Genetic Manipulation of Phytohormone Levels in Sugar Beet

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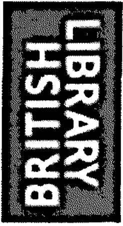

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ABSTRACT

The improvement of the commercial sugar beet crop by genetic modification will require the subtle regulation of expression of foreign genes in the transgenic plants. A strategy has been defined for production of high sucrose yield/ high juice purity/ low environmental impact varieties of sugar beet. This requires modification of the phytohormone levels in the outer cambial rings of the storage root. The auxin IAA has been implicated in the development of cambial rings in the sugar beet storage root. The hairy root transformation system was used to introduce the auxin biosynthetic genes (*iaaM* and *iaaH*) from *Agrobacterium rhizogenes* strain A4b. Agropine and mannopine were detected in all A4b hairy root clones tested, suggesting transfer (and functional expression) of genes from the Ri plasmid T_R T-DNA to the sugar beet genome. It was shown by DNA-DNA hybridisation experiments that part of the T_L and T_R T-DNA had been integrated into the sugar beet cv. Bella genome.

Altered levels of IAA were observed between transformed and non-transformed sugar beet seedling root material and also between different hairy root clones. Preliminary studies on the auxin binding proteins of A4b transformed sugar beet hairy root clones, suggested that there may be a regulatory system to compensate for the excess levels of IAA in transformed tissue. Those clones with the highest level of IAA, in general had lower ABP activity. The hairy root transformation system was a suitable model for introducing the auxin biosynthetic genes of *Agrobacterium* in order to alter plant growth regulator levels within the sugar beet cv. Bella genome.

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CHAPTER ONE

GENERAL INTRODUCTION

1.1 Sugar Beet, an Important Crop Plant

Sugar beet accounts for approximately one third of the world's sucrose production (Cook and Scott, 1993). It is grown widely in temperate zones, as a summer crop in cooler climates and as a winter crop in warmer areas such as South America and southern Europe. It is an important crop in Great Britain with some 165,000 hectares of land devoted to its cultivation in 1999. This land yields around nine million tonnes of beet per year. British Sugar process 8.5 million tonnes of sugar beet a year at the nine processing factories, producing about 1.4 million tonnes of sugar and 8000,000 tonnes of animal feed (that is around 10,000 tonnes of sugar per day) (British Sugar plc, 1999). Selective breeding since the 1890's and improved agricultural practices since the 1940's have increased fresh mass concentration of sucrose in sugar beet from 4 to 18% (Milford *et al.*, 1980). By 1990 the annual world sugar production from sugar beet was about 40 million tonnes (Cooke and Scott 1993).

Sugar beet is one of several sub-species of *Beta vulgaris*, it also includes other sub-species, the mangolds and fodder beet. Sugar beet is the most

widely grown sub-species but Swiss Chard (leaf chard) and mangold (fodder beet) are also commercially important, being grown for animal feed (Fick *et al.*, 1975).

In northern Europe sugar beet seeds are sown in late March, early April and the period from sowing to harvesting is 170-200 days. During the first growing season the storage organ stores large quantities of sucrose. Mature beets are harvested in September or early October of the same year. Such crops may yield up to 15 tonnes of sugar per hectare (Elliott and Weston, 1993). If left in the ground this energy supply would be used for flowering the following year.

Diploid multigerm varieties were first grown but these were replaced by higher yielding polyploid multigerm varieties. The biggest advances in sugar beet breeding have been the development of male sterility to allow controlled fertilisation and production of triploid and diploid monogerm varieties now grown by farmers (Hecker and Helmerrick, 1986; Winner, 1993).

As well as breeding for higher sugar yield, improvements have been made in percentage germination, bolting (premature flowering) resistance, processing quality and disease resistance.

New varieties of sugar beet are grown in trials at the National Institute of Agricultural Botany regional trials and then in main field trials. In order to assess growers' potential income several characteristics are noted, including, relative yield, (percentage sucrose per unit weight wet mass), quality of sugar (percentage sucrose measured against impurities), bolters per 1000 plants, size of top, resistance to certain diseases and percentage establishment.

1.1.2 Anatomy and Development of the Sugar Beet Storage Organ.

The storage organ comprises an enlarged hypocotyl and tap root and is conical in shape with two rows of thin lateral roots (Artschwager, 1926). It consists of a series of concentric cambial rings initiated early in development and present before the root reaches a diameter of 1 cm. Elliot *et al.* (1983) and Hosford *et al.* (1984) described comparative

studies with three subspecies of *Beta vulgaris*, sugar beet, mangold and Swiss Chard. All three have roots with a similar number of cambial rings.

Storage organ development can be divided into;

1) Cambial initiation
2) Differentiation and division of the products
3) Cell division into the parenchyma beyond the cambia, and
4) Expansion of parenchyma.

Cambial initiation occurs early in storage organ development in all three subspecies and more cambia are produced than subsequently develop. Attention has been focused on the remaining 3 phases, each of which contributes to differing extents to the growth of the storage organs of the three subspecies.

Up to twelve cambia may be initiated but usually only the first 5-6 develop to maturity and these make up 75% of the cross sectional area of the mature sugar beet. These rings develop simultaneously rather than sequentially (Elliott *et al.,* 1984). Subsequent expansion of the storage organ rings is both by cell division and cell expansion. The cambial rings expand in width as new cells are produced by continued division.

Existing parenchyma cells expand to create additional sites for sucrose storage. Some of the cells close to the cambia differentiate into xylem elements and sieve tubes embedded in small celled parenchyma to form discrete vascular zones. The cells between these zones remain as undifferentiated parenchyma, hence the characteristic ringed structure of the cross sectional area of the storage organ. See figure 1.1.

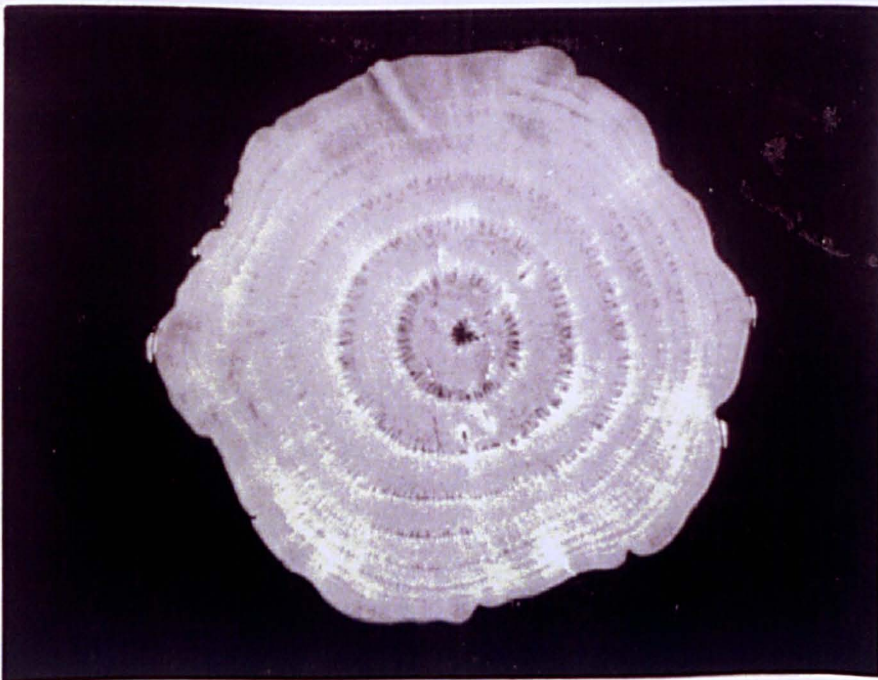


Figure 1.1 Transverse section through a mature sugar beet storage organ showing the characteristic ringed structure.

In sugar beet, continued cell division of the active cambia and parenchyma make an important contribution to overall growth. In contrast, chard produces much smaller storage roots than either sugar beet or mangold, because there is little division or cell expansion of the parenchyma after cambial initiation and more of its total dry matter is partitioned into shoot growth (Loomis *et al.*, 1977). In mangold the rate of cell division peaks earlier in development and root growth is predominately by the extensive expansion of the innermost four rings.

1.1.3 Sucrose Accumulation

Sucrose enters the root via the phloem of the vascular zone and is transported to the parenchyma cells where it is accumulated (Elliott *et al.*, 1983). As cell size increases up to 10-15 x 10^{-8} cm^3, sugar concentration per cell increases almost proportionally with cell volume. Above this size there is a less proportionate increase in sucrose concentration although the amounts of water and non-sugar dry matter in the cells continues to increase in direct proportion to cell volume (Elliott *et al.*, 1983).

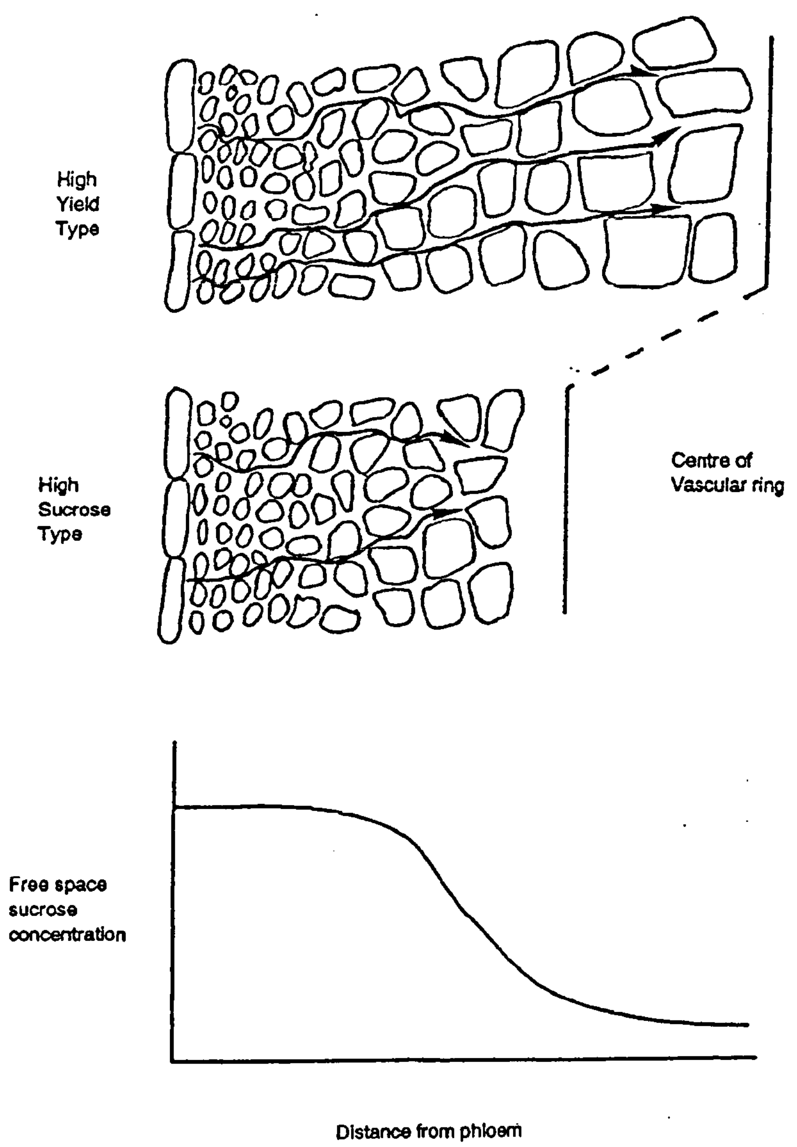


Figure 1.2 The phloem to storage cell sucrose transport path is longer in high yield type than in high sucrose type sugar beet storage roots (after Wyse, 1979)

Sugar concentration in parenchymatous zones composed of large cells is therefore lower than in those made up of more and smaller cells.

This strong negative correlation between sucrose concentration and dry root mass has meant that attempts to increase sugar concentration to more than 18% by conventional breeding and modern farming practices has failed (Elliott *et al.*,1986)

Wyse suggested that the factor controlling sugar uptake is not cell size per se but the length of the diffusion path and the diffusive resistance determined by the distance of the cell from the vascular system (Wyse, 1979). The cells closest to the phloem are younger and smaller, while those farther away are older and larger. The smaller cells would have more sucrose because they are close to the phloem and not because of any sucrose accumulating ability related to size. See figure 1.2.

So both high root yield and high sucrose content would result from the production of a large root containing more active cambial rings with short diffusion path length between phloem and accumulating cells and not from a large root whose size is due solely to enlarged cells. See figure 1.3

1.1.4 Phytohormonal Control of Cambial Activity

Milford and Lenton (1978) suggested that plant growth regulators could be utilised to bring about alterations in root structure. Auxin, cytokinin and sucrose have been strongly implicated in cambial initiation and activity and the differentiation of cambial derivatives (Elliott 1982, Torrey and Loomis 1976, Roberts 1976, Shiningar 1979). It has been suggested that auxin and sucrose act as morphogens in cambial initiation in wound callus and that the direction of radial gradients of these compounds determines the formation of phloem and xylem (Warren-Wilson 1978). Auxin and cytokinin have been implicated in cambial initiation and activity in storage organs of radish (Torrey and Loomis 1976, Ting and Wren 1980), turnip (Peterson 1973) and sugar beet (Elliott *et al.*, 1984).

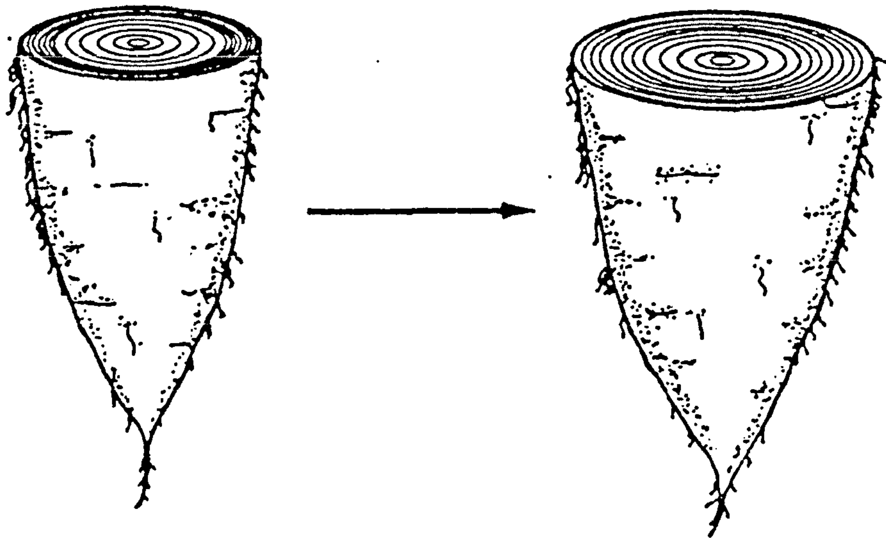


Figure 1.3 Activation of the outer cambial rings of the sugar beet storage root to produce a structure with more evenly spaced vascular rings combining high sucrose concentration and high dry matter yield. (After Elliott and Weston, 1993)

There is evidence that auxin, giberellins, cytokinins and ABA are involved in control of cambial initiation. Reports concerning storage organ growth implicate the requirement for IAA and cytokinin for the induction of vascular cambia (Shiningar 1979, Sachs 1981) and maintenance of cambial activity (Peterson 1973). It seems certain that these developmental processes are regulated by subtle localised changes in hormone balance.

All of this data led to a proposal that the outer cambial rings might be activated by using plant growth regulators (Elliott *et al.*, 1983; Hosford *et al.*, 1984; Thomas *et al.*, 1993).

Elliott *et al.* (1983) described experiments in which levels of IAA, ABA, cytokinins and gibberellins in the storage organ of sugar beet throughout the period between sowing and harvest were measured. During the period of cambial initiation levels of IAA, cytokinins and gibberellins were found to be high, while the level of ABA was low. At the end of this developmental period IAA, cytokinins and gibberellin levels sharply decline as ABA levels began to rise. Just prior to the period of rapid cell expansion gibberellin levels rose sharply, ABA levels were constant at the increased level and IAA and cytokinin levels remained at decreased

levels. During the phase of maximal cell production, cell division increases exponentially. Before this phase begins levels of IAA, cytokinins and ABA increased, reaching their peak levels during maximal cell production then declined. Gibberellins sharply declined at the start of this period and remained constant for the rest of the growth period. After this growth period all plant growth regulators declined and remained at very low levels (figure 1.4).

Data obtained from synchronised cell suspension cultures of sugar beet and *Acer pseudoplatanus* also suggest that changes in cytokinin and IAA levels cause developmental changes (Elliott *et al.*, 1986; Elliott *et al.*, 1987; Elliott and Weston, 1993).

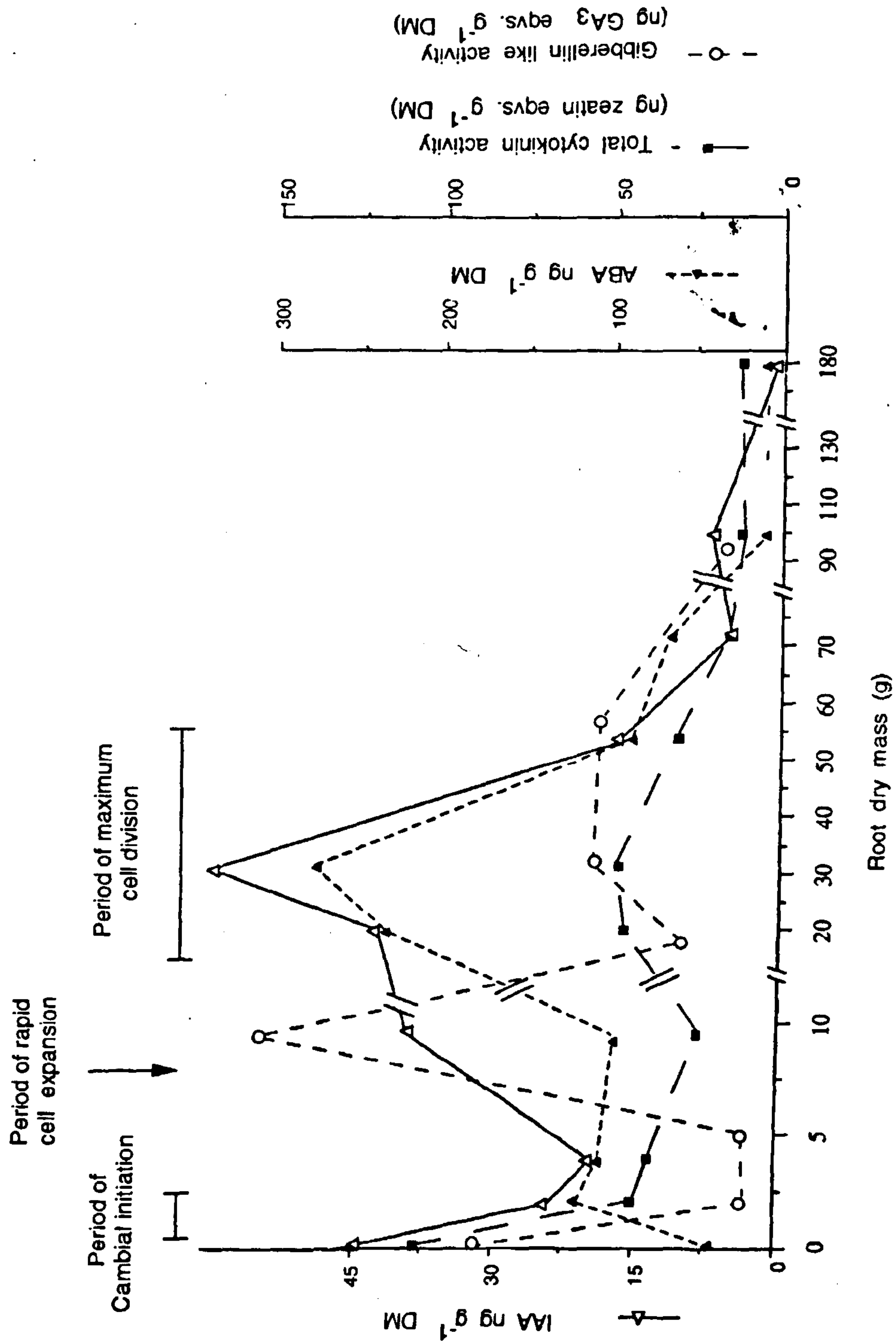


Figure 1.4 Levels of plant growth regulators during the growth period of the sugar beet storage organ.

This data was hoped to provide the bases for plant growth regulator regimes to be applied in the field to reduce cell expansion and reduce sucrose transport path lengths. Hosford (1984) suggested that activation of the outer cambial rings might be achieved by application of cytokinins and auxin before the 2g dry mass root stage. Restriction of enlargement of the parenchyma cells in the rings might be achieved by application of an inhibitor of gibberellin synthesis at about the 5-8g dry mass root stage.

A foliar application of paclobutrazol, an inhibitor of gibberellin synthesis, resulted in restriction of cell expansion in the storage root (Elliott *et al.*, 1986). Further evidence to support this was supplied by Keithly *et al.* (1990) who applied the gibberellin synthesis inhibitor 2(3,4-dichlorophenoxy) triethylamine (DCPTA) to sugar beet plants. When DCPTA was applied to sugar beet plants at 30μmol dm^{-3} at the four true leaf stage it led to storage roots with a greater fresh mass, but with a maintained sucrose content compared to untreated plants. This amounted to an 87% increase in sucrose yield/root over untreated plants.

A plant growth regulator regime would need to subtly modify the auxin and cytokinin levels in the target cells (cambia) in the target tissues (outer

cambial rings) of the target organ (storage organ) at the optimal stage (in early cambial development) (Elliott *et al.*,1988; Elliott and Weston, 1993; Thomas *et al.*, 1993). This would be difficult to apply specifically, would not be cost effective and would have environmental implications. A more likely approach would modify levels and or ratios of plant growth regulators in specific areas of specific organs at specific periods of growth during storage organ development of sugar beet.

It is possible that this may be achieved using plant genetic manipulation, especially the introduction of cytokinin and auxin biosynthetic genes of the *Agrobacterium* Ti or Ri plasmid (see section 1.3).

1.2 Methods of Gene Transfer in Plants

A range of methods have been developed to introduce useful genes into a wide variety of plant species (Weising *et al.*,1988; Uchimiya *et al.*,1989; Hall *et al.*, 1996). These methods are outlined below.
DNA can be easily introduced into protoplasts (obtained by the removel of the plant cell wall) following treatment with polyethyleneglycol (Davey *et al.*,1980; Werr and Lanz 1986). Paszkowski *et al.* (1984) used this method to produce transgenic tobacco plants.

DNA can also be introduced into protoplasts using electrical pulses, known as the electroporation technique (Fromm *et al.*, 1986). This causes pores to be formed in the plasmamembrane of the protoplasts. DNA can pass through these pores during a short pulse of high voltage D.C. and remains inside when the pores close. The DNA can then be stably incorporated into the chromosomes. In both cases the protoplasts are then allowed to regenerate a cell wall which, following repeated cell division, forms callus in some species. However, protocols to allow plant regeneration are not available in all species (Flavell 1990).

Electroporation has been used with sugar beet protoplasts and cells in various field strengths, temperatures and number of pulses (Lindsey and Jones, 1987a). This led to the introduction and transient expression of the chloramphenicol acetyl transferase (*cat*) gene into sugar beet protoplasts and intact cells (Lindsey and Jones, 1987b). Kanamycin resistance (from the *npt*-II gene) was stably incorporated into sugar beet protoplasts by the same method (Lindsey and Jones, 1989) but it was not possible to regenerate plants from these transformed cells.

A protocol for regeneration of sugar beet protoplasts has since been developed but appears to be cultivar dependent (Krens *et al.*, 1990). More recently, a procedure for transformation and regeneration of sugar beet guard cell protoplasts has been developed (Hall et al., 1996).

Regeneration of whole plants from single transformed cells is very difficult for a great many species. Several other methods have been used to introduce DNA into cells of organised structures, e.g. embryos. Micro-injection of DNA has been successful in species such as oil seed rape and micro-injection of DNA into floral tillers of rye has produced transformed plants (de la Pena *et al.*, 1987). This method though has proven not to be reproducible (Potrykus, 1991).

Shooting tungsten particles coated in DNA through cell walls has been used to transform cells of maize, soybean, rice, wheat and onion (Klein 1987, Wang 1988). Here DNA is precipitated onto gold or tungsten particles and fired at high velocity into plant cells. Transformed soybean plants have been obtained by introducing DNA coated gold particles, accelerated by electric discharge (McCabe *et al.* 1988).

The methods of transformation described so far are mainly used for transient gene expression. This is where DNA is introduced into the plant cell, transcribed and translated but not integrated into the genome. These methods are also useful for transformation of monocotyledonous species, which are not susceptible to infection by *Agrobacterium.*

The soil born pathogenic bacteria *Agrobacterium tumefaciens* and *A. rhizogenes* provide a natural system for introducing desirable genes into many (mainly dicotyledonous) plant species.

1.3 *Agrobacterium*

1.3.1 History and Biology

The Gram –ve soil born bacteria *Agrobacterium tumefaciens* and *A. rhizogenes* have an efficient natural system for transferring genes into plant cells. Transformation of plant cells results from the integration of T-DNA (transferred DNA) from the Ti (tumour inducing) or Ri (root inducing) plasmids into the genome of susceptible plants. *A. tumefaciens* is capable of infecting most dicotyledonous plants (DeCleene and Deley 1976) and an increasing number of monocotyledons and gymnosperms. Far less work has been carried out with *A. rhizogenes* but many plant species have been found to be susceptible (De Cleene and Deley 1981, Birot *et al.* 1987). Expression of T-DNA genes induces physiological changes in transformed cells, which modify normal plant development to produce the proliferating characteristics of crown gall (*A. tumefaciens*) and hairy root disease (*A. rhizogenes).*

Crown gall disease was first described almost 100 years ago and the involvement of bacteria was recognised in 1907 (Smith and Townsend, 1907). Since then a great deal of work has been carried out on these

organisms. It was shown in the 1930's that *A. rhizogenes* was responsible for production of adventitious roots when inoculated into a wounded plant stem (Riker *et al.* 1930) but it is only in recent times that interest has been revived (Moore *et al.* 1978).

In nature the presence of a plant wound is required for infection to take place (Braun 1947). Initial binding or attachment of *A. tumefaciens* to the exposed cell wall is governed by the expression of two Agrobacterial chromosomal loci *chr* A and *chr* B (Douglas *et al.*, 1985). Both crown gall tumours (Braun 1958) and hairy roots (Chilton *et al.* 1986) are capable of growth *in vitro* in the absence of the inciting bacteria and without addition of plant growth regulators to the medium.

There are numerous classes of both Ti and Ri plasmids but they share certain characteristics. They are large (200-280 kbp) and contain 2 regions necessary for tumorigenesis. These include the T-DNA region and the *vir* (virulence) region, which is not transferred into plant cells but which contains genes encoding proteins involved in the transfer of DNA. The expression of these genes is induced by the exudate from wounded plants. Phenolic compounds are the most potent inducers of these gene products (Zupan *et al.*, 2000). T-DNA genes in both *A. tumefasciens* and

A. rhizogenes encode genes that induce infected plant cells to synthesise unusual amino acid-sugar conjugates called opines. The plant is unable to metabolize these compounds but the inciting bacteria can use them as a sole carbon and nitrogen source. Ti and Ri plasmids can be classified due to the principle opine produced. There are four different classes of opines in Ti plasmids octopine, nopaline, agropine and agrcinopine. The most thoroughly characterised of these are the octopine and nopaline classes. There are three major types of opine produced in hairy root tissue, agropine, mannopine and cucumopine (Petit *et al.* 1983, 1986, Petit and Tempe 1985; Dessaux *et al.*, 1993).See figure 1.5.

In *A. tumefaciens* and *A. rhizogenes* the T-DNA harbours genes responsible for plant growth regulator biosynthesis resulting in the growth of infected cells to form unorganised tumours.

The rest of this discussion will be involved primarily with the Ri plasmid of *A. rhizogenes* and in particular strain A4 which has an agropine type Ri plasmid.

1.3.2 Organisation of Ri Plasmids

Functional maps of Ri plasmids are almost entirely based on homologies with Ti plasmids. Homology has been localised to areas associated with the origin of replication, plasmid stability and copy number incompatibility (Jonanin *et al.* 1985) and some of the virulence functions (Risuelo *et al.* 1982).

Hooykaas *et al.* (1984) showed that Ri plasmid functions are capable of complementing at least some of *A. tumefaciens* virulence mutant strains. Ri and Ti virulence genes although showing common functions are probably not identical. The Ri virulence genes are able to induce the transfer of both Ri T-DNA (Vilaine and Casse-Delbart 1987) and Ti T-DNA (Hammill *et al.* 1987). T-DNA's of mannopine and agropine type Ri plasmids are very different although both types possess 25 bp near perfect repeat border sequences (Slightom *et al.* 1986). Mannopine Ri plasmids possess a single T-region which has no homology to Ti plasmid hormone biosynthetic genes (Lahners *et al.*1984). In agropine type Ri strains the T-DNA consists of 2 different transferring elements, T_L-DNA and T_R-DNA (DePoalis *et al.* 1985, White *et al.* 1985). These segments are in neighbouring non-continuous regions of the Ri plasmid. Auxin

biosynthetic genes homologous to genes on the Ti plasmid T-DNA have been localised on the T_R-DNA (Carderelli *et al.* 1985).
This homology has shown to be functional as well as structural (Offringer *et al.* 1986). There are two genes involved in the synthesis of IAA in these *Agrobacterium* strains, *iaaM* and *iaaH* (formally known as *tms*1 and *tms* 2. The first gene codes for the enzyme tryptophan-2-monooxygenase, which converts tryptophan to indole-3-acetamide (IAM). The second, iaaH encodes the enzyme indole-3-acetamide hydrolase which catalyses the convertion of indole-3-actetamide to indole-3-acetic acid (IAA).

The T_L-DNA has no extensive homology with the Ti T-DNA genes but is strongly homologous to the T-region of mannopine strain Ri plasmids (Filetici *et al.* 1987, Combard 1987).

The T-DNA of Ti plasmids also harbours a gene involved with cytokinin biosynthesis called *tmr* or gene 4 which encodes the protein isopentyl transferase (Akujoshi *et al.* 1984). No homology has been observed between the *tmr* locus of Ti plasmids and any Ri plasmids.

The fact that only agropine type Ri plasmids possess genes responsible for auxin biosynthesis and that none possess the ability to synthesise cytokinins suggests that Ri plasmids alter development by mechanisms that differ significantly from those employed by Ti plasmids.

1.3.3. Induction of Hairy Roots

The T_L-DNA and T_R-DNA of agropine type Ri plasmids are each capable of inducing hairy root production, though neither region alone produces a response as strong as that of the wild type. This suggests that the two regions act co-operatively (Vilaine and Casse-Delbart 1987b). Auxin provided by T_R-DNA genes may trigger differentiation of auxin responsive T_L-DNA containing cells, bringing about hairy root production (Carderelli *et al.,* 1987a). In the case of mannopine and cucumopine type *A. rhizogenese* strains that have no auxin biosynthetic genes, plant auxin may play a part in hairy root induction. Evidence for this is provided by the asymmetric virulence called polarity exhibited by mannopine and cucumopine and agropine type *A. rhizogenese* strains.

$NH = C(NH_2) - NH - (CH_2)_3 - CH - COOH$

$|$

NH

$|$

$CH_3 - CH - COOH$

Octopine

$NH = C(NH_2) - NH - (CH_2)_3 - CH - COOH$

$|$

NH

$|$

$HOOC - CH_2 - CH_2 - CH - COOH$

Nopaline

CH_2

$HOH_2C - (CH\ OH)_3 - CH \quad NH$

$| \quad |$

$O \quad CH - (CH_2)_2 - CONH_2$

CO

Agropine

Figure 1.5 Chemical structures of nopaline, octopine and agropine. Figure redrawn from Grierson and Covey (1988).

In these cases almost no virulence is shown when they are inoculated onto the basal surface (facing root base) of carrot root discs (Ryder *et al.*, 1985) but can induce hairy root production when inoculated onto the apical surface. Wild type agropine *A. rhizogenese* strains can induce hairy root production when applied to either surface. These responses may be explained as being due to auxin depletion on the discs basal side resulting from an active flow of auxin towards the root apex (Carderelli *et al.* 1985).

The production of hairy roots by Ri plasmid mutants containing only T_R-DNA is thought to be due to auxin induced root formation (Offringa *et al.* 1986). The mechanism by which T_L-DNA or mannopine and cucumopine T-DNA's induce hairy roots formation is poorly understood. Extensive mutagenesis studies of the T_L-region of agropine type Ri plasmids has revealed 4 loci *rol* A, B, C and D which effect the morphology of root formation by *A. rhizogenes* on various host plants (White *et al.* 1985). These loci do not show any homology with T-DNA of Ti plasmids (Huffmann *et al.* 1984). Slightom *et al.* (1986) sequenced the T_L-DNA of A4 (agropine type Ri plasmid) and identified 18 open reading frames (ORF's). Four of these ORF's 10, 11, 12 and 15 coincide with loci *rol* A, B, C and D.

Tobacco plants transformed with a portion of the T_L-DNA containing these loci, but lacking ORF's 1-7 and T_R-DNA displayed traits characteristic of such transformed plants (Jouanin *et al.* 1987). These traits include reduced apical dominance of both stem and roots, shortened internodes, excessive and reduced geotropic root system and wrinkled leaves (Tepfer 1984, Oomes *et al.,* 1985). The 3 loci *rol* A, B and C are individually capable of stimulating root formation in tobacco. Their capacity to do so differs in different hosts and their effects may be co-operative (Spena *et al.* 1987). In tobacco *rol* B is more efficient than *rol* A or C. In Kalanchöe *rol* B is the only locus able to stimulate root production by itself and then only if auxin is supplied (Spena *et al.*, 1987).

It has been suggested that the T_L-loci influence root induction by rendering transformed cells sensitive to auxin (Carderelli *et al.*, 1987a) possibly by intervention or interaction with normal growth hormone controlled differentiation mechanisms (Spena *et al.,* 1987).

The expression of Ri T-DNA genes is highly variable. Organ specific Ri Ti gene expression has frequently been observed in tobacco (Durand-

Tardif *et al.*, 1985) and in potato (Oomes *et al.*, 1986). This organ specificity is not always constant among different transformants of the same species (Jouanin *et al.*, 1987). In contrast Ti T-DNA gene expression is generally constant (with the exception of the observation of organ specific expression of gene 5 from an octopine plasmid (Koncz and Schell 1986).

An improvement in the usefulness of *Agrobacterium* for plant transformation was the development of the binary vector system (Hoekema *et al.*, 1983). This made use of the trans acting nature of the *vir* gene products and the fact that any DNA between the T-DNA borders is transferred to plant cells (reviewed in Christie , 1997). Bevan (1984) constructed the vector pBin19 which encodes a selectable marker gene between the T-DNA borders. The *npt* –II coding region from the transposon TnS was flanked by the nopaline synthase (*nos*) promoter and terminator to confer resistance to the antibiotics kanamycin and G418 upon transgenic plant cells. It also included a multiple cloning site from mBmp19 which provided 8 unique restriction sites into which foreign genes could be ligated. PBin19 has a RK2 origin of replication permitting maintanance of the plasmid in both *Escherichia coli* and *Agrobacterium*

strains and prokaryotic kanamycin resistance gene allowing selection of bacteria harbouring the plasmid.

As *Agrobacterium* mediated transformation is relatively simple it has been used widely for the production of transgenic plants. Commercial species transformed and regenerated using this system include tomato, tobacco and petunia (Horsch *et al.*, 1985), carrot (Scott and Draper 1987), lettuce (Michelmore *et al.*, 1987), soybean (Chee *et al.*, 1989), oil seed rape (Maloney *et al.*, 1989), potato (Visser *et al.*, 1989), apple (James *et al.*, 1989), flax (McHughen and Jordan 1989), poplar (Brasileiro *et al.*,1991), plum (Mante *et al.*, 1991), melon (Dong *et al.*, 1991) and sugar beet (Lindsey and Gallois 1990; D'Halluin *et al.*, 1992) and more recently rice and other cereals have been transformed in this way.

1.4 The role of indole-3-acetic acid in plant development.

The auxin IAA, has a major role in the regulation of plant development. It has been shown to be involved in phototropism, gravitropism, apical dominance, as well as cell division, enlargement and differentiation (Thimann, 1988). Auxins, though, at high concentration are phytotoxic

and synthetic auxins such as 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) have been made use of as weed-killers.

Application of IAA at about 10^{-6} mol dm^{-3} causes elongation of isolated epicotyl, hypocotyl and coleoptile sections accompanied by changes in the levels of some abundant mRNAs and newly synthesised proteins (Parthier, 1989). Walker and Key (1982) observed an increase in mRNA level of an auxin induced gene 15 minutes after application of 2.3×10^{-5} mol dm^{-3} 2,4-D to excised, IAA depleted soybean hypocotyl sections. A similarly rapid response was observed by Theologis *et al.* (1985) in pea epicotyl tissue, who showed that the induction of transcription of genes corresponding to three cDNA clones was IAA specific, 2 with no effect caused by anaerobiosis, heat stress, cold stress, GA, kinetin, ABA or ethylene. As well as inducing transcription of specific mRNAs within as little as five minutes, some are inhibited by 2,4-D (Key, 1989). The alterations in gene expression have not been shown to cause elongation, but the response is sufficiently rapid for that to be a working hypothesis. The effects of auxins have largely been studied by application to excised plant material. Alteration of endogenous IAA levels by the introduction

of the *iaaM* and *iaaH* genes has allowed studies of the role of IAA in plant development which have until recently not been possible.

1.5 Aim

The overall aim of this research project was to genetically manipulate sugar beet so as to alter phytohormone profiles. It was hoped that levels of auxin in the sugar beet cells would be increased by inserting genes iaaM and iaaH responsible for auxin biosynthesis from T-DNA of *A. rhizogenes.* The working hypothesis was that manipulation of auxin levels in this way would result in a storage organ comprising of more active cambia separated by small storage cells. A storage organ of this type is predicted to have a higher sucrose concentration and content .

CHAPTER TWO

TISSUE CULTURE OF SUGAR BEET

2.1 Introduction

The term plant tissue culture has generated into an all encompassing, convenient term to describe all types of sterile plant culture procedures pertaining to the growth of plant protoplasts, cells, tissues, organs, embryos and plantlets. The techniques involved are extremely useful for studying growth of higher plant cells, as many of the complexities present in whole plant growth analysis are eliminated (Elliott *et al.*, 1977). Tissue culture methods have been used on many agronomically important plants for *in vitro* propagation, shoot tip propagation, virus elimination, secondary product synthesis and haploid plant production.
These techniques can be further exploited to establish callus or liquid cell cultures for genetic manipulation following mutagenesis or cellular manipulations.

In 1939 White and Gautheret separately showed that the parenchymatous wound cells which frequently form at the exposed surfaces of organ segments could be removed and grown independently as a relatively undifferentiated tissue or callus culture. Callus cultures can be initiated from a wide variety of plant organs or specific cell types e.g. pollen. In

general younger tissues are more suitable for callus initiation than mature ones (Dixon 1984), but the choice of explant is obviously to a large extent dictated by the aims of the research. In the experiments described in this report the overall aim was to alter the size and number of parenchyma cells in the storage root of sugar beet. It was therefore desirable to produce callus of root origin.

Nutrient media for the growth of plant tissues is basically a mixture of inorganic salts composed of macro- and microelements and a carbon source, which is usually sucrose. The majority of plant tissues also require an iron source, vitamins and growth regulators. Murashige and Skoog's medium (MS) (Murashige and Skoog 1962), Shenk and Hildebrant's medium (SH) (Schenk and Hildebrant 1972) and Gamborg's B5 medium (GB5) (Gamborg *et al.*, 1968) are commonly used for general plant tissue culture.

Despite the fact that sugar beet is an economically important crop plant there is surprisingly little literature published on the tissue culture of this plant. Sugar beet is considered as a recalcitrant species, so application of plant tissue culture techniques for sugar beet improvement has been retarded due to the lack of efficient methods for production of large

numbers of plantlets by organogenesis or by somatic embryogenesis (Tètu *et al.*, 1987).

Several media have been utilised in sugar beet tissue culture, including Murashige and Skoog medium (Tètu *et al.*,1987, Lindsey and Gallois 1990, Richie *et al.,* 1988), PGO medium (van Geyt and Jacobs 1985), Hooker and Nabors medium (Elliott *et al.*, 1987) and several novel media (Freytag *et al.*, 1990).

Previously root derived sugar beet cell suspension cultures have been used for detailed studies of the roles of endogenous phytohormones in this laboratory (Elliott *et al.* 1986) using Hooker and Nabor's medium but more recently Murashige and Skoog medium has been employed for several types of sugar beet cultures (Tetu *et al.*, 1987; Masuda *et al.*, 1988).

2.1.2 Aim

It was important for the aims of the project to produce large quantities of sterile sugar beet material suitable for infection with *A. rhizogenes* plasmid A4b (henceforth to be called A4T). To produce sugar beet root-derived cell suspension cultures. These could then be used for studies to measure difference in growth patterns between sugar beet root cells and those transformed with *Agrobacteria* containing either the A4 plasmid (containing auxin biosynthetic genes and *rol* genes) or a plasmid containing the auxin biosynthetic genes only (see Chapter 3). It was hoped to produce a reliable protocol to allow regeneration of plants from seedling root material.

2.2 Materials and Methods

All chemicals used were of analytical grade and obtained from Sigma, Poole, Dorset; Fisons, Loughborough; or BDH, Poole, Dorset. All tissue culture media and glassware was sterilised by autoclaving at 18-lbs. inch $^{-2}$ pressure for 20 minutes. All aseptic transfer and subculture was carried

out in a laminar airflow cabinet swabbed down with ethanol. Instruments were sterilised by flaming with ethanol or by autoclaving.

2.2.1 Seed Sterilisation and Germination

Sugar beet seedlings cv. Bella (Hilleshog UK Ltd, Docking, Kings Lynn, Norfolk) were obtained as follows:

Seeds were washed in 1% (v/v) Teepol, rinsed thoroughly with deionised water and transferred to a sterile flask containing 50% (v/v) Domestos (Lever Bros.) and shaken for 120 minutes. Seeds were then washed three times for 20 minutes each with sterile distilled water and then either briefly a further five times to remove any remaining bleach or rinsed with 70% ethanol and then briefly a further five times in sterile distilled water to remove any remaining bleach or ethanol.

These seeds were germinated on either sterilised vermiculite (autoclaved 18-lbs. inch^{-2} for 20 minutes), sterilised moistened filter paper or Murashige and Skoog medium (MS) (Murashige and Skoog 1963) containing 3% (w/v) sucrose and 0.8% (w/v) agar. They were germinated at 25°C in darkness. When seeds had germinated they were transferred to artificial daylight conditions (2500 lux) 16 hours day length at 25°C.

2.2.2 Petiole Cultures

Two weeks after germination, seedlings were removed aseptically and the roots and cotyledons removed. The petioles from apical segments were transferred to MS medium supplemented with 0.5-mg dm^{-3} 6-benzyladenine (BAP), 3% (w/v) sucrose and 0.8% (w/v) agar (MSBA). The petiole segments were grown for three weeks before transferring to large tubs. Four weeks later these petioles were large enough to be split and placed on fresh medium. The resulting petiole cultures were transferred to fresh medium every four weeks. MS medium supplemented with 0.5-mg dm^{-3} BAP was used, as this was a well-established protocol developed in this laboratory (Grieve, 1990)

2.2.3 Seedling Root Cultures

Roots excised from two-week-old sterile seedlings were placed on Gamborgs B5 medium supplemented with 3% (w/v) sucrose and 0.6% (w/v) agar. They were grown at 25°C in darkness and the root tips were sub-cultured every four weeks.

2.2.4 Liquid Seedling Root Cultures

1cm lengths of sterile seedling roots that had been grown on at least one passage on GB5 medium were removed and placed in 250 cm^3 flasks containing 50cm^3 liquid GB5 medium supplemented with 3% (w/v) sucrose. Four pieces of root were used for each flask.

2.2.5 Seedling Root Callus Cultures

Roots excised from sterile two-week-old seedlings were placed on MS medium supplemented with 3% (w/v) sucrose, 0.8% (w/v) agar and one of a matrix of plant growth regulator regimes using both natural and synthetic auxins and cytokinins. A range of plant growth regulator regimes, including the use of the anti-auxin TIBA was used to induce shoot regeneration. Cultures were also grown at 25°C and 30°C as described by Ritchie *et al.* (1989)

2.2.6 Liquid Cell Cultures

Seedling root derived callus that had been through at least two subcultures was used for initiation of liquid cell cultures. Both MS and GB5 media were used with all the methods described.

a) Callus was broken up and spread across the surface of solid media, 15 cm^3 of the same medium without agar was then poured on top. This was then shaken gently on an orbital shaker.

b) Callus was sub-cultured onto medium supplemented with decreasing concentrations of agar until no agar was added to the medium.

c) Callus was broken up and placed in liquid medium.

d) Callus was left in large clumps and added to liquid medium.

All of the above were used with or without anti-phenolic chemicals PVPP or citric acid.

2.3. Results

2.3.1. Seed Sterilisation and Germination

The highly stringent method used for sugar beet seed surface sterilisation produced up to 75% successful sterilisation (table 2.1 and table 2.3). The main contamination was fungal, but a pre-treatment with the antifungal preparation, Benylate did not give noticeably increased sterilisation rates. Sterilisation rates were calculated by surface sterilising 500 seeds per trial and each sterilisation regime was repeated four times.
Increasing the concentration of Domestos (Lever Bros.) increased the percentage of germinated seedlings free from contamination. Ethanol treatment after bleach did not significantly improve the efficiency of seed sterilisation but was adopted in the method.

These seeds were germinated on Murashige and Skoog (MS) medium (Murashige and Skoog, 1963) containing 3% w/v sucrose and 0.8% agar at 25^{0}C in darkness. When seeds had germinated they were transferred to artificial daylight conditions (2500 lux) 16 hours day length at 25^{0}C.

Table 2.1– Sterilisation using various concentrations of Domestos followed by a rigorous sterile distilled water rinse.

	Concentration of Domestos (% w/v)			
	20	**30**	**50**	**70**
Mean % of Contaminated Seeds	70.5 +/-19.2	44.3 +/-19.4	30.8 +/-11.9	25.3 +/- 9.0

Table 2.2 – Sterilisation using various concentrations of Domestos followed by a 70% ethanol rinse then rigorous sterile distilled water rinse.

	Concentration of Domestos (% v/v)			
	20	**30**	**50**	**70**
Mean % of Contaminated Seeds	66.3 +/-8.8	42.8 +/-12.6	35.3 +/-9.4	21.3 +/-12.8

Table 2.3 – Sterilisation using various concentrations of silver nitrate followed by distilled water rinse.

	Concentration of Silver Nitrate (% w/v)			
	2	**5**	**10**	**15**
Mean % of Contaminated Seeds	81.1 +/-8.2	75.5 +/-13.5	76.8 +/-11.9	69.3 +/-14.6

Table 2.4 – Germination rates at varying concentrations of Domestos.

	Concentration of Domestos (% w/v)			
	20	**30**	**50**	**70**
Mean % Germination	85.4 +/-10.4	73.0 +/-16.8	70.3 +/-19.4	49.8 +/-8.7

Pre-treatment with Benylate gave no significant difference in sterilisation efficiency.

MS medium supplemented with 0.8% agar was used for the germination trials as when germinated on vermiculite or sterilised filter paper those contaminated could not be immediately identified (until sub-cultured onto MS medium, by which time the whole vessel was contaminated).

The optimum conditions chosen for seed sterilisation and germination were 50% w/v Domestos solution. This was followed by a 70% ethanol rinse and rigorous sterile distilled water rinse.

2.3.2. Petiole Culture Production

Seedling petioles from apical segments were grown for about 3 weeks before transferring to large tubs. Four weeks later these were large enough to be split and placed on fresh medium (see figure 2.1). MS medium was used as it supported their growth well. Petiole sections from these cultures were subsequently used for infections with *A. tumefaciens* C58C1 2.1. All petiole material that originated from each sterile seedling was cultured separately providing clonal material.

Figure 2.1 Sugar Beet CV Bella petiole culture 2 weeks after subculture on MS medium containing 0.5mg dm^{-3} BAP.

Figure 2.2 Induction of callus on Sugar Bett cv Bella seedling roots after 2 weeks on MS medium containing 0.5mg dm^{-3} 2,4-D and 0.5 $mgdm^{-3}$ kinetin.

2.3.3 Seedling Root Cultures

Seedling roots were maintained on GB5 medium supplemented with 3% w/v sucrose and 0.8% w/v agar. This medium was chosen as previous experiments had shown that this was the most suitable growth medium for transformed hairy roots and this material was to be used for starting material for callus and control material. Initially growth was slow but after several subcultures at 4 week intervals the roots looked healthy, white with fine hairs and produced a mat of root material.

2.3.4. Liquid Seedling Root Cultures

To increase growth rates inorder to provide material for auxin analysis transformed roots were transferred to liquid medium. For comparison seedling roots were also grown on liquid half strength GB5 medium supplemented with 3% w/v sucrose. Growth rates were slower than for transformed material but the roots produced considerably more branching than those grown on solid medium.

At first growth was very slow and the roots browned quickly. After three subcultures at four-week intervals the growth rate increased and the roots were white in colour.

2.3.5 Seedling Root Callus Cultures

Callus appeared along the length of seedling roots within two weeks of being placed on callus initiating medium (Fig 2.2). After four weeks on this medium callus was removed from the explant material and maintained on the same medium under similar culture conditions. Callus was creamy white in colour and at first hard but became more friable after the first sub-culture (table 2.5 and table 2.6).

Table 2.5 The effect of 2,4-D and Kinetin on the initiation of callus from sugar beet seedling roots.

2,4-D $mgdm^{-3}$	Kinetin $mgdm^{-3}$	Appearance of roots
0.5	0.5	38/50 explants produced pale brown callus.
1.0	1.0	43/50 produced pale brown callus. 7 explants contaminated.
0.5	1.0	46/50 produced pale brown callus, 3 died, 1 contaminated.
1.0	0.5	Callus appeared along the length of the root after 2-3 weeks.
5.0	1.0	11/50 explant material died, 39/50 produced callus as above.
1.0	5.0	3/50 explants died, 47/50 produced callus as before.
1.0	2.0	37/50 explants produced callus, 13/50 contamiated.
2.0	1.0	41/50 explants produced callus, 7/50 died, 2 contaminated.

Table 2.6 The effect of IAA and Kinetin on the production of callus from sugar beet seedling roots

IAA ($mgdm^{-3}$)	Kinetin ($mgdm^{-3}$)	Appearance
1.0	1.0	Produced many roots covered in root hairs.
1.0	0.5	As above.
0.5	1.0	As above.
2.0	1.0	As above.
1.0	2.0	Many spindly roots covered in root hairs.
5.0	1.0	Produced many short roots covered in root hairs.
1.0	5.0	Produced short fat roots covered in root hairs.

These results led to the use of MS medium supplemented with 0.5 $mgdm^{-3}$ 2,4-D and 0.5 $mgdm^{-3}$ kinetin to initiate callus from sugar beet cv Bella seedling roots.

To try to induce shoot production, callus was placed on MS medium containing 2,4-D and BAP in varying concentrations and grown in artificial daylight conditions. Results are shown in table 2.7

Table 2.7-The effect of different concentrations of 2,4-D and BAP on sugar beet seedling root derived callus grown in artificial daylight conditions.

	$Mgdm^{-3}$ BAP				
$Mgdm^{-3}$ 2,4-D	0	0.1	0.5	2.0	5.0
0	Callus became darker in colour.	Callus as in dark	Callus pale green, soft and friable	Callus became pale green.	Callus became pale green in colour
0.1	Callus became light brown in colour	Callus became light brown in colour	Callus had some pale green patches, was soft	The edges of the callus became green	Darker green than above, soft.
0.5	Callus pale brown in colour. Dark brown after 9 days.	Callus pale brown, compact and hard	Callus had small patches of green and was very soft	Callus became green and was soft	Callus green, friable.
2.0	Callus was pale brown and friable	Callus pale brown	Callus was soft and turned brown	Callus was green, brown around the edge.	Callus became green in colour
5.0	Callus turned brown	Callus turned brown	Callus turned brown	Callus turned brown	Callus turned brown.

The inclusion of TIBA in the media described above (table 2.7), was shown to have no effect on these cultures. Each of the media above were also used to incubate the callus at 30°C. Again no difference was seen between these, and those incubated at 25°C.

2.3.6 Liquid Cell Cultures

All methods described in 2.2.6 failed to produce a cell suspension culture. No visible growth of the callus, or cells, if the callus had been broken-up took place. All of the attempts to grow the cells in liquid medium resulted in both the cells and the medium becoming dark brown and it became obvious after 3 subcultures that the cells were dead.

2.4. Discussion

The callus produced was to be used for the production of a cell suspension culture. There is at present no root derived sugar beet suspension culture in the laboratory. Cell suspension cultures provide a model system for studying many aspects of cellular physiology and biochemistry. When obtained it was to be used to compare the growth

parameters with those of similar cell cultures of sugar beet cells transformed with rol/auxin genes and with plasmids containing the auxin genes only. It was hoped that the transformed cells would show altered growth patterns that may provide insights into the control of cell division and storage root development.

In this study callus was only produced when 2,4-D was added to the medium. At first the callus was very hard and brown in colour but after several subcultures it became more friable and lighter in colour. It was noted by DeGreef (1978) that when 2,4-D was added to the medium that the callus produced was much more homogenous than that produced when using IAA. He found that when IAA was added to the medium, callus formation was accompanied by intense root formation. This was similar to the results described here with next to no callus being produced on medium with IAA. Hooker and Nabors (1977) stated that, in general high concentrations of auxin (10-25mgdm^{-3}) promoted callus initiation best, but that 2,4-D showed inhibitory effects at concentrations greater than 1mgdm^{-3}. This was not found in this study. Butenko *et al.* (1972) also showed that more callusing occurred when 2,4-D was added to their high salt – high sucrose medium. Butenko *et al.,* (1972) also suggested that sugar beet tissue cultures should be grown on media that contained

high salt concentrations (due to sugar beets tolerance to salt) and high sucrose concentrations to mimic parent plant tissues. Mohamed and Collins (1979) found that no callus growth occurred at sucrose concentrations above 10% (w/v) and that maximum growth occurred at 4% (w/v). Increased salt supply also had no stimulatory effect. These findings were confirmed in this laboratory (T. M. Grieve, 1990).

Tétu *et al.,* (1987) produced seedling root derived callus using a medium called A_3, MS medium supplemented with 0.5mgdm^{-3} IAA and 5.0mgdm^{-3} BAP. In their study 96% of the explant material produced callus. This PGR regime also produced callus but at a lower frequency.
Attempts at producing a cell suspension culture from the seedling root derived callus were all unsuccessful. After 2-4 days in liquid medium all cultures, including those containing anti – phenolic compounds became dark brown and no growth of the callus/cells occurred. It was concluded that after several sub cultures that the cultures had died.

In the organogenesis studies, the data presented here agree with the recalcitrance of sugar beet in culture reported by other workers (Anatassov, 1986; Ford-Lloyd and Bhat, 1986; Krens and Jamar, 1989). Seedling root callus could only be induced to produce callus on medium

containing 2,4-D, but once initiated would grow on media containing BAP and cultured in artificial daylight conditions. When BAP was included in the medium and cultures grown in light the callus became green but no shoots could be initiated.

Despite difficulties encountered in early experiments to induce shoot regeneration in sugar beet, several reports indicate that procedures for plant production from callus are now available. Saunders and Dolby (1986) reported a system in which 1mgdm^{-3} BAP was used to induce callus and regeneration from leaves. Tétu *et al.*, (1987) obtained higher shoot morphogenesis from petioles, roots and shoot tips callus when TIBA was applied. In this study the use of TIBA did not induce the production of shoots from callus. Freytag *et al.*, (1988), Van Geyt and Jacobs (1985) and Saunders *et al.*, (1992) all reported shoot regeneration from petioles. It appears that organogenesis frequency is low and genotype dependant Jacq *et al.*, (1993), Catlin (1990), Chun Lai Zhang (unpublished data 1996).

Seedling root explants could only be induced to produce callus on media containing 2,4-D. Organogenesis could not be achieved from this callus by inclusion of BAP or the anti auxin compound TIBA. It was not

possible to produce a liquid cell suspension culture from the callus produced from the seedling roots. The callus either failed to disperse in the liquid medium and/or produced what was thought to be phenolic compounds, which resulted in the tissue, and media turning black and the cells died.

The petiole cultures were very successful and provided large amounts of petiole material for transformation experiments with Ri plasmids containing *rol* and auxin genes (*iaaM* and *iaaH*) or auxin genes only. The production of seedling root material was also successful and was used for auxin analysis and auxin receptor analysis (see Chapter 6).

Chapter 3

Construction of Plant Transformation Vectors

And

Transformation of Sugar Beet

3.1 Introduction

The ability to cleave DNA molecules at specific sites and join them together in new recombinant sequences underpins the whole of gene manipulation technology. This was made possible by the discovery of enzymes known as restriction endonucleases which recognize specific short sequences of bases within long double stranded DNA molecules. Restriction endonucleases are divided into three types, I, II and III. Type I enzymes recognise a specific sequence and then track along the DNA molecule for 1000 to 5000 bases before cleaving a DNA strand (Old and Primrose 1985). Type III enzymes cut DNA 24-26 base pairs in the 3' direction from the recognition site. Type II enzymes break bonds at or near the recognition site in a more predictable manner and so are much more useful for the production of recombinant DNA molecules. Some enzymes leave overhanging single stranded DNA of a few nucleotides on the 5' (e.g. EcoRI) or 3' (e.g. Pst I) strand, while some leave no overhanging strand creating blunt ends on restriction fragment termini (e.g. Eco RV). This is shown in figure 3.1.

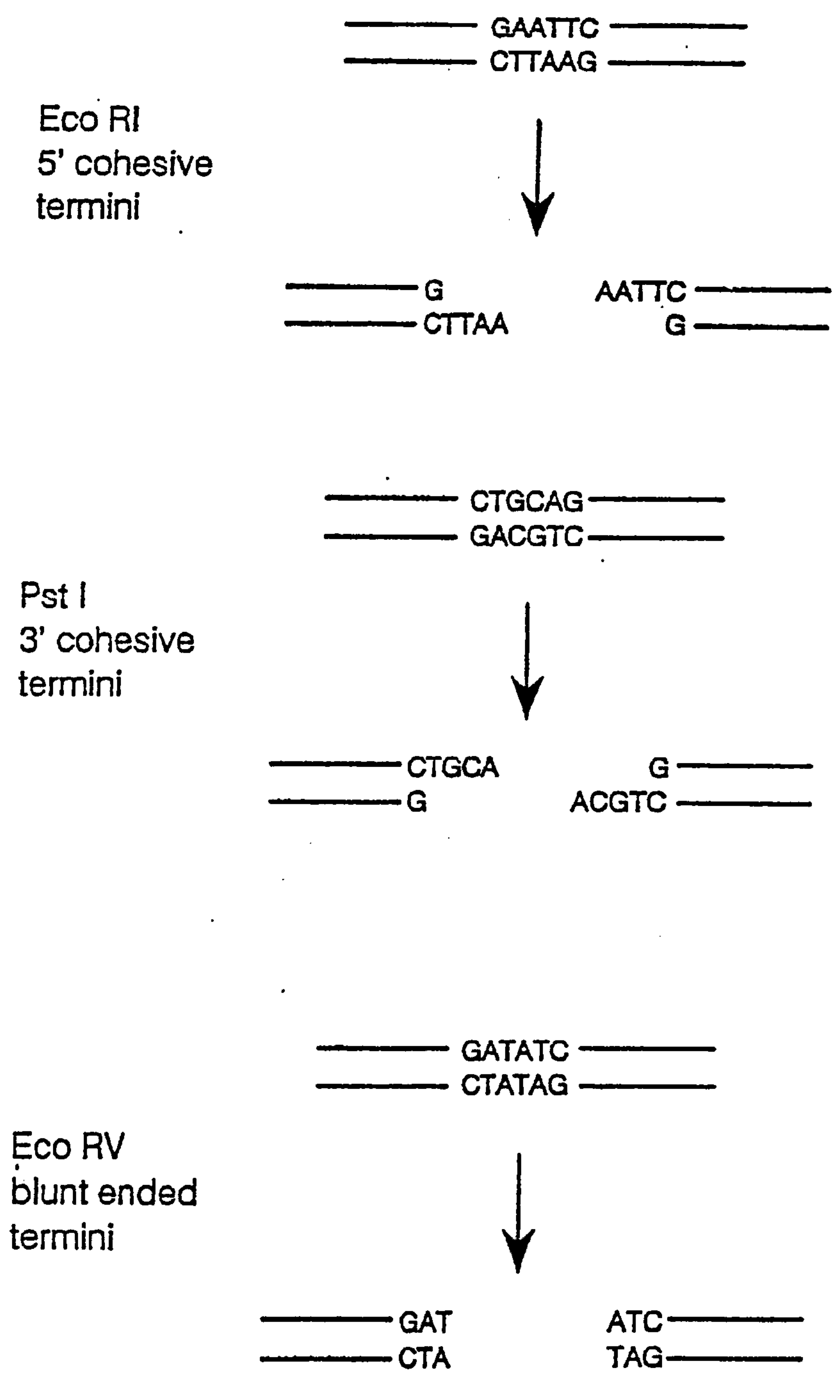


Figure 3.1 Termini resulting from digestion by various type II restriction endonucleases.

The ability to splice DNA in a defined manner is complemented by the ability to join the cleaved products to form recombinant molecules. This is normally achieved by the use of DNA ligase isolated from either *E. coli* or the bacteriophage T4. T4 DNA ligase aso requires ATP as a cofactor. ATP is split to form an AMP-ligase complex which binds to the 5' phosphate and 3' hydroxyl group of adjacent nucleotides. The two DNA strands are then joined by a covalent bond. Ligation is more efficient with DNA molecules with overhanging cohesive end groups of complementary bases than for blunt ended molecules. Thus enzymes which create cohesive ends are used in cloning strategies where possible.

To prevent re-circularisation of vector molecules, which may cause a high background of non-recombinant molecules within a typical ligation reaction alkaline phosphatase is used. This removes phosphate groups from 5' ends of DNA making the DNA unsuitable as a substrate for ligase (figure 3.2.

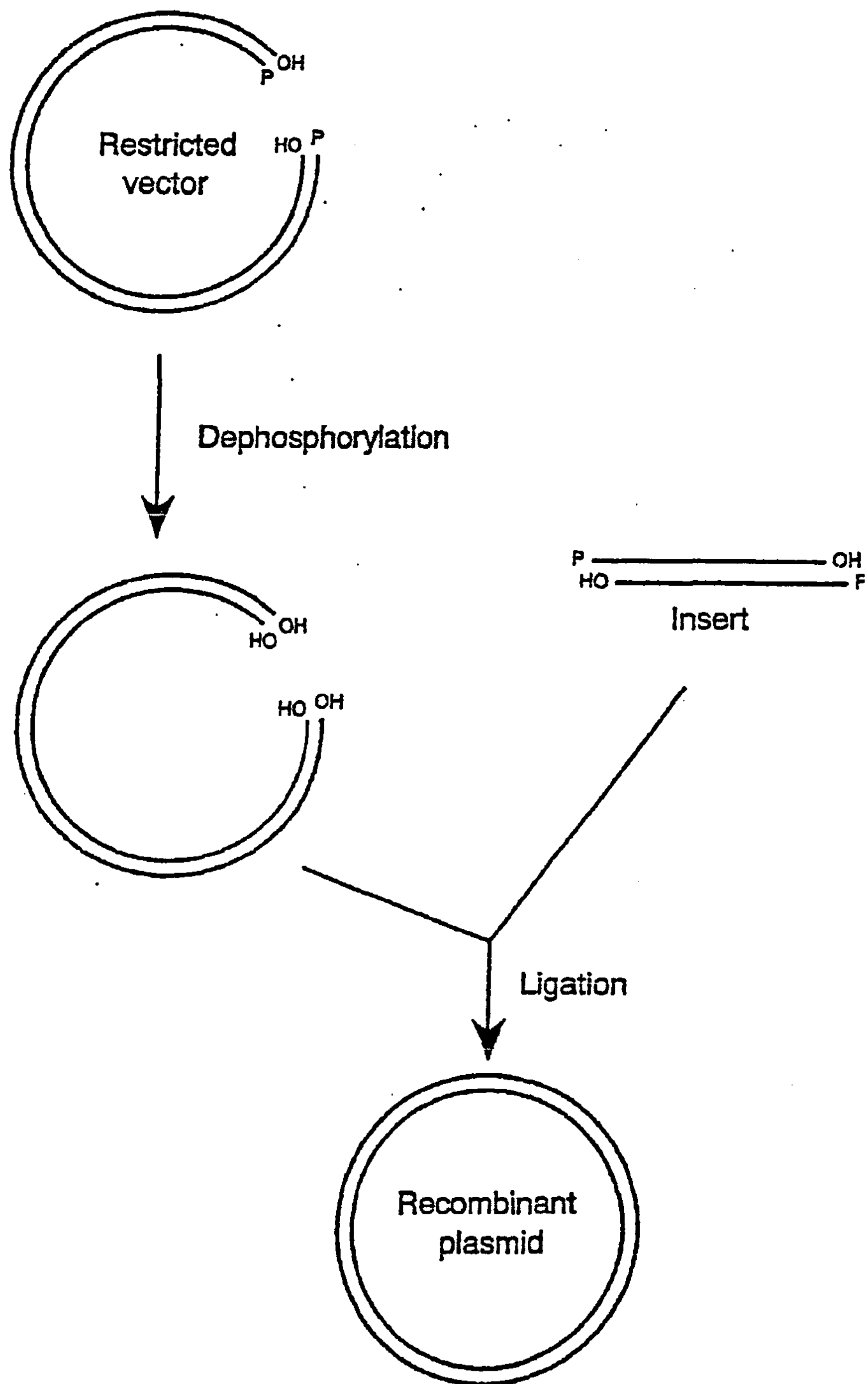


Figure 3.2 Use of phosphate prevent recircularization of vector molecules.

to form hydroxyl termini (OH, HO), to increase efficiency of recombination.

Usually a ligation reaction mixture will include a restricted vector as well as a restriction fragment for insertion into the vector. During the cloning procedure it is necessary for the vector and insert to form a linear molecule, and then for that recombinant molecule to circularize before it can be used to transform *E.coli.*

The transformation of *Agrobacterium* with the plasmid vector is a relatively inefficient process with respect to colonies formed per μg DNA. Hence *E. coli* was first transformed using the products of the ligation reactions in order to amplify the number of copies of plasmid DNA. These were subsequently isolated and screened for correct construction before being used in the transformation of *Agrobacterium.*

3.1.2 Aim

The aim of this work was to produce plant transformation vectors to enable the introduction of specific genes from *Agrobacterium* into the sugar beet cv Bella genome. Particular attention was paid to the genes contained within the Ri plasmid A4b (*iaaM* and *iaaH*, and the *rol* genes), and the auxin biosynthetic genes from the Ti plasmid pTiAch5.

3.2 Materials and Methods

3.2.1 Large Scale Plasmid DNA Preparation from *E.coli*

5cm^3 of nutrient broth was inoculated with *E. coli* containing the desired plasmid and incubated overnight at 37°C with shaking under appropriate antibiotic selection. The overnight culture was used to inoculate 1dm^3 Terrific Broth (Tartof and Hobbs., 1987) with the antibiotic selection.

Terrific Broth

A – 100cm^3 of:

0.17 moldm^{-3} KH_2PO_4

0.72 moldm^{-3} K_2HPO_4

B – 900cm^3 of:

12g bactotryptone

24g yeast extract

4cm^3 glycerol

This culture was incubated at 37°C in an orbital shaking incubator until the culture was at mid-log phase ($A_{600nm} = 0.6$). At this stage 150 mg dm^{-3} chloramphenicol was added to the culture to amplify the plasmid DNA, but prevent further growth of bacteria. The culture was left overnight at 37°C in an orbital shaker.

The culture was centrifuged in 250 cm^3 bottles at 10,000rpm in a Beckman J2-21 centrifuge. This was repeated 4 times, laying pellets on top of each other. These pellets were re-suspended in 2.5cm ^{3} 25% (w/v) sucrose, 50mmoldm^{-3} Tris HCl pH8 on ice and transferred to a sterile 250cm^3 conical flask. 4 cm^3 of lysozyme solution (5 mmoldm^{-3} Tris HCl pH8, 10 mgcm^{-3} lysozyme) was added, swirled and left on ice for 5 minutes. After 5 minutes 20 cm^3 10% (v/v) Triton solution was added and swirled gently for 30 seconds, left on ice for 20 minutes and then shaken gently for a further 30 seconds.

The mix was centrifuged at 18000 rpm for 60 minutes at 4°C in a Beckman J2-21 centrifuge. 30 cm^3 of supernatant was transferred into a

sterile 150 cm^3 flask and 1 gcm^{-3} CsCl was added. This was mixed gently until all of the CsCl dissolved then 0.75 cm^3 of 10 g dm^{-3} ethidium bromide solution was added and mixed in. This was left in the dark for 30 minutes.

The mixture was centrifuged at 10000 rpm for 30 minutes at 20^0C in a Beckman J2-21 centrifuge. The supernatant was transferred to vertical rotor ultracentrifuge tubes through a syringe and 21G needle.

Samples were centrifuged at 40000 rpm for 18 hours at 20^0C in a Europa ultracentrifuge.

After this centrifugation two bands of DNA could be seen clearly in UV light. The upper band was of linear bacterial DNA and nicked plasmid DNA. The lower band contained the closed circular plasmid DNA. To obtain this band the centrifuge tube was pierced at the top with a sterile hypodermic needle, size 21G. The tube was pierced just below the lower band of DNA and this was carefully drawn off into a syringe and transferred to a sterile test tube.

Ethidium bromide was removed by extracting into TE buffer (10 $mmoldm^{-3}$ Tris HCl pH8, 1 $mmoldm^{-3}$ EDTA) saturated 1-butanol. Equal

volumes of butanol solution were added to the plasmid solution and mixed gently and left to settle out.

The top phase was discarded. This was repeated several times until no pink colour remained. The remaining aqueous phase was dialysed against several changes of TE buffer pH8 at 4°C to remove the caesium chloride. Dialysis tubing was prepared by twice autoclaving in 5% (w/v) sodium carbonate 5 mmoldm^{-3} EDTA then stored in sterile water at 4°C.
DNA was precipitated by the addition of 0.1 volume of 3 mol dm^{-3} sodium acetate (pH 5.2) and 1 volume of propan-2-ol at –20°C for 16 hours in siliconized 30 cm^3 Corex tubes. DNA was collected by centrifugation at 10 000 rpm at 4°C for 15 minutes and the supernatant carefully discarded. The tubes were left inverted to drain for several minutes and the DNA was rinsed in 10 cm^3 cold 75% (v/v) ethanol, the pellets were broken up at this stage by pipetting in and out with a sterile Pasteur pipette. DNA was again collected by centrifugation, the supernatant poured off and the pellet dried under reduced pressure. The pellets were dissolved in TE at 37°C.

3.2.2. Agarose Gel Electrophoresis

Separation of DNA fragments was routinely achieved using agarose gel electrophoresis according to Maniatis *et al.* (1982) and Sambrook *et al.* (1989).

Agarose or low melting point agarose was heated in buffer on a heated stirrer until uniformly melted by boiling for a few minutes. Buffers used were usually 0.5 x TBE (45 mmol dm^{-3} Tris-borate, 1 mmol dm^{-3} EDTA) made up from a 5 x stock solution or when recovery of the DNA was required 1 x TAE (40mmol dm^{-3} Tris-acetate, 1 mmol dm^{-3} EDTA, pH 8.0) made from a 50 x stock solution:

1 dm^3 5 x TBE stock solution: 54 g Tris base
275 g Boric acid
20 cm^3 0.5 mol dm^{-3} EDTA (pH 8.0)

1 dm^3 50 x TAE stock solution: 242 g Tris Base
57.1 cm^3 Glacial acetic acid
100 cm^3 0.5 mol dm^{-3} EDTA (pH 8.0)

The range of lengths of DNA molecules which can be effectively separated by agarose gel electrophoresis is dependent upon the concentration of agarose in the gel. Larger molecules are separated most efficiently in relatively low percentage gels while smaller molecules

require higher percentage gels (Sambrook *et al.*, 1989). Hence different concentrations of agarose were used depending on the size of the DNA fragments to be resolved. For large plasmids (2.5-6.0 kb) about 1.0% (w/v) agarose was used and for smaller fragments of about 0.1 kb 2.0% agarose (w/v) was routinely used. After agarose had cooled to about 50^oC, 10 mg cm^{-3} ethidium bromide was added to a final concentration of 0.5 mg dm^{-3} to enable DNA to be detected by fluorescence under a UV trans-illuminator. Molten agarose was poured into a casting tray fitted with a comb to form wells. When set the comb was removed, the gel placed into an electrophoresis tank and similar buffer containing 0.5 mg dm^{-3} ethidium bromide was added to a depth of 1 mm above the surface of the gel.

Loading buffer was added to DNA samples to be separated on the gel from a 6 x concentration stock solution:

6 x Loading buffer	0.25% (w/v) Bromophenol blue
	0.25% (w/v) Xylene cyanol FF
	30% (w/v) Glycerol

Autoclaved and stored at 4^oC.

To ensure adequate separation, particularly of phage lambda DNA Hind III restricted fragments used as markers, DNA with loading buffer was heated to 68°C for 3-5 minutes and transferred to ice. Samples were loaded into wells in the gel and a direct current passed through the gel, The DNA moving in the direction of the positive terminal. Voltages used depended on the size of the fragments to be resolved, typically large fragments on a 15 cm long 0.5% (w/v) agarose gel were run at no more than 50 V, while small (0.1 kb) fragments on a 8 cm long 2.0% (w/v) agarose gel were run at 100-150 V for 30-60 minutes.

When TAE buffer was used the buffer was circulated from the negative terminal to the positive terminal end of the tank using a peristaltic pump.

Gels were photographed when fluorescing under UV light using Polaroid 667 instant film or a UVP video copy processor using Mitsubishi HD thermal paper (K65HM).

3.2.3 The use of Electroelution to Purify DNA

DNA was purified, by running on a 0.7% w/v agarose gel (see

section 3.2.2) together with Hind III cut λ DNA as markers. This separated the plasmid DNA from contaminating chromosomal DNA. The bands corresponding in size to those of the plasmids were cut from the gel and the DNA removed by electroelution:

Gel pieces were placed in dialysis bags with 0.5 cm^3 half-strength TBE buffer (see section 3.2.2). The dialysis bags were then placed in an electrophoresis tank containing half-strength TBE and DNA was eluted from the gel at 200 volts.

When no ethidium bromide was left in the gel the current was reversed for 30 seconds to release DNA from the side of the dialysis tubing. The DNA solution was transferred to microfuge tubes and purified by phenol/chloroform extraction and precipitated with NaAc and ethanol.

Plasmid DNA was then linearised with restriction enzymes (see section 3.2.4)

3.2.4 Restriction Digest of DNA

Restriction enzymes and their buffers were obtained from NBL (Cramlington, Northumberland).

The required units of enzyme for each digest were obtained using the equation below.

$$\text{Units of enzyme} = \frac{\mu g\ \text{DNA x total volume/20 x 4}}{\text{Reaction time (hours)}}$$

DNA to be cut was transferred to a sterile micro-centrifuge tube and the appropriate volume of 10x digestion buffer (specific for each enzyme) added and mixed. The required number of units of enzyme were added and the tube incubated at 37°C (or appropriate temperature) for the required time. The reaction was stopped, by adding 0.5 moldm^{-3} EDTA pH7.5 to a final concentration of 10 mmoldm^{-3}. DNA was purified by extracting with phenol/chloroform and precipitated. DNA was re-suspended in sterile H_2O and stored at –20°C.

3.2.5 Dephosphorylation of Restricted DNA

One unit of alkaline phosphatase is the enzyme activity which hydrolyses 1 μmol of 4-nitrophenyl phosphate in 1 minute at 37°Cin standard assay conditions (Boehringer Mannheim catalogue, 990/91).

Calculation of the amount of calf intestinal phosphate (CIP, Boehringer-Mannheim) required:

0.05 units enzyme activity will remove 5' terminal phosphate groups from 1 pmol of terminal at 37°C in 30 minutes in 20 mm^3 (1 pmol ends = 1.6 μg of a 5 kb fragment. Armitage *et al.*, 1988)

3.2.6 Ligation of Vector and Insert DNA

One unit of T4 DNA ligase is the amount of enzyme activity that converts 1 nmol [^{32}P] from pyrophosphate into Norit absorbable material in 20 minutes at 37°C (Boehringer Mannheim catalogue, 1990/91)(see table 3.1).

Table 3.1- A typical range of reactions set up to ligate an insert DNA fragment into a cloning vector

Tube	A	B	C	D	E	F	Z
		(mm^3)					
Vector DNA	4	4	2	2	5	5	4(uncut)
Insert DNA	2	0	4	0	1	0	0
Ligation buffer	1	1	1	1	1	1	0
20 mmol dm^{-3} ATP	1	1	1	1	1	1	0
10 mmol dm^{-3} Hex.	1	1	1	1	1	1	0
Ligase	1	1	1	1	1	1	0
Sterile H_2O	0	2	0	4	0	1	6

3.2.7 Preparation of Competent *E. coli* Cells for Transformation.

Competent cells of *E. coli* HB101 were prepared according to the protocol of Hanahan (1985). A single colony of *E. coli* was inoculated into 10 cm^3 SOB medium in a Universal bottle and incubated for 15 hours 30 minutes shaking at 150 rpm at 37°C.

SOB Medium

2% w/v Lab M tryptone

0.5% w/v Lab M yeast extract

10 mmol dm^{-3} NaCl

2.5 mmol dm^{-3} KCl

10 mmol dm^{-3} $MgCl_2$

10 mmol dm^{-3} $MgSO_4$

This culture was poured into 1 dm^3 SOB medium in a 2 dm^3 flask and incubated as before. After 3 hours 40 minutes (or until the culture had an OD_{550} of about 0.5) the culture was harvested at 3000 rpm, 4°C for 15 minutes, re-suspended in 330 cm^3 RF1 30 minutes and left on ice for 2 hours 30 minutes. Cells were centrifuged again and re-suspended in 80 cm^3 RF2. Cells were incubated for 15 minutes on ice and 0.6 cm^3 aliquots of competent cells were flash frozen in liquid nitrogen and stored at -70°C until required.

RF1

100 mmol dm^{-3} RbCl

50 mmol dm^{-3} $MnCl_2$

30 mmol dm^{-3} potassium acetate

10 mmol dm^{-3} $CaCl_2$

15% v/v glycerol

pH 5.8

RF2

10 mmol dm^{-3} 3-[N-morpholino] propane-sulphonic acid (MOPS)

10 mmol dm^{-3} RbCl

75 mmol dm^{-3} $CaCl_2$

15% v/v glycerol

pH 6.8

3.2.8. Transformation of *E. coli* HB101 Cells with Plant Transformation Vectors

The $CaCl_2$/RbCl transformation method (Maniatis *et al.*, 1982) was used to transform *E. coli* HB101. 50 cm^3 nutrient broth in a 100 cm^3 conical flask was inoculated with 0.5 cm^3 of an overnight culture of *E. coli*

(shaken at 120 rpm at 37°C for 16 hours). This was incubated in the same conditions above for 2 hours 30 minutes or until the OD_{550} was about 0.5. The culture was then harvested by centrifugation and re-suspended in 1 cm^3 10 mmol dm^{-3} MOPS, 10 mmol dm^{-3} RbCl, pH 7.0 and left on ice for 10 minutes. Cells were collected as before and re-suspended in 0.2 cm^3 MCR and left on ice for 15 minutes.

MCR

0.1 mol dm^{-3} MOPS (pH 6.5)

50 mmol dm^{-3} $CaCl_2$

10 mmol dm^{-3} RbCl

Cells were spun down again and re-suspended in 0.2 cm^3 MCR. DNA to be inserted was added (between 50 and 200 ng) and the cells were incubated for 30 minutes on ice. After heat shock, 44°C for 30 seconds, 1 cm^3 nutrient broth was added and the tubes were incubated without shaking for 60 minutes at 37°C. 150 mm^3 were plated on nutrient agar plates containing the appropriate antibiotic selection (e.g.50 mg dm^{-3} kanamycin for pB1023-19). Plates were incubated at 37°C until colonies were clearly visible.

3.2.9. Selection of Transformed Colonies and Isolation of DNA from *E. coli* Transformants.

Colonies which had grown on the selective plates after transformation were streaked for single colonies and incubated as before. Single colonies from these plates were grown in 10 cm^3 Terrific Broth (Tartof and Hobbs, 1987) with selection for 16 hours at 37°C. Cells were harvested from 1.4 cm^3 culture by centrifugation (12000 rpm for 1 minute). The small scale plasmid DNA isolation method of Alter and Subramanian (1989) followed by agarose gel electrophoresis through a 0.5% (w/v) agarose, TBE (Maniatis *et al.*,1982) gel containing 0.5 cm^{-3} ethidium bromide was used to analyse the plasmids.

3.2.10. Transformation of *Agrobacterium*

Agrobacterium tumefaciens LBA 4404 (harbouring pAL4404), (Hoekema *et al.*, 1983) and A4T were transformed using the method described by An (1987). Bacterial strains were grown in 5 cm^3 PYE(see below) medium at 28°C, shaken at 150 rpm in Universal bottles overnight.

PYE medium

4g dm^{-3} peptone

1g dm^{-3} yeast extract

2mmol dm^{-3} $MgSO_4$

250 cm^3 flasks containing 50 cm^3 PYE medium were inoculated with 25 mm^3 aliquots of the overnight cultures. These were incubated at 28°C, shaking at 150 rpm, for about 16 hours. The OD_{600} was measured for each culture against a PYE medium reference. The culture with OD_{600} closest to 0.5 was harvested by centrifugation at 5000 rpm, 20°C for 5minutes. The pellet was re-suspended in 10 cm^3 0.15 mol dm^{-3} NaCl and collected again by centrifugation as before. The pellet was re-suspended in 1 cm^3 20 mmol dm^{-3} $CaCl_2$ pre-chilled on ice. The cell suspension was divided up into 200 mm^3 aliquots and put into sterile micro-centrifuge tubes on ice. About 1μg plasmid DNA was added to each tube, the contents gently mixed and the tubes were incubated on ice for 30 minutes. The cells were frozen in liquid nitrogen for 1 minute and then thawed by placing the tubes in a 37°C water bath. The cells were diluted by adding 1 cm^3 PYE medium to each tube. The cells were allowed to recover and accumulate products of their antibiotic resistance gene for 3-4 hours shaking at 150

rpm, at 28°C. They were plated out and checked for colonies after 2-3 days.

3.2.11. Confirmation of Transformation of *Agrobacterium* strains

Putative *Agrobacterium* transformant colonies were streaked for single colonies on fresh selection plates. Colonies were inoculated into nutrient broth under appropriate selection conditions and grown for 42 hours before plasmid isolation by the method of Ebvert *et al.* (1987).

1.5 cm^3 of each culture were centrifuged in a micro-centrifuge for 1 minute at 13000 rpm and the pellets re-suspended by vortexing in 100 mm^3 GET.

GET

150 mmol dm^{-3} glucose

10 mmol dm^{-3} EDTA

25 mmol dm^{-3} Tris-HCL pH 8.0

3 mg cm^{-3} lysozyme

The tubes were incubated at room temperature for 10 minutes and then 200 mm^3 freshly made 0.2 mol dm^{-3} NaOH, 1% w/v SDS was added and the tubes shaken. 30 mm^3 alkaline phenol (phenol equilibrated with volume 0.2 mol dm^{-3} NaOH, twice) was added, the tubes vortexed for 1 second and 150 mm^3 3 mol dm^{-3} sodium acetate (pH 4.8) was added. The tubes were mixed by inversion and incubated for 15 minutes at -20°C. Cell debris was pelted by centrifugation for 10 minutes at 13000 rpm and the supernatants poured into clean tubes. 900 mm^3 absolute ethanol was added to precipitate the plasmid DNA and the tubes were left at -20°C for 2 hours. The tubes were centrifuged for 10 minutes at 13000 rpm and the resulting pellets rinsed in 70% v/v ethanol and dried under reduced pressure. Pellets were dissolved in 50 mm^3 TE and aliquots were examined by agarose gel electrophoresis as described in 3.2.2.

The same colonies used to isolate plasmid DNA were also used to inoculate lactose medium plates (1% w/v lactose, 0.1% w/v yeast extract, 2% w/v agar) and the ketolactose test for *A. tumefaciens* (Bernaerts and DeLey, 1963) was used to confirm the identity of the isolates.

3.2.12. Inoculation of Sterile Sugar Beet Petioles with *Agrobacterium* Strains Harbouring pRiA4b and pM1023-19.

Petiole explants from shoot cultures of the sugar beet cv Bella (see section 2.2.2) were inoculated with A4T strains of *Agrobacterium* harbouring pRiA4b or pPM1023-19. Bacterial cultures were incubated at 28^{0}C for 42 hours and drawn into a disposable sterile 19G needle fixed to a sterile 2cm^3 syringe. The basal end of each petiole was stabbed with the needle and the petioles placed base up into solidified MSO medium in sterile 175 cm^3 jars. Control petioles were not inoculated but stabbed with a sterile needle dipped in sterile nutrient broth. After 48 hours the inoculated and control petioles were transferred to MSO medium supplemented with 0.8% w/v agar and 1 g dm^{-3} carbenicillin.
As roots appeared at the site of inoculation they were removed and cultured each one separately on solidified B5/2 medium containing 0.5 g dm^{-3} carbenicillin. As the roots grew they were cultured as described in section 5.2.1. (See 5.3.4)

3.3 Results

3.3.1 Large scale plasmid DNA preparation

Results of the large scale plasmid preparation of plasmid pM 1023-19 (harbouring the auxin genes of *A. tumefaciens*) are shown in figure 3.3. A band of DNA at about 12 kb can clearly be seen in lanes 1, 2, 3, and 4 loaded with DNA from pM1023-19 plasmid preparation. In lane 6 can be seen the Hind III cut λ DNA markers.

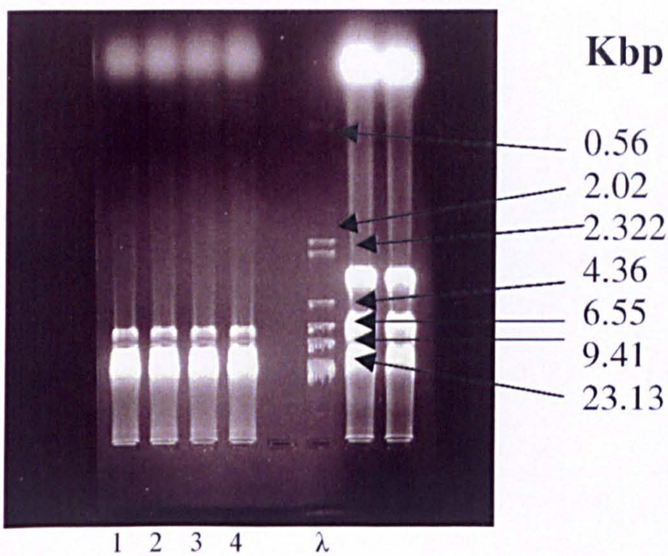


Figure 3.3. Large scale plasmid preparation of pM1023-19 with HindIII cut λ DNA markers

3.3.2 Restriction Digest of Plasmid DNA

Results of the restriction of vector SS1 with Eco R1 and PPT also restricted with EcoR1. The insert DNA shows a band of 1.3kb

Often when DNA that had been restricted or restricted and CIP treated was run on an agarose gel it appeared as a smear, indicating a mixture of randomly sheared fragments. This at first was thought to be due to contaminating endonucleases in the preparation. Phenol extraction and ethanol precipitation did not improve the results of digestion. It was suggested that the shearing may be due to physical disruption due to excessive shaking or use of fine bore disposable pipette tips (J. S. Gartland, pers. comm.). To minimise these effects, particularly with large fragments of DNA the ends of the pipette tips were removed and tubes were inverted several times to mix rather than shaking.

3.3.3 Transformation of *E. coli.*

Results of the transformations performed are shown in table 3.2

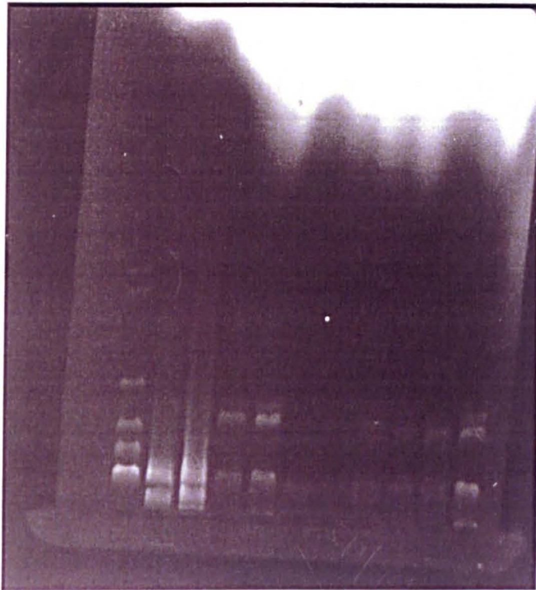


Figure 3.4 Plasmid preparation of a construct of the vector SS1 (12 Kbp) with a hygromycin resistance gene (PTT, 2 Kbp)

Ligation	Contents	mm^3 used	no. of colonies
		1	4
	SS1	1	0
a	&	1	0
	PPT	2	8
		2	0
		5	2
b	as above	1	0
		1	0
		2	0
		2	2
		5	2

Table 3.2- number of colonies formed after transformation of *E. coli* competent cells.

3.3.4 Infection of Sugar Beet Petioles with *Agrobacterium*

Un-Inoculated	A4T	pPM1023-19
0/45 (0%)	32/75 (43%)	
1/30 (3%)		28/52 (54%)

Table 3.4 Root formation of sugar beet cv Bella petioles 25 days after inoculation with *Agrobacterium tumefaciens* harbouring pRi A4b and plasmid PM1023-19

Note – Numbers of petioles infected and number of controls used in transformation study is shown below

A4T	100 inoculated	50 un-inoculated
	75 survived	45 survived
	32 produced roots	0 produced roots
1023-19.1	75 inoculated	35 un-inoculated
	52 survived	33 survived
	18 produced roots	1 produced roots

3.4 Discussion

Suitable controls for ligation reactions were often employed to give some idea of the degree of background transformation by non-recombinant plasmids. In some instances positive controls were also used to check the ligation steps. These measures were extremely useful and should be used whenever possible in cloning experiments. They can highlight the step that was ineffective if no tranformants are produced, which can be a frequent occurrence where there are so many important steps involved.

The plasmid DNA and large fragments of DNA were very fragile and prone to shearing. Some of this may have been due to impurities in the reaction reagents or contamination from the isolation of the DNA. This was kept to a minimum by using sterile equipment and wearing gloves for all stages of the DNA manipulation. A large proportion of the destruction may have been due to physical stress on the DNA during the manipulation procedures. To minimise this, care was taken to invert samples several times rather than shake them when mixing was required. Also a large improvement was made when the fine end of the disposable pipette tips were removed (J. S. Gartland pers. com.).

The transformation efficiencies achieved with the *E. coli* competent cells were low. This may be due to one or all of several factors. Problems with the CIP enzyme could result in the phosphate groups not being removed from the DNA fragments to be joined, resulting in the DNA pieces remaining separate, and no plasmid recombination. If the ratio of insert DNA to vector DNA is incorrect then the probability of the two different pieces of DNA joining together is reduced and the probability of the vector DNA re-joining is increased.

The number of infected petioles which formed roots was relatively high with both the pRiA4b and pPM1023-19 with only one out of 85 control petioles over both sets of transformations producing roots.
The roots produced from inoculation with pRi A4b when removed from the initial explant material became hairy and soon growth was rapid. The roots that appeared on the control petiole when cultured from the parent material did not produce as many root hairs, their growth was slow and they soon died on selective medium.
The roots that formed as a result of infection with pPM1023 –19 when cultured from the original explant material were not as uniform as those produced with pRiA4b. Some of the roots produced callus as well as ` hairy roots' on medium containing no plant growth regulators.

Unfortunately time did not allow for infections to be carried out with constructs containing the hygromycin resistance gene. This may have shown to be a more suitable selective agent for transformed sugar beet than other antibiotic resistance genes.

Chapter 4

Transformation of Sugar Beet

and

Molecular Confirmation of Stable Transformation

4.1 Introduction

Agropine type *A. rhizogenes* (plasmid A4) T-DNA consists of two different transferring elements, T_L DNA and T_R DNA (see Section 1.3.2). Auxin biosynthetic genes homologous to genes on the Ti plasmid T-DNA have been localised on the T_R DNA (Cardarelli *et al.*, 1985 and Figure 4.2).The first of these genes *iaaM* 1 encodes the enzyme tryptophan-2-monooxygenase, which converts tryptophan to indole-3-acetamide (IAM). The second gene, *iaaH* 2 encodes the synthesis of indole-3-actamide hydrolase which converts indole-3-acetamide to indole-3-acetic acid (IAA) (figure 4.1)

iaaM *iaaH*

Tryptophan-------------→IAM ----------------------→IAA

Tryptophan-2-monooxygenase IAM hydrolase

Figure 4.1 Auxin biosynthetic pathway in *Agrobacterium.*

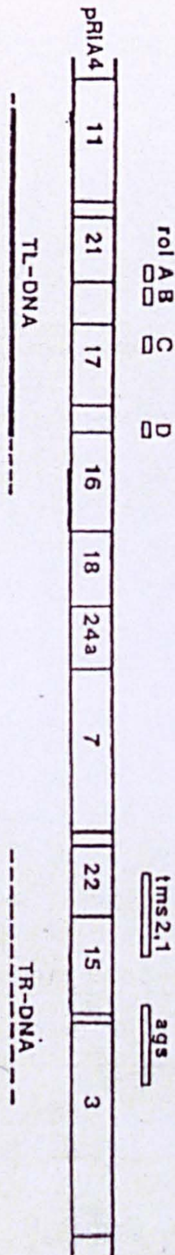


Figure 4.2. Plasmid map of RiA4 showing T_L and T_R portions and position of auxin biosynthetic genes (*iaaM* and *iaaH*) agropine synthesis (ags) and the *rol* genes.

This pathway is believed not to be utilised by higher plants.

The T_R DNA of A4 also harbours genes responsible for biosynthesis of agropine (see section 1.3.2).
The T_L DNA of pRiA4 has been sequenced (Slightom *et al.,* 1986) but the function of individual genes is still not clear, although some may be responsible for sensitising plant cells to auxin action (Cardarelli *et al.*, 1987).

Several workers have introduced auxin biosynthetic genes from *A. rhizogenes* or *A. tumefaciens* into plants and found altered levels of IAA in transformed tissue compared with control tissue of the same type (Klee *et al.*, 1987, Ondrej *et al.*, 1989).

4.1.2 Aim

The aims of these experiments were to provide evidence of the incorporation of A4T T-DNA into the sugar beet cv. Bella genome by DND/DNA hybridisation and to analyse some of the effects of its incorporation on the cellular biochemistry of sugar beet cv.

Bella, such as the acquired ability to synthesise agropine (see section 1.3.1). Alteration of IAA levels are reported in Chapter 6

4.2 Materials and Methods

4.2.1 Agropine Testing

Agropine tests were carried out based on the method of Morgan *et al.* (1987). 200 mg of hairy root or seedling root material was homogenised with 50 mm^3 0.1 $moldm^{-3}$ HCl in an Eppendorf tube with a shaped glass rod. Tubes were spun at 1200 rpm for 10 minutes in a micro-centrifuge to remove debris. 15 mm^3 of each sample was spotted onto Whatman 3MM paper. The paper was carefully dampened with running buffer and electrophoresed towards the anode at 400 volts for 60 minutes.

Table 4.1 Solutions Used in Agropine Analysis

Running Buffer	**% (v/v)**
Formic Acid	5
Glacial Acetic Acid	15
Distilled Water	80

Stain A Alkaline Silver Nitrate

0.625g $AgNO_3$

50cm^3 Acetone

$AgNO_3$ was dissolved in 5 drops of water and acetone was added to this. Water was then added drop wise until the white silver precipitate dissolved.

Stain B

10cm^3 20% w/v Sodium Hydroxide

90cm^3 Methanol

Stain C

3% w/v Sodium Thiosulphate

The chromatogram was first air dried then dipped in Stain A. the paper was again air dried then dipped in Stain B and Stain C, and air dried in between. After the final stain the paper was dried and rinsed in running de-ionised water for several minutes. Chromatograms were kept out of the light to prevent fading.

4.2.2 Plant Genomic DNA Extraction

DNA was extracted from sugar beet cv Bella hairy root and seedling root material based on a method described by Shure *et al.*, (1986).

Extraction Buffer (to make 400 cm^3)

6.25cm^3	5 $moldm^{-3}$ NaCl
5.00cm^3	1 $moldm^{-3}$ Tris HCl pH8
4.00cm^3	0.5 $moldm^{-3}$ EDTA pH8
5.00cm^3	20% w/v Sarkosine
379.00cm^3	Sterile distilled water
42.00g	Urea (do not autoclave)

Autoclaved stocks of NaCl, Tris, and EDTA were used.

Equilibrated Phenol

An equal volume of water to the weight of phenol was added and the phenol was dissolved in a 40°C water bath, 8-hydroxyquinoline was added to prevent oxidation. When dissolved, an equal volume of

1moldm^{-3} Tris pH 8 was added and mixed well. When settled the aqueous top layer was removed and an equal volume of TE buffer (10 mmoldm^{-3} Tris HCl pH 8, 1 mmoldm^{-3} Na_2 EDTA Tris pH8) was added and mixed. Again the layers were allowed to settle and the aqueous layer removed. This was repeated once more. The phenol was stored frozen at –20°C. If required, an equal volume of chloroform was added. This was also stored at –20°C.

Extraction

12g of plant material were frozen with liquid nitrogen and ground to a fine powder. This was transferred to a 50cm^3 centrifuge tube and 18cm^3 extraction buffer added. This was shaken well and 18cm^3 phenol/chloroform added and shaken again. This was left for 15 minutes, then spun at 2000rpm for 10 minutes at 4°C. The aqueous phase was removed and filtered through autoclaved miracloth into a fresh centrifuge tube. Acid washed PVPP (polyvinyl polypyrollidone) was added and the tube shaken well. PVPP was centrifuged out at 3000rpm for 10 minutes at 4°C. DNA was precipitated by the addition of $^1/_6$ volume of 4 moldm^{-3} NH_4AC and an equal volume of ice-cold isopropanol and mixed gently. The DNA was spun down at 3000rpm for 10 minutes at 4°C. The

resulting pellet was dried under reduced pressure and re-suspended in $500mm^3$ TE buffer. DNA was cleaned by partitioning with phenol/chloroform then chloroform, and then precipitated with 1/10 volume 3 $moldm^{-3}$ NaCl and 2.5 volumes of ice-cold absolute ethanol at –20°C overnight. DNA was spun for 10 minutes at 13000rpm in a micro-centrifuge. The pellet was washed with ice-cold 75% v/v ethanol and dried under reduced pressure. The DNA was re-suspended in $100mm^3$ sterile distilled water and stored at –20°C.

4.2.3 Quantification of DNA

Several methods were employed to quantify DNA.

a) Optical Density (OD) measurements were taken at 260nm and 280nm after diluting $10mm^3$ DNA sample with $990mm^3$ sterile distilled water. The concentration of DNA was obtained using the following equation :

$$OD_{260nm}\ 1 = 50\mu g\ cm^{-3}\ \text{DNA for 1cm pathlength}$$

b) Diphenylamine Test

Solution A 3N Perchloric acid

Solution B $198cm^3$ Glacial acetic acid

$20mm^3$ Paraldehyde

8g Diphenylamine

Reaction – added in the following order:

$10mm^3$ DNA sample

$290mm^3$ sterile water

$300mm^3$ Perchloric acid

$360mm^3$ Diphenylamine solution (B)

Tubes were incubated at $25^{\circ}C$ for 20 hours then measured in a spectrophotometer at 660nm. A standard curve of DNA concentration 0-0.5 μg cm^{-3} was obtained to compare sample readings.

c) Hoechst Dye Method

A stock of the dye H-33258 at concentration 200μg

cm^{-3} was made and stored at 4°C. This was diluted just prior to use, to 100ng cm^{-3} with a solution of 1mmoldm^{-3} EDTA pH8, 10mmol dm^{-3} Tris pH8 and 100mmol dm^{-3} NaCl. One mm^3 DNA sample was diluted in 99mm^3 sterile distilled water and 300mm^3 dye solution added. Samples were read in a fluorimeter at excitation 350nm, emission 455nm. A calibration curve of DNA concentration 0-300ng was obtained to compare sample DNA readings.

d) Ethidium Bromide Colour Comparison

A 1% (w/v) agarose gel containing 0.5 μgcm^{-3} ethidium bromide was made (see section 3.2.2). Onto this was spotted known amounts of DNA. The gel was observed under UV light and the brightness of each spot compared to unknown concentration of samples.

4.2.4 Large Scale Plasmid DNA Preparation

Plasmid DNA to be used for hybridising to putatively transformed sugar beet DNA was prepared as described in section 3.1. These were pPM4404 1023-19 (harbouring *iaaM* and *iaaH* from *A. tumefaciens* pAch5) and 21 (harbouring *rol* C from the T_L T-DNA of A4T).

4.2.5 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to separate, identify and purify DNA fragments (Maniatis *et al.*, (1982). As described in section 3.2.3

4.2.6 The use of Electroelution to Purify DNA

DNA was purified by running on a 0.7% w/v agarose gel (see section 3.2.4) with Hind III cut λ DNA as markers. This separated the plasmid DNA from contaminating chromosomal DNA. The bands corresponding in size to those of the plasmids were cut from the gel and the DNA removed by electroelution as described in section 3.2.5.

Plasmid DNA was then linearised with restriction enzymes (see section 3.2.7), before labelling for use as a probe (see section 3.2.8).

4.2.7 Restriction Digest of Probe DNA.

Restriction enzymes and their buffers were obtained from NBL (Cramlington, Northumberland).

The required units of enzyme for each digest were obtained using the equation below.

$$\text{Units of enzyme} = \frac{\mu\text{g DNA x total volume/20 x 4}}{\text{Reaction time (hours)}}$$

DNA to be cut was transferred to a sterile micro-centrifuge tube and the appropriate volume of 10x digestion buffer (specific for each enzyme) added and mixed. The required number of units of enzyme were added and the tube incubated at 37°C (or appropriate temperature) for the required time. The reaction was stopped by adding 0.5 moldm^{-3} EDTA pH7.5 to a final concentration of 10 mmoldm^{-3}. DNA was purified by extracting with phenol/chloroform and precipitated. DNA was re-suspended in sterile H_2O and stored at –20°C.

4.2.8 Dephosphorylation of Restricted DNA

One unit of alkaline phosphatase is the enzyme activity which hydrolyses 1 µmol of 4-nitrophenyl phosphate in 1 minute at 37°Cin standard assay conditions (Boehringer Mannheim catalogue, 1990/91). Calculation of the amount of calf intestinal phosphate (CIP, Boehringer-Mannheim) required:

0.05 units enzyme activity will remove 5' terminal phosphate groups from 1 pmol of terminal at 37°C in 30 minutes in 20 mm^3 (1 pmol ends = 1.6 µg of a 5 kb fragment). (Armitage *et al.*, 1988)

4.2.9 Transfer of DNA to Nylon Membrane

a) Dot Blot

DNA to be analysed was denatured by heating for 10 minutes at 95°C and chilled quickly on ice and spotted onto nylon membrane (Hybond N, Amersham), and dried to prevent the DNA spot spreading. 5µg DNA per sample was spotted. DNA was fixed onto the membrane by treating with UV light for 5 minutes. These filters were then treated in the same

manner as Southern blot filters for hybridisation (see section 4.2.10 and 4.2.11).

b) Southern Blotting

b1) 10 μg Hind III restricted DNA of each sample to be analysed was run on a 1% w/v agarose gel (see section 3.2.2) together with λ DNA digested with Hind III restriction enzyme as molecular weight markers. Gels were run until markers were well separated. The distance of the bands from the wells were noted and a calibration curve (1/distance and log molecular weight) produced.

b2) **Table 4.2** - Solutions Used in Blotting

Buffer 1	1.5 $moldm^{-3}$ NaCl, 0.5 $moldm^{-3}$ NaOH
Buffer 2	0.5 $moldm^{-3}$ Tris HCl pH7.4, 3 $moldm^{-3}$ NaCl
20 x SSC	3.0 $moldm^{-3}$ NaCl, 0.3 $moldm^{-3}$ NaCitrate pH7

The gels to be blotted (see section 4.2.8b1) were placed on a UV light box for 20 minutes. They were then shaken in buffer 1 for 40 minutes, rinsed in distilled water 2 times and shaken for 60 minutes in buffer 2.

Gels were finally rinsed in distilled water. DNA was then transferred to nylon membrane, Hybond N as follows:

Whatman 3MM paper was placed as a wick over a glass plate on a raised platform in a large plastic box and the wick dampened with 20 x SSC. The gel was then placed upside down on this with a piece of Hybond N the same size as the gel on top. Onto this was placed 2 layers of Whatman 3MM paper and a thick layer of paper towels, trimmed to the gel size. 20 x SSC was added to the box to a depth of 5 cm. A glass plate and a 500g weight was placed on top of the towels and left undisturbed for 15 hours. The wet paper towels were replaced with dry towels and left a further 8 hours. The nylon membrane was then checked on the UV light box to see that the ethidium bromide had transferred and then left 5 minutes on the UV light box to fix the DNA to the membrane.

4.2.10 Labelling of DNA for Use as a Probe.

The plasmid DNA to be used as a probe was linearised using restriction enzymes (section 4.2.7). Plasmid pPM4000 102319 (harbouring the auxin genes *iaaM* and *iaaH*, B Hess *et al.*, 1989) was cut with Bsc1 and

plasmid pPM4000 80121 (open reading frame 12, *rol* c, S. Sugaya *et al.*, 1989) with Bam H1.

4.2.11 Non Radioactive Labelling of DNA

This method utilised the DNA labelling and detection kit from Boehringer Mannheim. Labelled DNA is generated with Klenow Fragmenmt DNA polymerase by random primed incorporation of Digoxigenin labelled deoxyuridine-triphosphate (dUTP). The dUTP is linked via a spacer arm to the steroid hapten digoxigenin (DIG-dUTP). After hybridisation to the target DNA the hybrids are detected by enzyme linked immunoassay using an antibiody conjugate (anti-digoxigenin alkaline phosphatase conjugate (DIG-AP)). A subsequent enzyme catalysed colour reaction with 5-bromo-4 chloro-3-indolyl phosphate (X-phosphate) and nitrobluctetrazolium salt) (NBT) produces an insoluable blue precipitate, which allows visualisation of hybrid molecules.

To label, the DNA was denatured by heating for 10 minutes at 95°C and chilled quickly on ice. For every 1μg DNA, 2mm^3 hexanucleotide mixture 2mm^{3}dNTP labelling mixture and 1mm^3 Klenow enzyme in

$20mm^3$ volume was added. This was incubated at 37°C for 16 hours. The reaction was stopped by adding $2mm^3$ 0.2 $moldm^{-3}$ EDTA pH 8.0 and DNA precipitated with $2mm^3$ $4moldm^{-3}$ LiCl, and $60mm^3$ ice cold ethanol. DNA was left at –20°C overnight and then spun in a micro-centrifuge to form a pellet. This was washed with 75% ice-cold ethanol and dried under vacuum. The DNA was then re-suspended in TE buffer and stored at –20°C until required.

4.2.12 ^{32}P Labelling of DNA

This method made use of the random primed DNA labelling kit from USB (Cleaveland, Ohio). A mixture of all possible hexanucleotides was hybridised to the DNA to be labelled and the complementary strand is synthesised from the 3 hydroxyl termini of the hexanucleotide primers using Klenow enzyme. Labelled deoxynucleotide triphosphates (in this case ^{32}P) present in the reaction mixture are incorporated into the newly synthesised complementary strand. This kit can be used for labelling DNA in solution (i) or isolated in a low melting point agarose gel (ii).

4) (i) Labelling DNA in Solution

DNA was denatured by heating for 10 minutes at 95°C then chilled rapidly on ice. 25 mg of this was added to a fresh microfuge tube and then $3mm^3$ of dGTP, dTTP, dCTP mixture, $2mm^3$ reaction mixture and $5mm^3$ of ^{32}P dATP was added and the volume adjusted to 19 mm^3. 2units of Klenow enzyme was added and the mixture incubated at 37°C for 1 hour. The reaction was stopped by adding $2mm^3$ $0.2moldm^{-3}$ EDTA pH 8.0.

(ii) Labelling DNA isolated in low melting point Agarose Gels

DNA fragments were separated by electrophoresis on a 1% w/v low melting temperature agarose gel containing $0.5mgcm^{-3}$ ethidium bromide (see section 3.2.2) in a buffer of $40mmoldm^{-3}$ Tris-acetate pH 8.0. 5 mmol dm^{-3} sodium acetate. The desired band was cut out from the gel and weighed. Water was added to the gel at a ratio of $3cm^3$ H_2O per gram of gel. This was boiled for 7 minutes to dissolve the gel and denature the DNA. This was labelled in a similar manner to DNA in solution except the reaction volume was increased to $50mm^3$ and the volumes of reaction

mixture and dGTP, dTTP,dCTP mixture were increased to $5mm^3$ and $7.5mm^3$ respectively. The reaction was terminated in the same manner.

DNA labelled with ^{32}P was chemically denatured, by adding 0.1 volumes $3moldm^{-3}$ NaCl and mixing for 5 minutes at room temperature. It was then transferred to ice for 1 minute before adding 0.05 volumes of $1moldm^{-3}$ Tris HCl pH 7.5 and 0.1 volumes of $3moldm^{-3}$ HCl. It was then left on ice until required. It was then added to the hybridisation solution.

4.2.13 DNA-DNA Hybridisation

a) With non radioactive labelled probe

Solutions Used in DNA-DNA Hybridisation

A) **Hybridisation solution -** 5 x SSC, 0.5% w/v blocking reagent (as supplied by Boehringer Mannheim non radioactive DNA-DNA hybridisation kit), 0.1% w/v N. lauroylsarcosine, 0.02% w/v SDS

B) **Solution 1 -** 2 x SCC, 0.1% w/v SDS

C) **Solution 2** - 0.1 x SSC, 0.1% w/v SDS

Filters were pre-hybridised in a sealed plastic bag with $20cm^3$ per $100cm^3$ hybridisation solution for 4-16 hour at 68^oC in a shaking water bath. This was then replaced with $2.5cm^3$ per $100cm^2$ filter of hybridisation solution containing (10-15 ng) labelled DNA. Filters were then incubated for 16 hours at 68^oC in a shaking water bath then washed twice in solution 1 for 5 minutes each and twice in solution 2 for 15 minutes each at 68^oC.

b) With ^{32}P Labelled DNA

Solutions Used in DNA-DNA Hybridisation with ^{32}P Labelled Probe

A) Pre-hybridisation solution - 50% v/v de-ionised formamide, 5 x SSC, $50mmoldm^{-3}$ $NaPO_4$ pH 7.0, 5 x Denhardts solution (Denhardt *et al*), 0.5% w/v SDS, $250mgcm^{-3}$ sheared salmon sperm DNA.

B) Hybridisation solution

50% v/v de-ionised formamide. 5 x SSC, 1 x Denhardts solution, 0.5% w/v SDS, 20mmoldm^{-3} $NaPO_4$ pH 7.0, 100mgcm^{-3} salmon sperm DNA (sheared and denatured).

C) Wash solution 1

5 x SSC, 0.1% w/v SDS.

D-Wash solution 2

0.3 x SSC, 0.1% w/v SDS.

Filters were pre-hybridised with pre-hybridisation fluid for 4-14 hours in sealed plastic bags at 42°C in a shaking water bath. This was replaced with hybridisation solution containing labelled probe DNA and filters incubated for 36 hours at 42°C in a shaking water bath.

After hybridisation, filters were washed two times at room temperature with agitation. Filters were then washed twice for 30 minutes each in wash solution 1 in a shaking water bath at 68°C. The final two washes were also in a shaking water bath in wash solution 2 for 30 minutes each.

4.2.14 Detection of Hybrid Molecules

a) Using Non Radioactive Detection

Solutions Used in Non Radioactive Detection

Buffer 1

100mmoldm^{-3} Tris HCl pH 7.5, 150 mmoldm^{-3} NaCl

Buffer 2

0.5 % w/v blocking reagent in buffer 1

Buffer 3

100mmoldm^{-3} Tris HCl pH 9.5, 100mmoldm^{-3} NaCl, 50mmoldm^{-3} $MgCl_2$.

Buffer 4

10mmoldm^{-3} Tris HCl pH 8, 1mmoldm^{-3} EDTA

Colour Solution

45mm^3 NBT solution, 35mm^3 X-phosphate solution in 10 cm^3 buffer 3.

Filters were washed for 1 minute in buffer 1 then incubated for 30 minutes with buffer 2. Filters were rinsed again in buffer 1 then incubated for 30 minutes with diluted antibody conjugate solution (1in 5000 dilution in buffer 1). Unbound antibody conjugate was removed by washing twice in buffer 1 for 15 minutes then sealed in a plastic bag with $10cm^3$ of colour solution and placed in the dark for 8 hours. The reaction was stopped by washing the filters in buffer 4, for 5 minutes.

b) With ^{32}P Labelled DNA

Hybridised molecules labelled with ^{32}P were detected by exposing the filter to photographic film (Hyperfilm MP- Amersham International) in a cassette with intensifiying screen at $-70^{o}C$ overnight or up to 4 weeks. Film was developed in developing solution (Photosol, Basildon, Essex) for 5 minutes, rinsing in water then placing in fixing solution (Photosol, Basildon, Essex) for 5 minutes. Film was then rinsed thoroughly and air-dried. Films were kept in the dark to prevent fading.

4.3 Results

4.3.1 Agropine Tests

Paper electrophoresis as described in section 4.2.1 showed the presence of both agropine and mannopine in each of eight sugar beet hairy root clones (Initiated as described in section 3.2.12). Neither agropine nor mannopine was detected in sugar beet cv Bella seedling root or leaf material during these experiments (figure 4.3).

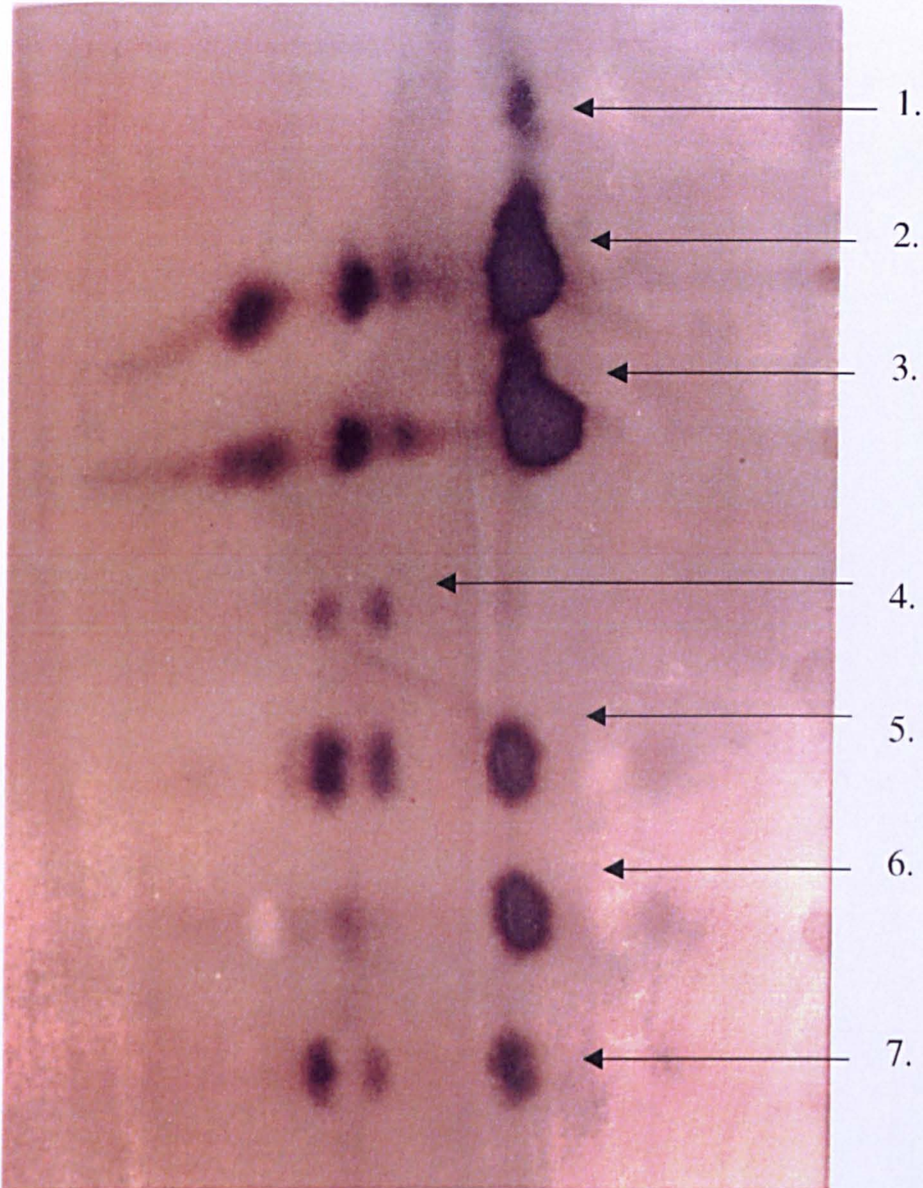


Figure 4.3 Agropine test of Sugar Beet cv Bella Hairy Roots and Seedling Roots.

Track 1. Seedling Root
Track 2. Hairy Root Clone 3
Track 3. Hairy Root Clone 7
Track 4. Agropine (A) and Mannopine (M) standards
Track 5. Hairy Root Clone 5
Track 6. Hairy Root Clone 11
Track 7. Hairy Root Clone 4

4.3.2 DNA Preparation and Quantification

Concentrations of DNA from genomic DNA preparations as described in section 3.3.2 were measured using the Hoescht Dye method (see section 3.3.3).

Sample	Fresh Mass	$ngmm^{-3}$ DNA
Sugar Beet cv Bella Leaf	19.0g	9.0
Sugar Beet Hairy Root Clone 5	9.0g	2.6
Sugar Beet Hairy Root Clone 4	10.0g	1.2
Sugar Beet Hairy Root Clone 3	8.7g	2.1
Sugar Beet Hairy Root Clone 7	8.4g	1.8

4.3.3 Comparison of methods of DNA concentration determination

a Optical density measurements at 260nm and 280nm (described in section 3.2.3a) were useful for a rapid estimation of DNA concentration but measurements can indicate a higher concentration than the actual concentration due to contamination of RNA and proteins.

b The diphenylamine test (see section 3.2.3b) did not produce any useable results and the method was time consuming.

Any impurities in the reagents rendered them useless and they had to be remade frequently.

c The Hoescht dye method (section 3.2.3c) was the most accurate and sensitive method tested here. This method was used for DNA concentration measurements in all relevant experiments described.

d Comparison of brightness of colour of ethidium bromide stained DNA (section 3.2.3d) could be used as a rapid rough estimate of DNA concentration.

4.3.4 Dot Blot Hybridisation with Plasmid pPm4000 1023-19

Figure 4.4 shows a dot blot hybridised with ^{32}P labelled plasmid pPM4000 1023 19 probe. It shows hybridisation with all four sugar beet hairy root clones analysed 3,4,5 and 7 and to the plasmid pPM4000 1023 19 positive control. There was no hybridisation to the negative control untransformed sugar beet cv Bella DNA.

4.3.5 Southern Blot Hybridisation with pPM40008021

Figure 4.5 shows a Southern hybridisation of ^{32}P labelled plasmid pPM4000 8021 with hairy root DNA. Bands of hybridised DNA could be seen in each of the hairy root clones 3,4,5 and 7 but no such band was seen in the untransformed sugar beet cv Bella DNA.

When probe labelled by the non radioactive labelling kit from Boehringer was used it was not sensitive enough to detect hybridisation.

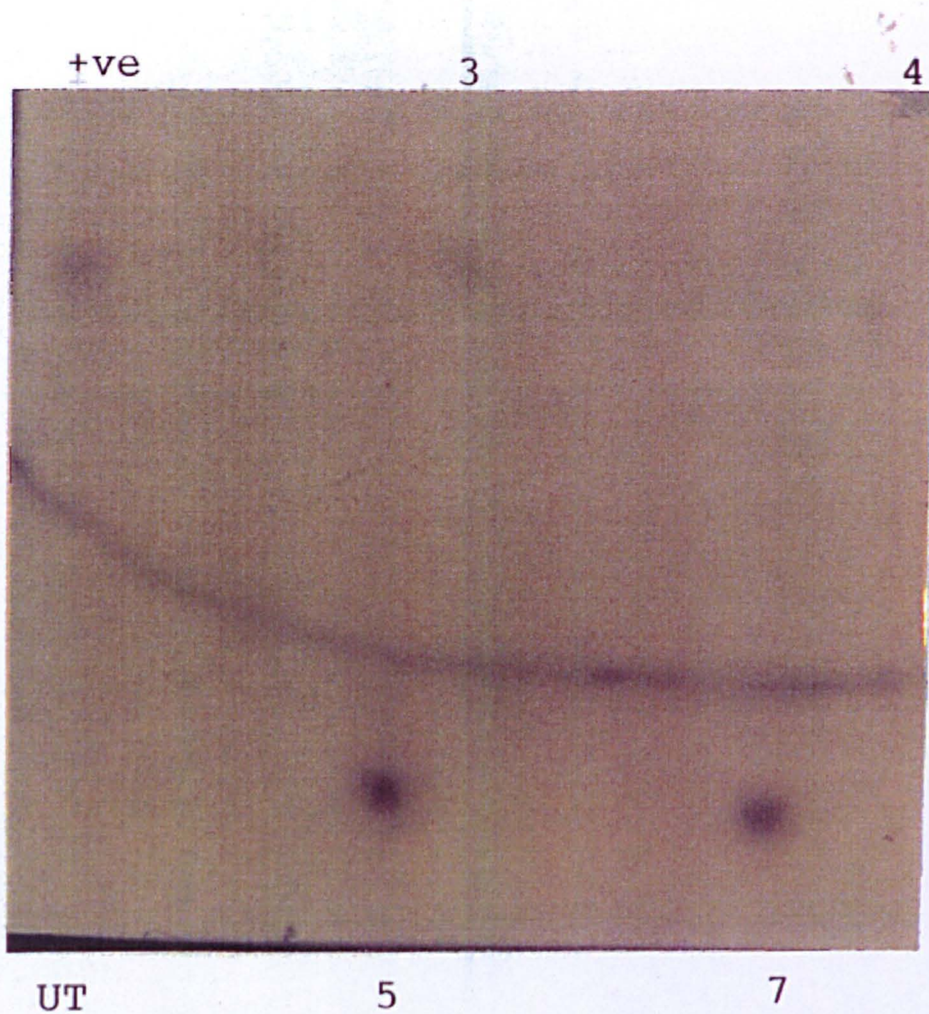


Figure 4.4 A dot blot of hairy root clones 3, 4, 5 and 7 and untransformed sugar beet cv Bella DNA (UT) hybridised with ^{32}P labelled plasmid pPM4000 1023 19.

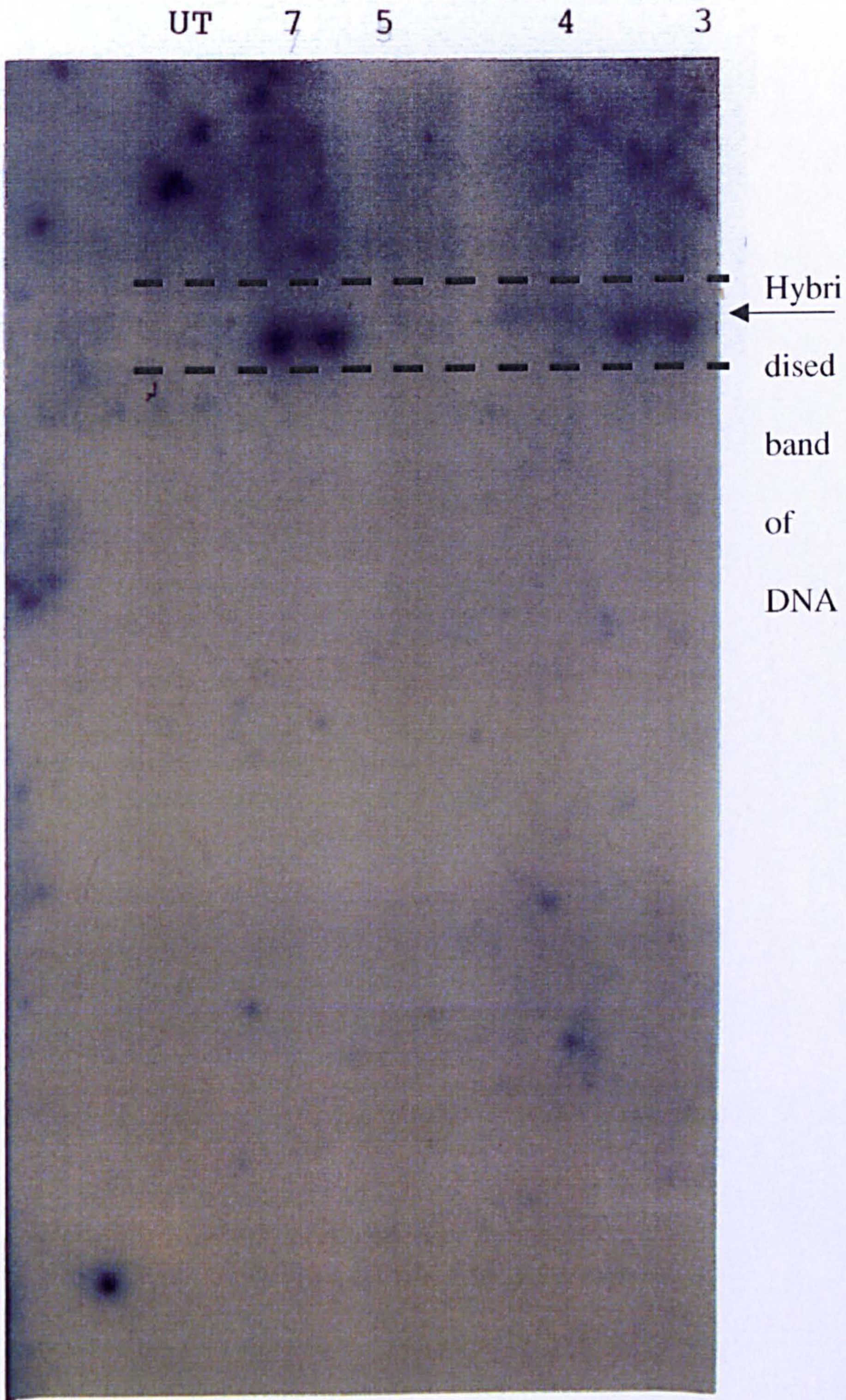


Figure 4.5 Southern blot of sugar beet hairy root clones 3, 4, 5 and 7 and untransformed sugar beet cv Bella DNA (UT) hybridised with ^{32}P labelled plasmid pPM4000 80121.

4.4 Discussion

The non-radioactive DNA labelling and detection kit was not sensitive enough to produce the expected bands of hybridised molecules on the nylon membrane. Bands (or dots in the case of a dot blot) did appear when the same probe DNA labelled with ^{32}P was used. The use of this non-radioactive kit should be further investigated by altering labelling and hybridisation conditions. A non-radioactive method is safer for the worker and is less expensive.

Opines were shown to be an accurate indicator of transformation, each clone that gave positive result for agropine also had incorporated genes that hybridised to the probe DNA. Molecular conformation of transformation is necessary, as non-transformed sugar beet tissue `escapes' are capable of growth on relatively high levels of selection. The material used for transformation was clonal i.e. all the petioles infected by A4T had been derived from the same seed. This was to ensure that any differences seen between the hairy root clones were due to the transformation event and not any genetic variability in the initial plant material used for infection.

There was no hybridisation of control non-transformed sugar beet cv. Bella DNA and either of the sequences of probe DNA.

There appeared to be some variation in the amount of hybridisation seen between the hairy root clones and the genes used to probe the transformed sugar beet DNA. In the Southern blot of transformed DNA hybridised to the *rol* C gene from the T_L T-DNA, clone 7 showed a darker band than any other clone. This should not be due to differences in the amount of DNA loaded onto the gel, as the same amount of DNA of each clone and non-transformed DNA. It may be due to incorporation of more copies of these genes into the hairy root clone 7 genome.

When the auxin biosynthetic genes were hybridised to the control and hairy root DNA, the dots of clone 5 and clone 7 produced a darker spot than clone 3 or clone 5. Again this may be due to the incorporation of more copies of these genes into those hairy root genomes.

CHAPTER FIVE

TISSUE CULTURE OF TRANSFORMED

SUGAR BEET

5.1 Introduction

The method of gene transfer used in the experiments described here concentrated upon the use of *Agrobacterium* Ri plasmids rather than Ti plasmids. This was for several reasons. The work is concerned with the alteration of auxin levels by the introduction of genes responsible for the indole-3-acetic acid biosynthesis. Both *A. tumefaciens* and some strains of *A. rhizogenes* have such genes (see section 3.1) but *A. tumefaciens* also harbours genes involved in cytokinin biosynthesis, the levels of which were not to be directly altered in this programme (see sections 1.3.1 and 1.3.2).

Transformation with Ri plasmids results in a proliferation of hairy roots at the site of inoculation (see Section 1.3.3 Induction of Hairy Roots). These roots have altered cambial development and rapid growth, providing large amounts of material for analysis in a short period of time. All the material is genetically identical, if clonal material derived from individual hairy root tips is used.

5.1.2 Aim

The aim of the transformation work was to alter auxin levels in sugar beet tissue by introducing the auxin biosynthetic genes from *A. rhizogenes*. It was hoped that this would alter growth patterns in the sugar beet storage organ to produce more active cambia separated by numerous small cells. To produce large amounts of transformed plant tissue for DNA and auxin analysis optimal growth parameters were to be established. To test for any alteration in growth patterns it was desirable to produce a cell suspension culture from transformed tissue and measure growth parameters in these and untransformed sugar beet cells.

If possible, establish an efficient protocol for regeneration of the transformed tissue. This would allow the mature sugar beet storage organ to be studied in plants regenerated from transformed tissue from either hairy root or callus tissue.

5.2 Materials and Methods

Sugar beet cv Bella hairy roots were obtained by stabbing petiole sections with a 21G needle dipped in a culture of *A. tumefaciens* C58C1 harbouring pRi A4b (A4T) described in section 3.2.12. Individual hairy root tips were excised and cultured. See fig 5.1

5.2.1 Hairy Root Cultures

Transformed roots were maintained in the dark on half-strength Gamborgs B5 medium (B5/2, Gamborg *et al.*, 1968) supplemented with 3% w/v sucrose, 0.8% w/v agar and 0.5mg dm^{-3} carbenicillin to remove inciting bacteria. Roots were sub-cultured every four weeks. After several passages on carbenicillin containing medium the antibiotic was omitted. See figure 5.2

5.2.2. Effect of Medium Composition on Hairy Root Growth

Several medium compositions were tested for their affect on growth rate of sugar beet hairy root cultures. The media tested were half strength Gamborgs B5 (B5/2) Gamborg *et al.*, 1968, Murashige and Skoog (MS) Murashige and Skoog 1963, Schenk and Hildebrant (SH) Schenk and Hildebrant1979 and Whites medium (WH) White 1963. Four growing root tips were placed in each jar of medium. Ten jars of each treatment were weighed aseptically after 0, 21 and 35 days. Each medium was supplemented with 3% w/v sucrose and 0.8 % w/v agar.

5.2.3. Effect of Sucrose concentration on Hairy Root Growth

Roots were inoculated as described in 4.2.2 and grown on half-strength Gamborgs B5 medium supplemented with 0.8% w/v agar and either 1%, 2%, 3% or 4% w/v sucrose. Ten replicates of each treatment were inoculated and weighed aseptically on day 0, 21 and 35.

5.2.4. **Effect of Agar Concentration on Hairy Root Growth**

Roots were inoculated as described in 5.2.2. They were grown on B5/2 medium. This was supplemented with 3% w/v sucrose and agar concentration of 0.4% w/v, 0.6% w/v, 0.8% w/v, 1.25% w/v, 1.5% w/v or 2.0% w/v. Ten replicates of each treatment were inoculated and weighed aseptically on day 0, 21 and 35.

5.2.5. **Liquid Hairy Root Cultures**

After at least two subcultures in antibiotic free media hairy roots were transferred to liquid medium. Five 1cm long growing root tips were inoculated in 50 cm^3 B5/2 medium with 3% w/v sucrose to promote rapid growth. Cultures were incubated at 25^{0}C and 60 rpm shaking. Growth of cultures was measured aseptically by weighing at days 0, 7 and 14. Ten flasks were used.

5.2.6 Callus Initiation

Calli from sugar beet hairy root cultures were obtained by placing 1 cm^2 mats of roots on MS medium containing 0.5 mg dm^{-3} 2,4-D, 0.5 mg dm^{-3} kinetin and 0.8% w/v agar. Cultures were incubated in the dark at 25°C.

5.2.7 Organogenesis of Hairy Root Cultures

Once callus was established it was placed on several plant growth regulator regimes to try to induce regeneration. Callus was grown on MS medium with 3% w/v sucrose, 0.8% w/v agar and one of the following plant growth regulator combinations described in Table 5.1

All the media tested were incubated at 25°C and 30°C to analyse any effects temperature alteration had on the ability to induce shoot organogenesis.

Table 5.1. - Plant Growth Regulator Regimes for Regeneration of A4T Transformed Sugar Beet Callus

	BAPmgdm^{-3}	2,4-Dmgdm^{-3}	Kinetin mgdm^{-3}	*TIBA mgdm^{-3}
A	0.5	0.5	0.5	
B	1.0	0.5	0.5	
C	5.0	0.5	0.5	
D	5.0	1.0	0.5	
E	0.5	0.5	0.5	1.0
F	1.0	0.5	0.5	1.0
G	5.0			

* TIBA (2,3,5 triiodobenzoic acid) – a known inhibitor of auxin transport

5.2.8. Suspension Cultures

Several methods were employed in order to obtain a cell suspension culture of A4T transformed sugar beet. The callus used was grown on MS medium with 0.5 mg dm^{-3} 2,4-D, 0.5 mg dm^{-3} BAP, 0.5 mg dm^{-3} kinetin and 3% w/v sucrose.

a) Friable callus was inoculated in 30 cm^3 liquid medium of the same composition without agar and shaken 120 rpm at 25°C in the dark and artificial daylight conditions (500 lux).

b) 10 cm^3 liquid medium was added to the top of callus grown on solid medium and shaken at 60 rpm, 25°C in either darkness or artificial daylight conditions (500 lux).

c) Callus was subcultured onto decreasing levels of agar from 0.8% w/v to 0.4% w/v and then into liquid medium shaken at 120 rpm, 25°C in either darkness or artificial daylight conditions.

d) Callus was homogenised and transferred to liquid medium and shaken at 120 rpm at 25°C in darkness or artificial daylight conditions.

5.3. RESULTS

5.3.1. Growth of Hairy Roots on Infected Petioles

The roots that appeared at the site of inoculation when removed were capable of rapid growth in the absence of added plant growth regulators. The roots were white and covered in dense white fluffy hairs. (see figure 5.1 and 5.2).

5.3.2. Effect of Medium on Growth of Hairy Roots

The composition of growth medium had a great affect on the growth of sugar beet hairy roots. Growth on half strength Gamborgs B5 medium was by far the greatest. There was far less growth on MSO medium, while roots showed no growth on either Whites or Schenk and Hildebrant medium and died. (see figure 5.3).

Figure 5.1 Hairy Roots appearing at the site of infection with A4T on sugar beet cv Bella petiole.

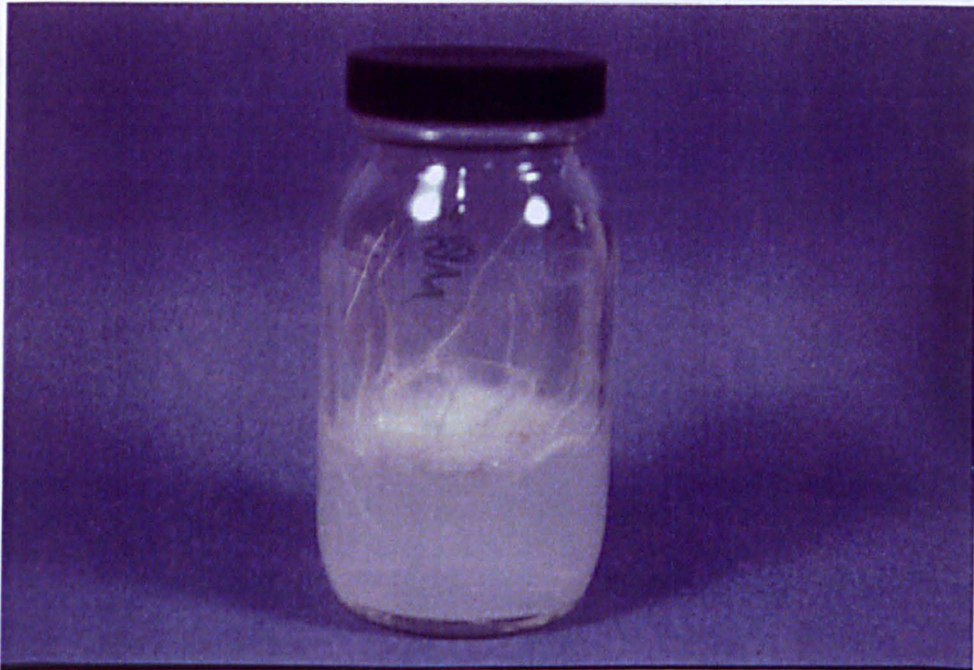


Figure 5.2 21 day old hairy root culture initiated by infection of sugar beet (cv Bella) with *A. tumefaciens* strain C58C1 containing pRiA4b (A4T)

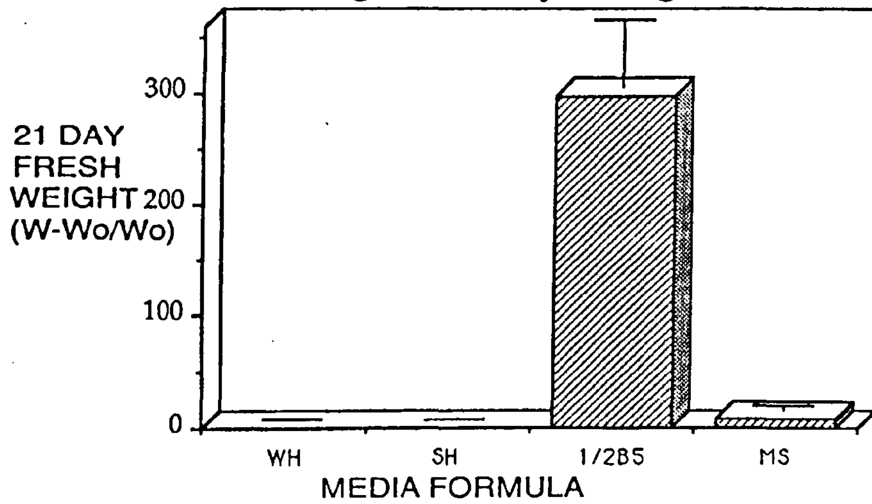


Figure 5.3

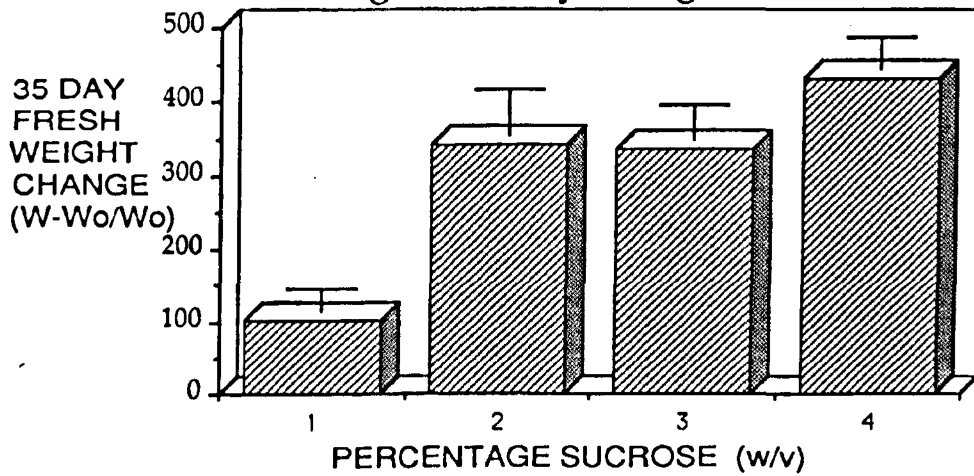


Figure 5.4

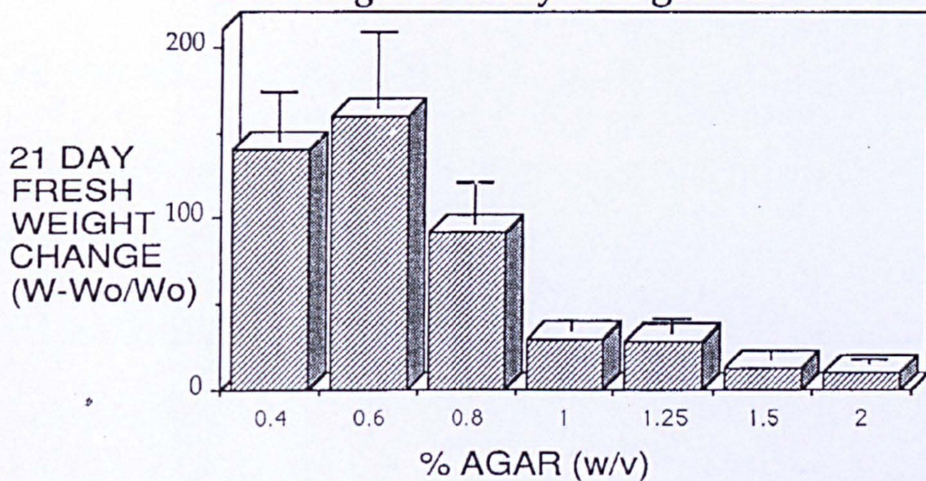


Figure 5.5

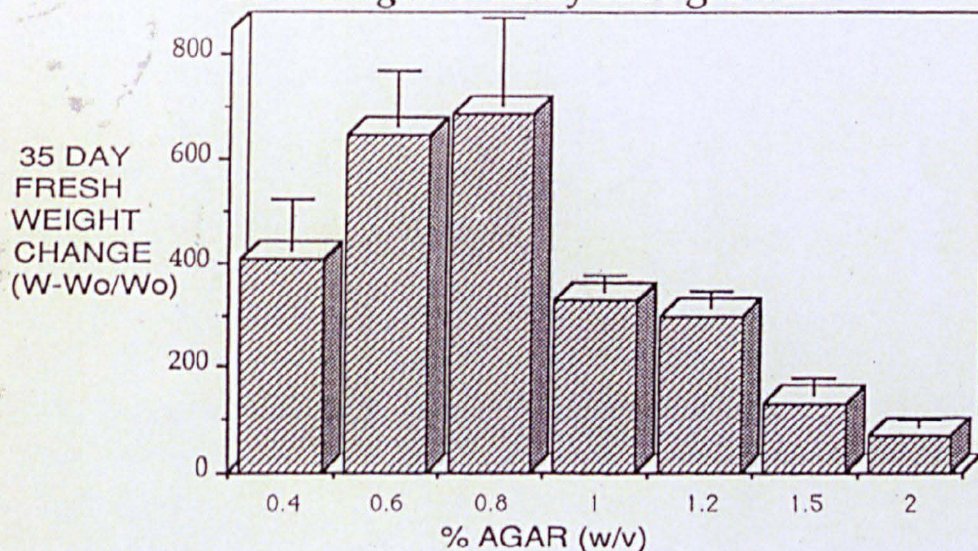


Figure 5.6

5.3.3 Effect of Sucrose on Growth of Hairy Roots

After 21 days roots grown on 4% sucrose showed by far the greatest growth rate with 2% showing a higher growth rate than 3%. After 35 days 4% w/v sucrose still showed a slightly higher growth but 2% and 3% w/v sucrose were similar. Cultures on 2% and 3% w/v sucrose continued to grow and were more " healthy " in appearance than roots grown on 4% w/v sucrose which browned very quickly. (see figure 5.4).

5.3.4. The Effect of Agar Concentration on Growth of Hairy Roots.

After 21 days cultures grown on 0.6% w/v agar showed the greatest rate of growth with slightly less on 0.4% w/v agar. Growth on concentrations above 0.8% w/v agar was very slow. After 35 days growth rate on 0.8% w/v agar was greatest with 0.6% w/v agar only a little behind. (See figure 5.5).

Using the results of these experiments it was decided to grow sugar beet hairy roots on half strength B5 medium supplemented with 3% w/v sucrose and 0.6% w/v agar for a 21 day growth period.

5.3.5. Growth of Hairy Roots in Liquid Medium

Growth in liquid was at first slow and the roots turned brown quickly but after two subcultures the growth rate increased and the roots grew far more rapidly in liquid than on solid medium (see figure 5.7). In seven days there was an 11-fold increase in fresh weight and after 16 days there was a 20-fold increase in fresh weight from day 0. The results for growth of clone 7 are shown in table 5.2.

Table 5.2-Growth of Sugar Beet Hairy Roots in Liquid Medium

Mean Mass of root material (g) per flask		
Day 0	Day 7	Day 14
0.013 +/- 0.08	0.467 +/- 0.161	10.789 +/- 2.853

Figure 5.7 10 day old culture of sugar beet cv Bella hairy roots infected with A4T grown in B5 liquid medium.

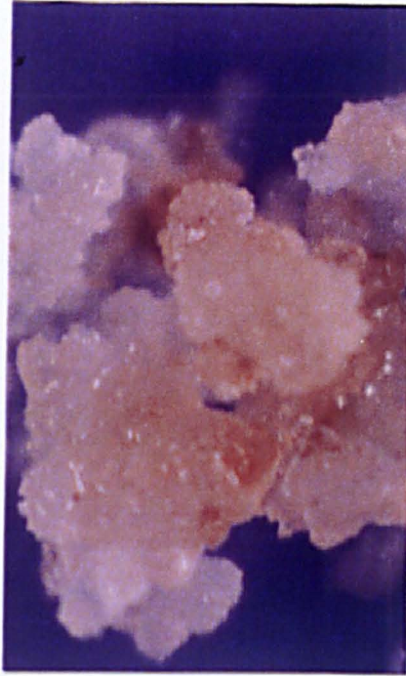


Figure 5.8 Callus of sugar beet cv Bella hairy roots harbouring A4T grown in the dark on MS medium containing 0.5 $mgdm^{-3}$ 2, 4-D, 0.5 $mgdm^{-3}$ kinetin.

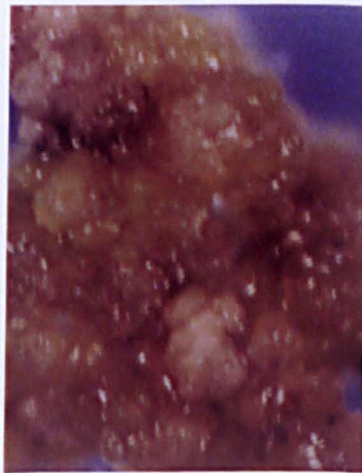


Figure 5.9 Callus of sugar beet cv Bella hairy roots harbouring A4T grown in artifical light on MS medium containing 0.5 $mgdm^{-3}$ 2, 4-D, 0.5 mgdm-3 kinetin and 0.5 $mgdm^{-3}$ BAP.

5.3.6. Callus Initiation

Callus appeared all over the surface of the mat of hairy roots after two weeks on initiating medium (MS supplemented with 0.5 $mgdm^{-3}$ 2,4-D, 0.5 $mgdm^{-3}$ kinetin, 3% (w/v) sucrose and 0.6% (w/v) agar). After 4 weeks callus was removed and placed on fresh medium. At first growth was very slow and the callus was hard and brown. After 3 subcultures the callus became friable and white or pale cream in colour. See figure 5.7.

5.3.7. Induction of Regeneration of Shoots in Hairy Root Callus

When BAP was added to the growth medium and the callus was cultured in artificial daylight conditions the callus turned green. In some cultures hard green nodules appeared but no shoots were produced. There was no noticeable morphological differences in growth of callus on any of the media tried. All produced green friable callus with occasional nodular structures but no regeneration of shoots was observed. Growth was rapid and callus had to be sub-cultured every 3 weeks. If callus was left for longer, both it and the medium turned dark brown and the callus died. In these experiments the anti auxin TIBA had no effect on the attempts to induce shoots from the sugar beet hairy root callus. There were no

noticeable differences in callus incubated at 25°C as compared with 30°C (figure 5.8).

5.3.8 Cell Suspension Culture of Sugar Beet Hairy Roots

In all the methods tested to obtain a cell suspension culture of hairy root callus the liquid medium used was MS supplemented with either 0.5 $mgdm^{-3}$ 2,4-D and 0.5 $mgdm^{-3}$ kinetin or 0.5 $mgdm^{-3}$ 2,4-D, 0.5 $mgdm^{-3}$ kinetin and 0.5 $mgdm^{-3}$ BAP. None of the methods described in 3.2.8 were successful in producing a cell suspension culture. In all cases the callus turned dark brown within 1 day and died.

The use of the anti-phenolic compound PVPP did not reduce the production of substances that turned both the callus and the medium black. Cultures grown in darkness were no different to those grown in artificial daylight conditions.

5.4 Discussion

Several laboratories have reported regeneration in sugar beet from callus. Saunders and Doley (1986) reported a system in which 1 mg dm^{-3} BAP was used to induce callus and regeneration from leaves. Tetu *et al.* (1987) obtained higher shoot morphogenesis from petioles, roots and shoot tip callus when TIBA was applied in the regeneration regime. This was not

found in this study, where the use of TIBA in the regeneration medium had no visible effects and no shoots were produced. Freytag *et al.* (1988) reported shoot regeneration from petioles using 0.4 mg dm^{-3} IBA as the sole hormone with combinations of several amino acids and vitamins. The regeneration of sugar beet transgenic plants transformed using the *Agrobacterium* system has proved to be difficult (Krens *et al.* 1988). A few plants have been obtained from transformed shoot base tissues or embryonic callus (Lindsey and Gallois 1990; D'Halluin *et al.*, 1992). The procedures appear to be highly genotype dependant and probably cause somatic variation.

No transgenic sugar beet plants were produced during this study although a protocol similar to one previously used to produce transgenic shoots in this laboratory (Elliott *et al.*, 1992) was used. Elliott *et al.* (1992) found that the cultivar Salohill gave the most efficient regeneration of shoots from petiole explants. This may suggest either a cultivar dependant effect and or explant material dependance. Elliott *et al.*, (1992) used hygromycin for the selection of transgenic cells while kanamycin was used in the present study. Yenofsky *et al.* (1990) showed that the *nptII* gene present in pBin19 (therefore in the binary vectors described in this

study) encodes a mutant protein which is less active than the wild type enzyme.

D'Halluin *et al.* (1992) found that inoculation of 3000 sugar petiole explants resulted in only transformed, compact non-morphogenic callus and non-transformed shoots.

The selective agents used to select for transformed cells may also have a deleterious effect on regeneration ability (Jarl and Bornman, 1986; Scott *et al.*, 1995). Other workers have suggested that herbicides are more useful as selective agents (D'Halluin *et al.*, 1992).

More recent publications since this work, concerning the regeneration of transformed sugar beet are generally inconclusive. Many reports show limited success or are difficult to reproduce outside the laboratory that carried out the original work (Krens et al 1996). It has been suggested that the stomatal guard cells are the only progenitors of totipotent protoplasts in sugar beet (Kuspa and Loomis 1992, Hall *et al* 1995). Hall *et al.* (1996) described a transformation and regeneration protocol using sugar beet stomatal guard cells, but this was still relatively inefficient.

Chapter 6

Auxin Analysis

6.1 Introduction

Several workers have introduced auxin biosynthetic genes from *A. rhizogenese* or *A. tumefaciens* into plants and found altered levels of IAA in transformed tissue compared with control tissue of the same type (Klee *et al.*, 1987, Ondrej *et al.*, 1989, Melford and Klee *et al.*, 1989).

In this chapter the effects of the introduction of the pRiA4b genes into the sugar beet cv Bella genome is considered. Levels of IAA and IAM were measured in order to understand the kinetics of the tryptophan to IAM to IAA pathway in transgenic plants (figure 6.1). This knowledge is important in experiments where the precise control of auxin levels is required.
Auxin levels per se are not the only determinant of activity and earlier work in this laboratory has shown that interaction with receptor systems are important (Elliott *et al.*, 1988). There is abundant circumstantial evidence that auxin binding proteins (ABP) and naphthylphalamic acid (NPA) binding proteins (regulatory proteins of auxin efflux carriers) can be determinants of the final biological effect of auxins (Napier and Venis. 1991; Rubery, 1987).

The interaction between IAA levels and the auxin receptor systems has been studied in roots of sugar beet cv Bella transformed with the Ri plasmid A4b, which introduces the auxin biosynthetic genes *iaaM* and *iaaH*.

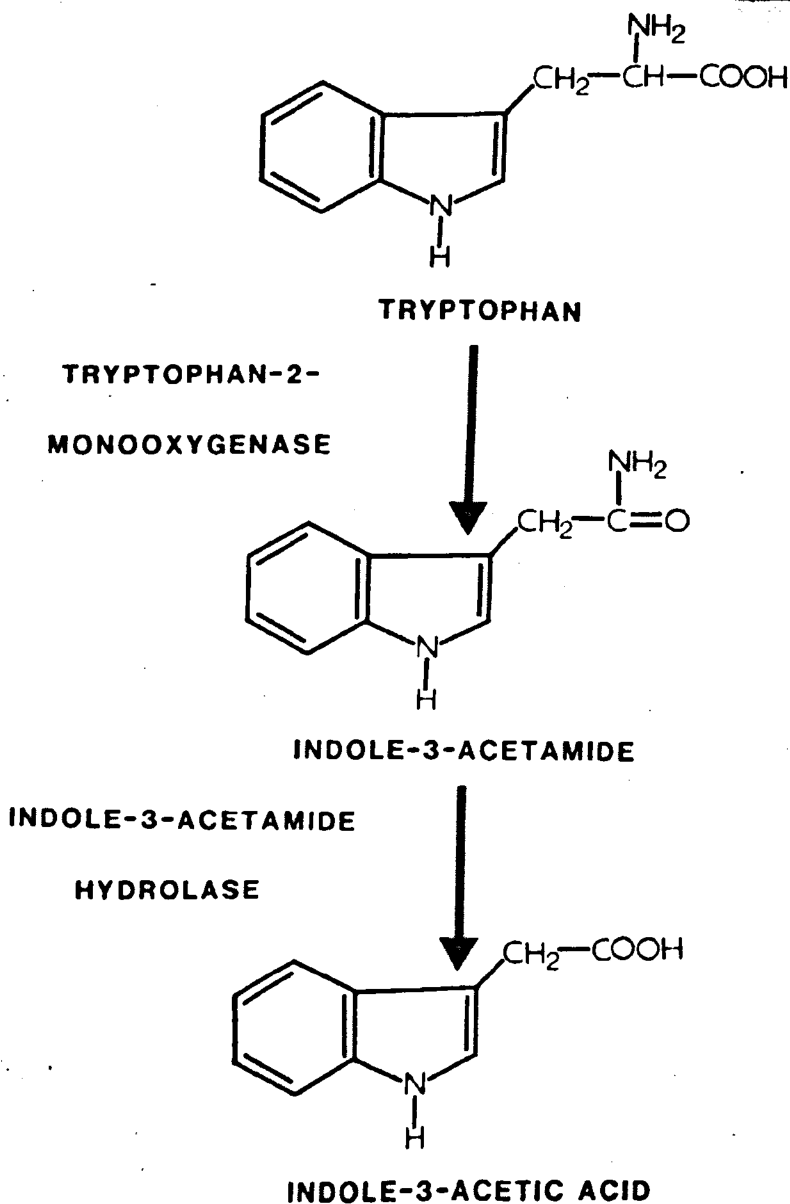


Figure 6.1 IAA biosynthetic pathway in agropine type *A. rhizogenes*.

6.1.2 Aim

The aim of these experiments was to analyse the effects of incorporation of A4T T-DNA into the sugar beet cv Bella genome. The effects on the cellular biochemistry of sugar beet cv Bella were investigated, including the acquired ability to synthesise IAM, alteration of IAA levels and effects on membrane bound and non membrane bound IAA binding proteins.

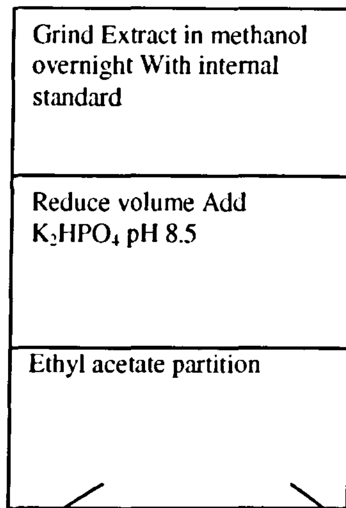
6.2 Materials and Methods

6.2.1 Preparation of Glassware and Solvents

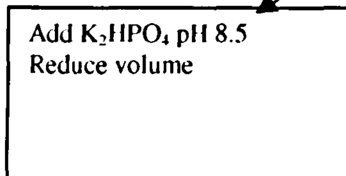
All glassware used in plant growth regulator analysis was routinely soaked in 5% v/v Decon 90 (BDH) overnight then vigorously brushed and thoroughly rinsed in tap water. It was then soaked in chromic acid, rinsed thoroughly in tap water followed by deionised water. Glassware was then oven dried and treated with Repelcoat (BDH) to reduce absorption of material onto the surface of the glass. After again oven drying, glassware was rinsed thoroughly with deionised water.

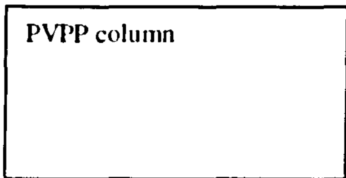
Organic solvents used in the extraction process were redistilled to remove any impurities or peroxidases present. HPLC solvents and solutions were filtered through a 2.0µm pore size Millipore filter (Millipore) and degassed by stirring under reduced pressure.

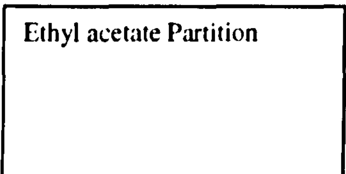
IAA/IAM ANALYSIS

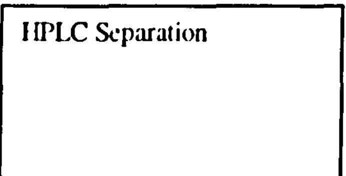


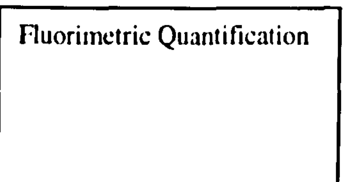
IAM



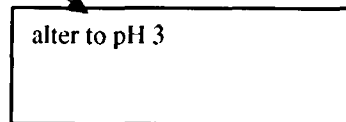


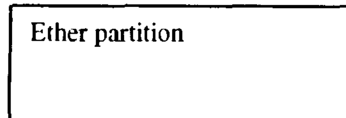


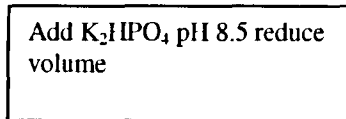


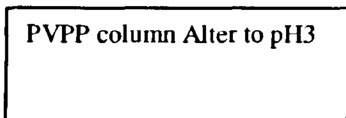


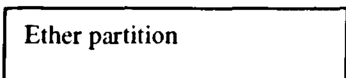
IAA

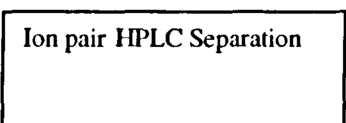












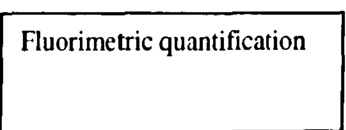


Figure 6.2 Flow diagram of IAA and IAM extraction from the same starting material.

6.2.2 Extraction and Analysis of IAA

Hairy root material (3 flasks ground and split into 3 and analysis done in triplicate) and seedling root material were frozen in liquid nitrogen and ground in methanol. Known activities of ^{3}H IAA (Amersham) and ^{14}C IAM were added as internal standards and samples were shaken overnight at 4°C. Extracts were filtered under vacuum and the volume reduced to 1cm^{3} at 35°C under pressure. 50 cm^{3} 0.5 moldm^{-3} K_2HPO_4 pH8 was added and samples partitioned three times against ethyl acetate to extract IAM (see section 6.2.5 and Figure 6.2). Aqueous samples were rapidly adjusted to pH3 with 6 moldm^{-3} HCl and partitioned three times against diethyl ether to extract IAA. 1 cm^{3} 0.5 moldm^{-3} K_2HPO_4 pH8 was added to each sample and the diethyl ether removed under reduced pressure at 35°C, leaving the aqueous phase.

Samples were applied to a 10 cm x 1 cm poly-N-vinyl polypyrrolidone (PVPP, Polyclar AT) column equilibrated with 0.1 moldm^{-3} K_2HPO_4 to pH8. 150 cm^{3} eluent was collected from the column and repartitioned 3 times with diethyl ether after adjusting the pH to 3 with 6 moldm^{-3} HCl.

Extracts were stored at –70°C to freeze out any water which was removed by filtering through Whatman IPS filter paper and reduced to 1 cm^3 at 35°C under reduced pressure, transferred to vials and stored at –20°C prior to purification by reverse phase ion pair HPLC.

6.2.3 Purification of IAA Samples by Reverse Phase Ion Pair HPLC

The method used was that described by Blakesley *et al.* (1984). Samples were reduced to dryness under a stream of nitrogen and taken up in 50 mm^3 methanol. Samples were then injected onto a preparative Hypersil 5 ODS column (150 x 22.5 mm id) fitted with a 55 x 5 mm pre-column packed with Li Chroprep RP 18 (Merck). Detection was via a spectrophotofluorimeter with excitation wavelenth 280 nm and emission wavelength 350 nm.

The mobile phase was a gradient of 15% to 35% methanol in 0.01 $moldm^{-3}$ K_2HPO_4 (ph 6.6) at a rate of 1% per minute for 20 minutes, held at 35% for 5 minutes before returning to 15% at 2% per minute. Flow rate was 1 cm^3min^{-1}.

The sample fraction with the same retention time as an IAA standard retention time was collected and 50 cm^3 0.1 $moldm^{-3}$ K_2HPO_4 pH 8 added. The pH was adjusted to 3.0 with 6 $moldm^{-3}$ HCl and partitioned three times against ethyl ether. Water was removed by freezing and filtering as described in section 6.2.12b?. Samples were reduced to 1 cm^3 under reduced pressure and stored at –20°C prior to quantification.

6.2.4 Quantification of IAA

The method for quantification of IAA was that described by Blakesley *et al.* (1983). Quantification was performed by HPLC of the 2 methyl indolo-2,2,3',4' pyr-6-one (2-MIP) derivative of IAA. This is formed by the reaction of IAA with acetic anhydride and trifluroro-acetic acid (TFA).

Samples were dried under nitrogen and then 30 mm^3 of 1:1 v/v mixture of acetic anhydride and TFA were added mixed well and left at room temperature for 5 minutes. After 5 minutes the sample was injected into the HPLC onto an analytical Hypersil 5ODS column (250mm x 5mm id) fitted with a 55mm x 5mm precolumn packed with Hypersil 5 ODS. Detection was by a spectrophotofluorimeter with excitation wavelength

445nm, emission wavelength 480nm. The mobile phase was a gradient of methanol and water (adjusted to pH 3.5 with glacial acetic acid).

The 2-MIP was eluted by a gradient of 50% to 100% methanol to water (pH 3.5) at a flow rate of $1.5cm^3min^{-1}$, rising with a gradient of 1% methanol to water per minute for 5 minutes, 2% methanol to water for 10 minutes and 5% methanol to water (pH 3.5) per minute for 5 minutes.

The sample fraction with the same retention time as a 2-MIP derivatised IAA standard retention time was collected and $10cm^3$ Aqua phase scintillation fluid was added (Amersham International).
Samples were counted in liquid scintillation counter for 1 minute per sample.

6.2.5 Extraction of IAM

IAM was extracted from the same starting material as IAA until it was separated by ethyl acetate partitioning (see section 6.2.2 and figure 6.2). $1cm^3$ 0.5 $moldm^{-3}$ K_2HPO_4 pH 8.0 was added to the ethyl acetate samples. The samples were then reduced to the aqueous phase under reduced pressure at 35°C. Each sample was then applied to a PVPP

column (as described in section 6.2.2) and $150cm^3$ eluent was collected from the column and repartitioned 3 times with ethyl acetate. Water was removed from samples by freezing at –70°C and filtering (as in section 6.2.2) and then reduced to $1cm^3$ under reduced pressure. Samples were then transferred to vials and stored at –20°C prior to quantification by HPLC.

6.2.6 Quantification of IAM

IAM was quantified by ion pair HPLC. Samples were reduced to dryness under a stream of nitrogen and taken up in $50mm^3$ methanol. Samples were then injected onto a preparative Hypersil column as described in section 6.2.4. Detection was via a spectrophotofluoimeter with excitation wavelength 280nm, emission wavelength 350nm.

The mobile phase was a gradient of 15% to 45% methanol in 0.01 $moldm^{-3}$ TAC, 1.0 $moldm^{-3}$ K_2HPO_4 pH 6.6 at a rate of 1.5 % per minute for 20 minutes and held at 45% for 5 minutes before returning to 15%. The flow rate was $1.5cm^3min^{-1}$. The sample fraction with the same retention time was collected and $10cm^3$ aqueous phase scintillation fluid

(Amersham International) was added. Samples were counted in a liquid scintillation counter for 1 minute per sample.

6.2.7 Auxin Binding Assays

10-15 g of plant material (either 2 week old hairy root material or sugar beet seedling root) was harvested and washed to remove any growth media.The plant material was then ground in PVPP and 50 mmol dm^{-3} boric acid extraction buffer, with the aid of acid washed sand, for 5-10 minutes on ice. This material was transferred to centrifuge tubes and centrifuged at 3000 rpm, $0^{o}C$ for 10 minutes. The supernatant was transferred to 50 cm^3 ultracentrifuge tubes and pairs of tubes carefully balanced. The tubes were then centrifuged at 21000 rpm (50000g), $4^{o}C$ for 45 minutes to bring down cell membranes.

The supernatant was discarded and the resulting pellets resupended in a total volume of 6 cm^3 assay buffer. Two pellets were combined by adding 5 cm^3 assay buffer to one tube and dislodging the pellet. The contents of this tube were added to the second tube and this pellet was also dislodged. This was then transferred to a homogeniser. The two tubes were washed

with 1 cm^3 assay buffer and this was also added to the homogeniser. The material was homogenised with the aid of a vortex mixer and kept cold. 0.5 cm^3 of this plant extract was added to each of 10 polycarbonate ultracentrifuge tubes. 5 of the tubes contained 0.5 cm^3 10^{-8} mol dm^3 IAA (for total binding) and 5 contained 0.5 cm^3 10^{-4} mol dm^3 IAA (for non-specific binding). Each of the 10 tubes also contained 1.45 cm^3 assay buffer and 50 mm^3 ^{3}H-IAA.

Each tube was mixed and left to incubate for 40 minutes, shaken at intervals. After 40 minutes the tubes were centrifuged at 38000 rpm (110000g), 4°C for 30 minutes. The supernatant was carefully poured away and the tubes inverted to drain. A small volume of assay buffer was used to carefully wash the tubes (opposite side to the pellet) two times and again the tubes were left top drain. When the tubes were drained, 1 cm^3 ethanol was added to each, caps fitted and the tubes left on their sides' overnight.

The ethanol was transferred to scintillation counting vials, and the top of each tube washed with 0.5 cm^3 ethanol and this also drained into the vial. The top of the tubes were dried with tissue and then the pellets were removed and put into scintillation counting vials together with the 0.5 cm^3 ethanol used to wash the corresponding centrifuge tube.

Scintillation fluid was added to each vial and the activity read in a scintillation counter.

6.3 RESULTS

6.3.1 IAA Analysis

Table 6.1 shows levels of IAA in five hairy root clones 3, 4, 5, 7 and 11and in seedling roots of sugar beet cv Bella.

Sample	ngIAA/g fresh weight
Hairy Root Clone 3	6.15 +/- 1.73
Hairy Root Clone 4	10.52 +/- 1.82
Hairy Root Clone 5	32.74 +/- 3.75
Hairy Root Clone 7	3.80 +/- 0.41
Hairy Root Clone 11	35.89 +/- 3.76
Seedling Root	5.20 +/- 0.92

Table 6.1 IAA Levels in Hairy Root Clones

These results clearly show a considerable variation in IAA levels between clones and between transformed and untransformed sugar beet.

6.3.2 IAM Analysis

The method used (described in section 6.2.1) allowed both IAA and IAM to be extracted and analysed from the same tissue. Table 6.2 shows the levels of IAM in sugar beet hairy root clones 3, 4, 5, 7 and 11 and in sugar beet cv Bella seedling root.

Sample	ngIAM/g Fresh weight
Hairy Root Clone 3	70.11 +/- 46.2
Hairy Root Clone 4	87.61 +/- 38.3
Hairy Root Clone 5	92.03 +/- 43.4
Hairy Root Clone 7	118.47 +/- 48.0
Hairy Root Clone 11	96.38 +/- 35.2
Seedling Root	1.21 +/- 0.8

Table 6.2 - Levels of IAM in Hairy Root Clones

These results suggested that IAM was present in untransformed seedling roots. This was not expected as it is not thought that the Tryptophan to IAM to IAA pathway is utilised by plants.

6.3.3 Specific Binding of ^{14}C-NAA and ^{3}H-NPA by Membrane Preparations.

Sample	Specific Binding (pmol/mg protein)	
	NAA	NPA
Clone 4	375	0.85
Clone 5	245	0.93
Clone 7	582	0.91
Clone 11	342	0.95
Seedling root	295	0.73

Table 6.3. Specific binding of ^{14}C-NAA and ^{3}H-NPA by membrane preparations from different hairy root clones and non-transformed seedling roots.

It is evident from these results that there are considerable differences between the clones in the specific binding of NAA, whereas that of NPA is less varied.

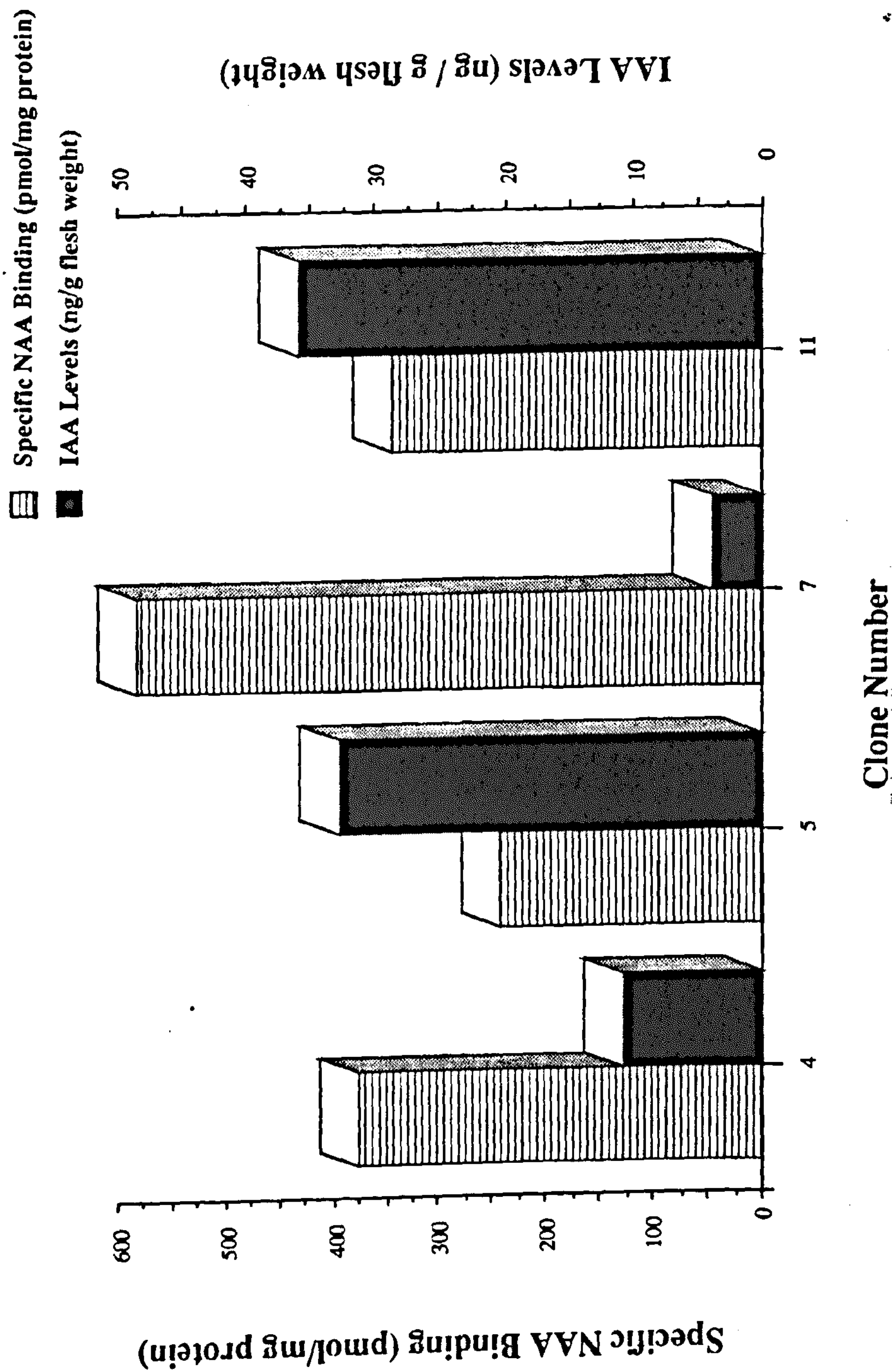


Figure 6.3 Comparison of the specific binding of NAA by membrane preparations and the levels of IAA in four different Ri plasmid transformed hairy roots and non-transformed seedling roots.

6.4 Discussion

Variations in expression of a foreign gene between independent transformants can be up to 200 fold (An, 1986). The eight-fold differences in the IAA levels seen here between clones 5 and 7 could be due to several factors including the number of copies of T_R-DNA genes 1 and 2 integrated into the sugar beet cv Bella genome, site of integration within the genome, chromatin folding patterns or/ and tissue culture effects (Gelvin, 1987). The methylation state of these genes may also affect auxin levels. Highly methylated sequences, particularly in plants, are thought to be partially switched off compared with unmethylated ones. Cytosine methylation has been shown to suppress expression of T-DNA genes in plants. Hepburn *et al.* (1983) showed that 22-24 copies of a *nos* gene in a flax tumour line were inactive when extensively methylated at cytosine residues. It is found that octopine synthase and agropine and mannopine synthesis were activated by 5-azacytidine treatment of tobacco tumours.

Methylation of endogenous plant genes has been implicated in the somaclonal variation observed in many plants subjected to plant tissue techniques (Brown, 1989). Reduction of methylation of

phytohemaglotinin genes which encode seed storage proteins in cotyedons of *Phaseolus vulgaris* has been shown to correlate with their expression in an organ specific mannor (Riggs and Chrispeels, 1990). Methylation then, is probably used as a mechanism for control of endogenous plant genes as well as causing reduction in expression of T-DNA introduced foreign genes.

The site of T-DNA integration is more or less random. Dean *et al.* (1988) made an effort to reduce this but the variation in expression was not abolished.

Rearrangement of T-DNA may occur after integration while transformed material is in tissue culture (Peerbolte *et al.*, 1987). Somaclonal variation alters endogenous genes influencing the expression of the foreign genes. This might also cause variability between transformants. It would seem advisable to keep time in tissue culture short.

It may also be that the T_L-DNA of the Ri plasmid and in particular the *rol* genes influence the levels of free IAA. Guern has established that *rol* B expression can alter the number of auxin binding protein sites on the outer surface of tobacco plasma membranes. This sensitivity- increasing

effect, together with expression of *rol* A and *rol* C may exert some influence upon the T_R-DNA auxin biosynthetic circuits.
Unfortunately time did not allow for analysis of the roots transformed with T_R-auxin genes only. This would have allowed comparison of auxin levels and so the effects of the T_L-DNA.

There was a great difference in IAM levels between triplicate samples of the same clone. The results also suggested the presence of IAM in untransformed seedling root material. This would not be expected, as plants do not use the tryptophan to IAM to IAA pathway. The problem of analysis appeared to be that comigrating peaks, i.e. substances with the same retention time as IAM were interfering with the results.

Alternative HPLC programs have been investigated which included a purification step as in IAA analysis where reverse phase ion pair HPLC is used to 'clean up' samples. Also GC-MS analysis of seedling root samples would identify the presence or absence of IAM.
The same transformed clones, together with non-transformed seedling root material were analysed for NAA and NPA binding proteins (Table 6.3) . It is evident that there are considerable differences between the

clones in the specific binding of NAA, whereas that of NPA is less varied.

A comparison of IAA levels with ABP levels shows a tendency for clones with higher specific binding to have lower free IAA levels and vice versa (figure 6.3)

One interpretation of this preliminary data points to a negative feedback mechanism operating with elevated IAA levels leading to reduced receptor availability. One possible outcome of such a system would be maintenance of a particular level of occupancy of the ABP.

The influence of the T_L region *rol* loci must also be considered in these transformed roots. The effects of the *rol* loci are complex and still not fully understood but may involve anti-auxins, altered auxin receptor status or some other form of altered sensitivity to auxin in hairy roots. It has been established that *rol* B expression can alter the number of ABP sites on the outer surface of tobacco plasma membranes (Maurel *et al.*,1990).

By studying systems such as this it is hoped to gain sufficient understanding of the underlying mechanisms controlling plant development to enable a variety of beneficial modifications to be made to crop productivity including the sugar beet storage organ.

CHAPTER 7

GENERAL DISCUSSION

The aim of the research programme being pursued in this laboratory was to genetically modify phytohormone profiles, in particular IAA, in the sugar beet storage root, in order to modify cambial development. A larger storage organ containing more storage parenchyma zones comprised of smaller parenchymatous cells has been identified as a target for the genetic manipulation of sugar beet for increased sucrose storage. The aim of the experiments described in this thesis was to investigate the potential for hairy root cultures as a model system to determine the effects of the genetic manipulation of IAA levels on sugar beet.

The hairy root system of transformation has several advantages. Sugar beet cultivars studied in this project have a high level of resistance to kanamycin, however Hamill et al. (1987) found that hairy roots of *B. vulvaris* cv. Bolthardy did not grow in liquid medium containing 10 mg dm^{-3} kanamycin. The relatively kanamycin sensitive hairy root system therefore seemed a promising method for testing the expression of introduced genes. This system had certain advantages as a model system for gene expression. Hairy

roots were easily obtained by inoculation of sugar beet petioles with the *A. tumefaciens* strain C58C1 harbouring the root inducing plasmid pRiA4b (designated A4T) (Brown et al., 1990a and 1990b). They appear to have a similar structure to roots grown under similar culture conditions derived from uninoculated seedling root tips, although they grow much faster. The hairy root system meant that it was not necessary to obtain transgenic shoots, before inducing roots in order to analyse expression of the auxin biosynthetic genes in transgenic root tissue. The transgenic phenotype is very obvious, hairy roots appear in abundance on many inoculated petioles, whereas adventitious roots growing from uninoculated petioles are extremely rare and do not grow when excised and transferred to fresh selective medium. Hairy roots are fast growing and so a large amount of material may quickly be accumulated for plant growth regulator analysis.

There are also disadvantages, as with many model systems. To date there is not a regeneration regime to obtain plants from sugar beet hairy roots, although this is possible in some species.

The strategy of the overall programme required the regeneration of transgenic plants following genetic manipulation using Ri plasmids. This ambitious objective was not fully realized.

Establishment of Methods for the Production of Clonal Sugar Beet Material

Sterile sugar beet material cv Bella was produced for infection by *A. rhizogenes,* and to provide control material for comparison in experiments to confirm transformation and for auxin analysis. It was possible to produce large quantities of sterile, clonal (derived from the same seed) sugar beet material. A reasonably efficient seed sterilization procedure was developed to give a mean sterilization rate of around 65% with a germination rate of 70%. The sterile seedlings were used to produce petiole cultures. The material generated from each seed was cultured separately from each other, producing large quantities of genetically identical sugar beet material. This was important, so that any variation in auxin levels measured could be attributed to the transformation event, and not to any genetic variability in the initial infected material.

Problems in the Production of Callus and Cell Suspension Cultures

Sugar beet seedling root material rapidly produced callus when inoculated onto callus-inducing media. The callus was soft and friable and growth was rapid, requiring sub-culture every 2-3 weeks. However, this callus, although readily breaking up did not grow in a liquid medium and within just a few days both the callus and the medium became black. This was probably due to phenolic compounds produced by the cells. When the calli were placed in medium supplemented with BAP and incubated in artificial daylight conditions, some of the callus produced pale green patches but it was not possible to induce shoot formation. There have been reports of shoot regeneration from petiole or callus of various *Beta* species by several workers (Freytag *et al.*, 1988; Harms *et al.*, 1983; Hussey and Hepher, 1978; Richie *et al.*, 1989; Saunders, 1982; Tetu *et al.*, 1987; Yu, 1989; Grieve *et al.*, 1997) but with a relatively low frequency. Ritchie *et al.* (1989) described the effects of temperature, BAP concentration, time in culture of donor plants and cultivar on shoot regeneration from petioles. Several researchers have reported that BAP plays a key role in the process of shoot regeneration in sugar beet (Coumians-Gilles *et al.*, 1981., Freytag *et al.*, 1988.,Hussey and Hepher

1978., Saunders 1982) but that kinetin was only effective at higher concentrations (Saunders and Shin 1986) or was ineffective (Miedema *et al.*, 1980). Thomas *et al.* (1993) also reported that BAP is much more active than kinetin and that the optimum BAP concentration was between 0.5 and 1.0 $mgdm^{-3}$. They also reported cultivar differences in regeneration frequencies.

Other studies described some regeneration from tissue explants but there was a high degree of variability in the regeneration frequency from different explants of different genotypes (Jacq *et al.*, 1993). The use of BAP and IBA (Freytag *et al.*, 1988) promoted regeneration at genotype dependent frequencies of 55-70% in six North American varieties. Grieve *et al.*, (1997) describe work done in this laboratory where temperature, BAP and cultivar were seen to be important in regeneration levels in six commercially important, genotypically distinct European cultivars. The report implies a stimulatory effect of BAP at concentrations between 0.25 and 0.5 $mgdm^{-3}$ on shoot organogenesis at $25^{0}C$ similar to that found by Tetu *et al.*, (1987). They also report that an alteration in culture temperature may trigger organogenesis, as a $5^{0}C$ increase or decrease in temperature stimulated regeneration. This was in agreement with the

findings of Ritchie *et al.* (1989). There was also a significant difference in shoot regeneration between the six cultivars tested.

In this study BAP concentration and temperature had no effect on the sugar beet callus produced from sterile seedling root explant material. The cultivar used was Bella, which has not been recorded in other reports. It may be that this variety is particularly recalcitrant, in accordance with the reports that sugar beet regeneration is highly dependant on genotype. Another factor could be that regeneration capability is tissue dependent. Nearly all reports of regeneration use petiole of leaf tissue as the original explant materal, with regeneration direct or via callus. Indeed Hall et al. (1995) consider that the only cells of sugar beet capable of regeneration are the stomatal guard cells. If this is the case then any regeneration from root material is very unlikely.

Establishment of a seedling-root derived cell suspension culture would have been particularly useful in allowing fundamental studies of cell growth and division in sugar beet root-derived cells in relation to changes in exogenous plant growth regulators. It would also have provided a model system against which to compare the effects of incorporation of genes from *A. rhizogenes* on cell growth. One of the biggest problems

encountered was the production of phenolic compounds by the cells, although the addition of the anti-phenolic compounds PVPP or citric acid had no noticeable effect on the colour of either the cells or the medium. One of the problems often encountered in producing a cell suspension culture is that the callus used to initiate the culture is hard and will not break up in the medium. This was not a problem here, the callus was soft and broke up easily when put into liquid medium.

Transformation of Sugar Beet Tissue

Vectors were constructed to transform the sugar beet material. Unfortunately time did not allow for infection of sugar beet material during this study. It was hoped that the introduction of the hygromycin resistance gene (used for selection of transformed tissue) would be an improvement on those more commonly used such as kanamycin. It is thought that some anti-biotic selection markers may interfere with regeneration of transformed plants. Sugar beet petioles cv Bella were infected with *Agrobacterium* harbouring the pRi A4b plasmid (A4T) and also pPM 4000 1023-19 (1023-19). The A4T transformations introduced the auxin biosynthetic genes, *iaaM* and *iaaH*, and the genes on the T_L T-DNA, including the *rol* genes. The 1023-19 infections transferred the

auxin biosynthetic genes only, without the *rol* genes. The morphology of the roots that were produced as a result of these two types of transformation were different. The roots that were produced from the A4T infections were slender, covered in numerous white root hairs and growth in culture was rapid. The roots produced by the insertion of the auxin biosynthetic genes without the rol genes produced `fatter' roots with numerous root hairs, several also produced callus. These roots had a slower growth rate than the A4T roots.

After several subcultures eight hairy root clones of A4T transformed sugar beet cv Bella were well established. These clones were tested for confirmation of transformation and auxin analysis. The 9 clones of 1023-19 transformed sugar beet cv Bella clones were not well established to produce enough material for such analysis before the end of this study.

The transformation of the hairy root clones produced by infection of sugar beet cv Bella with A4T was confirmed by opine assay and Southern blot hybridisation. Molecular conformation is essential for sugar beet transformation because non-transgenic "escapes" that are able to grow under a relatively high selection pressure occur at high frequency. As a quick test, all of the A4T clones (1, 2, 3, 4, 5, 7, 9 and 11) were tested for

the presence of the opines agropine and mannopine. The ability of these hairy root clones to produce these compounds indicated that the T_R – DNA of the plasmid pA4b had been incorporated into the sugar beet genome as the genes for synthesis of these compounds are situated here. A more reliable method of conformation of incorporation of the selected genes was used to confirm the presence of the specific sequences of DNA. This was confirmed in clones 3, 4, 5 and 7 by hybridising genes from the left and right sections of the Ri plasmid seperately to DNA extracted from the hairy root clones and control sugar beet DNA that had not been infected. The presence of at least part of the T_R-DNA was demonstrated with a dot blot. This showed hybridisation of the *rol*C gene from the *A. rhizogenes* plasmid A4b with DNA extracted from hairy root clones 3, 4, 5 and 7 but no hybridisation was seen with DNA extracted from untransformed seedling roots. The presence of at least part of the T_L-DNA was seen by hybridisation of the auxin biosynthetic genes *iaaM* and *iaaH* from the Ri plasmid A4b with DNA extracted from hairy root clones 3, 5, 7, and 11 but not with DNA extracted from sugar beet seedling roots not infected with *Agrobacterium*. The results can be seen in figure 4.5. The band on the lane containing DNA from clone 7 is darker than the other clones. This should not be due to more DNA of this clone being loaded onto the gel because the same amount of DNA in each

sample was loaded onto the gel. It is possible that more copies of the genes contained on the probe have been incorporated into the genome of this clone. The material is clonal as all the infections were of sugar beet material derived from a single sterile seedling, and then each root that appeared was cultured separately. This prevents any differences between different transformation events being due to genetic variability in the original plant material.

The growth of hairy root cultures

Growth conditions for the transformed tissue were optimised so as to be able to produce large quantities of material for auxin analysis.

It was found that all clones of sugar beet hairy roots grew rapidly in half strength B5 medium supplemented with 4% sucrose. Growth was even more prolific in liquid culture conditions of half strength B5 medium supplemented with 4% sucrose and shaken at 60rpm.

Attempts to regenerate shoots from hairy root tissue

The hairy root material produced callus on all of the plant growth regulator regimes tested, and in artificial daylight conditions the callus turned green and occasionally produced hard dark green nodules. The callus was at first hard and dark brown in colour but after 3 subcultures

had become soft and friable and if cultured in darkness, a pale cream in colour. Although this callus was soft and could be broken up easily it was not possible to produce a liquid cell suspension culture. In all cases the cells turned black and died within one day of being transferred to liquid medium. This dark colouration may have been due to phenolic compounds produced by the cells. Work on the sterile culture of grapevine material transformed via *Agrobacterium* also reported such problems. Attempts made to prevent the production of these compounds by the addition of the anti phenolic chemicals PVPP and ascorbic acid failed. These problems were similar to those encountered in the production of a seedling root derived cell culture but more pronounced.

It was not possible to induce organogenesis from either the hairy root clones directly or via callus. When cultured in artificial daylight conditions and the media supplemented with relatively high levels of BAP (2.0- 5.0 mgdm^{-3}) the callus turned green and occasionally produced hard dark green nodules. These nodules remained unchanged in the cultures for many sub-cultures and no shoot regeneration could be induced. The difficulty in regenerating plantlets from *Agrobacterium* mediated transformed material has been reported elsewhere (Sangwan *et al.*, 1992.; Draper *et al.*, 1988). The regeneration of transformed sugar

beet may be made more difficult due to its recalcitrant nature even before genetic manipulation. There have been several reports showing that regeneration is dependent on genotype, and there may be variability of response to culture components. Also selective agents can cause problems during regeneration (Jarl and Bornman, 1986; Scott *et al.*, 1995). D'Halluin *et al.* (1992) overcame some of these problems by using herbicides as selective agents and employing somatic embryogenesis to regenerate transformants. A protocol being developed in this laboratory has been described which involves a dual antibiotic-herbicide selection system for transformation of sugar beet and regeneration by somatic embryogenesis (Scott *et al.*,1995).

Other workers have used mannose selection for the production of transgenic plants (Joersbo *et al.*,1998). The mannose system employs the *Escherishia coli* phosphomannose isomerase (PMI) gene as the selectable marker gene and mannose as the selective agent. Mannose is phytotoxic , inhibiting plant growth by depletion of the intracellular ATP by accumulation of mannose-6-phosphate (Joersbo *et al.*, 1999). This selection method is very effective, with transformation efficiencies ten times those using kanomycin and is reported not to be genotype

dependent. It also has the advantage that it avoids the use of an antibiotic resistance gene.

Reports published since the work described here suggest that stomatal guard cells may be the only progenitors of totipotent protoplasts in sugar beet (Hall *et al.*, 1995). Hall *et al.* (1996) subsequently used guard cells to produce a protocol for transformation and regeneration although still at low levels. It was reported that plasmid uptake into the guard cells was 3-7% , but only 1% of these cells formed transformed callus and only 1-2% of these eventually regenerated.

Auxin Content of Hairy Roots

Analysis of the auxin content in five of the A4T transformed hairy root clones and sugar beet cv Bella seedling roots was completed.

The auxin biosynthetic genes from *Agrobacterium*, *iaaM* and *iaa*H synthesise IAA via a pathway which is not thought to be used by higher plants (see section 3.1 and 6.1). Tryptophan is converted to indole-3-acetamide (IAM), which is subsequently hydrolysed to IAA. The intermediate, IAM is not thought to exist in non-transformed plants. In

this study it was shown that it is possible to alter auxin levels in sugar beet material by insertion of these genes. There was a large difference in IAA levels both between non-transformed sugar beet seedling roots and transformed sugar beet hairy roots and also between different hairy root clones. Clones 5 and 11 had exceptionally high levels of IAA (32.74 ng/g FW and 35.89 ng/g FW) compared to all other clones and non-transformed material, whereas clone 7 had a low level of IAA (3.8 ng/g FW) even compared to non-transformed material (5.2 ng/g FW). The low level of auxin in clone 7 may be related to the fact that more copies of the T_L T-DNA could have been incorporated into this genome.

IAM levels proved more difficult to measure by HPLC. The IAM retention time coincided with some other compound that was very difficult to remove from the samples. Once it had been removed it was clear that those clones that had lower levels of IAA had higher levels of IAM. This may suggest some kind of control mechanism that regulates the conversion of IAM to IAA. This may involve the formation of IAA conjugates.

Transformation of tobacco to express the *iaa*H and *iaaM* genes under the control of their native promoters, as in this study, resulted in calli, which were selected for the presence of IAM. On analysis they contained on average twice as much free IAA and three times as much conjugated IAA

as wild type calli (Stibon *et al.*, 1991). Some plants regenerated from calli displayed apparently normal phenotypes (at least until the onset of flowering) and were found to have similar free IAA levels to untransformed plants but relatively higher levels of IAA conjugates. This finding suggests the presence of a system for controlling the endogenous levels of IAA via conjugation, thereby compensating for the increase in IAA biosynthesis due to the expression of the T-DNA genes.

However other studies have shown that this mechanism may be overcome if expression of the biosynthesis genes is sufficiently high. Sitbon *et al.* (1992) produced plants with abnormal morphology including small, narrow, curling leaves with occasional adventitious roots, apical dominance, elongated woody stems containing more secondary xylem and phloem and adventitious roots developing from the phloem parenchyma associated with elevated IAA levels. This was achieved by transforming plants expressing the *iaaH* and *iaaM* coding regions under the control of the strongly expressed CaMV 35S promoter.

Auxin Binding Proteins

Auxin binding proteins were studied in four of these clones and control seedling root material. The aims of this work were to correlate the altered auxin content of sugar beet hairy roots with their auxin binding activities.

The auxin binding data was only preliminary and time did not permit replication of these studies. In general the higher the level of IAA measured in the material the lower the auxin binding activity. Again this may reflect a control mechanism, ensuring that in conditions of excess IAA, fewer binding protein sites are available, making the cells less sensitive to the action of auxin.

Another factor that must be considered is that of the *rol* genes. These genes have been shown to be able to affect auxin sensitivity without the introduction of the auxin biosynthetic genes. At the time of this study very little was known about the effects of these genes on phytohormone profiles or plant sensitivity to particular phytohormones. Since then, more work has been carried out to elucidate the function of the *rol* genes. The *rol* B gene of *A. rhizogenes* encodes a β-glucosidase, which, it has been suggested, causes the release of IAA from a conjugated state (IAA-β-

glucoside, Estruch *et al.*, 1991c). However free IAA levels in leaves and internodes of tobacco plants transformed with a CaMV 35S promoted *rol* B construct were no different from those in wild type control plants (Nilson *et al.*, 1993). In that comparison *rol*B gene expression was found to have no effect on IAA metabolism and the plants displayed a phenotype very different from that observed for *iaaM* and *iaaH* expressing transgenics. A more recent study by Lemcke *et al.* (2000) hypothesised that *rol*B has tryptophen 2-monoxygenase activity. It was found, in both transformed seedling material and transformed *E. coli* that expression of *rol*B led to a significantly higher turnover rate of tryptophan to IAM.

The *rol* C gene encodes a cytokinin-β-glucosidase, which probably causes the release of free cytokinin from conjugates (Estruch *et al.*, 1991b). Analysis of tobacco transformed with a CaMV 35S promoter-*rol*C construct suggested a role for this gene in an altered response of seedlings to auxins, cytokinins, ABA, GA and 1-aminocyclo-propane carboxylic acid (a precursor of ethylene) as well as causing an up to four times increase in the content of some cytokinins in transgenic potato plants (Schmulling *et al.* 1993).

Aspen (*Populus tremula* L. x *tremuloides* Michx.) transformed with CaMV 35S promoted *rol*C were assessed for wood formation. They had reduced shoot growth and earlier bud break. It was suggested that dwarfism of the transgenic trees was likely to be due to reduction in cell number. Also the cells lacked both secondary walls and normal lignification (Gruenwald *et al.*, 2000).

Tuominen *et al.* (2000) transformed aspen with *rol*C promoter linked to the rate limiting *iaaM* gene and the reporter gene for β-glucuronidase (GUS) uidA to visualise the expression of the iaa genes. The *rol*C gene was shown to be cambial-region-specific. Analysis of the IAA concentration across the cambial region tissues showed an increase in IAA concentration of about 35% to 40%. No changes in the radial distribution pattern of IAA compared with wild-type plants. This increase did not result in any changes in the developmental pattern of cambial derivatives or the cambial growth rate. This emphasises the importance of the radial distribution pattern of IAA in controlling the development of secondary xylem, and suggests that a moderate increase in IAA concentration does not necessarily stimulate growth in trees.

Analysis of the promoter region linked to the GUS reporter gene system showed that it caused high levels of expression. This expression was seen specifically in root tips (Kamo and Blowers, 1999).

A possibly useful feature of the auxin biosynthetic pathway in *Agrobacterium* may be that two foreign genes are introduced into the sugar beet genome. They may be introduced either together or separately. This has the potential advantage that there are two transcriptional regulating events, which may be controlled.

Klee *et al.*, (1987) found that introduction of the *iaaM* gene alone, under the control of the highly expressed CaMV 35S promoter into petunia, led to levels of IAM between 2.8 and 25 μg g^{-1} fresh weight (FW) leaf tissue. This resulted, whether by enzymatic or spontaneous hydrolysis, in increased levels of IAA (110-120 ng g^{-1} FW) in transgenic plants compared with 10 ng g^{-1} FW in leaves from non-transformed plants. Transgenic plants with elevated IAA levels showed abnormal morphology (as described above). Abnormal morphology was heat inducible in petunia plants transformed with a maize hsp70 promoter-*iaaM* gene fusion (Medford and Klee 1989). The *iaaM* gene under control of its natural promoter, introduced alone into tobacco, resulted in IAM

production (261 ng g^{-1} FW) but in no abnormal morphology or increase in IAA levels (van Onckelen *et al.*, 1985).

One strategy would therefore be to use a strong promoter such as CaMV 35S to drive the *iaaM* gene alone in transgenic sugar beet. This could result in altered IAA levels without using both *iaaM* and *iaaH* genes. It is possible that conversion of IAM to IAA will not occur in sugar beet without the IAM-hydrolase enzyme. Even so it may be desirable to alter the expression of one gene at a time. If *iaaM* gene expression was held at a low level then IAM production would be limited and so increasing the expression of *iaaH* beyond a certain level would cause no further increase in IAA. However if *iaaM* production was at a high level and there was some conversion of IAM to IAA without *iaaH* gene product, then a build up of IAM and consequently IAA would occur.

Alteration of one phytohormone type in all tissues may have considerable effects on other phytohormones. Romano *et al* (1993) showed that tobacco and *Arabidopsis* transformed with a construct containing the *iaaM* coding region regulated by the CaMV 19S promoter had not only elevated levels of auxin but also higher rates of ethylene synthesis compared to wild-type plants.

Targeted Gene Expression

Together these studies highlight the need for strict regulation of the foreign genes used to control plant development if they are to be of use in producing a commercially useful sugar beet.

Promoters are required that can direct the specific spatial and temporal expression of genes which is necessary for activation of the outer cambial rings of the sugar beet storage root (Elliott *et al.*, 1988; Elliott and Weston 1993). Isolation of promoters that will direct such gene expression was begun in these laboratories by screening of cDNA libraries (Gartland *et al.*, 1990). Root and leaf cDNA libraries were probed with biotinylated root and leaf mRNA resulting in the isolation of two cDNAs which hybridised strongly to root mRNA but not to leaf mRNA. These root specific (or at least highly enhanced) clones, designated RS1 and RS2 have been sequenced (Slater *et al.*, 1994) and are currently being used to isolate their regulatory regions by genomic DNA library screening and polymerase chain reaction (PCR) techniques.

An even more specific tuning may be achieved if promoters that are outer cambial ring specific can be found. This should now be possible using PCR techniques. Very small quantities of DNA could be amplified to produce a library from cDNA to mRNA from the sugar beet storage organ outer cambial rings and screened to allow detection of outer cambial ring specific transcripts. Other root specific promoters have been isolated in Arabidopsis (Koncz *et al.*,1989), potato (Koster-Topfer *et al.*,1989) tobacco (Yamamoto *et al.*,1991) and French bean (Keller and Baumgartner, 1991).

The emphasis of this report has been on the use of gene manipulation techniques to enhance sucrose levels in the storage root of sugar beet. Another problem of high priority has been identified by breeders and growers in the developed world. When the sugar beet crop is harvested a great deal of soil is removed from the field with it. This soil is referred to as dirt tare, and can increase the costs of production in several ways. The soil is transported with the beets and must be cleaned before processing. This adds to costs for transportation and requires large volumes of water. Soil accumulates at the processing plants and must be removed at cost. In areas where beet necrotic yellow vein virus (BNYVV), the causative agent of the sugar beet disease rhizomania, is prevalent the soil must also

be treated to prevent the spread of the disease. The research group in Leicester is now putting the strategy developed for altering the development of sugar beet to increase sucrose yield (Elliott *et al.*, 1988) to use in solving this problem (Elliott and Weston, 1993). Some varieties of *Beta vulgaris*, such as fodder beet and table beet develop with their storage organs partly above the ground. If these varieties had a sucrose content close to that of sugar beet, while having a low level of impurities (which adversely affect the sucrose extraction procedure) then it would have advantages over the present sugar beet varieties whose storage roots develop very low in the ground. This would result in greatly reduced tare, cost of production and environmental impact.

The presence of RS1 transcripts have been demonstrated in these other phenotypes of *B. vulgaris* (Baldridge, 2000). This indicates that any genes inserted into these other genotypes under the control of RS1 non-coding regions should be expressed. In the study by Baldridge (2000) the results suggest that RS1is expressed mainly in the storage organ of different beet phenotypes, although levels of expression in Swiss chard petioles were moderate. Previously low levels of RS1 expression have been identified in sugar beet leaves. This is not inconsistent with other research into organ specificity; since there are many cases where

expression of particular organ specific genes/clones have been detected at low levels in other tissues (Becker *et al.*,1992; Hesse *et al.*,1995).

Not only specific sites of expression must be found, but also precise levels of gene expression are desirable, if growth and development are to be manipulated in a controlled manner.

Regulating the Levels of Expression of Transgenes

Detailed comparisons of the activities of different promoters or regulatory elements using reporter (or marker) genes in sugar beet will be needed if gene expression is to be so closely regulated.

The β-glucuronidase (GUS) reporter gene system may be used for such detailed analysis. Work in this laboratory (Phillips *et al.*, 1992) studied GUS activity in hairy root clones of sugar beet (cv Salohill). It showed that variations in levels of gene expression, up to 200 fold between independent transformants, were not due to variation in plant genotypic backgrounds, as was also found in this study. This system could be used with any site or developmental stage specific gene regulatory regions to assess promoter strength and site(s) of expression.

The regulation of genes to cause expression within the outer cambial rings of the sugar beet storage organ early in their development is of paramount importance for the long term aims of the programme. The identification of genes which will activate the cambia when so expressed is of equal importance. The work being carried out in this laboratory using cell cycle regulatory genes may achieve this (Fowler *et al.*, 1998). It is unlikely that such specific induction of cell division will be followed by cell enlargement and differentiation, which are also necessary. Control of phytohormone profiles may, however, be expected to bring about these changes in development (Elliott *et al.*, 1988; Elliott and Weston 1993).

ABBREVIATIONS

ABA	Abscisic acid
AMP	Adenosime monophosphate
$AgNO_3$	Silver nitrate
ATP	Adenosine triphosphate
BAP	6-benzylaminopurine
bp	Base pair
B5	Gamborgs B5 medium (Gamborg et al., 1968)
B5/2	Half strength Gamborgs B5 medium
CaMV	Cauliflower mosaic virus
CAT	Chloramphenicol acetyl transferase
cat	Gene encoding chloramphenicol acetyl transferase
cDNA	Copy DNA
CIP	Calf intestinal alkaline phosphatase
CsCl	Cesium chloride
dATP	Deoxyadenine triphosphate
DC	Direct current
DCPTA	2(3,4-dichlorophenoxy) triethylamine
dCTP	Deoxycytosine triphosphate
dGTP	Deoxyguanine triphosphate
Dig-ATP	Digoxygenin adenosine triphosphate

DNA	Deoxyribonucleic acid
dTTP	Deoxythymine triphosphate
dUTP	Deoxyuridine triphosphate
2,4-D	2,4-dichlorophenoxyacetic acid
EDTA	Ethylenediaminetetra acetic acid
GUS	β-D-glucuronidase
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
IAA	Indole-3-acetic acid
iaaH	Gene encoding indole-3-acetamide hydrolase
iaaM	Gene encoding tryptophan-2-monooxygenase
IAM	Indole-3-acetamide
ipt	Gene encoding isopentenyl transferase
Kb	Kilobase
K_2HPO_4	Dipotassium hydrogen orthophosphate
KH_2PO_4	Potassium dihydrogen orthophosphate
lbs	Pounds
LiCl	Lithium chloride
mRNA	Messenger RNA
2-MIP	2-methylindilo-2,3 4-pyr-6-one
MS	Murashige and Skoog (1962) basal medium

NaAc	Sodium acetate
NaCl	Sodium chloride
$NaPO_4$	Sodium phosphate
NBT	Nitroblue tetrazolium salt
NH_4Ac	Ammonium acetate
npt-II	Gene encoding neomycin phosphotranmsferase
OD	Optical density
ORF	Open reading frame
PVP	Polyvinyl pyrrolidone
PVPP	Polyvinyl polypyrrolidone
Ri	Root inducing
RNA	Ribonucleic acid
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
TCA	Tetra ethylammonium chloride
T-DNA	Transferred deoxyribonucleic acid
TFA	Trifluoroacetic acid
Ti	Tumour inducing
TIBA	2,3,5-triiodobenzoic acid
T_L-DNA	Left T-DNA
T_R-DNA	Right T-DNA

UV	Ultra violet
vir	Virulence region
v/v	Volume to volume
w/v	Weight to volume

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APPENDIX

APPENDIX 1
PLANT GROWTH MEDIUM

Murashige and Skoog Medium

Macronutrints	$mgdm^{-3}$	g/stock		cm^3dm^{-3}
NH_4NO_3	1650.0	33.00	}	
KNO_3	1900.0	38.00	}	
$CaCl . 2H_2O$	439.5	8.80	} in $1dm^3$	50
$MgSO_4 . 7H_2O$	370.6	7.40	}	
KH_2PO_4	170.0	3.40	}	
Micronutrients				
H_3BO_3	6.20	0.62	}	
$MnSO_4 . 4H_2O$	22.30	2.23	}	
$ZnSO_4 . 7H_2O$	8.60	0.86	}	
KI	0.83	0.083	} in $500cm^3$	5
$NaMoO_4 . 2H_2O$	0.25	0.025	}	
$CuSO_4 . 5H_2O$	0.025	0.025	}	
$CoCl_2 . 6H_2O$	0.025	0.025	}	
Vitamins				
Nicotinic acid	0.58	0.058	}	
Thiamine HCl	0.10	0.01	}	
Pyridoxine HCl	0.50	0.05	} in $100cm^3$	1
Glycine	2.00	0.20	}	
Iron Source				
FeNA EDTA	36.70	1.835	}	
MESO Inositol	100.00	5.00	} in $500cm^3$	10

Gamborgs B5 Medium

Macronutrients	$mgdm^{-3}$	g/stock			$cmdm^{-3}$
KNO_3	2500.00	50.00	}		
$CaCl . 2H_2O$	150.00	3.00	}		
$(NH_4)_2SO_4$	134.00	2.68	}	in $1dm^{-3}$	50
$MgSO_4 . 7H_2O$	250.00	5.00	}		
$NaH_2PO_4 . H_2O$	150.00	3.00	}		

Micronutrients

KI	0.75	0.015	}		
H_3BO_3	3.00	0.06	}		
$MnSO_4 . 4H_2O$	10.00	0.20	}		
$ZnSO_4 . 7H_2O$	2.00	0.04	}	in $500cm^3$	25
$Na_2MoO_4 . 2H_2O$	0.25	0.005	}		
$CuSO_4 . 5H_2O$	0.025	0.0005	}		
$CoCl_2 . 6H_2O$	0.025	0.0005	}		

Vitamins

Pyridoxine HCl	1.00	0.10	}		
Thiamine HCl	10.00	1.00	}	in $100cm^3$	1
Nicotinic acid	1.00	0.10	}		

Iron Source

FeNa EDTA	40.00	1.835	}		
MESO Inositol	100.00	5.00	}	in $500cm^3$	10

Schenk and Hildebrandt Medium

Macronutrients	**mgdm^{-3}**		**cm^{3}dm^{-3}**
$(NH_4)_2SO_4$	300.00	}	
KNO_3	2500.00	} in 1dm^3	50
$CaCl_2 . 2H_2O$	200.00	}	
$MgSO_4 . 7H_2O$	400.00	}	
Micronutrients			
$FeSO_4 . 7H_2O$	15.00	}	
$MnSO_4 . 4H_2O$	10.00	}	
$ZnSO_4 . 7H_2O$	1.00	}	
H_3BO_3	5.00	} in 500 cm^3	5
KI	1.00	}	
$Na_2MoO_4 . 2H_2O$	0.10	}	
$CuSO_4 . 5H_2O$	0.20	}	
$CoCl_2 . 6H_2O$	0.10	}	
Vitamins			
Nicotinic acid	5.00	}	
Pyridoxine HCl	0.50	} in 100cm^3	1
Thiamine HCl	5.00	}	
Iron Source			
$Na_2 EDTA . 2H_2O$	20.00	}	
MESO Inositol	1000.00	} in 500cm^3	10
$FeSO_4 . 7H_2O$	15.00	}	

Whites Medium

Inorganic Stock	g/stock		cm^3 for stock
A $CaNO_3 . 4H_2O$	144.00	} in $1dm^3$	20
KNO_3	40.00	}	
B KCl	32.50	} in $1dm^3$	20
$NaH_2PO_4 . 2H_2O$	10.75	}	
C $MnCl_2 . 4H_2O$	3.00	}	
$ZnSO_4 . 7H_2O$	1.325	} in $500cm^3$	10
KI	0.375	}	
D1 $Na_2SO_4 . 10H_2O$	113.35	} in $2dm^3$	80
$MgSO_4 . 7H_2O$	185.00	}	
D2 H_3BO_3	0.375	} in $500cm^3$	20
E MoO_3	0.0034	} in $1dm^3$	5
F $CuSO_4 . 5H_2O$	0.008	} in $1dm^3$	25

Vitamins

Thiamine HCL	0.05	}
Nicotinic acid	0.25	} in $500cm^3$
Pyridoxine HCl	0.05	}

Iron Source

NaFe EDTA	0.421	} in $1dm^3$

For 1 dm^3 Whites Medium use:

Inorganic Stock	100 cm^3
Vitamins	1 cm^3
Iron Source	10 cm^3