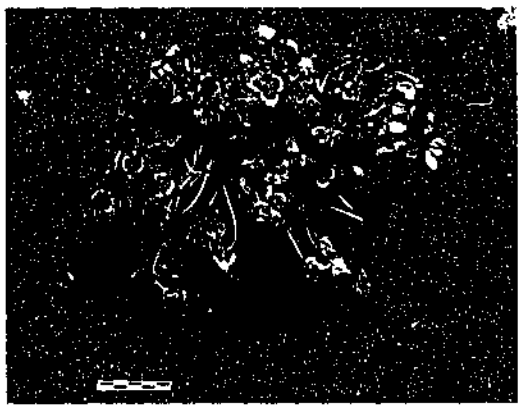
WT

Tr*rol*C3

Tr*rol*C1

Tr*rol*C4

Tr*rol*C2



Tr*rol*C5

Figure 4.10. Tr*rol*C transgenic and WT plants grown in soil. Note the extremely compact form of Tr*rol*C3 plant and the shorter stolon length of Tr*rol*C5 transformant (due to shortened internodes).

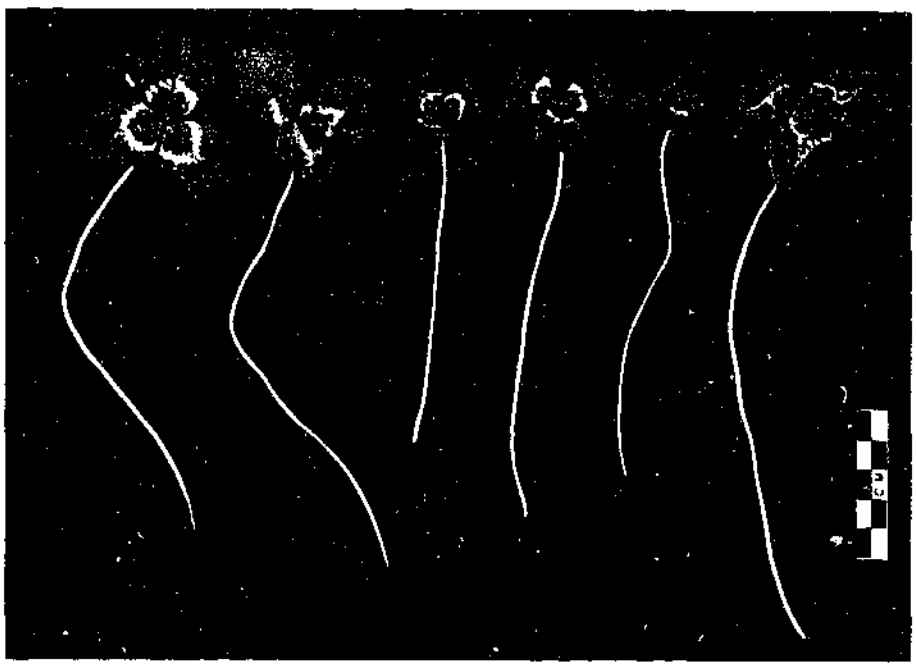
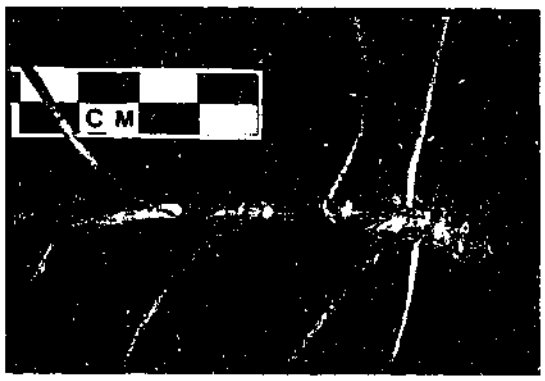
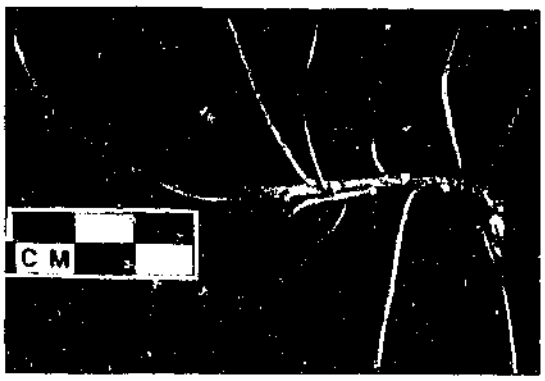


Figure 4.11. Single leaves of approximately same age from each Tr*rol*C and WT line, excised at the base of the petiole.

WT
stolon segment



Trrol C5
stolon segment



Trrol C3
stolon segment

Figure 4.12. Stolon segments of WT (top) and Trrol C5 (middle) plants, at the same scale. Trrol C5 stolons have shorter internodes and produce a greater density of leaves than WT stolons. Trrol C3 plants (bottom) have extremely shortened internodes, so that successive leaves often appear to grow from the same point, producing clumps of leaves. These transformants do not spread laterally like WT plants.

WT

T*rolC*5

Figure 4.13. Adventitious (nodal) root formation on stolons of soil-grown T*rolC*5 and WT plants. Canopy was lifted from the soil to show the underside for photographic purposes. The proliferation of adventitious nodal roots from stolons of the *rolC* transgenic plant is clearly visible, in contrast to the WT plant.

Root formation

T*rol*C5 plants produced a noticeably greater density of adventitious roots from stolons in comparison to WT plants (Fig. 4.13). No obvious differences in root morphology were apparent in any of the transformants.

Flowering

An increased tendency to flowering was a noted feature of T*rol*C1 transformants, which produced flowers more readily and under more variable environmental conditions than the WT and other transgenic plants. Both controls and other transformants either did not flower at all or flowered very rarely, under the long day (16 hr) conditions in which they were grown.

4.3.2.2. Quantitative phenotypic analysis of Tr*rol*C white clover lines

(a) Chlorophyll content

Reduced chlorophyll content is a well-documented feature of a number of *rol*C-transgenic species, including tobacco (Schmülling et al., 1988) and potato (Fladung, 1990). Although the colour of Tr*rol*C transgenic clover leaves did not appear noticeably paler by visual observation (Fig. 4.8), significant reductions in mean total chlorophyll contents were measured. Leaves of lines Tr*rol*C1, Tr*rol*C2 and Tr*rol*C3 had significantly lower levels of total chlorophyll than WT plants, with around 75% of WT levels ($p < 0.05$) (Fig. 4.14). Tr*rol*C4 and Tr*rol*C5 leaves did not have altered total chlorophyll levels compared to WT leaves.

Separate measurements of chlorophyll *a* (Chl *a*) and chlorophyll *b* (Chl *b*) (Fig. 4.15 A, B) indicate that reductions in overall chlorophyll content in Tr*rol*C1, Tr*rol*C2 and Tr*rol*C3 are mainly due to lower Chl *b* levels. This is also reflected in the significantly higher ratios of Chl *a* to Chl *b* (Chl *a/b*) calculated for Tr*rol*C1 and Tr*rol*C2, compared to WT (Fig. 4.16).

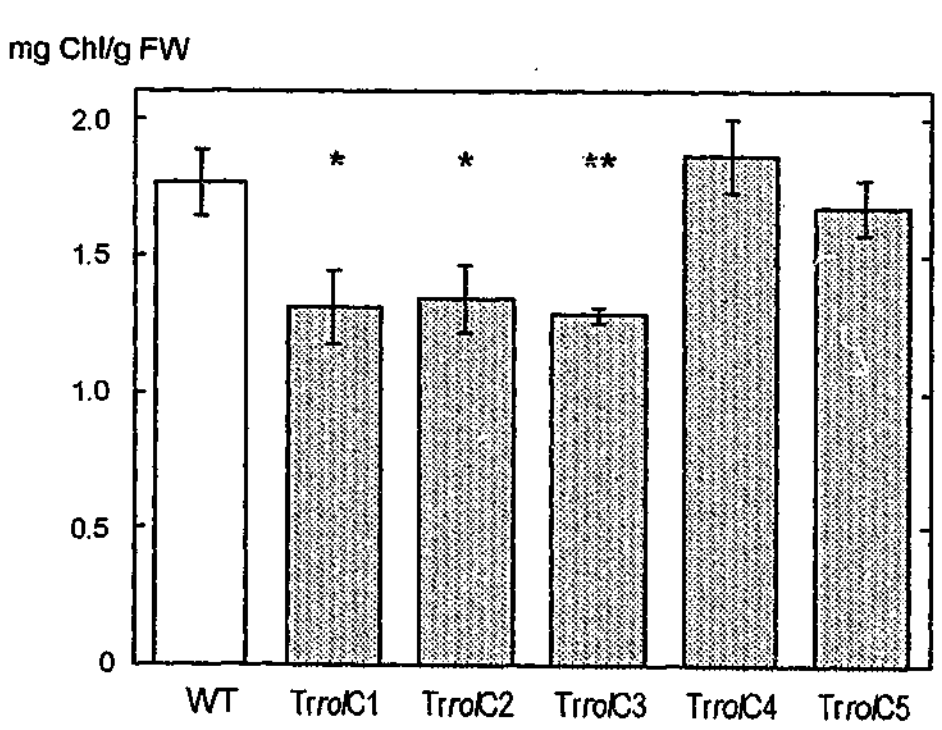


Figure 4.14. Comparison of total chlorophyll content of WT and transgenic Tr*rol*C white clover lines. Bars represent mean mg chlorophyll per gram FW, n = 8; vertical lines represent standard error of the mean. Statistical significance levels: * $p<0.05$; ** $p<0.01$. Abbreviations FW=fresh weight; Chl=chlorophyll.

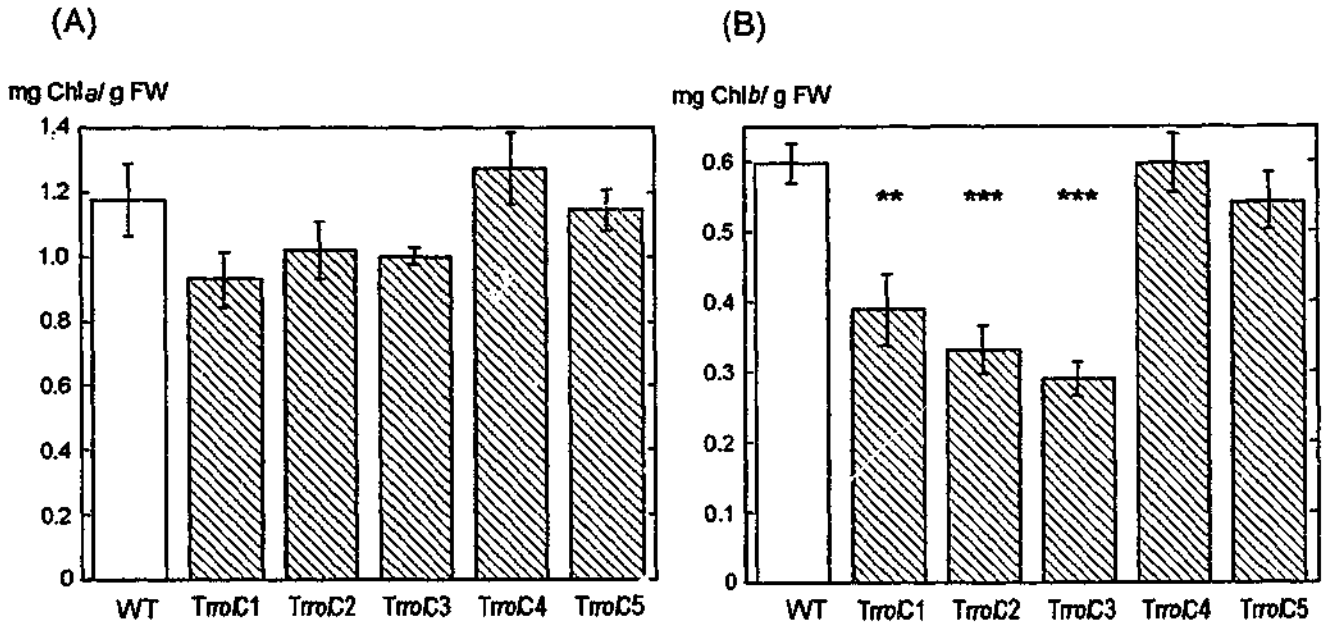


Figure 4.15. Mean chlorophyll *a* (A) and mean chlorophyll *b* (B) levels of leaves of WT and transgenic T*rrol*C white clover. Bars represent mean mg chlorophyll per gram FW, n = 8.

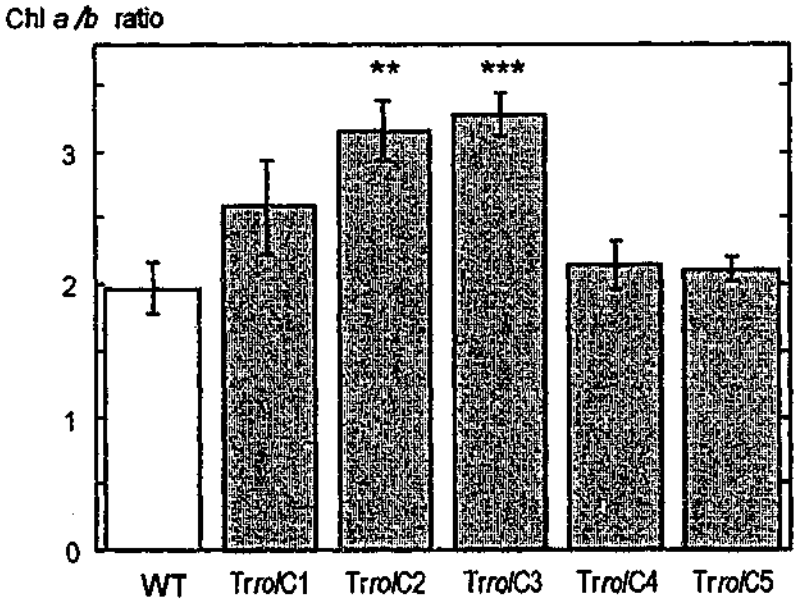


Figure 4.16. Mean chlorophyll *a*/*b* ratio of WT and transgenic T*rrol*C white clover leaves. Bars represent mean ratio of chl *a* to chl *b*, n = 8, vertical lines represent standard error of the mean. Statistical significance levels: ** p <0.01; *** p <0.001.

(b) Leaf soluble protein content

The soluble protein content of T*rrol*C1 and T*rrol*C4 leaves were not different from that of WT plants. In contrast, the soluble protein content level of T*rrol*C3 leaves was significantly lower (*ca* 60%) than that of WT plants (t-test, p <0.05). T*rrol*C2 and T*rroi*C5 leaves also had considerably reduced soluble protein levels (Fig 4.17), although these levels were not statistically significant (p >0.05).

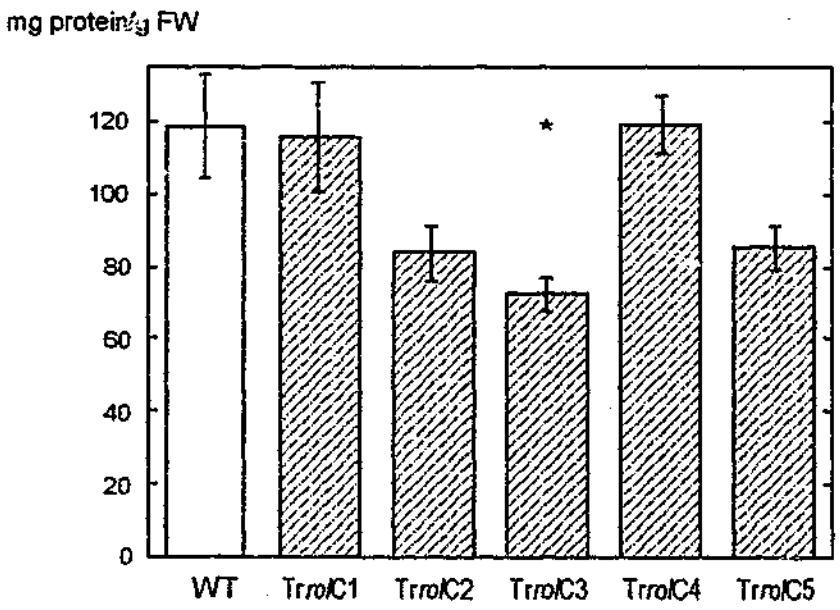


Figure 4.17. Comparison of the soluble protein content of WT and transgenic Trrol*C* white clover lines. Bars represent mean mg of soluble protein per gram of fresh weight, n = 8; vertical lines represent s.e.m. Statistical significance levels: * p <0.05. FW=fresh weight.

(c) Leaf dry weight

The dry weight of leaves of Trrol*C*2, C3, C4 and C5 were significantly lower than the dry weight of WT and Trrol*C*1 leaves (Trrol*C*4 p<0.01; Trrol*C*2, C3, C5 p<0.001) (Fig 4.18). There was no correlation between the leaf dry weight and chlorophyll levels or protein levels (p>0.05). The lower leaf weight of Trrol*C*2, C3, C4 and C5 transformants corresponded with observations of smaller leaf size in these plants.

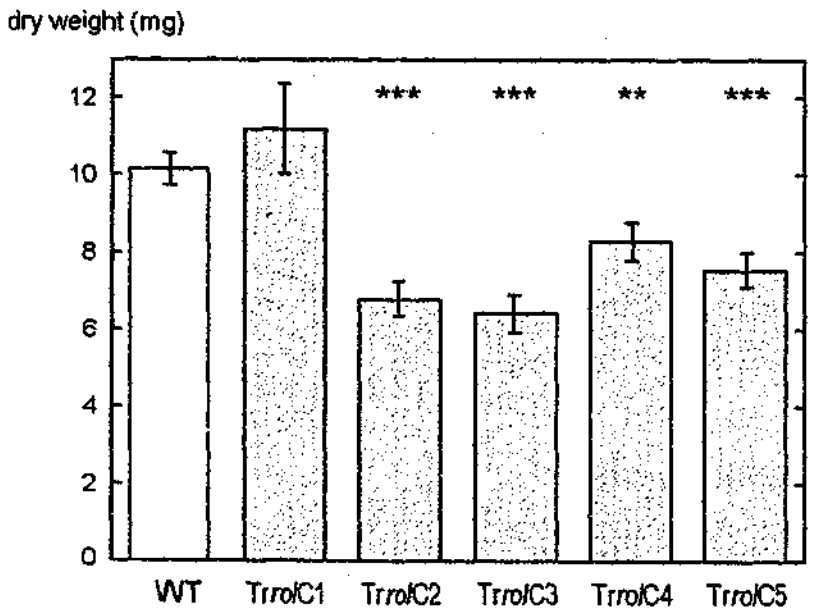


Figure 4.18. Dry weight of single leaflet of WT and transgenic Trrol*C* white clover plants. Bars represent mean dry weight, n = 8; vertical lines represent s.e.m. Statistical significance levels: ** p <0.01 *** p <0.001.

(d) Internode length

With the exception of Tr*rol*C5, the mean internode lengths of all transformed lines were similar to that of WT plants (Fig. 4.19). A highly significant difference between the internode lengths of Tr*rol*C5 and WT stolons ($p < 0.001$) was found, with the mean Tr*rol*C5 internode length less than half that of the mean WT internode distance. Overall stolon lengths are also noticeably shorter in these plants.

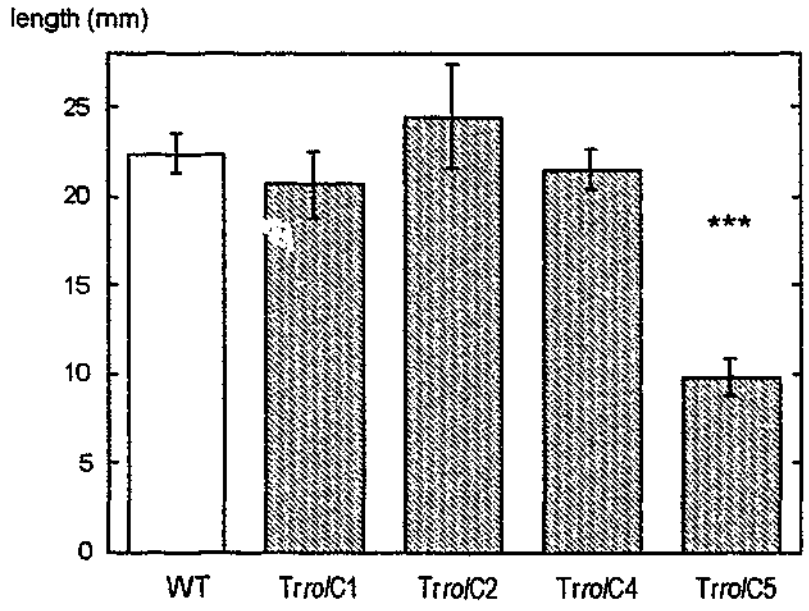


Figure 4.19. Comparison of internode length in WT and transgenic Tr*rol*C white clover plants grown in soil. Bars represent mean internode length in mm, n = 15-20; vertical lines represent s.e.m. Statistical significance levels: *** $p < 0.001$. Tr*rol*C3 transformants were not included in this analysis due to the lack of stolon growth.

(e) Adventitious root formation

Stolon segments with three nodes and three leaves were taken from the second- or third-last node from the end of the stolon. These segments of stolon were growing over the edge of the pots, not in contact with the soil and had not produced adventitious roots at the time of harvest. Segments were placed in soil and monitored for the appearance of adventitious roots. By day 4, most stolon segments had produced several roots, although Tr*rol*C4 stolons produced very few and most had not advanced past the stage of root node formation (Table 4.1). By day 7, however, Tr*rol*C4 stolons were similar to those of WT and Tr*rol*C2. Tr*rol*C1 and Tr*rol*C5 had produced significantly more adventitious roots after 7 days than other lines and the roots of Tr*rol*C5 stolons also branched into a greater number of lateral roots than roots from other stolons.

After day 7, it became impossible to accurately count the roots, particularly lateral roots. To compare further root growth, an experiment was conducted with a separate set of stolon segments and root growth measured as the dry weight of the total root system subsequently produced (see Fig. 4.21).

Table 4.1. Adventitious and lateral root formation on stolon segments: mean number of roots ± standard error of mean. n = 6-8. Significance levels of statistical differences between T*rrol*C transgenic lines and WT control line: * p <0.05 ** p <0.01.

Clover line	Day 4 mean no. adventitious roots	Day 7 mean no. adventitious roots	Day 7 mean no. LR per adventitious root	Day 7 approx no. root tips per stolon segment
WT	3.0 ± 0.2	3.2 ± 0.3	10.6 ± 2.8	34
T*rrol*C1	4.4 ± 0.4	6.9 ± 0.9 **	12.7 ± 3.7	90
T*rrol*C2	3.2 ± 0.4	3.7 ± 0.4	13.8 ± 2.8	50
T*rrol*C4	1.2 ± 0.5	3.8 ± 0.5	6.8 ± 4.2	26
T*rrol*C5	3.5 ± 1.2	10.5 ± 2.6 **	22.0 ± 12.5 *	230

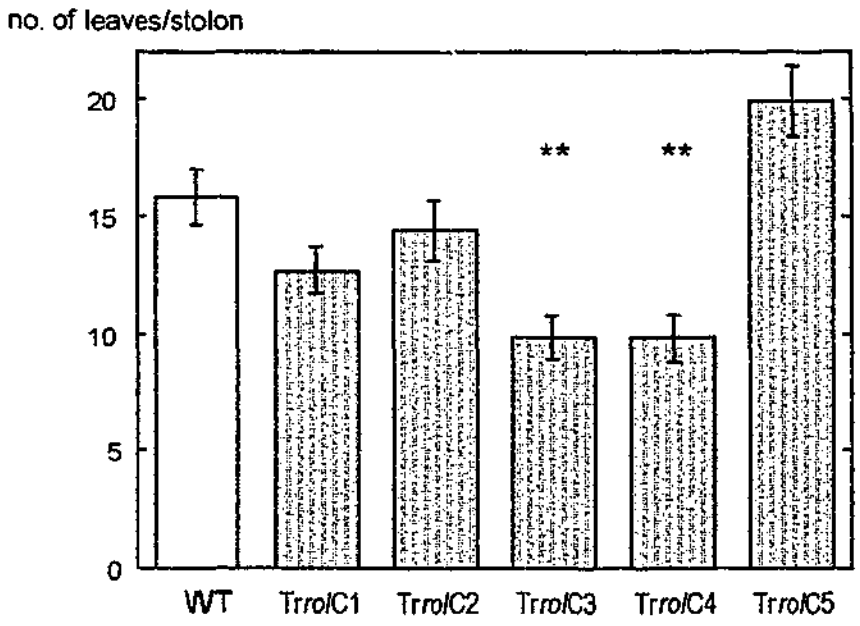
New root and leaf production from single node stolon segments

Stolon sections consisting of a single node with one leaf were cultivated in a hydroponic system. Plants were grown for five weeks. Leaf number, petiole length (height) and root and shoot dry weights were compared.

(f) Leaf number and petiole length

After five weeks of growth, the number of leaves produced by T*rrol*C1 and T*rrol*C2 plants were not different from WT, whereas T*rrol*C3 and T*rrol*C4 stolon segments produced significantly fewer leaves compared to the WT. In contrast, from both the data and from visual observation, T*rrol*C5 plants showed a tendency to produce slightly more leaves on average than WT plants. However, due to variation between samples, the difference was not statistically significant (p>0.05) (Fig. 4.20A). The average petiole lengths of leaves of T*rrol*C3 and T*rrol*C5 transformants were shorter than for leaves of all other lines (Fig. 4.20B).

(A)



(B)

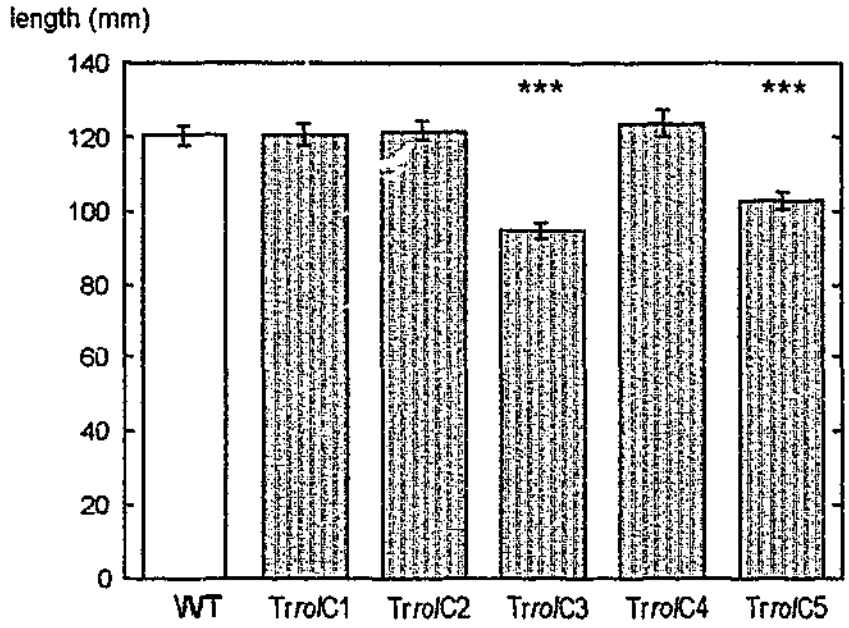


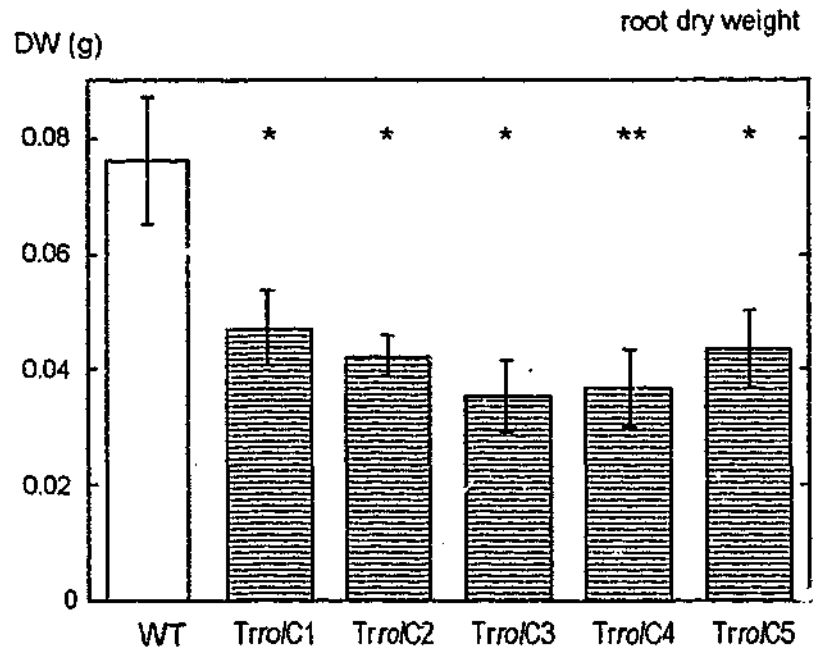
Figure 4.20. Comparison of WT and transgenic T*rrol*C white clover lines: (A) Mean leaf number (B) Mean petiole length. Plants were grown from stolon segment consisting of a single node and leaf, for five weeks in hydroponic nutrient solution. Bars represent mean of measurements from (A) 8-10 individual plants (B) 55-64 individual leaves, except T*rrol*C3, for which 22 leaves were sampled. Vertical lines represent s.e.m. Statistical significance levels: ** $p < 0.01$ *** $p < 0.001$.

(g) Root and shoot dry weights and r:s ratio

The balance between shoot and root dry weight were similar in all lines except T*rrol*C3, which showed a significantly larger r:s ratio than WT (Fig. 4.22A). However, the actual root and shoot masses are higher in WT than in all other lines (Fig. 4.21), indicating better overall growth under the conditions in which the plants were grown. As the actual root mass of T*rrol*C3 is similar to that of other transformant lines and less than WT, the

greater r:s ratio of T*rol*C3 plants is clearly due to a lower relative shoot mass (as shown in Fig. 4.21B). This is most likely due to the lack of stolon production in these plants, as stolons normally account for a substantial proportion of overall shoot weight. The relative dry weight to fresh weight ratio of leaves (expressed as a percentage) is significantly reduced in T*rol*C1, T*rol*C3 and T*rol*C5 lines, compared to WT (Fig. 4.22B), indicating a higher than normal relative water content (RWC) in fresh leaves of these transformants.

(A)



(B)

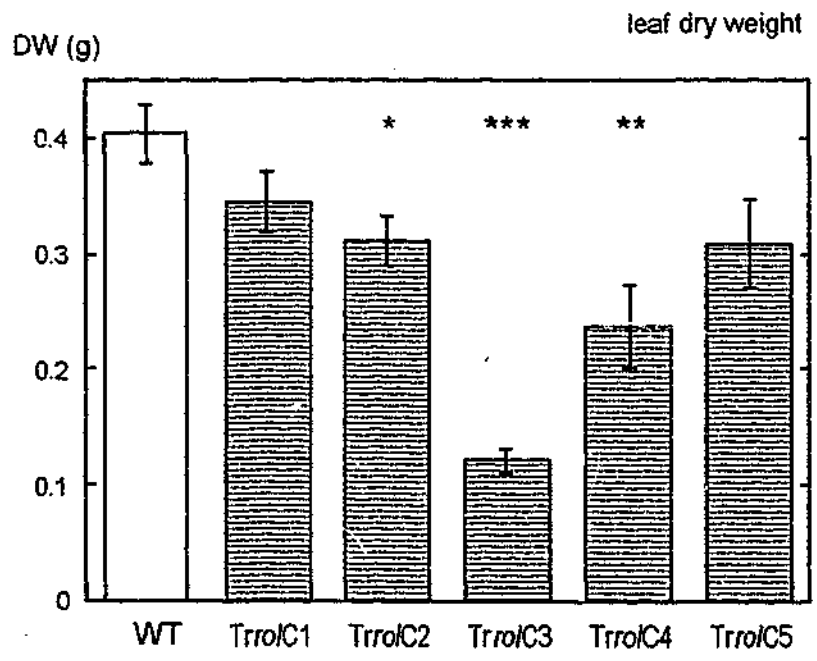
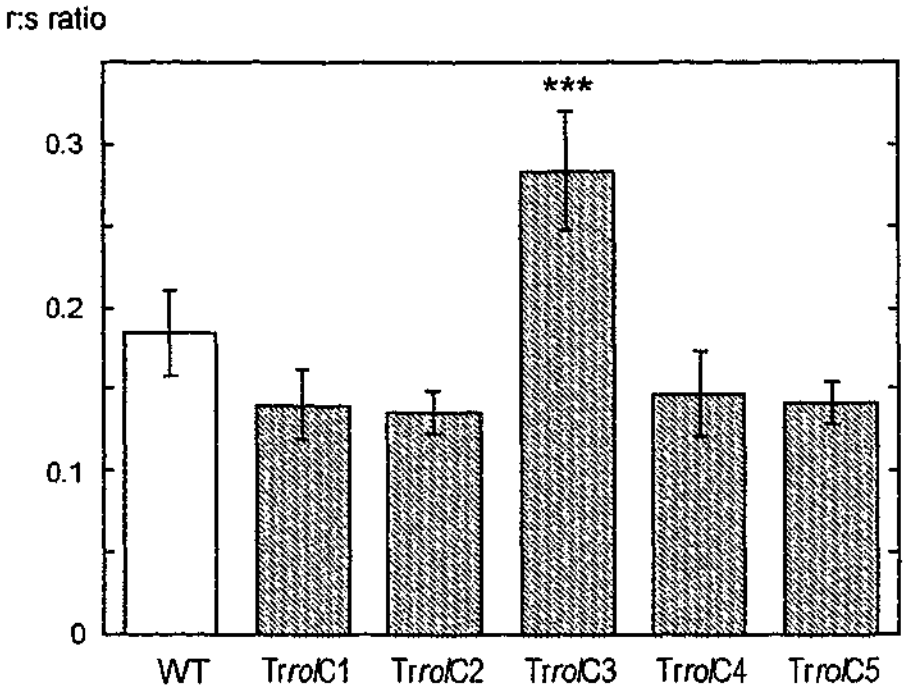


Figure 4.21. Comparison of (A) mean root dry weight and (B) mean shoot dry weight, of WT and transgenic *rol*C white clover lines. Plants were grown from stolon segments consisting of a single node and leaf, for five weeks in hydroponic solution. Bars represent mean dry weight of 8-10 plants; vertical lines represent standard error of the mean. Statistical significance levels: * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$.

(A)



(B)

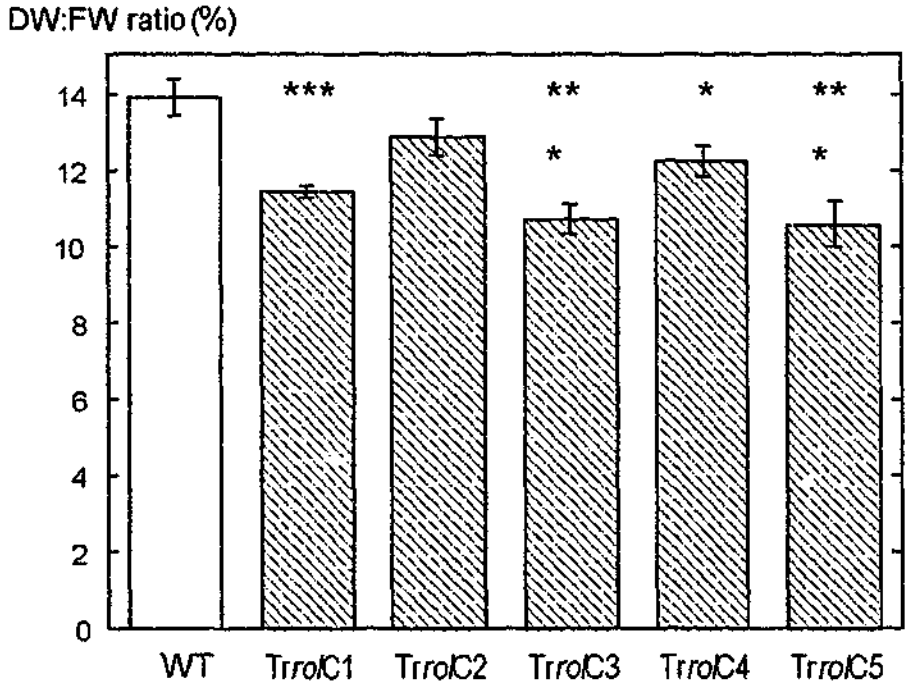


Figure 4.22. Comparison of WT and transgenic *rol*C white clover lines: (A) Mean root-to-shoot dry weight ratio. (B) Mean leaf dry weight relative to leaf fresh weight (%). Plants were grown from stolon segments consisting of a single node and leaf, for five weeks in hydroponic solution. Vertical lines represent s.e.m, n = 8-10. Statistical significance levels: * $p < 0.05$; *** $p < 0.001$. Abbreviations r:s = root:shoot ratio; DW:FW = dry weight to fresh weight ratio.

Table 4.2. Summary of phenotypic modifications identified in *rol*C transgenic white clover plants.

Line	Estimated copy no.	Transcripts detected +/-	Main phenotypic modifications compared to WT
Tr*rol*C1	2	+	- increased AR formation - flowers more readily - reduced DW:FW ratio in leaves - reduced total Chl
Tr*rol*C2	4	-	- smaller leaves - lower root and shoot DW - reduced total Chl - increased Chl *a/b* ratio
Tr*rol*C3	3	+	- lack of stolons - smaller, wrinkled leaves - reduced petiole length - reduced leaf number - lower root and shoot DW - reduced DW:FW ratio in leaves - reduced total soluble protein and total Chl - increased Chl *a/b* ratio in leaves
Tr*rol*C4	1	-	- smaller leaves, reduced no. leaves - lower root and shoot DW
Tr*rol*C5	3	+	- markedly increased AR formation - increased LR formation - shorter stolons with reduced internode length - smaller, lanceolate, wrinkled leaves - reduced DW:FW ratio in leaves - greatly reduced petiole length

4.4. DISCUSSION

4.4.1. Expression of *rol*C in transformed clover plants

The five independently transformed *rol*C white clover lines display a range of phenotypic modifications, some of which are reminiscent of *rol*C effects reported in other species. Several reasons may account for the variations in effects evident in each transformant line, including different transgene integration positions in the plant genome, differences in the genetic background of each individual line, and/or disparities in transgene copy number.

Between one and four *rol*C gene copies were present in the T*rrol*C transgenic white clover lines generated in this study. In other studies using a similar transformation technique, copy number in white clover transformed with a maize seed storage protein ranged from one to six (Sharma et al., 1998) and nine clover transformant lines expressing the ß-glucuronidase (GUS) and *nptII* genes all contained single copies of the T-DNA insert (Voisey et al., 1994).

Phenotype variations resulting from different integration sites were found between transgenic petunia lines transformed with a single functional copy of a gene involved in synthesis of flower pigment (Pröls and Meyer, 1992). The transgene integration regions of two lines were shown to differ in degree of methylation, resulting in variations in transcription levels and intensity of pigmentation. The transgene in a third transformed line was inactivated as a result of integration into a region of repetitive heterochromatic DNA (permanently condensed chromosomal region). Hobbs et al. (1990) studied the progeny of tobacco plants transformed with T-DNA carrying the GUS gene. No variations in GUS activity were found as a result of different integration positions, however T-DNA copy number was strongly negatively correlated with GUS activity. Environmental conditions and developmental stage are other factors potentially affecting transgene expression (Hobbs et al., 1990; Meyer et al., 1992). For example, the inactivation frequency of an introduced chitinase gene was lower in transgenic *N. sylvestris* plants grown from seed germinated in a greenhouse, compared to those germinated *in vitro* and then transplanted to greenhouse conditions (Hart et al., 1992). In the present analysis of *rol*C-transgenic white clover, growth conditions were kept consistent for all T*rrol*C white clover lines to avoid, as far as possible, these factors as a source of phenotypic variation between transformant lines.

The possibility that some phenotypic variations between the T*rrol*C transgenic lines are a result of differences in genetic backgrounds, rather than transgene expression, cannot be completely discounted. However, this seems more likely to apply

to more subtle differences, such as leaf size, rather than distinctive alterations such as a near absence of stolons or markedly shorter internode lengths. These striking modifications were associated with particular transformants and were never observed in any WT lines. Given that analogous phenotypes have been reported in *rol*C transgenic lines of other species, such as tobacco (Schmülling et al., 1988) and potato (Fladung, 1990), these observations can probably be assumed to be a consequence of *rol*C expression. To confirm this, however, it may be necessary to generate new transformants, using paired cotyledons from a single embryo to produce a transformant line and control line with identical genetic backgrounds. Alternatively, *rol*C transgenic plants could be crossed with WT plants to verify that the segregation of phenotypic traits correlates with the inheritance of the *rol*C gene.

Scott et al. (1998) demonstrated that the genetic background and allelic composition of a plant may influence the stability and expression of a transgene. This is particularly relevant for genetic modification of an outbreeding species such as *Trifolium repens*, in which there can be a high degree of genetic variability between even closely related individuals (Williams, 1987a). Investigations into the stability of introduced genes in various species have shown diverse results, with some reports of stable expression for many generations and others of transgene loss or silencing after one or two generations (Kilby et al., 1992; Schmülling and Röhrig, 1995; Duan et al., 1996; Srivastava et al., 1996; Scott et al., 1998; Fladung, 1999). In this study, phenotypic analyses of T*rrol*C transgenic white clover plants demonstrated the phenotypic modifications to be stable over several years of vegetative growth.

The issue of transgene expression stability is an important one in assessing the feasibility of genetic modification of a plant species. Unpredictable effects or instability may lessen the potential usefulness of introduced genes. Scott et al. (1998) noted the importance of progeny testing of white clover transformants to check transgene expression and stability in a variety of backgrounds, before release of transgenic species or cultivars. This may also reveal possible differences in expression effects resulting from random transgene integration positions or variable copy numbers.

Two of the T*rrol*C white clover lines (T*rrol*C2 and T*rrol*C4) were shown to contain one or more intact copies of the *rol*C gene but expression was not detected by Northern blot hybridisation. It is possible that transcript levels were below the limits of detection, which may be due to transgene inactivation, a commonly reported occurrence in transgenic plants (reviewed in Finnegan and McElroy, 1994; Matzke and Matzke, 1995; Stam et al., 1997). Transgene inactivation may be a defence mechanism by plants against possible detrimental effects of introduction of foreign DNA - for example, expression of genes that may have negative effects on the plant, or insertion of

transposable elements that may disrupt endogenous genes. Transgene inactivation is most frequently associated with the presence of multiple transgene copies and may occur by a number of different possible mechanisms. These include both *cis*- and *trans*-interactions between homologous endogenous and introduced genes (co-suppression), multiple repeats (repeat-induced gene silencing), allelic transgenes or non-allelic T-DNA inserts, and can occur as transcriptional or post-transcriptional events (Finnegan and McElroy, 1994; Flavell, 1994; Schmülling and Röhrig, 1995; Stam et al., 1997).

Ectopic pairing between DNA segments is one mechanism for inactivation of genes with homologous sequences, possibly by inducing DNA methylation. Many reports have documented correlations between multiple gene copies and increased methylation of promoter or transcribed regions, resulting in reduced expression (Linn et al., 1990; Assaad et al., 1993; Neuhuber et al., 1994; Matzke et al., 1994; Schmülling and Röhrig, 1995; De Neve et al., 1999). In addition to instability of expression, interaction of homologous sequences can also lead to physical instability of transgenes. In a study of gene stability in transgenic aspen lines transformed with *rol*C (Fladung, 1999), frequent phenotypic reversions occurring in one transgenic line were suggested to be due to the presence of a partial duplication of the T-DNA insert sequence adjacent to the complete T-DNA copy. Possible intrachromosomal homologous pairing of these sequences during mitosis would preclude proper replication of this region and thus lead to loss of the transgene sequence.

It is possible that the presence of four *rol*C gene copies in the Tr*rol*C2 clover genome may have resulted in inactivation of the transgene. Alternatively, as in other reported instances (Pröls and Meyer, 1992), the introduced T-DNA may have integrated into hypermethylated or heterochromatic regions of the Tr*rol*C2 and Tr*rol*C4 genomes. RNA transcripts of *rol*C were detected in Tr*rol*C1 and Tr*rol*C5 transformants that contained three copies of the *rol*C gene.

4.4.2. Phenotypic modifications in Tr*rol*C transformants and comparison with effects in other plant species

Most of the phenotypic modifications observed in the Tr*rol*C clover lines were analogous to those reported for *rol*C transformants in a range of other plant species. This implies that *rol*C may affect quite basic and conserved plant processes, which is consistent with the hypothesis that expression of the gene may influence auxin perception and/or responses to auxin and consequently the auxin/cytokinin balance of transformed cells (see Chapter 3).

4.4.2.1. Root growth and r:s ratio

Effects of *rol*C on root growth and development were apparent in two transformant lines, Tr*rol*C1 and Tr*rol*C5, with enhanced adventitious root production and lateral root branching observed in comparison to controls. Although the actual root dry weights of plants of these lines were not increased when compared to WT plants, after 5 weeks growth from single node segments, it is possible that increased root differentiation would have noticeable effects on root biomass at later stages, for example with the development of stolons with separate nodal root systems.

As discussed in Chapter 3, factors such as nutritive conditions and ontogeny play a large role in influencing plant growth. It is relevant to note that, in this study, white clover plants were grown with full nutrient provision and root and shoot growth were measured at a single time point and starting point (i.e. stolon segments with a particular number of nodes). The propensity of some of the *rol*C transformants to differentiate more lateral and adventitious roots may be less evident when there is no shortage of nutrients and no need for augmented root system differentiation and growth. If nutrient availability were more limited, however, the advantage gained from enhanced root growth may potentially be greater. The capacity for increased root growth of *rol*C plants may then be utilised and may possibly be apparent as measurable differences in root system growth.

Although an increased root:shoot ratio was observed for Tr*rol*C3 plants compared to WT, when grown in a hydroponic system, this may have more to do with production of a lower shoot mass rather than an increase in root mass *per se*. In fact, WT plants produced a significantly greater mass of both root and shoot than *rol*C transgenic plants, in the particular conditions in which they were grown. This is consistent with the generally reported dwarfed stature of *rol*C transgenic plants (Fladung, 1990; Winefield et al., 1999), with some *rol*C tobacco transformants calculated to have shoot weights less than half that of controls (Scorza et al., 1994).

Alterations to root system growth have been reported in alfalfa (*Medicago sativa*) transformed with the *rol*C gene (Frugis et al., 1995). An important forage legume, creeping-rooted strains of alfalfa are characterised by a lateral spreading growth style, with frequent root branching and subsequent development of shoot buds along the roots. Root production was found to be increased in *rol*C-transformed alfalfa plants, with a root mass 1.5-fold greater than in WT controls. Correspondingly, the number of stems produced along the roots was increased almost two-fold in *rol*C transgenics (Frugis et al., 1995).

4.4.2.2. Stolon modifications

Decreased internode length and an associated increased density of shoots and leaves, as observed in Trrol C5 plants, have been commonly reported features in many plant species transformed with *rol*C and 35S-*rol*C. These include the legume *Lotus corniculatus* (Pozárková et al., 1995), tobacco (Scorza et al., 1994; Guivarc'h et al., 1996), potato (Fladung, 1990), hybrid aspen (Nilsson et al., 1996b), petunia (Winefield et al., 1999) and pear (Bell et al., 1999). Shortened internodes and reduced plant stature have been correlated with a reduction in giberellic acid (GA) content (Atwell et al., 1999c). Exogenous applications of GA to the apical shoot of 35S-*rol*C tobacco transformants induced stem and internode elongation and restored normal plant height (Schmülling et al., 1993). In upper leaves of 35S-*rol*C tobacco plants, reductions in GA_1 to about 30% of WT levels were measured (Nilsson et al., 1993b). Schmülling et al. (1993) also noted up to 60% decreases in GA_1 in the apical shoot of strongly expressing 35S-*rol*C tobacco and potato plants. However, the alterations to GA levels are likely to be secondary effects of *rol*C expression, as other effects on phenotype, including reduced leaf chlorophyll, early flowering and male sterility, were not mitigated by GA treatment (Schmülling et al., 1993).

4.4.2.3. Leaf modifications

Reduced GA levels in *rol*C-expressing transformants have also been associated with the characteristic *rol*C features of smaller leaf and leaf cell size (Nilsson et al., 1993b). Smaller leaves were observed in the Trrol C2, C3, C4 and C5 clover transformants produced in this study, concurring with descriptions of smaller leaf size in *rol*C-transformed tobacco (Schmülling et al., 1988; Scorza et al., 1994), 35S-*rol*C transgenic hybrid aspen (Nilsson et al., 1996b) and some *rol*C transgenic petunia lines (Winefield et al., 1999). Decreased leaf size may be due to the presence of generally smaller and flatter leaf mesophyll cells, as observed in tobacco 35S-*rol*C transformants (Estruch et al., 1991c), although this was not the case in 35S-*rol*C hybrid aspen (Nilsson et al., 1996b).

The decreased dry weight-to-fresh weight ratios (DW:FW) of leaves of the clover transformants Trrol C1, Trrol C3 and Trrol C5, relative to controls, are also consistent with reported effects in tobacco. In *rol*C-transformed tobacco leaves, a lower DW:FW ratio and higher water turgor were found in comparison to control leaves (Nilsson et al., 1993b). A higher water turgor was also previously reported in *rol*ABC transformants by

Ooms et al. (1986). The different water status of *rol*C transgenic and control plants have been correlated with reduced ABA levels in the transgenic plants, a probable secondary effect of the *rol*C protein. For example, ABA levels in both 35S-*rol*C tobacco and potato leaves were found to be reduced by up to 50%, compared to control plants (Schmülling et al., 1993; Nilsson et al., 1993b) and reductions of similar magnitude have been measured in *rol*C and 35S-*rol*C hybrid aspen (Fladung et al., 1997). Nilsson and Olsson (1997) suggested that higher water turgor and DW:FW ratios in leaves of *rol*C-transformed plants may signify the presence of increased levels of sugars, and proposed a possible role for *rol*C in sugar metabolism and/or transport, in which *rol*C activity creates a strong sink for sugar by promoting sucrose unloading from the phloem.

The lower levels of chlorophyll assayed in leaves of the Trrol C1, Trrol C2 and Trrol C3 clover lines in comparison to WT clover are consistent with observations of reduced levels in *rol*C-expressing potato (Fladung and Ballvora, 1992; Fladung et al., 1993) and *Lotus corniculatus* (35S-*rol*C) (Pozárková et al., 1995). Chlorophyll levels were reported to be reduced by around 50% in *rol*C transformants of these species. Pale green leaves have also been observed in 35S-*rol*C transformants of tobacco, hybrid aspen and *A. belladonna* (Schmülling et al., 1988; Kurioka et al., 1992; Oono et al., 1993b; Nilsson et al., 1996b).

In this study, decreased total chlorophyll contents of Trrol C1, Trrol C2 and Trrol C3 clover transformants were mainly a result of reductions in Chl *b* levels, also resulting in an increased Chl *a/b* ratio. While both Chl *a* and *b* have light-harvesting activity, some Chl *a* forms are also involved in energy processing. Thus, a higher Chl *a/b* ratio usually occurs in leaves grown in strong light, to maximise the energy processing capacity of leaves. Conversely, in low light conditions, light harvesting is a greater priority and the relative abundance of Chl *b* is generally increased, resulting in a lower Chl *a/b* ratio (Atwell et al., 1999d). In this study, however, all white clover plants were grown under identical light conditions (16 hr photoperiod and photon flux density of 150 $\mu mol\ m^{-2}s^{-1}$). Thus, reductions in Chl *b* levels and therefore increased Chl *a/b* ratios in some of the Trrol C transgenic lines suggests that transformation with *rol*C may have induced alterations in the proportions of photosynthesis components in those lines.

These alterations in Chl *a/b* ratios in the clover transformants are in contrast with reported studies in potato and *L. corniculatus*, in which both Chl *a* and *b* were reduced proportionally. The Chl *a/b* ratios of leaves of untransformed and *rol*C-transformed diploid potato lines were not significantly different (between 3.14 and 3.21) (Fladung, 1990) and Chl *a/b* ratios of around 3 were similarly measured in both 35S-*rol*C and control *L. corniculatus* plants (Pozárková et al., 1995). A connection between cytokinin and reduced Chl in *rol*C transformed plants has been shown (as discussed below),

however few studies on chlorophyll content in *rol*C transgenic plants have reported measurements of Chl *a* and *b* separately. Alterations in Chl *a/b* do not seem to have been previously reported as an effect of *rol*C expression.

A role for cytokinin in promoting chlorophyll stability has been described (Grossmann et al., 1991) and demonstrated in plants transformed with the *A. tumefaciens* cytokinin biosynthesis gene, *ipt*, in which the overproduction of cytokinin results in darker green leaves (Beinsberger et al., 1991). This, and further evidence, suggested that *rol*C may have an influence on cytokinin levels, with respect to chlorophyll content. Hybrid tobacco plants produced from a cross between 35S-*rol*C (pale green leaves) and 35S-*ipt* cytokinin-overproducing plants (dark green leaves) were observed to have a *rol*C phenotype with the significant exception of normal coloured leaves (Schmülling and Schell, 1993). Apparently, *ipt*-induced cytokinin production was able to compensate for the effects of *rol*C on chlorophyll levels, indicating that lower chlorophyll levels in *rol*C-transformed tobacco leaves are likely to be directly due to reductions in cytokinin content brought about by expression of the *rol*C gene. In other respects however, the *rol*C phenotype in *rol*C-*ipt* hybrid plants has not been observed to be diminished by expression of the *ipt gene*, suggesting that other phenotypic changes in *rol*C plants are not directly due to lower cytokinin levels (Schmülling et al., 1993; Faiss et al., 1996).

4.4.2.4. Modifications to flowering

A tendency to flower more readily than usual was observed in one clover transformant line, Tr*rol*C1, which may be a similar phenotypic effect to the early flowering reported in *rol*C transformants of other species. Early flowering of tobacco has been frequently reported (Scorza et al., 1994; Faiss et al., 1996; Guivarc'h et al., 1996) and was also observed in the present study with *rol*C and 35SC-transformed tobacco (Chapter 3). Similarly, transgenic petunia lines expressing *rol*C were found to flower 10-12 days earlier on average than untransformed plants (Winefield et al., 1999). Promotion of flowering has also been observed in *A. belladonna* plants transformed with 35S-*rol*C (Kurioka et al., 1992), which produced more flower buds and also flowered under *in vitro* conditions in which untransformed plants were never observed to flower. In contrast, however, no alterations in flowering time were reported for *rol*C potato transformants, although a reduced flower size was noted (Fladung, 1990), which was similarly observed in tobacco (Schmülling et al., 1988; Scorza et al., 1994).

Particular variations in *rol*C effects have been observed in some species along with the common phenotypic effects. For example, in hybrid aspen, a woody perennial tree species, expression of *rol*C resulted in early release from dormancy (Fladung et al., 1997) and stem fasciation (Nilsson et al., 1996b). Distinctive phenotypic effects of the *rol*C-transgenic clover in this study include non-stoloniferous growth (Tr*rol*C3) and an increase in production of four-segment leaves (Tr*rol*C1).

4.4.2.5. Reported effects of *rol*C and Ri T-DNA genes on nodulation

Interestingly, in 35S-*rol*C transformants of the legume *Lotus corniculatus*, a significant reduction in nodule number and nitrogenase activity were found (Pozárková et al., 1995). However, these effects were not observed in *rol*AB or *rol*ABC transgenic *L. corniculatus* plants. Similarly, no alterations in nodule formation or capacity for nitrogen fixation were observed in Ri T-DNA-transformed white clover (Díaz et al., 1989) or *L. corniculatus* (Webb et al., 1990). On the other hand, Beach and Gresshoff (1988) reported that in three forage legume species - red clover, siratro and alfalfa - transformed with the wild-type *A. rhizogenes* mannopine strain 8196, nodulation by the relevant *Rhizobium* or *Bradyrhizobium* strain was inhibited by the presence of the T-DNA. Any alterations to the nodulation process or efficiency by *rol*C expression could clearly affect clover growth and consequently would have implications for the practical value of transformation of white clover with the *rol*C gene. An examination of root nodulation of the *rol*C-transgenic clover lines was not undertaken in this study but would be pertinent for further characterisation of these transformants.

As discussed previously (Chapter 3), parallels between the processes of LR and nodule formation are apparent, in terms of the influence of local auxin levels and auxin to cytokinin ratio. A high local level of auxin is required to stimulate division of nodule progenitor cells, although, unlike LRP initiation, nodule initiation is preceded by a temporary polar inhibition of auxin transport that leads to an accumulation of auxin at the site of nodule formation (Mathesius et al., 1998). Reports of alterations in nodulation in some *rol* gene-transformed legumes (Beach and Gresshoff, 1988; Pozárková et al., 1995) may be a reflection of a direct or indirect impact of *rol*C (and/or other *rol* genes) on hormone perception and relative cytokinin and auxin levels in transformed cells.

4.4.3. Phenotypic characteristics of Tr*rol*C transformants with potential agronomic advantages

Further characterisations of *rol*C-transgenic white clover are required to more fully evaluate the possible positive and negative effects of *rol*C, on growth in field conditions. As has been noted, key traits relevant to the agronomic performance of white clover include plant persistence in pasture, productivity (shoot yield) and nutritional value of the shoot matter produced (Mason, 1993; Lane et al., 1997; Frame et al., 1998). Although the phenotypic modifications observed in the *rol*C-transformed clover in this study are moderate, some may have potential for enhancing traits advantageous to growth.

One potentially beneficial effect is an increased production of adventitious roots, as observed in Tr*rol*C1 and Tr*rol*C5 plants. As indicated by Blaikie and Mason (1993) and Caradus and Woodfield (1998), root growth is an important factor in promoting productivity and persistence of white clover pastures. Improved nodal root growth may provide plants with an advantage in obtaining water and soil nutrients, as well as promoting establishment of stolons and subsequent persistence in the field. A somewhat higher density of leaf production was observed in one transformant line (Tr*rol*C5), a characteristic that could also be beneficial to clover productivity.

Decreased internode length, as observed in the Tr*rol*C5 line, may present both benefits and disadvantages to the plants. With decreased stolon extension, it is likely that the plant will not be able to spread as far, possibly reducing competitiveness by limiting exploration and utilisation of soil and aerial environments. However, it has been shown that shorter internodes can have benefits to the carbon economy of plants, which would be of importance during conditions of winter stress and low sunlight (Frankow-Lindberg, 1999). White clover plants adapted to winter stress (Swedish cultivars) were shown to have shorter internodes than unadapted plants, due to much slower stolon extension rates and less reduced rates of leaf appearance. It is suggested that plants with shorter internodes may require a relatively reduced allocation of biomass to stolons and roots, thereby raising the source:sink ratio for carbon, compared to plants with longer internodes. This is consistent with observations that winter-adapted plants have an increased ability to maintain non-structural carbohydrates during winter stress, which is correlated with good growth immediately after winter (Frankow-Lindberg and von Fircks, 1998). The adaptive benefits of shorter internodes to winter stress may thus have some relevance to development of white clover cultivars able to survive in cold climates and alpine environments.

In examining the potential agronomic suitability of *rol*C transgenic white clover, modifications advantageous to clover growth and persistence as well as potentially negative effects of the *rol*C phenotype would need to be considered. Use of traditional breeding methods may help to minimise negative effects while maintaining desirable traits in these *rol*C-expressing transgenic clover lines.

4.4.4. Experimental limitations and aspects for further consideration

Although the number of *rol*C transformants that have been produced and characterised in this study is not large, these plants give some indication of the phenotypic effects that can be expected from expression of *rol*C in white clover. Evidently, there is considerable variability among the transformants and further work would clearly involve the generation of a greater number of independent transformants, to assess the consistency and reproducibility of the modifications observed and identify any further effects of *rol*C expression. In terms of potential agronomic applications, the high genetic variability of an outbreeding species such as white clover raises the importance of testing different plants and progeny of transformants, to identify suitable genetic backgrounds for transgene stability and appropriate expression (Scott et al., 1998).

Data on phenotypic features and growth measurements were collected from white clover plants raised in a growth environment that was as uniform as possible. Difficulties were experienced in obtaining seed from Tr*rol*C clover transformants, due, in part, to a deficiency of flowers for most plants grown under the prevailing greenhouse conditions and the unsuccessful manual outcrossing of plants that did flower. As attempts to produce non-*rol*C transgenic plants as controls were not successful in this study, it was necessary to make comparisons with WT plants derived from the same seed batch. As Tr*rol*C transformants were regenerated via shoot organogenesis from cotyledonary tissue, it is possible that somaclonal variation could also have contributed to differences between the WT and transgenic plants. To address this concern as far as possible within the experimental confines of this study, WT and transformed plants were propagated by vegetative subculturing at intervals of 3-4 months for a period of approximately two years, before phenotypic and growth analyses were undertaken. With repeated vegetative culturing for this length of time, it was considered likely that any variability caused by the differences in origin would be minimised as far as possible. Furthermore, as an alternative course to growing all plants from seed, growth

measurements were made with plants grown, as far as possible, from equivalent starting points - from stolon sections of a single leaf node of approximately the same age with no roots attached. Further studies of these transformed clover lines would aim to include comparisons with WT control plants regenerated from cotyledons and to confirm that the difference in origin does not constitute a source of variation.

As discussed, the potential for *rol*C to improve growth and developmental attributes has been considered in a number of species and the effects of the gene in white clover suggest some potential for applications in this species. While these particular Tr*rol*C transformants may be limited in terms of productive improvements, some of the induced modifications may be useful if enhanced or combined. Clearly, further work would need to be done to fully analyse the possible agronomic benefits of introducing the *rol*C gene into white clover.

Aspects for further investigation

Investigation of potential applications of the *rol*C gene to white clover growth could be expanded by modification of *rol*C expression levels and patterns. In particular, root-specific expression of *rol*C may allow increases in overall root growth without generating potentially adverse or complicating phenotypic effects on the aerial organs. As discussed in Chapter 3, a number of root-specific promoters have been reported, some of which may be useful in conferring expression of *rol*C specifically or predominantly to roots.

Given that *rol*C may influence the hormonal balance of the plant, it would be essential to investigate in detail the long-term growth effects and physiological changes which may not be immediately apparent. In particular, the possible effects of *rol*C on infection of transgenic roots with *Rhizobium* and subsequent nodulation would be an important aspect for investigation. As discussed above, inhibition of nodulation in some Ri T-DNA transformed legumes have been reported, although in other cases no differences were apparent (Beach and Gresshoff, 1988; Díaz et al., 1989; Webb et al., 1990; Pozárková et al., 1995).

Further analyses are also required to investigate potential effects on growth of *rol*C clover transformants resulting from alterations in photosynthetic apparatus, i.e. Chl *a/b* ratio. In particular, assessing the influence of factors such as light levels or photoperiod on growth would be relevant to such studies. As some of the Tr*rol*C lines have higher Chl *a/b* ratios than WT plants, an effect sometimes observed in plants adapted to high light conditions, it could be speculated that there may be benefits for growth of these Tr*rol*C white clover transformants in conditions of high light or long

photoperiod. Other developmental features noted in some sun-adapted plants, have included reduced leaf area and reduced r:s, as high amounts of available light reduce the need to maximise the area of photosynthetic tissue (Atwell et al., 1999d).

Reduction in male fertility is another commonly observed trait induced by *rol*C expression and may have relevance to the management of genetically modified crops, particularly for a long-lived perennial species such as white clover. Controlling the potential spread of transgenes may be facilitated by the prevention of cross-pollination with other populations of white clover.

Another conceivable approach to the improvement of clover may be the transformation of clover with the *rol*B gene. The role of *rol*B in stimulating adventitious root formation (Spena et al., 1987) suggests that expression of this gene may have the potential to improve the growth of white clover because of the manner in which the plants spread vegetatively, via stolon extension and adventitious root formation from stolon root nodes. Transformation of alfalfa with *rol*B induced substantial increase in the dry weight of the root system and corresponding increases in stem number (Frugis et al., 1995), although these improvements were offset by the reduced capacity for regeneration of *rol*B-transformed tissue.

Combined expression of *rol*B and *rol*C genes may also have potential for improvement of adventitious and lateral root induction. Increased AR production from tobacco leaves transformed with both *rol*B and *rol*C has been documented (Spena et al., 1987; Aoki and Syōno, 1999), although increased AR production was not observed in tobacco leaves in this work (Chapter 3). However, an increased capacity to initiate LR in response to auxin was evident in 35SB+C tobacco seedlings. Frugis et al. (1995) found that the presence of both the *rol*B and *rol*C genes in transgenic alfalfa plants resulted in significant increases in root dry weight compared to controls, although these increases were not greater than caused by *rol*C or *rol*B alone.

Key roles for *rol*C and *rol*B in the induction of adventitious roots by *A. rhizogenes* are described in a model proposed by Nilsson and Olsson (1997). With both genes expressed in phloem cells, *rol*C was suggested to have a role in sucrose unloading and *rol*B in increasing sensitivity of cells to auxin, leading to the stimulation of AR formation. Thus, it is conceivable that combined expression of these two genes in stolons of white clover plants may be effective in enhancing the induction of adventitious nodal roots. Furthermore, the increased propensity of *rol*C+*rol*B transformed roots to form lateral roots in response to auxin (Chapter 3) may also have potential to further augment growth of white clover root systems.

Thus, both the *rol*C and *rol*B genes may be relevant to further research into the potential influence of *rol* genes on growth of white clover. A particular consideration would be the effects of these genes on initiation of LR and AR and the capacity of the root system to form functional nitrogen-fixing nodules in response to infection with *Rhizobium*. Again; in terms of the root system, potential advantages may be enhanced by root-specific expression of the relevant genes. In addition, possible shoot phenotypic modifications induced by *rol*B or *rol*C+*rol*B must be considered, to determine whether these may be detrimental or advantageous to agronomic objectives for white clover cultivation.

CHAPTER 5

SURVEY OF LOCAL ENVIRONMENTS FOR THE PRESENCE OF *AGROBACTERIUM RHIZOGENES*

5.1. INTRODUCTION

5.1.1. *Agrobacterium* taxonomy and nomenclature

The *Agrobacterium* genus is comprised of four well-established taxa and a number of new strains isolated more recently (Kersters and De Ley, 1984; Sawada et al., 1993; Bouzar et al., 1995; Ridé et al., 2000). The taxonomy of the *Agrobacterium* genus was originally based on phytopathogenic characteristics, i.e. disease symptoms, caused by the presence of a particular plasmid, to distinguish between different *Agrobacterium* species. Thus, *A. radiobacter* was the designation for non-pathogenic strains, *A. tumefaciens* for tumorigenic Ti-plasmid strains and *A. rhizogenes* for strains carrying Ri-plasmids. However, as plasmids can be lost or are transferable to *Agrobacterium* strains lacking them, they clearly do not provide a stable basis for taxonomic classification (Kersters and De Ley, 1984; Young et al., 2001). Furthermore, the morphology, physiology and genotype of some strains, allocated to different species, were indistinguishable, whereas other strains grouped by pathogenicity were completely different in terms of other characteristics. For example, the high level of DNA-DNA relatedness between strains originally known as *A. radiobacter* and *A. tumefaciens* suggests that these are closely related and should be considered as the same species (Sawada et al., 1993).

Thus, subsequent revisions of the definition of *Agrobacterium* species have generally grouped strains into biovars or species, on the basis of chromosome-encoded characteristics and independently of pathogenicity related to plasmid type. Four main taxa are generally defined: biovar 1 strains (*A. tumefaciens* and *A. radiobacter*); *A. rhizogenes* (previously biovar 2); *A. vitis* (biovar 3) and *A. rubi* (a small group, originally isolated from *Rubus* spp, e.g. blackberry) (Kersters and De Ley, 1984; Ophel and Kerr, 1990; Sawada et al., 1993; Bouzar, 1994).

Biovar 1 strains are generally referred to as *A. tumefaciens* and a prevalent view is that biovar 1 strains should be named *A. tumefaciens*, with pathovar designation according to pathogenicity; thus, *A. tumefaciens* pv. *tumefaciens* for tumour-inducing strains and *A. tumefaciens* pv. *rhizogenes* for rhizogenic strains (Kersters and De Ley, 1984; Bouzar, 1994). Most rhizogenic strains are in the species *A. rhizogenes* which also includes some tumorigenic strains carrying Ti-plasmids (designated as *A. rhizogenes* pv. *tumefaciens* by this nomenclature) (Kersters and De Ley, 1984). Recently, further revision of the *Agrobacterium* and *Rhizobium* genera has been proposed, suggesting an amalgamation of all *Agrobacterium* species into the genus *Rhizobium* (Young et al., 2001). The basis for this proposal is the close phylogenetic relationship of *Agrobacterium* to *Rhizobium*, based on 16S rRNA sequence analyses, as well as the unresolved inconsistencies in the formal *Agrobacterium* nomenclature. Under this proposed revision, *A. tumefaciens*, *A. rhizogenes*, *A. rubi* and *A. vitis* would be renamed *Rhizobium radiobacter*, *R. rhizogenes*, *R. rubi* and *R. vitis*, respectively (Young et al., 2001).

In addition, a number of unusual marine bacterial strains isolated from the northeastern Atlantic Ocean were suggested to have sufficient similarity to be classified as *Agrobacterium*, based on low-molecular-weight RNA profiles. However, placement of all of these strains in the *Agrobacterium* genus is not conclusive (Rüger and Höfle, 1992). Other strains not conforming to the four defined species have also been identified (Sawada et al., 1993; Bouzar et al., 1995).

5.1.2. Classification of *Agrobacterium* strains

The overall *Agrobacterium* genus classification is based on results of analyses including numerical taxonomy of phenotypic characteristics, biochemical and physiological characteristics, DNA-DNA relatedness (homology) and thermal stability of DNA-DNA hybrids, electrophoregrams of soluble proteins and comparison of 16S rRNA sequences (Kersters and De Ley, 1984; Sawada et al., 1993; Bouzar et al., 1995; Ridé et al., 2000). Examples of variations in biochemical and physiological characteristics between species include requirements for growth factors, utilisation of certain substrates and formation of reaction products. For example, biovar 2 strains are able to utilise citrate and L-tyrosine and can use erythritol as a sole carbon source, whereas biovar 1 strains can utilise citrate but not L-tyrosine or erythritol (Kersters and De Ley, 1984; Sawada et al., 1993).

As well as these more classical methods, other techniques such as sequencing or RFLP analysis of rRNA genes are now commonly applied for identification and classification of *Agrobacterium* species, as well as species of the closely related *Rhizobium* genus (Bouzar et al., 1995; Burr et al., 1995; Terefework et al., 1998). Analysis of fatty acid profiles has also become a widely employed method for classification of bacteria (Mahaffee and Kloepper, 1997). Bouzar et al. (1993) tested strains for carbon-source utilisation and fatty acid content profile, both methods utilising rapid automated identification systems. From 95 different carbon sources, several were identified which could be used to distinguish between species and analysis of fatty acid content indicated two acids of diagnostic value for differentiating between *Agrobacterium* species. Cluster analysis of relationships between the strains based on these two systems generally confirmed the division of strains into four distinct species groups.

5.1.3. Comparison of *A. rhizogenes* strains

In terms of similarity of strains within the species, *A. rhizogenes* (biovar 2) strains are highly homogeneous, with DNA homology of 80-100% between most *A. rhizogenes* strains, based on DNA/DNA heteroduplex hybridisations (Kersters and De Ley, 1984). In contrast, biovar 1 strains are a heterogeneous group, with about 45-50% DNA homology between subgroups. It has also been demonstrated that agropine strains of *A. rhizogenes* appear to have almost identical Ri plasmids (Costantino et al., 1981; Jouanin, 1984), although differing from Ri plasmids of strains encoding other opines (Costantino et al., 1981). However, a comparison of Ri plasmids from agropine, mannopine and cucumopine strains (1855, 8196 and 2659 respectively) identified two highly homologous regions of Ri T-DNA present in all three plasmids (Filetici et al., 1987). In relation to agropine Ri T-DNA, one region of homology covers the TL-DNA region including ORFs 8, 9 and 10 (*rol*A) and the second region incorporated ORFs 13 and 14. The intervening region between these conserved segments, comprising the *rol*B and *rol*C genes, showed much weaker homology between strains. In another study, T-DNA heteroduplex formations were analysed to identify regions of Ri T-DNA homology between the same strains (Brevet and Tempé, 1988). Conclusions were generally consistent with the former study, with two exceptions - the *rol*A ORF was found to be located outside of the homologous region and ORF13 was not completely within the second homologous region.

5.1.4. *Agrobacterium* species in Australia

To date, most of the research on *Agrobacterium* species in Australia has related to crown gall-inducing bacteria, *A. vitis* and *A. tumefaciens*. *A. tumefaciens* was identified as a major pathogen in nursery and orchard species such as peach, almond and plum, with plant damage and stunted growth resulting from tumour formation presenting serious and economically significant problems (Kerr, 1969; Keane et al., 1970). Also prevalent in Australia, *A. vitis* predominantly infects grapevines, causing crown gall tumour formation and tissue necrosis (Burr and Katz, 1983; Ophel and Kerr, 1990). A serious problem in viticultural areas worldwide, *A. vitis* is not thought to be indigenous to Australia and was probably introduced from other grapevine sources, including from California, USA (Gillings and Ophel-Keller, 1995). As the bacteria can survive systemically in the vines with no visible gall formation (Burr et al., 1987), latent infections were previously not recognized or detectable and therefore probably unknowingly spread by distribution of propagated rootstocks and cuttings (Gillings and Ophel-Keller, 1995).

The presence of *A. rhizogenes* in Australia does not appear to have been reported to date. Earlier work on developing selective media and biochemical tests for *Agrobacterium* was generally conducted with the aim of identifying pathogenic tumour-inducing *Agrobacterium* (Keane et al., 1970; New and Kerr, 1971; Brisbane and Kerr, 1983). Until later, investigations relating to *A. rhizogenes* were relatively uncommon.

5.1.5. Potential role for *A. rhizogenes* in promotion of plant growth

In addition to soil organisms such as mycorrhizal fungi and nodule-forming bacteria, which form symbiotic relationships with plants, asymbiotic microorganisms also constitute a significant presence in the rhizosphere. Many of these naturally occurring soil bacteria have been demonstrated to be capable of colonizing roots and having stimulatory effects on plant growth, and have been termed plant growth promoting rhizobacteria (PGPR) (Chanway, 1997). Greenhouse experiments with *Agrobacterium* spp. have indicated the possibility of some promotion of plant growth when applied to pine and beech trees (Leyval and Berthelin, 1989;. 1993).

Although often described as a plant pathogen, the effects of infection by *A. rhizogenes* on plants could be considered as more like that of a symbiotic or commensal relationship between the plant and bacteria. The root-inducing effects of

A. rhizogenes have potential benefits for horticultural, agronomic and forestry applications (Strobel and Nachmias, 1985; Lambert and Tepfer, 1991; Häggman and Aronen, 2000), including promotion of root system size or branching, and induction of roots on cuttings of normally recalcitrant species.

5.1.6. Influence of soil environment factors on rhizosphere bacterial populations

Soil characteristics and plant-related influences are factors that may affect rhizosphere microbial populations, and therefore the presence of *Agrobacterium* species, in certain environments. Soil characteristics have been shown to influence the colonisation of rhizosphere environments by microorganisms and can be correspondingly important in determining the size and composition of rhizosphere populations (Alabouvette and Steinberg, 1995; Chiarini et al., 1998). These influences may include abiotic and biotic soil factors, such as soil texture and type, pH and chemical profile (organic and inorganic elements).

Plants also affect rhizosphere populations by secretion of root exudates (Curl and Truelove, 1986). Accumulating in the rhizosphere as a rich source of nutrients and biologically active chemicals, root exudates have a key role in stimulation of microorganisms. In addition, a distinct class of root cells, known as root border cells, may have a significant role in influencing rhizosphere microbial populations and their interactions with plant roots. Root border cells are healthy somatic cells that have differentiated from root cap cells and are programmed for release from the root tip, in response to environmental and endogenous signals (Hawes et al., 1998). Differentiation of root border cells is accompanied by modifications in gene expression and a subsequent synthesis and extracellular export of low molecular weight proteins. In addition, chemicals with known biological activity, such as nodulation gene-inducing signals, are also released (Brigham et al., 1995; Hawes et al., 1998).

Root border cells have been shown to have the capacity to influence the expression of certain genes in soil microorganisms necessary for establishing plant-microbe interactions (Hawes and Brigham, 1992; Zhu et al., 1997). Considerable increases in expression of *nod* genes from two *Rhizobium* spp. were observed in response to root border cells from alfalfa (*Medicago sativum*) and, to a lesser degree, from pea (*Pisum sativum*) root border cells (Zhu et al., 1997). In the same study, slight increases in expression of *vir*E from *A. tumefaciens* were also observed, in response to

root border cells from the two species tested - pea, an *A. tumefaciens* host species, and corn (*Zea mays*), a non-host species.

Thus, via physiological and chemical factors, including those arising from root exudates and root border cells, plants may influence the composition of rhizosphere microbial populations by establishing certain soil environment conditions that are conducive to specific microorganisms. For example, fluorescent pseudomonad populations isolated either from roots of tomato and flax plants or from uncultivated soils were found to differ in ability to utilise certain organic compounds (Lemanceau et al., 1995). Changes in the developmental stage of plants can also affect the diversity of microbial populations in the rhizosphere (Di Cello et al., 1997; Chiarini et al., 1998).

Specific selective advantage can be conferred to particular bacteria by engineering of plant exudates, as has been demonstrated with opine-utilising bacteria and transgenic plants producing the corresponding opine. The term "biased rhizosphere" has been applied to describe these effects (O'Connell et al., 1996). As observed in several studies, growth of opine-utilising bacteria is specifically favoured in the rhizosphere of plants producing that opine (Guyon et al., 1993; Oger et al., 1997; Savka and Farrand, 1997). For certain opine-utilising pseudomonad strains, a catabolic bias was found to remain even several months after the removal of transgenic opine-producing *Lotus corniculatus* plants (Oger et al., 2000).

This chapter presents results of a survey for the presence of *A. rhizogenes* in locations in Melbourne and regional Victoria, conducted to investigate the possibility that free-living *A. rhizogenes* may be present in local soils. It is conceivable that the presence of *A. rhizogenes* may not have been noticed to date, as the rhizogenic effects are not necessarily deleterious to plant growth. Furthermore, if *A. rhizogenes* strains were able to colonise the rhizosphere of plants growing in their vicinity, these bacteria may have the potential to play a role in the growth and survival of the local vegetation.

The possible presence of *A. rhizogenes* in the surrounding environment may also have implications for potential agronomic applications of transgenic plants containing *rol* genes, such as white clover. The release of transgenic plants into the environment, even controlled agricultural environments, is viewed with apprehension in the general community. Although a remote possibility, the prospect of *rol* genes transferring from decaying plant roots to soil microbes is likely to be an issue of some concern. The prior presence in the rhizosphere of bacteria containing such genes may

help to alleviate concerns about the use of genetically engineered crops transformed with these genes.

As described above, creation of a biased rhizosphere, by release of certain plant exudates or root border cells from transgenic plants, may alter the ecology of rhizogenic bacteria (O'Connell et al., 1996; Oger et al., 2000). For plants transformed only with *rol* genes, a similar basis for an influence on surrounding micro-organisms does not seem likely, as these genes do not appear to encode production of particular nutritional sources, such as opines. It is conceivable, however, that some other factor, perhaps hormone-related, resulting from the presence of *rol* gene-transformed plants, may affect rhizogenic agrobacteria populations and subsequently alter microbial balance in the rhizosphere. Conversely, it is possible that agrobacteria may interact with *rol* gene transgenic plants and influence plant growth in unforeseen ways.

These are possibilities that would need to be investigated in more detail, with comprehensive testing, as important considerations relating to the use of transgenic plants. Thus, a relevant preliminary step is to ascertain the likelihood of the presence of *A. rhizogenes* in the local environment.

5.2. MATERIALS AND METHODS

5.2.1. Soil sampling locations

A total of 45 soil samples were collected (from 39 different geographical locations) around metropolitan Melbourne and Victoria (Fig 5.1, 5.2). Samples were collected during the spring season, when plants are most likely to be actively growing. Approximately 25g samples were taken from 5-10cm under the soil surface, where the soil was moist. Samples were collected into sterile glass containers and conveyed to the laboratory for analysis within 2-3 days.

Most samples were collected from cultivated garden or farm areas, near plants and/or fruit trees (Table 5.1). 17 samples were from less developed, natural bush environments, with surrounding native trees and scrub. These also included locations beside a creek, in national parkland (native bush) and open native grass paddocks.

Table 5.1. General environment from which soil samples were collected.

Description of location	No. of samples
Cultivated garden, orchard or farm with surrounding vegetation	28
Natural / bushland environment near native trees or scrub	17

5.2.2. Isolation of putative *Agrobacterium* strains from soil

Following the method of Moore et al. (1988), 1.5 g of each soil sample was weighed out and mixed with 5 ml of a 1M phosphate buffer solution (88% (v/v) 1M NaH_2PO_4; 12% (v/v) 1M Na_2HPO_4; pH 6), shaken vigorously and then allowed to settle. Plates of selection medium 2E (see below) were spread with 0.1 ml of undilute soil suspension or 1/5 and 1/10 dilutions of the mixture.

5.2.3. Selection medium 2E

A selective medium, 2E, developed by New and Kerr (1971) and modified by Brisbane and Kerr (1983) and Moore et al. (1988) was used (composition specified in §2.3). This medium selects for biovar 2 *Agrobacterium*, which includes most of the *A. rhizogenes* species tested (Kersters and De Ley, 1984). The basis of selection is erythritol, which is the sole carbon source provided and which biovar 1 strains cannot utilize(New and Kerr, 1971; Brisbane and Kerr, 1983). Erythritol is an intermediate in the tricarboxylic acid carbon cycle. Inclusion of sodium selenite inhibits growth of other soil microbes, including rhizobia (New and Kerr, 1971).

After soil samples were spread on the medium, the plates were placed at 25°C. Sterile soil samples inoculated with *A. rhizogenes* strain A4 were also included. Colonies were sufficiently grown for observation after 4-5 days. *A. rhizogenes* colonies are typically convex, circular and glistening, i.e. quite slimy, as growth on carbohydrate-containing media is usually accompanied by copious extracellular polysaccharide slime (Kersters and De Ley, 1984). Selected colonies with the typical appearance of *Agrobacterium* were repatched onto new plates of selective media for confirmation and purification.

Figure 5.1. Soil sample collection sites in regional Victoria - locations as indicated on the map.

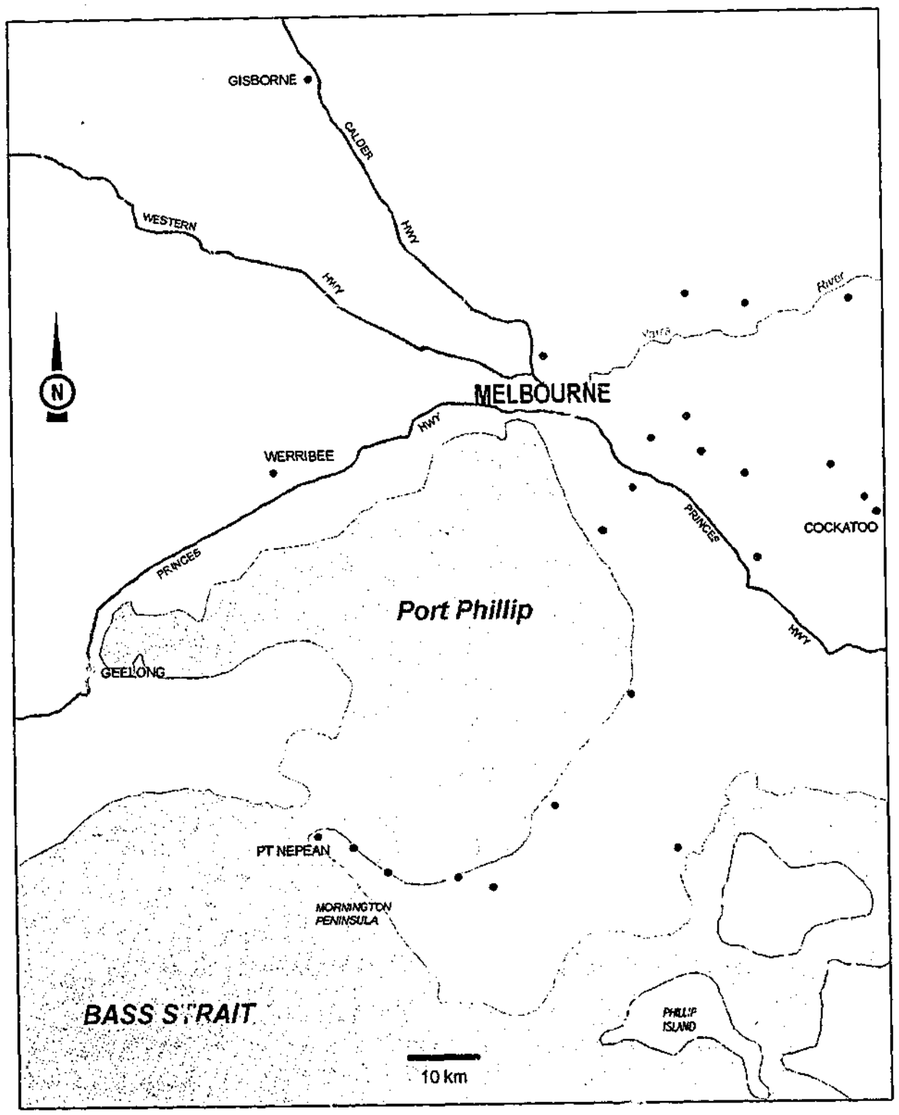


Figure 5.2. Soil sample collection sites in the Melbourne metropolitan area, marked by circles. Sites at the outermost locations are labelled on the map.

5.2.4. Colony nybridisation

Putative *Agrobacterium* strains isolated from selection medium 2E were numbered for identification and patched onto solid YMB medium to allow vigorous growth. As *Agrobacterium* colonies generally extrude a viscous polysaccharide slime, the most controlled way to transfer bacteria was to dab small amounts of bacterial colonies onto relevant positions onto the Hybond N^+ nylon membrane, using sterile toothpicks. DNA was then denatured and fixed using standard procedures for colony blots (see §2.1.1). The membrane was probed under medium stringency conditions with a fragment of a highly conserved region of Ri plasmid as described below. *A. rhizogenes* strains A4 and 9402 were used as positive controls. Negative controls were *A. tumefaciens* strain T37 and an *E. coli* strain DH5α.

5.2.5. Probe for colony blot hybridisation

The probe fragment was chosen from conserved regions present in the Ri T-DNA of three Ri plasmid types, agropine, mannopine and cucumopine (Filetici et al., 1987). Two zones of very high sequence homology were identified in all three plasmid types. One of the strongly conserved regions is a 4kb region that, according to the map of Filetici et al. (1987), includes all of the agropine TL-DNA *Eco*RI fragment 40 and a portion of the *Eco*RI fragment 15, including the *rol*A gene (see Fig. 5.3). DNA fragments corresponding to this region may be useful for identifying any *A. rhizogenes* strains possessing Ri-plasmids present in soil samples, as they may also be likely to contain these highly conserved regions. A fragment from this region was prepared for use as a probe (Fig. 5.3) and was isolated as follows: *Bam*HI fragment 8 of *A. rhizogenes* strain A4 T-DNA (Jouanin, 1984) had been previously subcloned into a pGEM3Z plasmid. This fragment was re-isolated and then digested with *Eco*RI. Three fragments result from this digest - with sizes of 6 kb, 1.9 kb and 1.43 kb. The 1.9kb fragment, denoted B8/E2, overlaps with the right-hand end of one of the two homologous Ri-plasmid regions (Filetici et al., 1987). This 1.9 kb B8/E2 fragment was used as a probe for identification of putative *A. rhizogenes* colonies.

5.2.6. DNA extractions and restriction enzyme digests

Total genomic DNA was extracted from *Agrobacterium* strains according to the protocol outlined in §2.1.1. Restriction enzyme digests of extracted DNA were carried out according to standard procedures (see §2.1.1). Several reference *A. rhizogenes* strains were analysed in comparison; these were agropine strains A4 and 9402, mannopine

strains 2626 and 8196 and cucumopine strains 2628 and 2655 (from *A. rhizogenes* collection, Department of Biological Sciences, Monash University).

5.2.7. Hybridisation of restriction fragments to T-DNA probes

The digested DNA was probed with each of the probe fragments B8/E2, B5 and B20 as described below (Fig. 5.3), under medium stringency hybridisation conditions. Each radiolabelled probe was removed before the next hybridisation was carried out (see §2.1.1).

Probe1: **B8/E2**, the same 1.9 kb fragment as used for probing colony blots.

Probe2: **B5**, the 12 kb fragment 5 released by *Bam*HI digest of agropine Ri plasmid (Jouanin, 1984). (NB this *Bam*HI fragment was designated as fragment 7 in Filetici et al., (1987)). This fragment, denoted here as B5, covers the second conserved region in the three Ri plasmid types (Filetici et al., 1987), including ORFs 13 and 14.

Probe3: **B20**, a TR-DNA fragment of agropine Ri plasmid (Jouanin, 1984), which includes part of one of the *aux* genes. This is a 4.3 kb fragment corresponding to fragment 20 of the *Bam*HI digest of pRi A4.

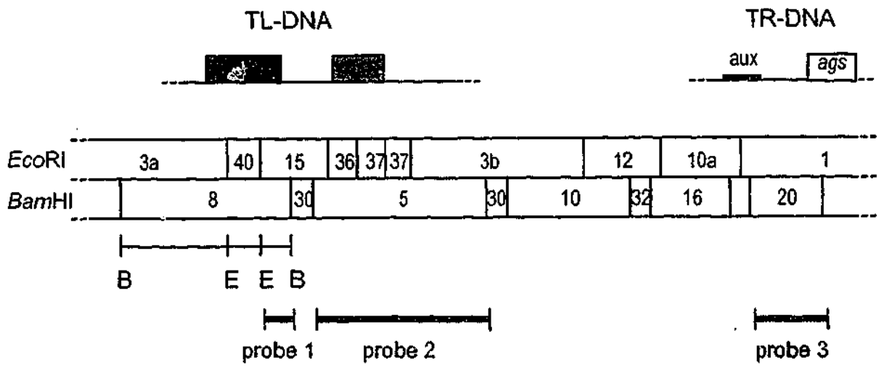


Figure 5.3. Restriction map of relevant regions of A4 T-DNA and fragments corresponding to probes 1, 2 and 3 (modified from Filetici et al., 1987). It was noted by Jouanin (1984) that Ri plasmids of agropine strains are essentially identical to each other. The map represents relative locations of fragments but fragment sizes are not exactly to scale. Probe 1 (red) = *Bam*HI 8/EcoRI; probe 2 (green) = *Bam*HI 5; probe 3 (blue) = *Bam*HI 20. Conserved T-DNA regions are represented by striped boxes; the box marked *ags* indicates approximate location of agropine synthesis genes; the bar marked *aux* indicates auxin synthesis genes. Restriction enzyme cleavage sites: B, *Bam*HI; E, *Eco*RI.

5.2.8. Root induction tests

Tests for rhizogenic potential of putative *Agrobacterium* strains were conducted with carrot and beetroot, obtained from a local fruit market. Carrots and beets were peeled and surface sterilised in a 10% (v/v) solution of household bleach (5% available hypochlorite) for 30 minutes, then rinsed 4 times in sterile distilled water in sterile laminar air flow cabinet. The outer surfaces of roots were then again cut away to remove bleached, damaged tissue.

Transverse slices or discs of beet and carrot were cut, approximately 0.5cm thick and ensuring all pieces included cambial tissue. Beetroot slices were cut into wedge-shaped segments. Carrot and beetroot pieces were approximately 2.5 – 3 cm^2 in area. Vegetable pieces were placed on M+S medium containing 2% sucrose (w/v) and 1 mg l^{-1} 6-benzylaminopurine (BAP) in divided 9 cm petri dishes and inoculated with fresh bacterial suspensions spread over the cut surface. 6-9 individual pieces were inoculated for each strain. Carrot discs were inoculated on the apical side, to ensure any rhizogenic capacity of bacteria was not overlooked as a result of polar root induction. In the absence of exogenous auxin or *aux* genes, such as in mannopine and cucumopine strains, root induction from carrot discs is polar, forming only from the apical side (Cardarelli et al., 1985). This is due to auxin-depletion of the basal side, as a result of the directional flow of auxin from root base to apex. For sections of beetroot tissues, inoculation of either apical or basal surfaces with *A. rhizogenes* strains has been observed to be equally effective for root induction (J.D. Hamill, pers. comm.).

After two days, plant tissues were transferred to fresh M+S medium with 2% sucrose and 500 mg l^{-1} ampicillin, to suppress bacterial overgrowth. Thereafter, tissues were transferred every 2-3 days to fresh media, supplemented with either ampicillin (500 mg l^{-1}) or cefotaxime (500 mg l^{-1}).

5.3. RESULTS

5.3.1. Selection of putative strains

An ample coverage of colonies appeared on most selection plates, with one or two main morphological types predominating. Many of these resembled colonies of the A4 control strain, which grew on the selection medium, and the general descriptions of the typical appearance of *Agrobacterium* (Kersters and De Ley, 1984). A large number of these colonies were chosen and reselected, with most growing very vigorously on the fresh

selection plates. Some colonies were not repatched successfully or may have been false-positive selections in the first screen.

5.3.2. Colony blot hybridisation

1200 different bacterial colonies, representing 45 different soil samples, were screened for hybridisation to the conserved Ri plasmid region corresponding to probe 1 (B8/E2) (Fig. 5.3) (Filetici et al., 1987). The A4 and 9402 reference strains hybridised strongly with the probe, while no hybridisation signals were observed for the *A. tumefaciens* or *E. coli* negative controls. A range of signal intensities was observed for the putative *Agrobacterium* strains (Fig. 5.4). Although there was some variation in the amount of bacterial DNA present on the membrane, the presence of any signal suggested the possibility of some homology with the conserved probe sequence.

Colonies that showed a reasonable level of hybridisation to the probe were identified and purified on YMB plates for further analysis.

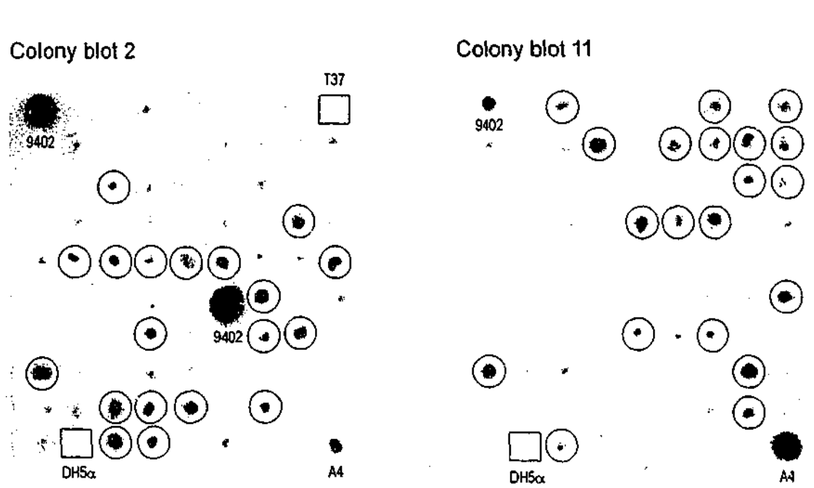


Figure 5.4. Colony blots of putative *A. rhizogenes* strains. 1200 different colonies obtained from selection medium 2E were numbered and blotted onto membranes then probed with the B8/2E probe. Selected autoradiographs (2 of 13) are shown, displaying examples of hybridization signals obtained with the B8/E2 probe. The A4 and 9402 strains of *A. rhizogenes* were used as positive controls, as labeled on the figure. *E. coli* strain DH5α and *A. tumefaciens* strain T37 were included as negative controls, with positions indicated by boxes on the figure. Signals considered as possible positive hybridizations are marked by circles. Positive signals were present on all (13) autoradiographs, with no particular trend apparent in terms of geographic location or environment type.

5.3.3. DNA extractions and restriction digest.

DNA extracted from 28 positively hybridising strains was digested with *Eco*RI for comparison with restriction patterns of several reference strains. These were A4 and 9402 (agropine), 2626 and 8196 (mannopine), 2628 and 2655 (cucumopine).

Photographs of agarose gels show the digested DNA of controls and test strains (Fig 5.5A, B). A degree of variation is apparent in the amount of DNA present; of the positive controls, the quantity of cucumopine strain 2628 DNA appears relatively low.

5.3.4. Analysis of restriction fragments

The digested genomic DNA was blotted and probed separately with the 3 different T-DNA probes, B8/E2, B5 and B20. Probes hybridised with DNA of reference strains but no hybridisation was evident with any of the putative *Agrobacterium* strains (Fig. 5.6 and 5.8). Only autoradiographs corresponding to Gel 1 (Fig. 5.5A) are shown, as no signals were evident for any strains present on Gel 2.

A second gel and Southern blot of the same reference strains were also carried out to confirm hybridisations with Probe 1; these are referred to as Gel 1-2 and Blot 1-2 (Fig. 5.5B, 5.6B). In the reference strains, thesizes of hybridising bands were consistent with expected sizes, as calculated from plasmid maps (Jouanin, 1984; Filetici et al., 1987). Negative controls did not hybridise with any of the probes.

The lack of hybridisation of any of the probes to the DNA of putative *A. rhizogenes* strains was not likely to have been due to problem with DNA, gels or blots, as DNA from the positive control (reference) strains were able to bind probes. Thus, the absence of hybridisation under medium stringency conditions suggests that the isolated bacteria were not *A. rhizogenes*, or at least not of a type sharing conserved sequences with other known *A. rhizogenes* strains.

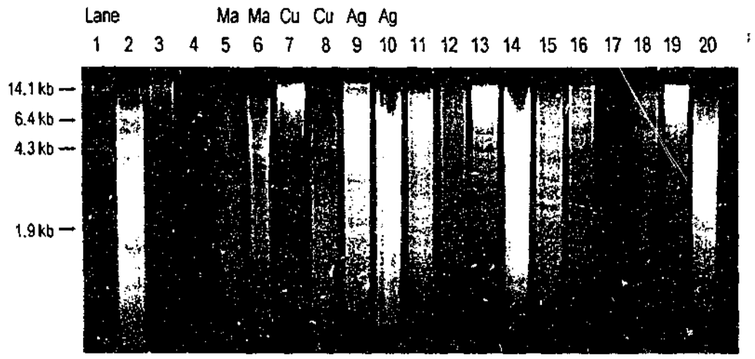
The absence of hybridisation of genomic DNA with the B8/E2 probe (Probe 1) is also inconsistent with the results of the colony screen, in which positive hybridisations of these strains to this same probe were detected, to various extents. The fact that digested genomic DNA did not hybridise to the same probe may reflect the technical accuracy of probing extracted and digested genomic DNA compared to screening bacterial colonies directly. However, as neither of the other two T-DNA probes hybridised to genomic DNA of any putative strains, this appears to confirm the outcome of the B8/E2 probe and indicate the absence of conserved Ri plasmid sequences in these strains.

Figure 5.5. *(Facing page)* Agarose gels with digested DNA of putative *Agrobacterium* strains and reference strains prior to blotting. Strains are as listed below, numbers represent site number/colony number.

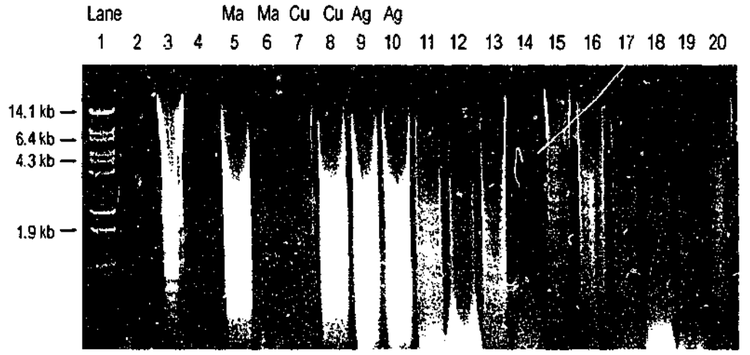
(A, B) Gels 1-1 and 1-2: Positive controls - lanes 5-10, ma=mannopine; cu=cucumopine, ag=agropine. Negative controls - lane 2, *E. coli* strain DH5α (not included on gel 1-2); lane 3, *A. tumefaciens* strain T37. Putative *Agrobacterium* strains - lanes 11-20. (C) Gel 2: Putative *Agrobacterium* strains - lanes 3-20.

Lane	Strain	
	Gels 1-1 and 1-2	Gel 2
1	DNA markers	DNA markers
2	DH5α	-
3	T37	27/12
4	-	27/16
5	2626 (ma)	35/4
6	8196 (ma)	35/20
7	2628 (cu)	38/2
8	2655 (cu)	43/22
9	9402 (ag)	46/7
10	A4 (ag)	49/29
11	3/17	51/10
12	7/18	52/30
13	12/10	54/14
14	13/15	56/4
15	14/3	56/5
16	15/4	56/16
17	15/11	57/13
18	16/26	58/19
19	25/21	59/8
20	27/12	59/28

(A) GEL 1-1 Reference strains and putative *Agrobacterium* strains



(B) GEL 1-2 Reference strains and putative *Agrobacterium* strains



(C) GEL 2 Putative *Agrobacterium* strains

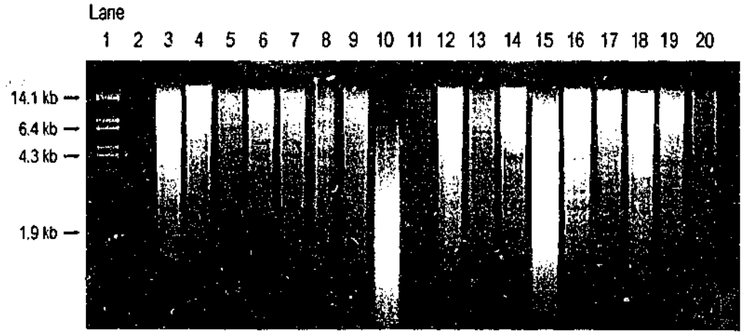


Figure 5.6. (Facing page) Southern blot analysis of putative *A. rhizogenes* strains: hybridisation of Probe 1, B8/E2, to reference strains. (A) Blot 1-1 (from Gel 1-1); (B) Blot 1-2 (from Gel 1-2).

For Blots 1 and 2, bacterial DNA was digested with *Eco*RI and probed with the B8/E2 fragment of TL-DNA from agropine strain A4. Hybridisations were carried out at 60°C with medium stringency washes (1xSSC; 0.5% (w/v) SDS). DNA loadings on Gel 2 for mannopine strain 8196 and cucumopine strain 2628 were much lower than for the other reference strains.

Agropine strains (lanes 9, 10) - According to the agropine restriction map (Jouanin, 1984), Probe 1 would be expected to hybridise to the 4.3 kb *Eco*RI fragment 15 of 9402 and A4 T-DNA (see Fig. 5.7 below). In (A) this corresponds with the strongest band for 9402 DNA and the band in the A4 DNA.

The smaller hybridisation band apparent in 9402 corresponds in size to the fragment *Eco*RI 36 (1.9 kb), which is to the immediate right of *Eco*RI 15 (see Fig. 5.7 below) and may have cross-hybridised due to a slight overlap with the probe sequence. The larger band (5.5 kb) may possibly represent the *Eco*RI 3a fragment, which overlaps with *Bam*HI 8. Again, this may be due to cross-hybridisation, although *Eco*RI 3a is not immediately adjacent to the probe region. As Probe 1 was originally cut from fragment *Bam*HI 8 (see Fig. 5.3), it is possible some of *Bam*HI 8 may have still been present and therefore hybridised to the *Eco*RI 3a fragment. However, on Blot 1-2 (B), hybridisation of 9402 and A4 T-DNA with Probe 1 was specific to the 4.3 kb fragment, confirming this as the correct fragment. Due to experimental variations, hybridisation conditions may have been slightly less stringent for Blot 1-1, resulting in some non-specific binding.

Mannopine strains (lanes 5, 6) - (A) Probe 1 appeared to hybridise to a fragment approximately 5.5 kb in size, as expected (see Fig. 5.7 below). This corresponds to *Eco*RI fragment A. For Blot 1-2, it can be seen from the agarose gel (Fig. 5.5B) that the amount of 8196 DNA is insufficient; however, DNA of strain 2626 is adequate and also shows a clear hybridisation band corresponding to a 5.5 kb fragment (B).

Cucumopine strains (lanes 7, 8) did not show hybridisation with Probe 1 on Blot 1-1, although on Blot 1-2, Probe 1 hybridised successfully with 2655 DNA, binding to a band approximately 5.5 kb in size. An insufficient amount of 2628 DNA on both Gels 1-1 and 1-2 (Fig. 5.5A, B) is likely to be the reason for the lack of hybridisation with this strain.

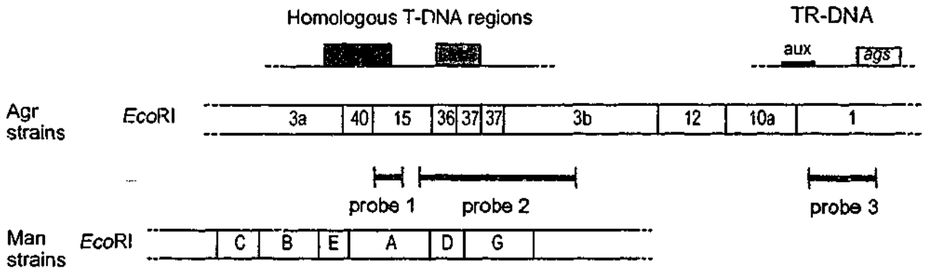
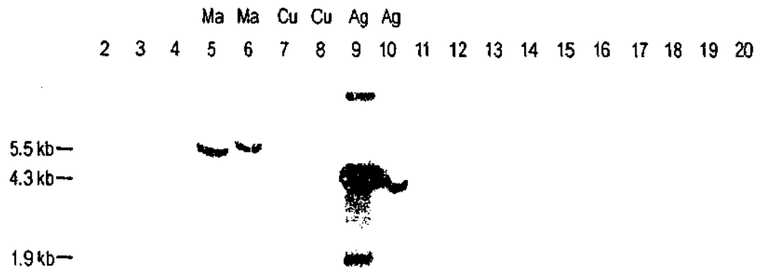
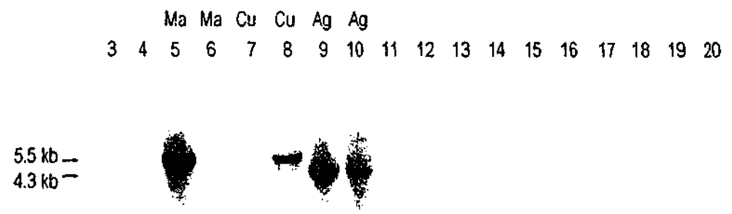


Figure 5.7. Restriction map of relevant sections of agropine and mannopine T-DNA, indicating the respective *Eco*RI fragments expected to hybridise to probes. T-DNA map modified from Filetici et al. (1987) and Jouanin (1984).

(A) PROBE 1 BLOT 1-1



(B) PROBE **1** BLOT 1-2



Lanes 2, 3 - negative controls DH5α and T37; lane 4 - blank; lanes 5, 6 - mannopine strains 2626 and 8196; lanes 7, 8 - cucumopine strains 2628 and 2655; lanes 9, 10 - agropine strains A4 and 9402; lanes 11-20 unknown strains. Negative control DH5α was not included on Blot 1-2 or Gel 1-2.

Figure 5.8. *(Facing page)*. Southern blot analyses of *A. rhizogenes* strains (A) hybridisation of Probe 2, B5 (B) hybridisation of Probe 3, B20. These DNA blots correspond to Gel 1-1.

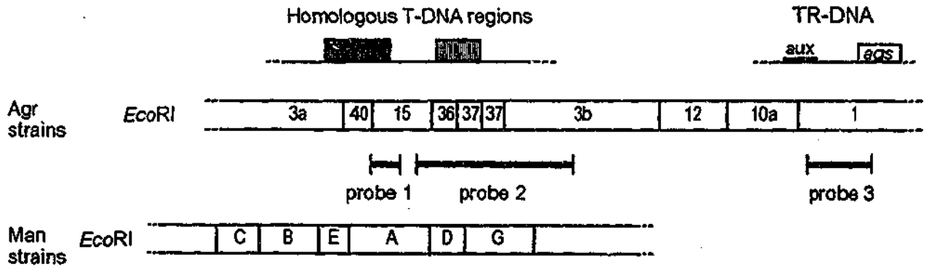
Bacterial DNA was digested with *Eco*RI and probed with relevant fragment of TL-DNA from agropine strain A4. Hybridisations were carried out at 60°C with medium stringency washes (1xSSC; 0.5% (w/v) SDS).

(A) Agropine strains (lanes 9, 10) - Probe B5 hybridised as expected to four regions of *Eco*RI-digested 9402 and A4 DNA - fragments 3b, 15, 36 and 37 (see T-DNA map below), with sizes of 12.9, 4.3, 1.85 and 1.65 kb respectively (Jouanin, 1984).

Mannopine strains (lanes 5, 6) - Probe 2 hybridised to three fragments from the two mannopine strains as expected, although hybridisation was much weaker than for agropine strains and are difficult to view clearly on the autoradiograph. These correspond to fragments with approximate sizes of 5.5 kb, 4 kb and 1.65 kb - fragments A, G and D respectively, as indicated below.

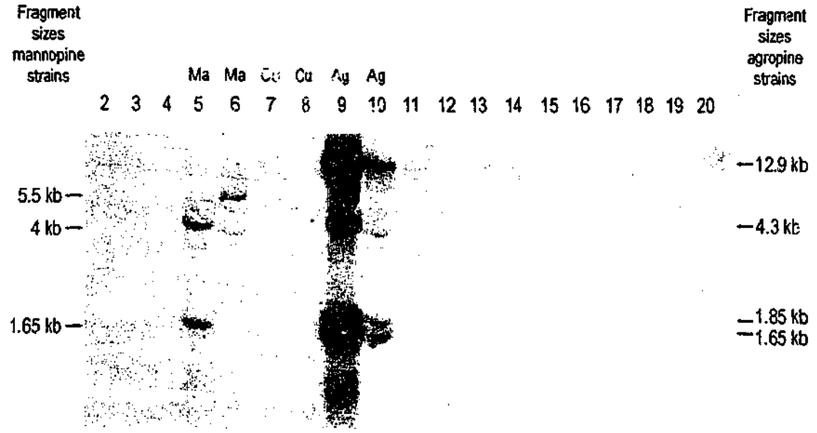
Cucumopine strains (lanes 7, 8) - No hybridisation of Probe 2 was evident with cucumopine strains, which may be due to low loading levels of DNA of these strains on the agarose gel (Gel 1-1, Fig. 5.5A). (This is also consistent with the lack of hybridisation of these strains on Blot 1-1 with Probe 1, whereas on Blot 1-2, Probe 1 was able to detect fragments in 2655 DNA).

(B) Probe 3 appears to have hybridised to *Eco*RI fragment 1 (19.5 kb) of agropine strain 9402, as predicted from restriction maps (see T-DNA map below), although hybridisation to strain A4 is not apparent. For the two mannopine strains, hybridisation was not evident with Probe 3, as this probe corresponds with the TR-DNA region of agropine strains, with which the mannopine strains do not share homology (Filetici et al., 1987; Brevet and Tempé, 1988). Similarly, as expected, hybridisation with Probe 3 was not observed for cucumopine strains.

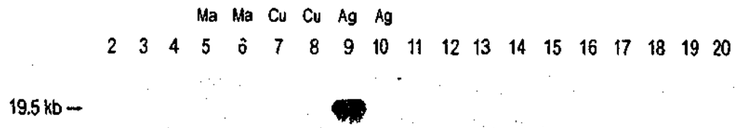


The above figure is a duplicate of Figure 5.7 - restriction map of relevant sections of agropine and mannopine T-DNA, indicating the respective *Eco*RI fragments expected to hybridise to probes.

(A) PROBE 2



(B) PROBE 3



Lanes 2, 3 - negative controls DH5α and T37; lane 4 - blank; lanes 5, 6 - mannopine strains 2626 and 8196; lanes 7, 8 - cucumopine strains 2628 and 2655; lanes 9, 10 - agropine strains A4 and 9402; lanes 11-20 unknown strains.

5.3.5. Induction of roots from carrot and beetroot

Strong root responses were not induced on carrot or beetroot by any of the putative agrobacterial strains. The most common effects observed, if any, consisted of various amounts of callus formation. Several strains (6) induced formation of a small number of roots from carrot pieces; a number of other strains (49) induced some callus formation. From beetroot tissues, no root formation was observed and callus formation was induced by a few strains only. Therefore, these results are not suggestive of a strong rhizogenic capacity, for any of the strains. Of the control strains, mannopine strains 8196 and 2655 in particular were able to induce abundant root formation on carrot discs.

Overall sample numbers, particularly of beetroot, were reduced due to considerable amounts of bacterial overgrowth, despite the presence of antibiotics and frequent transfers to fresh media. Thus, it may be valuable to further investigate the rhizogenicity of isolated strains with a second root induction assay, utilising increased sample sizes and focusing on the particular strains demonstrating responses in the initial assay. In addition, another possible method to confirm the presence of Ri T-DNA may be to assess the ability of excised roots to grow in liquid culture *in vitro* without phytohormones.

5.3.6. Potential identification of strains by PCR amplification

PCR analysis of putative *A. rhizogenes* strains was considered as a possible additional approach to identification of isolated strains. A preliminary PCR analysis was carried out, utilising primers based on the sequence encoding the endonuclease domain of the *virD2* gene (Haas et al., 1995). This domain is responsible for cleaving the T-DNA border sequences and is highly conserved in virulent strains of *Agrobacterium*. PCR amplification of this region detects pathogenic agrobacteria but does not distinguish between *A. rhizogenes* and other species. Therefore, the potential usefulness of another region was also examined, with PCR primers corresponding to the TL-DNA region between the *rol*C and *rol*B genes.

Following amplification, strong bands of the expected size for the *virD2* endonuclease region were evident for a number of strains. Many reactions also produced additional amplification products of various sizes, which may be due to sub-optimal timing or temperature conditions. A potential complication, however, was the presence of false positives, as PCR products of apparently correct sizes were evident for the negative control *E. coli* strain DH5α, for both primer sets. Though great care was

taken in setting up these PCR experiments, the fact that *A. rhizogenes* 9402 has been used for several years in this laboratory may mean that plasmid DNA is widespread, despite all efforts to ensure materials remained uncontaminated. Clearly, for further application of PCR for the identification and analysis of potential *Agrobacterium* strains, optimisation of conditions and minimisation of false positive results would be required.

5.4. DISCUSSION

Analysis of soil samples from various locations suggest that *A. rhizogenes* is not prevalent in Victoria. Putative *Agrobacterium* strains were initially noted on the basis of certain biochemical properties, by growth on selective medium. Positive hybridisation of selected colonies with conserved Ri-plasmid sequences (Filetici et al., 1987) suggested the possibility that these strains might have contained similar regions; however, a subsequent lack of hybridisation of conserved Ri T-DNA probes with DNA of these strains did not support this conjecture. Furthermore, tests for rhizogenicity did not indicate strong root-inducing responses.

5.4.1. Initial selection of putative strains

The selective basis of medium 2E (Brisbane and Kerr, 1983) is the ability of biovar 2 strains to utilise erythritol as a sole carbon source, which biovar 1 and 3 strains are not able to utilise (New and Kerr, 1971; Bouzar et al., 1993). Although utilisation of erythritol is not a categorical basis for selection, particularly with the continuing isolation of novel *Agrobacterium* strains, it is regarded as an effective initial selection method. Many of the other types of microbes generally present in soil samples are inhibited by the medium, such as rhizobia (New and Kerr, 1971), thus increasing the probability of finding *Agrobacterium* strains, if present. Equivalent selective media are also commonly utilised for isolation and detection of *A. tumefaciens* and *A. vitis* (Burr et al., 1987; Moore et al., 1997).

Other diagnostic tests that have been devised to distinguish between biovars include many based on the utilisation of various carbon substrates and biochemical responses to other substances; for example, the production of certain reaction products or changes in indicator colours. Like the use of selection media, these types of tests are not definitive and are limited by possible exceptions to normal responses or the presence of novel strains that may not conform to characteristics of known strains.

Examples of exceptions to the biovar 2 classification criteria have been demonstrated, although strains are still classed as biovar 2. Of 35 biovar 2 strains identified by Bouzar and Moore (1987), 8 were not detected on selective medium 2E, with some unable to grow on erythritol and another group unable to utilise L-tyrosine.

Novel *Agrobacterium* strains were isolated from aerial tumours of fig trees (*Ficus benjamina* L.) (Bouzar et al., 1995). Results of diagnostic tests on tumorigenic strains did not fit in with responses of other biovars, with differences in ability to utilise carbon substrates and composition of fatty acids. Furthermore, phylogenetic analysis based on 16S rRNA gene sequences placed these tumorigenic fig strains in a new *Agrobacterium* species. Similarly, from *Agrobacterium* strains isolated from grapevine galls, Ridé et al. (2000) identified 3 strains which were atypical for a number of characteristics and did not conform phenotypically to any of biovars 1, 2 or 3.

As noted by Bouzar and Moore (1987), isolation of strains which do not match taxonomical characteristics of established biovars is likely to continue to become more common as agrobacteria are identified from novel environments, outside of customary target locations such as nurseries and orchards. Another example of this was demonstrated by the isolation of putative *Agrobacterium* strains from ocean sediments (Rüger and Höfle, 1992).

5.4.2. Colony blot hybridisation of putative *A. rhizogenes* strains

In this analysis, putative *Agrobacterium* colonies identified from selection media were probed with a conserved Ri plasmid sequence, as a means of readily detecting possible *A. rhizogenes* strains. A number of colonies hybridised to this probe, with various intensities, suggesting some may contain common Ri plasmid genes.

The strong similarity of the T-DNA gene sequences between different Ri plasmid types (Filetici et al., 1987; Brevet and Tempé, 1988) implies an involvement in important functions and therefore the reasonable likelihood that *A. rhizogenes* strains isolated from soil samples may also possess these DNA regions. Presumably, these highly conserved regions relate to the rhizogenic functions of *A. rhizogenes*. Regions strongly homologous to these Ri regions were not identified in Ti plasmids by DNA hybridisation studies (Risuleo et al., 1982), although amino acid sequence comparisons indicated weak similarities between the central portion of Ri TL-DNA and some Ti T-DNA genes, particularly the Ri ORF8 and Ti *tms1* gene (Levesque et al., 1988).

Interestingly, the first homologous Ri T-DNA region (left region), as established by Filetici et al. (1987), incorporates the *rol*A gene from agropine strains but not *rol*B,

which has a more prominent role in adventitious root induction. The *rol*A ORF was not placed in the region of homology by Brevet and Tempé (1988). In both studies, the conserved region also includes ORFs 8 and 9, which when transferred alone are not capable of inducing adventitious root formation in tobacco leaf discs (Lemcke and Schmülling, 1998). Similarly, the second region of homology includes ORFs 13 and 14, which also do not have independent capacity to induce root formation (Capone et al., 1989). Thus, Ri T-DNA genes which are not essential for the induction of hairy roots appear to be more highly conserved across different *A. rhizogenes* strains than the key *rol* genes. However, ORFs 8, 13 and 14, have been shown to have important accessory roles with regard to root induction, and in some cases facilitate root induction by *rol* genes in the absence of exogenous auxin (Capone et al., 1989; Lemcke et al., 2000). It is possible that these genes are highly conserved because they are required to ensure the effectiveness of the *rol* genes in inducing rhizogenesis.

5.4.3. Genotypic analysis of putative *Agrobacterium* strains

It is possible that bacterial strains isolated in this study may include novel strains of *A. rhizogenes*, which do not have the conserved Ri T-DNA regions and therefore would not be detected on this basis. Likewise, speculation that these genes may be conserved due to requirement for rhizogenic function suggests that they would not necessarily be present in nonpathogenic strains. The absence of hybridisation of any of the probes to genomic DNA of test strains may support these possibilities. Although the colony screen suggested the presence of sequences hybridising to B8/E2 probe, subsequent probing of genomic DNA with this and two other probes suggested that these did not contain these conserved Ri T-DNA sequences. One possibility that may reconcile these apparently contradictory observations is that bacterial strains may have initially possessed Ri plasmids, which may have subsequently been lost during culture in the laboratory. Although never observed in our experience with standard laboratory strains such as A4 and LBA9402 (cultured at 25°C on YMB medium), plasmid loss may happen as a result of exposure to high temperatures (Fullner and Nester, 1996) or possibly due to culture in nutrient-rich medium, which may conceivably reduce selective pressure for retention of plasmid functions (i.e. opine metabolism, as a C and N source).

A somewhat analogous situation was noted with rhizobia isolated from root nodules of *Phaseolus vulgaris* L. (Mhamdi et al., 1999). Some strains were found to be unable to renodulate the original hosts from which they were isolated; it was suggested that this may be due to the loss of symbiosis genes during lab culture. Also analogous to

the present study, rhizobia strains isolated from *Phaseolus vulgaris* L. were analysed by restriction analysis of genomic DNA, utilising a 16S rDNA sequence and genomic DNA from known reference strains to probe digested genomic DNA fragments and group isolates into types based on the RFLP patterns (Mhamdi et al., 1999). In another example, novel *A. tumefaciens* strains isolated from tumours of fig tree (Bouzar et al., 1995) and chrysanthemum plants were tested for similarity to known strains by probing *Bam*HI-digested genomic DNA with T-DNA and *vir* regions from various reference strains (Vaudequin-Dransart et al., 1995). Positive hybridisations of DNA from chrysanthemum strains to these probes were observed, however strains isolated from fig trees appeared to have little homology to reference strains. No hybridisation was evident with a number of different probes for these strains and no PCR product was amplified using primers corresponding to conserved Ti plasmid regions.

5.4.4. Analysis of rhizogenicity

The testing of strains for pathogenicity, by assessment of ability to induce hairy root, did not reveal any strongly rhizogenic strains. Although testing was restricted to two host species, carrot is a common *Agrobacterium* host and frequently utilised for root induction assays (Kersters and De Ley, 1984). Thus, any capacity for rhizogenic function in the putative *A. rhizogenes* strains would be likely to induce a root-formation response in carrots.

A possible reason for the absence of strong rhizogenic responses may be that the isolated strains are predominantly non-pathogenic or have a very narrow host range. This was observed to be the case with *Agrobacterium* strains isolated from a natural savanna environment (Bouzar and Moore, 1987). It was reported that none of the strains isolated were able to infect tomato seedlings, another commonly utilised host species for pathogenicity tests. Earlier work with stone fruit species also showed that nonpathogenic *Agrobacterium* were highly prevalent over pathogenic strains in the rhizosphere of host plants (Kerr, 1969; New and Kerr, 1972).

A low capacity for root induction by the putative agrobacterial strains may also be consistent with the apparent absence of conserved Ri T-DNA sequences, as it is reasonably likely that a function of these sequences is related to the rhizogenic capacity of *A. rhizogenes*.

5.4.5. Possible approaches for further analysis of putative *Agrobacterium* strains

Other possible diagnostic tests include a characterisation of opines catabolised or produced by potential *Agrobacterium* strains and to identify whether known Ri plasmid-encoded opines are involved. However, as discussed below, opine catabolism is not restricted to *Agrobacterium* species and the capacity for synthesis of a particular opine and its catabolism are not always strictly associated (Moore et al., 1997), so this would not be a definitive identification test for *A. rhizogenes*.

PCR amplification of particular regions is another potential approach to isolation of agrobacterial strains from soil bacteria. The *virD2* region and, most likely, other *vir* loci, are suitable identification sequences for *Agrobacterium*, as these are required for pathogenicity of agrobacteria and are highly conserved (Haas et al., 1995). Along similar lines, Sawada et al. (1995) targeted regions of the *virC* gene for analysis by PCR. However, as the presence of *vir* gene regions does not distinguish between different *Agrobacterium* species, identification of *A. rhizogenes* strains would also require detection of sequences specific to rhizogenic bacteria. For example, the root-inducing (*rol*) genes, or the conserved Ri plasmid regions (Filetici et al., 1987; Brevet and Tempé, 1988) may be appropriate targets, although this would also limit detections to strains in which these sequences were sufficiently conserved to be amplified by PCR. In an analogous example, Haas et al. (1995) performed PCR analysis on strains using primers sets corresponding to both the *virD2* domain and the *A. tumefaciens* cytokinin synthesis gene, *ipt*, to identify pathogenic *A. tumefaciens* strains.

Other well-established methods for phylogenetic placement of *Agrobacterium* and *Rhizobium* species include sequencing of rRNA genes (e.g. Sawada et al., 1993; Bouzar et al., 1995; Burr et al., 1995) and also RFLP fingerprinting of genomic DNA or PCR-amplified sequences (e.g. Khbaya et al., 1998; Momol et al., 1998; Terefework et al., 1998). In one example, *A. vitis* strains from grapevines in three Australian states were compared by fingerprinting of genomic DNA (Gillings and Ophel-Keller, 1995). The identification of unique strains in each state suggested the occurrence of multiple introductions of the bacterium into Australia. Furthermore, DNA fingerprint patterns matched these Australian isolates with strains from a major grapevine area in California, U.S.A, indicating they were most likely introduced with imported grapevine cuttings from that region. In a similar analysis to categorise *A. vitis* strains originating from USA and Europe, the 5'-end of the 23S rRNA gene from isolates were characterised by fingerprinting (Momol et al., 1998). In addition, strains were analysed by use of Random Amplified Polymorphic DNA (RAPD) markers, generating fingerprints of randomly

amplified PCR products to be compared for polymorphisms. These techniques were found to be effective for characterising strains and assessing genetic diversity.

5.4.6. Presence of *Agrobacterium* in natural environments

In general, *Agrobacterium* strains have been isolated from cultivated soils and galls of infected plants in nurseries, orchards and vineyards, in which the pathogenic effects of the bacteria frequently cause problems. Thus, the presence of *Agrobacterium* in nonagricultural environments has not been widely studied.

Bouzar and Moore (1987) investigated the occurrence of *Agrobacterium* in an undisturbed, uncultivated oak savanna and tallgrass prairie in Minnesota, U.S.A. Interestingly, *Agrobacterium* species appeared to be endemic in these soils and were found at levels greater than often observed in cultivated soils. Predominantly biovar 2 strains were isolated and all or most were considered likely to be non-pathogenic, as none were able to infect tomato seedlings, a generally good test species for pathogenicity. Furthermore, only a small proportion of the isolated microbial strains were able to utilise nopaline or octopine as a sole carbon and nitrogen source; of these, most were not *Agrobacterium* species.

The diversity of opine-utilising non-*Agrobacterium* strains has been progressively found to be quite large, including fluorescent and non-fluorescent pseudomonads, coryneforms, *Arthrobacter* and some fungal species (Nautiyal and Dion, 1990; Canfield and Moore, 1991). However, with no evidence of *Agrobacterium*-induced crown gall infections, the only identified source of nopaline and octopine, the significant presence in uncultivated soils of non-agrobacterial strains able to utilise these substrates suggested the possibility of an independent ecological niche for these bacteria, in competition with *Agrobacterium* (Bouzar and Moore, 1987).

These, and further observations made by Moore et al. (1997), diverge from the general basis of the opine concept, which proposes that agrobacteria create a niche for themselves by inciting tumours that produce opines specific to their own requirements (Tempé et al., 1979). Thus, the extent to which opine utilisation confers an advantage to *Agrobacterium* strains may be less clear, or more variable, than previously inferred, particularly in less established environments. Furthermore, Moore et al. (1997), investigating the occurrence of opines and opine-utilising bacteria from naturally occurring (field) tumours of various plant species, found that the correlations between opine catabolic capability and presence of opine in the tumours were not as specific as generally assumed. In some cases, agrobacterial or pseudomonad strains catabolising

certain opines were isolated from tumours, even though no opines of the same types were detected in the tumours. Another highly unusual finding was the discovery of novel strains that could utilise both mannopine and nopaline; furthermore, in laboratory infections, these strains could induce tumours producing either of these opines, but not both. The significance to *Agrobacterium* of a capacity to induce more than one type of opine is not clear, although an ability to utilise a wider range of substrates may have evolutionary benefits in expanding the range of tumours available for colonisation. Further diversity was observed in pathogenic strains able to induce opines from laboratory tumours that were different to the opines present in field tumours from which they were isolated.

Investigations of *Agrobacterium* in different environments have thus indicated that the ecology of *Agrobacterium* and other opine-utilising bacteria may be different, or more complex, in natural and undeveloped environments than in cultivated soils or laboratory and greenhouse situations. Furthermore, as soil composition and plant type have a significant role in influencing rhizosphere microbial populations (Chiarini et al., 1998), further investigation of environments with different soil and vegetation types, as well as the possible presence of novel *A. rhizogenes* strains, may be of interest.

The prevalence of *A. rhizogenes* in natural environments does not appear to have been widely examined in other countries. For example, it is interesting to note that, of strains isolated from uncultivated savanna soils by Bouzar and Moore (1987) most were biovar 2 strains, now designated *A. rhizogenes*, a group that includes most of the known rhizogenic strains. Although a capacity for catabolism of nopaline and octopine was examined, utilisation of opines corresponding to Ri plasmids, such as agropine or mannopine was not tested. It could be speculated that some of the strains may have been rhizogenic strains carrying Ri plasmids, rather than Ti plasmid strains. Thus, although no crown gall was evident in this nonagricultural environment, it is possible that *A. rhizogenes* strains may have been present. As the biological effects of *A. rhizogenes* may not be deleterious in the same way as crown gall, its presence in plant environments may not be as conspicuous.

It is possible that *A. rhizogenes* may be present in the local environments in Victoria but in small numbers that are not readily detectable, particularly as it is most likely not native to Australian soil. However, as strains corresponding with biovar 2 seemed to be reasonably prevalent in soil samples from diverse locations, this may support speculation that *A. rhizogenes* could also be present in the soil. Further investigation utilising additional identification methods, as discussed above, may result in discovery of such strains.

The colonisation of rhizosphere environments by introduced microorganisms is often restricted by the natural suppressiveness of soils and the previous establishment of environmental niches (Alabouvette and Steinberg, 1995). This has been observed in studies relating to the release of biologically active antipathogenic bacteria (e.g. *A. radiobacter* K84) or plant growth promoting rhizobacteria (PGPR) (Whipps, 1997). Attempts to eradicate soil-borne plant pathogens can be hindered by the long-term ability of the pathogenic bacteria to survive in particular soil environments, which can be quite strain specific. Conversely, establishment of introduced strains, such as those with anti-pathogen activity, can be inhibited by poor survival in soil.

Alabouvette and Steinberg (1995) suggest that introduced microorganisms are likely to survive in soil at low densities; however, due to the highly competitive environment and large diversity of other microorganisms, it is not likely that introduced populations will become dominant. Thus, it may be difficult for bacteria introduced for specific biological purposes to be established at sufficient population densities to be effective. Application of selection pressures, i.e. creating a biased rhizosphere, is one approach to increasing the dominance of target rhizobacteria; the cultivation of opine-producing transgenic plants has been demonstrated to have this effect on opine-utilising rhizosphere pseudomonad species (Oger et al., 2000).

CHAPTER 6

FINAL DISCUSSION

The potential for modification and improvement of root system growth and function has possible wide-ranging agricultural, horticultural and ecological applications. Root system function is a particularly important consideration for agricultural crops in environments deficient in water or soil nutrients. Growth, persistence and productivity of crop plants in these environments are key aspects that may benefit from improved root system function and enhancement of these features may also help to extend the range of possible regions that can be farmed or forested. Improvements in root system growth and architecture may also have potential applications for reduction of soil erosion and land slippage. Aspects such as these will become increasingly important in forthcoming decades as worldwide environmental degradation and climate changes continue and as human population numbers increase, placing greater demand on resources. The value of relatively rapid, safe and novel biotechnological solutions for some of these problems seems unquestionable.

The root-inducing properties of *A. rhizogenes* have previously been considered in terms of potential agricultural applications in a few species, including olive, almond and apple (Strobel and Nachmias, 1985; Lambert and Tepfer, 1991). Significant increases in root growth were measured as a result of inoculation of olive and almond bare rootstock with virulent *A. rhizogenes* and these increases were also correlated with increased shoot growth (Strobel and Nachmias, 1985). In addition, the use of *A. rhizogenes* to induce adventitious root growth from micropropagated cuttings has been explored for numerous tree species, and found to facilitate the establishment of root systems from otherwise recalcitrant species (Häggman and Aronen, 2000).

Due to the central role played by the *rol* genes in determining the capacity of *A. rhizogenes* to induce hairy root formation, the particular use of these genes may be a viable strategy for the engineering of improved root growth or root-shoot balance in agriculturally and horticulturally important species. However, although effects of transformation with *A. rhizogenes rol* genes on shoot phenotypes have been extensively described in tobacco and in numerous other species, few studies have examined in depth the specific effects of *rol* genes on the root system itself or the relationship

between root and shoot systems. The *rol*C gene in particular has previously been reported to cause visible increases in lateral root branching and root mass (Schmülling et al., 1988; Tepfer, 1994; Scorza et al., 1994), although actual quantifications of these changes or further analyses have been for the most part absent. A notable example of the potential for *rol*C to induce larger root system growth was demonstrated in chimaeric apple trees, with *rol*C-transformed microcuttings demonstrating a greater ability to form roots, compared to untransformed clones or cuttings inoculated with *A. rhizogenes* (Lambert and Tepfer, 1991).

Using the model species *N. tabacum*, in which *rol* gene effects have predominantly been characterised, the present study has focused on the effects of *A. rhizogenes rol* genes on root initiation and growth and the balance between root and shoot growth. The results, presented in Chapter 3, have suggested that expression of *rol*C and also *rol*B, singly and in combination, may have the capacity to stimulate increased lateral root formation by enhancing sensitivity to auxin and thereby triggering relevant cells to initiate LRP. Although the precise biochemical functions of *rol* genes have not yet been elucidated, the effects on auxin sensitivity evident from these experiments are consistent with previous observations of *rol* gene effects in transgenic plants.

It is interesting that both *rol*C and *rol*B expression in tobacco roots affect LRP initiation in response to auxin to a similar extent, even though experiments with promoter-reporter gene fusions have suggested *rol*C to be specifically expressed in phloem cells but not in pericycle cells of tobacco roots (Guivarc'h et al., 1996). If this is in fact the case, it suggests that the expression of *rol*C in phloem cells of tobacco seedling roots may influence the sensitivity of pericycle cells to LRP initiation signals, by some as yet undetermined mechanism. Insight into this effect will clearly require further investigation into the possible mechanisms of *rol*C and *rol*B in increasing sensitivity to the LRP stimulating effects of auxin, and also the role of auxin itself in stimulating pericycle cell division. The genetic regulation of LR initiation and development of primordia is not completely understood as yet, particularly the mechanisms determining which pericycle cells will be triggered to re-enter the cell cycle and initiate formation of LR primordia. Investigation of expression of auxin-induced genes in roots of *rol*C, *rol*B and *rol*C+*rol*B transgenic plants may also conceivably shed light on changes in auxin sensitivity in these plants. Study of auxin-related genes involved in root initiation would clearly need to be part of a much wider program to elucidate the role of auxin in essential

plant processes and the mechanisms of plant hormones and receptors in signal transduction.

While the dwarfing and reduced male fertility often associated with expression of *rol*C may have specific horticultural applications, e.g. in petunia (Winefield et al., 1999) and fruit trees (Bell et al., 1999), the usefulness of this and other *rol* genes in many commercial and agricultural contexts may be limited by the pleiotropic phenotypic effects induced by these genes in transgenic plants. This raises the possibility of using tissue-specific promoters for more precise targeting of genetic modifications in *rol* gene transgenic plants. Furthermore, changes to upper plant phenotype or functions such as dwarfed stature or reduced chlorophyll content may not be favourable to root growth and may result in redirected partitioning of resources to shoots, thereby reducing relative partitioning to the root system. In this study, plants transgenic for the *rol*C gene had overall reduced biomass compared to controls not expressing *rol*C. Growth measurements of grafted plants in this study have however suggested the potential advantages of achieving root-specific expression of the *rol*C or the *rol*B gene, for the modification of biomass allocation patterns and increase in root:shoot ratios. In particular, *rol*C and *rol*B in combination appear to have potential for increasing relative root biomass and overall plant biomass. As discussed in Chapter 3, various root-specific promoters have been reported in the literature, some of which may be suitable to obtain expression of *rol*C or *rol*B in appropriate root cell types, particularly cells from which LR or AR are initiated.

Effects of *rol*C expression on growth of white clover

Due to the importance of white clover as a pasture crop in Australia and worldwide and the need to improve persistence and drought tolerance in pastures, improvements to root growth may potentially be highly beneficial in agricultural and commercial terms. In this study, experiments were initiated to explore the potential advantages of adding the *rol*C gene to the white clover genome, with regard to these characteristics.

Given that the number of transformant white clover lines evaluated in this study was small, the conclusions that can be drawn are somewhat preliminary. However, the results presented in Chapter 4 have suggested that there may be potential value in using *rol*C to induce improvements in root growth, with nodal and LR initiation increased in two T*rrol*C transgenic lines in comparison to WT plants.

As discussed in Chapter 4, some of the shoot phenotypic effects produced in the T*rol*C transgenic lines, such as shortened internodes or increased density of leaf formation, may conceivably have relevance to agronomically desirable traits. While other phenotypic effects may not necessarily be appropriate in an agricultural context, transformation of white clover with *rol* genes has not been reported to date and *rol*C-induced alterations of white clover leaf and stolon form are therefore of interest, adding to information about the effects of *rol*C expression in various plant species. Clearly, an important aspect to consider for future studies with *rol* gene transgenic white clover lines would be the generation of a greater number of independent transformant lines, to examine more fully the extent of expression levels and phenotypic variations that may be produced.

As previously discussed, analyses of root growth of plants transgenic for *rol* genes under different nutrient and/or water conditions and at different stages of development are also important considerations. A large array of soil and aerial environmental factors can influence root and shoot growth and root:shoot partitioning and are therefore significant in agricultural terms. Of particular interest are the control of root responses to the presence or lack of nutrients and/or other environmental factors, a notable example being the work of Zhang and Forde (2000) regarding the regulation of root elongation in response to nitrate availability. Potential production of transgenic clover plants with the aim of achieving improved root growth would necessitate the identification of such factors that may particularly influence the performance of the transgenic crops in the field.

As nodal root systems are functionally the major root systems of white clover, nodal adventitious root formation, as well as the subsequent branching of LR, may be appropriate targets for improvement. Identification of mutants specifically affected in nodal root formation (Hetz et al., 1996; White et al., 1998) suggested that there may be elements of the genetic control of adventitious root production that are unique to nodes. Changes to other root system characteristics, such as LR elongation or root morphology may also affect root system growth and function and could also be considered for engineering improvements to root growth of plants for agricultural applications.

If the results of experiments involving tobacco can be extrapolated to clover, the observations made in this study suggest that the use of the *rol*C gene in combination with *rol*B, particularly if expressed under the control of root-specific promoters, is a prospect that warrants further investigation for potential white clover crop improvement.

Presence of *Agrobacterium* in Victorian soils

Studies described in Chapter 5 of this thesis, while not conclusive, did suggest that strains of *A. rhizogenes* may be present in soils sampled from various sites in and around Melbourne. The possible presence of *A. rhizogenes* in Victorian soils, and elsewhere in Australia, may have implications for the potential utilisation of *A. rhizogenes* genes in transgenic plants (or the possible use of *A. rhizogenes* itself). As previously discussed, the pre-existence of *A. rhizogenes* in agricultural or local native environments may alleviate foreseeable concerns regarding the generation of transgenic *rol* gene crops and risks of introduction of *A. rhizogenes* genes into the environment. In addition, if *A. rhizogenes* were found to exist in the local environment, it may be of interest to investigate possible effects of its presence on plant growth. One relatively unexplored area is the potential use of soil organisms, particularly nonsymbiotic rhizobacteria (PGPR), to promote plant growth. Stimulatory effects of different PGPR have been attributed to a number of potential mechanisms, including production of phytohormones or promotion of nutrient uptake and nitrogen fixation (Chanway, 1997). *Agrobacterium* strains have been demonstrated to have some growth-promoting activity when applied to beech and pine trees, under greenhouse conditions, by promoting the uptake of certain nutrients (Leyval and Berthelin, 1993). Further investigations into the potential for growth promotion or root-stimulation of plants by *A. rhizogenes* may therefore be of interest. If *A. rhizogenes* were found to have plant-growth promoting properties, the deliberate introduction of *A. rhizogenes* into soil to promote growth of certain crop species may be one prospective application. As highly controlled environments such as greenhouses will naturally be different from a field environment, any potential applications relating to crop growth in field would clearly require extensive testing under appropriate conditions. Consideration of potential effects of *A. rhizogenes* on other rhizosphere microbe populations may also be necessary.

Conversely, it may also be pertinent to examine possible effects that *rol* transgenic plants may have on *A. rhizogenes* and other rhizosphere microbial populations. The concept of creation of a biased rhizosphere has been explored with opine-producing transgenic plants, which have been shown to influence rhizosphere microbial populations of opine-utilising bacteria (O'Connell et al., 1996; Oger et al., 2000). Although unlikely to exert a specific influence on agrobacteria, due to a lack of opine synthesis genes, it is not inconceivable that modifications in root growth induced by *rol* gene expression may have effects on *A. rhizogenes* (if present) and other soil

microorganisms. For example, roots could potentially influence the rhizosphere by inducing accelerated depletion of nutrients and water, and also by altering rhizosphere properties such as pH or chemical balance by the release of root-derived compounds.

In conclusion, a full appreciation of the potential effects of *rol* gene expression on root and shoot growth, and possible agricultural applications for transgenic plants containing these genes, would clearly involve an integrated study of many aspects of root biology, encompassing physiological and genetic aspects of root growth and root system interactions with shoots and the rhizosphere environment.

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