

**Examination of the Role of Proline in Environmental Stress
Tolerance through Genetic Manipulation of Forest Tree
Cultures**

**A thesis submitted to Dublin City University in fulfilment of the requirements for
the Degree of Doctor of Philosophy**

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Declaration

I hereby declare that this material which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my own work.

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Publications

‘Environmental stress tolerance in forest species’

Environmental Sciences Association of Ireland, Conference 2001, Dublin City University

‘Production of salt, cold and frost tolerant forest species via *Agrobacterium*-mediated transformation’

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‘Investigation into stress tolerance in Larch (*Larix X leptoeuropaea*) via genetic manipulation’

Abstract

The amino acid proline has been proven to play an important role in plants exposed to environmental stress (cold, salt and freezing). The interaction of exogenous proline with both NaCl and chilling temperature (4°C) on the growth of embryogenic cell lines of Larch (*Larix X leptoeuropeae*), Sitka spruce (*Picea sitchensis* (Bong.) Carr.) and Oak (*Quercus robur* L.) was investigated. Increasing the exogenous proline concentration increased the growth rate of the cells, both with chilling and salt stress. Freezing tolerance, quantified by K⁺ leakage, was increased by exogenously added proline. Plasmids expressing P5CS (Δ^1 -pyrroline-5-carboxylate synthetase), the rate limiting enzyme in cellular proline production, were introduced separately into *Agrobacterium tumefaciens* LBA4404. pBI-P5CS contained proline-overexpressing *Vigna aconitifolia* P5CS cDNA. pBI-P5CSF129A was a feedback-insensitive variant of this. Both vectors contain the NPTII and GUS coding regions that were used for selection of transgenic plants on kanamycin and as a reporter of transformation, respectively. Embryogenic calli of Larch, Sitka spruce and Oak were transformed with these plasmids, a number of transgenic lines isolated, and these examined for proline production, cold, salt and frost tolerance.

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Abbreviations

AAS	Atomic absorption spectrometry
ABA	Abscisic Acid
AC	Activated Charcoal
ASF	Adventitious Shoot Formation
ATP	Adenosine Tri Phosphate
BAP	Benzylaminopurine
bp	Base pairs
CaMV	Cauliflower Mosaic Virus
CAT	Chloramphenicol Acetyl Transferase
<i>codA</i>	Choline oxidase gene
<i>Des9</i>	$\Delta 9$ -desaturase gene
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetraacetic acid
EPSP	5-enolpyruvyl shikimate-3-phosphate
ESM	Embryo Suspensor Masses
<i>Et al.</i>	And others
EU	European Union
<i>g</i>	gravity
GK	γ -Glutamyl Kinase
GM	Genetically modified
GPR	γ -Glutamyl Phosphate Reductase
GSA	Glutamic Semialdehyde
GSADH	Glutamic Semialdehyde Dehydrogenase
GUS	β -glucuronidase
h	Hour (s)
HCl	Hydrochloric acid
IBA	Indoyl Butric Acid
kb	Kilo base pairs
LEA	Late Embryogenesis Abundant

L-Glu	L-Glutamic Acid
Log	Logarithm (common)
min	Minute
MS	Murashige and Skoog Medium
MSV	Maize Streak Virus
<i>MiLD</i>	Mannitol-1-phosphate dehydrogenase gene
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NB	Nutrient Broth
NPTII	Neomycin Phosphotransferase II
OAT	Ornithine amino transferase
OD	Optical Density
PAT	Phosphinotricin acetyl transferase
P5C	Pyrroline-5-Carboxylic Acid
P2C	Pyrroline-2-Carboxylic Acid
P5CDH	Pyrroline-5-Carboxylate Dehydrogenase
P5CR	Δ^1 -Pyrroline-5-Carboxylate Reductase
P5CS	Δ^1 -Pyrroline-5-Carboxylate Synthetase
PDH	Proline dehydrogenase
PEG	Polyethylene Glycol
Proline DH	Proline Dehydrogenase
RBCL	Ribulose-1,5-bisphosphate carboxylase
RBCS	Ribulose-1,5-bisphosphate carboxylase
RNA	Ribonucleic acid
Rnase	Ribonuclease
ROS	Reactive Oxygen Species
RPM	Revolutions per Minute
S	Second (s)
SCV	Settled Cell Volume
SDS	Sodium Dodecyl Sulphate
SE	Somatic Embryogenesis

SODS	Superoxide Dismutases
TCA	Tricarboxylic Acid Cycle
TGMV	Tomato Golden Mosaic Virus
TPSI	Trehalose-6-phosphate synthase subunit
Tris	Tris (hydroxymethyl) aminoethane
Vir	Virulence region
X-Glu	5-bromo-4-chloro-3-indoyl β -D-glucuronic acid

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Chapter 1
Introduction

1.1 The Importance of Wood

1.1.1 Historical background of Irish forests

As the ice sheets retreated northwards at the end of the last glaciation period 10,000 years ago, Ireland began to be colonised by trees migrating from the continent. Ash and Elm migrated across Britain into Ireland, while Oak, Alder and Pine are thought to have come across a land bridge from France. Between 5000 and 7500 years ago, woodland probably covered 80% of the land surface (Joyce et al. 1998). Towards the end of this period Neolithic farmers began a limited clearance. As the population increased during the Bronze Age, agricultural pressure on the forests became more widespread. It is accepted that there was a considerable reduction in forested areas, right up to the 17th century. The latter half of the 18th century saw an attempt to restore woodlands. By 1907 the estimated area of woodland had diminished to 1.5% of the land area.

1.1.2 Irish forests today

Total forest cover in Ireland is now between 7 and 9% of the land area (ENFO, www.dcu.ie/~enfo/fs/fs18.htm). Ireland is still the least forested country in the European Union (EU), where the average is over 30%. The EU produces three times more food than it needs, but only 25% of its timber requirements. After oil, timber is the second largest import into Europe. Ireland's soils and climate are highly favourable for growing trees and plantations. Afforestation programs have in the past concentrated almost exclusively on Sitka spruce to the extent that it accounts for approximately 60% of the forest estate as against 24% for other coniferous species (Lodgepole pine, Douglas fir and Norway spruce) and 16% for broadleaves (Oak, Beech, Ash and Hawthorn). In the past, Government policy only allowed land that was unsuitable for agricultural purposes to be purchased for forestry. Consequently, sites suitable for the planting of broadleaves were only available in very limited areas and most of the land acquired was of poorest soil and site types, much of it on exposed hillsides. However, although broadleaf cover is low in comparison to most European countries, broadleaf afforestation has increased substantially in recent years.

Trees are the largest living organisms in the world, and they are primary producers. They produce food in the leaves by photosynthesis, taking carbon dioxide

from the atmosphere, combining it with water and the energy of sunlight. This food, which forms the beginning of the food chain, is essential for all other forms of life. Trees provide beautiful and varied landscapes, shelter from harsh weather and improved air quality. They help prevent flooding, keep the soil healthy and curb soil erosion, and they provide many and varied habitats for birds and other wildlife.

1.1.3 Benefits of forestry

Wood is a very versatile raw material. It is used as sawn wood for rafters, floorboards, joinery, furniture, boats and as poles for electricity and telecommunications. It is also used as pulpwood for particle and fibre wood and also as chemical pulp for the paper industry. It is a source of energy for industry and public use. Approximately 13,000 people are employed in Ireland, either directly or indirectly by the forestry industry. Each additional 1000 hectares planted generates 100 new jobs (ENFO). Trees also make a valuable contribution to environmental quality, and trees help control soil erosion, flooding, avalanches and they help balance the earth's atmosphere, improve the landscape and also support a variety of flora and fauna.

1.1.4 *Larix* genus

The genus *Larix* belongs to the Pinaceae family, and has 10 species and a large number of subspecies and hybrids. It is widely distributed across North America, Asia and Europe. Larches are deciduous conifers and their bright autumn colours have a great aesthetic value in tourist areas. Larch wood is used for construction, furniture, paneling, pulp and paper and many other purposes (Keegan et al. 1992). Hybrid Larch is fast gaining favour in Ireland as a minor species, particularly for planting in mixture plantations (Harper and O'Reilly 2000/1999). Coillte are presently involved in a European wide Larch Wood Chain project aimed at setting up a cooperative-like breeding program across the EU for Larch, ensuring their preservation and exploiting their uses. This project is focused on the genetics, propagation, silvi-culture and wood processing of the cultivated larch of the lowland of Western Europe (www.skogforsk.se/www/EU-projekt/larch/larch.htm).

Larch species such as *Larix occidentalis* Nutt., *Larix laricina* (Du Roi) K. Koch, *Larix decidua* Mill., *Larix X leptoeuropaea* and *Larix X eurolepis* A. Henry

are important in North America and Europe for commercial forestry. Furthermore, Larches are interesting because of their deciduous habit, high CO₂ fixation rate, good wood quality, rapid growth and affinity for interspecific hybridisation (New Brunswick Forest Advisory Committee, 1986). However, their use for reforestation is limited because of low seed set and poor germinability. The use of tissue culture techniques such as somatic embryogenesis would permit the large scale propagation of elite trees for reforestation and accelerate traditional tree improvement programs by reducing the long time required to produce genetically improved propagules (Cheliak and Rogers 1990, Charest et al. 1992). Larches generally have poor soil salt tolerance and high water demand. They are also easily damaged by spring frosts, which leads to losses of young plantlets each year (www.gcw.nl/dryad).

1.1.5 *Picea* genus

The genus *Picea* belongs to the Pinaceae family, and has seven species. Sitka spruce (*Picea sitchensis*) is a monoecious, wind-pollinated, cross-fertilising species showing a wide genetic variation (Faulkner 1987). It is the most important forest tree in Ireland today. It occurs naturally along a narrow strip of the North Pacific coast of North America, and was introduced into Ireland shortly after 1831. Sitka spruce utilises the oceanic climate of Great Britain and Ireland to advantage to produce increased growth rates. It is very suited to our moist climate and thrives on wet mineral soils.

Sitka spruce is an evergreen tree, grown for its ornamental and commercial value. The timber of Sitka spruce has been accepted by industry as suitable for building construction and for pallet making. It is also an ideal raw material for the production of panel boards and paper pulp. The rotation age of a Sitka spruce crop is approximately 50 years, with thinnings at 15-20 years and every 4-7 years thereafter.

Norway spruce (*Picea abies*) is an important tree in the forests of central and northern Europe. In the past it was preferred to Sitka spruce in frosty areas, being less susceptible to damage because its buds open later (O'Carroll 1992). Sitka spruce also has a high water demand and poor soil salt tolerance, as with Larch and grows best in reasonably fertile soils (www.gcw.nl/dryad).

1.1.6 *Quercus* genus

The most important native Irish species are the Oaks (*Quercus robur* L. and *Quercus petraea*). The genus *Quercus* includes about 450 species with a large number of varieties and hybrids. The Oaks are widely distributed throughout the temperate regions of the Northern Hemisphere, in Europe, North America and Asia. In Europe, Oaks represent about 9% of the growing stock in forests, and cover 25-30% of the forested areas in some countries (France, UK, Romania, Belgium and Greece). In Ireland, Oak is linked to the ancient woodlands and the number of place names with 'derry' (the Irish for Oak) points to its widespread distribution. In the past, Oak constituted a significant part of the native woodland, which covered a large part of the country. Today, only a small area of this woodland survives.

Oaks are monoecious, deciduous trees and provide fine hardwood, valued not only for its great strength and durability, but also for its beauty. The wood of Oaks is hard, heavy and strong, and has a pronounced growth ring figure (Chapula 1995). Oak wood is used for furniture, paneling, house and ship construction. Only a small proportion of the land available for forestry is capable of growing broadleaf trees to timber size. Oak (also Ash, Beech and Chestnut) require reasonably fertile mineral soil at low elevation to produce good timber crops. The optimum soils for *Quercus* are deep, fertile, fine textured and slightly acidic.

1.2 Desirable Traits for Forest Trees

Forest trees have undergone relatively little domestication, consequently tree-breeding programmes could potentially have a great impact on forestry and forest products, much as happened with agronomic crops. However, biotechnological approaches, such as *in vitro* propagation, gene transfer and marker-assisted breeding have done much to bring the genetic improvement of forest trees to a level comparable to that of agronomic species. Some of the most problematic barriers to genetic improvement of forest trees, such as their large size and long breeding cycles, can be circumvented by the application of these new techniques. The trunk is the major harvested organ in forest trees, and breeding programs are usually aimed directly at improving trunk performance and wood quality. This includes biochemical modification of wood characteristics and trunk structure, increasing its growth rate

and altering its shape. Additional breeding targets include pest resistance, herbicide resistance and tolerance to abiotic stresses.

1.2.1 Genes for modifying lignin content and composition

Lignin represents about 25% of the global wood biomass and consists of three different basic molecules that are inter-linked and are also connected to other molecules in wood, such as cellulose. It is an important component of all vascular plants and occurs in high quantities in the secondary cell walls of fibres, xylem vessels and tracheids, providing them with mechanical support, playing a role in vascular conductivity and helping in the plant's defence against pathogens (Boudet et al. 1995). However, lignins represent a serious obstacle to efficient paper and pulp production, for which they must be removed in an energy-consuming process that involves the use of polluting chemicals (Biermann 1993). On the other hand, the chemically complicated lignins are energy rich relative to other wood components and a high lignin content is thus an advantage when wood is exploited as an energy source. Genetic modification to reduce lignin content in wood, or alter its chemistry to enhance extractability, are expected to increase the quality and efficiency of pulping and decrease mill effluents (Halpin et al. 1994). The presence of syringyl units, carrying two methoxy groups, makes hardwoods, in general, a better choice for paper production because the lignin is more easily extractable using chemical pulping techniques (Chiang et al. 1988). This is in contrast to the high proportion of condensed *para*-hydroxyphenyl units in the compression wood of softwoods, which decreases lignin hydrolysis efficiency. Thus, changes in lignin composition are also desirable, in addition to a reduction in lignin content.

1.2.2 Genes for altering tree form, quality and performance

The world's wood supply is currently shifting from old-forest resources to relatively new, intensively managed and fast-growing plantations. This transition is resulting in lower wood quality and yield, because of a reduction in tree age, a smaller stem diameter and a higher incidence of wood defects (Zobel 1984). The definition of wood quality varies according to the end use of the wood (e.g. structural lumbar for construction requires a high wood density, while such density is less desirable for packaging lumbar), but foresters usually define wood quality in terms of density,

absence of knots and uniformity (Zhang et al. 1997). In many cases, bacterial genes have been used to alter plant form and performance (Gaudin et al. 1994). The use of auxin biosynthetic genes from *Agrobacterium tumefaciens* significantly affected several wood characteristics in transgenic hybrid aspen, the transgenic trees were generally smaller than the controls, exhibiting reduced growth rate, leaf size and stem diameter (Tuominen et al. 1995). Although these changes seem undesirable for the improvement of wood quality, other changes, such as reduction in the number of side shoots following decapitation and changes in the xylem structure and composition, show some potential for transgenic trees expressing these genes.

1.2.3 Herbicide resistance

Herbicide-resistant transgenic crops are considered one of genetic engineering's major successes. In herbaceous crops, weeds compete directly with the crop for nutrients and water, and there is a constant need for weed control. Although such direct competition also exists in tree species, it is most important early in the tree's life cycle (1-3 years after planting). A mutant transgenic *Populus* species was created to exhibit a lower sensitivity to the herbicide glyphosate. This low-toxicity herbicide, which blocks 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase in the aromatic amino acid biosynthesis pathway, has a broad spectrum and is the active ingredient in many commercial herbicides (Fillatti et al. 1987). Overexpression of the *bar* gene, encoding the PAT enzyme (which directly inactivates the herbicide phosphinothricin) in transgenic poplar trees conferred resistance to the herbicide Basta (De Block 1990).

1.2.4 Insect resistance

Insects are responsible for substantial losses in forest-tree species and their damage can be a limiting factor for tree growth and survival (Klopfenstein et al. 1993). Different insects feed on different tree parts, e.g. bark beetles are the most damaging forest pest to mature trees, while spruce budworms prefer to attack buds. In practice, the use of insecticides is rather limited in forestry, owing in part to the large forest areas and tree sizes, to the development of resistance by insects and the environmental impact of the insecticides. Genetic engineering for insect control has been achieved in several annual plants, using either a Bt toxin (from *Bacillus*

thuringiensis) or insect-digestive-system-inhibitor genes (Shah et al. 1995). Feeding experiments with Bt-transgenic European Larch trees showed a decrease in the average weight of larvae fed on transformed needles relative to those fed on untransformed plants. Also, needle consumption was less, although no increase in larval mortality rate was observed (Shin et al. 1994).

1.2.5 Abiotic-stress tolerance

Cold, drought, salinity and heavy-metal toxicity are the main stresses specifically affecting trees, which are subjected to many annual changes in their life cycle. Genetic engineering for cold tolerance has been evaluated in several transgenic plants expressing an ice-nucleation gene from bacteria (Baertlein et al. 1992), antifreeze genes from fish (Georges et al. 1990) and altered lipid composition in their cell membranes (Murata et al. 1992). Cold tolerance in trees would enable the use of cold-sensitive species in northern areas as well as providing better protection of native plants from chilling damage. Drought and salinity tolerance is particularly important for forestation in arid and semi arid areas, where rain fall may be well below that required for tree growth.

1.3 Environmental/Abiotic Stress

Plant water deficit is a consequence of several different stresses, all of which affect uptake and conductance of water, thereby severely limiting plant growth (Bohnert and Sheveleva 1998). Drought, salinity and freeze-induced dehydration constitute direct osmotic stresses; chilling can indirectly cause osmotic stress via reduced water uptake and/or water loss. Plant response to these stresses is controlled by the expression of an array of genes associated with cellular protection. Such genes may offer protection by producing proteins which act as molecular chaperones, by increasing the production of osmoprotective compounds and/or by affecting membrane stability.

The cell membrane serves as an impermeable barrier to macromolecules and to most substances of low molecular mass. When extracellular solute concentrations are altered or extracellular ice forms, there is a flux of water from the cells, causing a reduction in turgor and a consequent increase in the concentration of intracellular

solutes, which exerts a strain on the membrane (Lichtentaler 1995). There are many plant responses to this stress, including production of osmoprotectant compounds and stress-inducible proteins, as well as changes in cell membrane and cell wall components. Plant cells also contain antioxidant enzyme systems, such as peroxidases and superoxide dismutases which scavenge reactive oxygen intermediates and reduce cellular damage under oxidative stress. Many attempts have been made to understand the molecular basis of tolerance to certain abiotic stresses. Enzymes responsible for the production of compatible solutes and radical scavenging have been cloned and characterised and used for genetic transformation of stress susceptible genotypes (Tarczynski et al. 1993, Kishor et al. 1995, Yoshida et al. 1995, Holmström et al. 1996, Lilius et al. 1996, Xu et al. 1996, Romero et al. 1997).

1.3.1 Osmotic Effects on Cells

Most microorganisms have optimal growth at low osmotic pressures, but even so are often able to grow, although more slowly, in the presence of above normal concentrations of environmental solutes. The membranes that encompass cells are readily permeable to water but present a more effective barrier to most other solutes (Csonka and Hanson 1991). Therefore, exposure of cells to high external osmolarity results in an efflux of water from the interior (Csonka 1989) and results in a reduction in the turgor pressure and a shrinkage of the cytoplasmic volume. As a consequence of the reduction in the cytoplasmic volumes, the concentrations of all the intracellular metabolites increase. In the absence of active osmotic adjustment by the cell, the cytoplasmic volume would shrink until the water activity of the interior equaled that of the exterior (Csonka 1989). Many organisms respond to osmotic stress by increasing the concentrations of a limited number of solutes (Csonka 1989). Consequently cell volume and turgor can be returned near their original values. Since the molecules that are accumulated during conditions of osmotic stress are not greatly inhibitory to cellular processes, they have been termed compatible solutes (Brown and Simpson 1972).

Most plants grow in environments that are, to a considerable degree unfavourable to plant growth. The National Academy of Sciences of the USA includes salinization of soils and waters as one of the leading processes contributing to a worldwide biological catastrophe. The deleterious effects of environmental stresses result from

both dehydration, which can denature many proteins or membranes, and ion displacement, in which the accumulating chemical compound replaces inorganic co-factors needed for some enzymes to work efficiently (Beatriz Garcia et al. 1997). Unlike most toxins and herbicides, excess NaCl or insufficient water has no single cellular target (Beatriz Garcia et al. 1997). The set of responses observed depends upon severity and duration of the stress, plant genotype, developmental stage and environmental factors providing the stress. (Bray 1993).

Plants require many salts for growth but the optimum concentration in the root medium is rather low for many plants, of the order of 10 molm^{-3} or less (Hazem Kalaji and Pietkiewicz 1993). Concentrations above this, even of essential salts, may cause a salt stress state, which reduce growth and yield (Nieman 1988). Salt tolerance is the ability of a plant to maintain the principle physiological process, especially growth, and confine its life cycle on highly salinized media.

Osmotic adjustment, one of the most important adaptive mechanisms assuring the maintenance of turgor (Morgan 1984), occurs under both salt and water stress, and was first observed in salinized plants (Flowers et al. 1977). As the cell membrane is permeable to water, it is not possible for cells growing in aqueous environment to avoid the osmotic stress of their surrounding solution. The first response to salinity is a loss of turgor and if this is severe enough, a loss of the ability to grow. Osmotic stress is one type of water deficit stress and can be of two types: (1) dehydration avoidance and (2) dehydration tolerance (Levitt 1980b). Dehydration tolerance allows survival with the loss of turgor, but the cell is in a non-growing state. Dehydration avoidance permits rehydration of the cell, return of cell turgor and recommencement of cell growth. This is only possible as a result of an increase in cell solute content. The increase must be sufficient enough to lower osmotic potentials (measured in the turgid state) below that of the aqueous environment. This process is called osmoregulation, osmotic compensation, or osmotic adjustment (Levitt 1980a).

1.3.2 Osmoprotective Compounds

Plants are often exposed to environmental stresses that result in water deficit. They deal with this problem through a number of responses, the primary response being the accumulation of compatible solutes to levels sufficient to maintain equal water

potential with the environment (Bohnert and Jensen 1996, Holmström et al. 1996, Hare et al. 1998 and references therein). It is generally accepted that the increase in cellular osmolarity, which results from the accumulation of compatible solutes, provides the turgor necessary for cellular expansion. However, since all subcellular substances must exist in an aqueous environment, tolerance to cellular dehydration also depends on the ability of cells to maintain membrane integrity and prevent protein denaturation.

Typically, compatible solutes are hydrophilic, giving rise to the view that they could replace water at the surface of proteins, protein complexes or membranes (Bohnert and Shen 1999). Recent observations indicate that compatible solutes also function in the protection of enzyme and membrane structure and in the scavenging of radical oxygen species. Amino acids and their derivatives, sugars, acyclic and cyclic polyols, fructans and quaternary amino and sulfonium compounds frequently act as compatible solutes (Levitt 1980, McCue and Hanson 1990, Delauney and Verma 1993, Bartels and Nelson 1994, Bohnert and Jensen 1996).

Compatible solutes act as osmolytes in response to stress, that is, they accumulate to high concentrations to alleviate the effects of high ion concentrations on protein stability and enzyme activity. However, some solutes, such as trehalose, do not respond to stress by accumulating to high amounts, but are protective even at low concentrations (Mackenzie et al. 1988, Holmström et al. 1996). When present at low, osmotically insignificant concentrations, such solutes may function in mechanisms that are non-osmotic in nature, such as radical oxygen scavenging (Shen et al. 1997a, b). While the net increase of solutes lowers the osmotic potential of the cell which supports the maintenance of water balance under osmotic stress, the net lowering of the solute potential may not be the only, or even the essential function of a compatible solute.

A common effect of many stresses is to cause oxidative damage, i.e. production of reactive oxygen species (ROS) including superoxide, H_2O_2 , hydroxyl radicals and singlet oxygen. ROS are toxic and their action can result in oxidative damage of proteins, membrane lipids and other cellular components (Asada 1994). Hydroxyl radicals are often associated with causing oxidative damage during drought or chilling stress (Shen et al. 1997b). It has been reported that proline, mannitol, sorbitol and pinitol are active scavengers of ROS, while glycine betaine is not

effective in ROS scavenging (Smirnoff and Cumbes 1989, Orthen et al. 1994). The accumulation of osmoprotectants has long been a target for plant genetic engineering, and in many cases introduction of a single foreign gene into a transgenic plant has led to accumulation of a particular osmoprotectant and a consequent increase in stress tolerance. These osmoprotectant compounds include proline, glycine betaine, mannitol and trehalose, all of which will be discussed in detail later in the text

1.3.2.1 Proline

1.3.2.1.1 Introduction

Proline belongs to the class of 'glutamate family' amino acids, whose biosynthesis depends on the ability of the carbon skeleton of glutamic acid to give rise to glutamine, proline, arginine and ornithine (Conn et al. 1987). A specific system of enzymes with special properties has evolved to mediate the metabolism of proline. These metabolic interconversions among proline, ornithine and glutamate have pyrroline-5-carboxylic acid (P5C) as the sole common intermediate. Accumulation of proline is a late adaptive response in plant tissues under salt stress and proline production has been proposed to have a multi-component effect in stress tolerance (Delauney and Verma 1993, Hare and Cress 1997). Under high osmotic conditions, proline acts as a mediator of osmotic adjustment (Handa et al. 1986), as a stabiliser of subcellular structures (Schobert and Tschesche 1978), as a scavenger of free radicals (Smirnoff and Cumbes 1989), and as a stress-related trigger for plant biosynthetic pathways (Werner and Finklestein 1995). The accumulation of proline may be part of a general adaptation to adverse environmental conditions such as low temperature, nutrient deficiency, exposure to heavy metals and high acidity. Many of these stresses do not have a significant osmotic component and although both chilling and freezing reduce the availability of water, it may be that increases in proline levels are independent of changes in tissue water balance, although there is no clear consensus on the precise role(s) of proline. Besides its stabilising effect on cellular structure, proline can also detoxify free radicals by forming adducts with them (Smirnoff and Cumbes 1989). Oxidative stress is likely to result whenever environmental conditions block the normal dissipation of the light-induced high-energy state. The reduction in the rate of CO₂ assimilation under adverse environmental conditions results in exposure of chloroplasts to excess excitation energy and increases the rate

of formation of ROS (Smirnoff 1993). It has been shown that proline enhanced the photochemical electron transport activities of isolated membranes of *Brassica juncea* by arresting photoinhibitory damage via its ability to scavenge free radicals (Alia et al. 1991).

1.3.2.1.2 Pathways for the biosynthesis and metabolism of proline (See Figure 1.1)

In bacteria, such as *E.coli*, *P.aeruginosa*, *S.typhimurium*, proline synthesis from glutamate is catalysed by three enzymes: γ -glutamyl kinase (GK), glutamic semialdehyde dehydrogenase (GSADH) (also called γ -glutamyl phosphate reductase (GPR) and Δ^1 -pyrroline-5-carboxylate reductase (P5CR) encoded by genes proB, proA and proC, respectively (Csonka and Baich 1983, Deutch et al. 1982, 1984, Hayzer and Leisinger 1980, 1981, 1983, Smith et al. 1984). The pathway begins with the ATP-dependent phosphorylation of the γ -carboxy group of L-glutamic acid (L-Glu) by GK. The product of GK is reduced to glutamic- γ -semialdehyde (GSA) by GSADH, with which GK forms an obligatory enzyme complex. GSA cyclises spontaneously to form pyrroline-5-carboxylate (P5C), which is finally reduced to proline by P5CR. In *E.coli* proline synthesis is regulated by allosteric inhibition of GK, the first enzyme in the pathway, by the end product, proline. Various mutations in proline overproduction have been mapped to the proB gene (Csonka 1989) and some of these mutations confer enhanced resistance to osmotic stress in bacteria. The mutation proB74, which resulted in the most pronounced osmotolerance, was shown to involve a single nucleotide change in the proB gene (Csonka et al. 1988, Dandekar and Uratsu 1988) leading to the synthesis of a mutant GK enzyme 200-fold less sensitive to end-product inhibition (Smith 1988).

The accumulation of proline in wilted plant tissues was first observed by Kimble and Mac Pherson (1954) working with Rye grass. It has been suggested that, in plants, proline is synthesised either from glutamate or ornithine and that the pathway from glutamate is the primary route for the synthesis of proline under conditions of osmotic stress and nitrogen limitation, while the pathway from ornithine predominates at high levels of available nitrogen (Delauney and Verma 1993, Kishor et al. 1995). The ornithine pathway proceeds via two routes, both involving transamination of ornithine followed by cyclisation and reduction. If the α -amino group of ornithine is transaminated, the product is α -keto- δ -aminovalerate

which cyclises to Δ^1 -pyrroline-2-carboxylate (P2C) and is then reduced to proline. Alternatively, transamination of the δ -amino group yields GSA, which is converted to proline via P5C. In the glutamic acid pathway, the GK and GSADH functions are contained in a single enzyme, Δ^1 -pyrroline-5-carboxylate synthetase, P5CS. Since GK is the rate-limiting enzyme in bacteria, interest has focused on the P5CS gene. Proline is synthesised from glutamate via Δ^1 -pyrroline-5-carboxylate synthetase (P5CS) and P5CR. A cDNA clone for P5CS was isolated from mothbean (*Vigna aconitifolia*) by complementation of a mutant of *E.coli*, and recombinant P5CS protein, expressed in *E.coli*, had both GK and GSA activities (Hu et al. 1992). In plants, the control of proline biosynthesis is therefore complex since there are two pathways which may be regulated at the level of both enzyme activity and gene expression. In comparison with most other amino acids, proline has the metabolic advantage of being the terminal product of a relatively short and highly regulated pathway. Proline accumulation therefore affects fewer metabolic reactions than the build-up of multi-use substrates such as glutamate, which are participants in many equilibrium reactions central to intermediary metabolism. Proline and its immediate precursor P5C are not interconverted by a single reversible enzyme, but by two distinct enzymes with different mechanisms and in different subcellular compartments, therefore the final product of the proline biosynthetic pathway is not necessarily in equilibrium with its immediate precursor. The biosynthetic pathway from glutamate involves an extremely high rate of consumption of reductants and also proline degradation is capable of high-energy output. The accumulation of proline appears to be an excellent means of storing energy since the oxidation of one molecule of proline can yield 30 ATP equivalents (Atkinson 1977).

The second important factor that controls levels of proline in plants is the degradation or metabolism of proline. L-proline is oxidised to P5C in plant mitochondria by proline dehydrogenase (PDH) (oxidase), which is an oxygen-dependent flavoprotein, and P5C is converted to L-glutamate by Δ^1 -pyrroline-5-carboxylate dehydrogenase, P5CDH. Both PDH and P5CDH are bound to the matrix side of the inner mitochondrial membrane. Proline accumulation induced by stress conditions is mediated by increased synthesis and reduced oxidation of the amino acid. Two P5CDHs have been found in plant mitochondria. One oxidises P5C

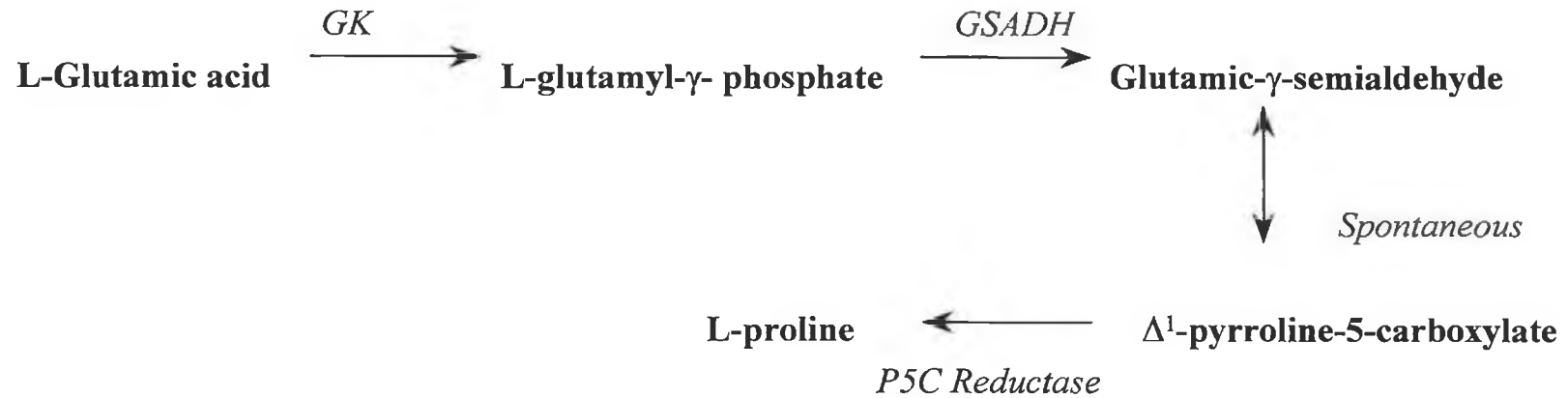
derived from proline and the other oxidises P5C from ornithine. Proline oxidation is believed to be responsible for transferring electrons into the first portion of the electron transport chain (Elthon and Stewart 1981, 1982). Oxidation of proline is inhibited during the accumulation of proline under water stress and is activated in re-hydrated plants (Rayapati and Stewart 1991). PDH and P5CDH catalyse reactions that are the reverse of those catalysed by P5CS and P5CR, respectively, in the biosynthesis of proline.

1.3.2.1.3 Transcriptional regulation of the level of proline under water stress

The gene for P5CS is induced by dehydration but is repressed by rehydration. Expression of the gene for P5CR is slightly up-regulated by dehydration. These results indicate that the induction of the gene for P5CS plays a major role in the biosynthesis of proline. The plant hormone ABA accumulates under environmental stresses such as drought, high salinity, and low temperature and it is involved in responses and tolerance to dehydration (Giraudat et al. 1994). Many genes that respond to water stress are also induced by the exogenous application of ABA (Bohnert et al. 1995, Ingram and Bartels 1996, Bray 1997). It appears that water stress triggers the production of ABA which, in turn, induces various genes. Because the gene for P5CS is also induced by the exogenous application of ABA, it seems likely that this gene is one of the many ABA-inducible genes. However, evidence for the ABA-independent expression of the gene for P5CS under dehydration conditions was also suggested by studies of an ABA-deficient mutant (Yoshida et al. 1995). These observations indicate, therefore, that the expression of the gene for P5CS is induced by two different pathways, an ABA-independent and ABA-dependent pathway, under dehydration conditions.

Proline is known to play an important role both as an osmoticum and as a substrate for the TCA cycle during recovery from stress, while the interconversions between proline and its precursors may be involved in the regulation of cellular pH and redox potential. Although turgor maintenance is the driving force for cell expansion and organ growth, it is important to note that these processes are under metabolic control. The rather energetically expensive process of proline accumulation might thus simultaneously maintain water balance while ensuring homeostasis necessary for the continuation of metabolic processes.

1. Bacteria



2. Plants

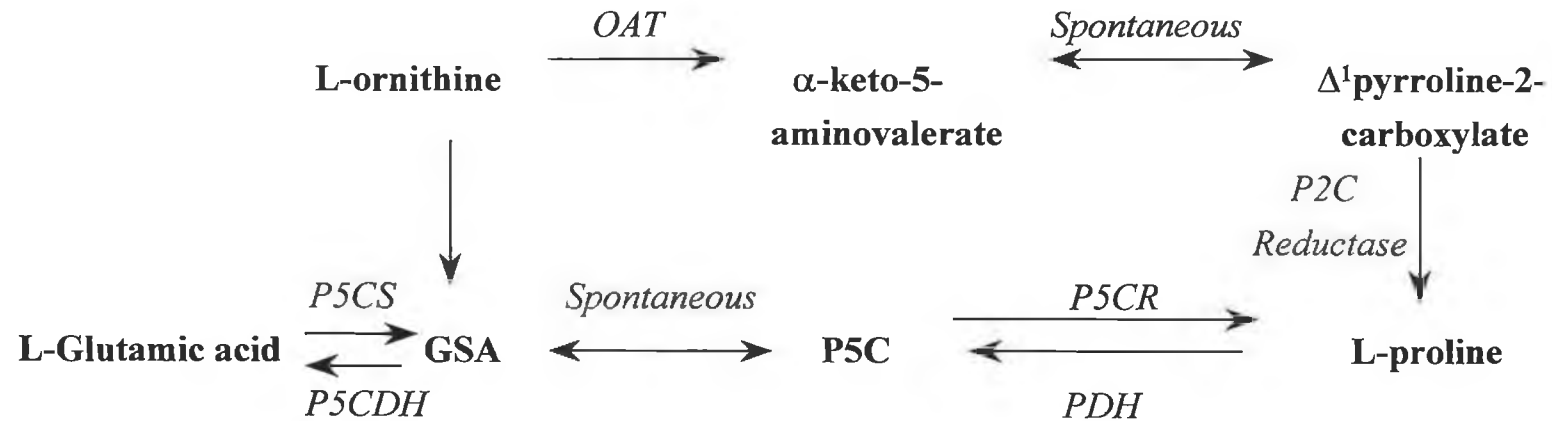


Figure 1.1 Proline Pathway

1.3.2.2 Glycine Betaine

Glycine betaine is synthesised in the chloroplast stroma through a two-step oxidation of choline via the unstable intermediate betaine aldehyde (Rhodes and Hanson 1993). It accumulates in the cytoplasm of many plants under osmotic stress (Wyn Jones and Storey 1981, Lilius et al. 1996, Hayashi et al. 1997) and may have a stabilising and protecting effect on proteins, enzymes and membranes under stress conditions. Glycine betaine is also involved in protection against low temperatures (Coughlan and Heber 1982, Zhao et al. 1992, Kishitani et al. 1994, Rajashekar et al. 1999), and has been shown to protect photosynthetic enzymes (Coughlan and Heber 1982), proteins and thylakoid membranes (Zhao et al. 1992) and electron flow in the Photosystem II complex (Naidu et al. 1991).

1.3.2.3 Trehalose

Trehalose does not respond to osmotic stress by accumulating to high concentrations, but is protective even at low concentrations (Mackenzie et al. 1988, Holmström et al. 1996). Although the trehalose protection effect remains unclear at the molecular level, evidence suggests that trehalose stabilises proteins and membrane structures under stress (Iwahashi et al. 1995). Trehalose has protein and membrane stabilisation properties superior to those of other sugars, and it is thought that this may be the dominant mode of action, rather than acting purely as an osmoprotectant.

1.3.2.4 Mannitol

Mannitol is accumulated by a wide range of species in response to drought and salinity (Stoop et al. 1996). Abiotic stress often leads to the overproduction of ROS causing extensive cellular damage and inhibition of photosynthesis. ROS can result in oxidative damage of proteins, membrane lipids and other cellular components (Asada 1994). The chloroplast is usually a rich source of ROS (removal of ROS is essential for chloroplast function), and mannitol has been recognised as a hydroxyl radical scavenger *in vitro* (Dhariwal et al. 1998).

1.3.3 Cold Acclimation

Many plants develop resistance to freezing temperatures during exposure to a period of low but non-freezing temperature. This complex process is known as cold or frost

acclimation and involves a number of biochemical and physiological changes. Analysis of these alterations is necessary in order to understand the metabolic basis of freezing tolerance of plants. Frost hardy species are distinguished from frost-sensitive species in that they have evolved numerous mechanisms to allow them to tolerate ice formation within their tissues. The woody perennial birch is an example of a plant exhibiting increased frost tolerance after cold acclimation (cold hardening). Whereas nonacclimated trees are severely injured or killed by temperatures around -10°C , trees that are fully cold acclimated can survive experimental freezing temperatures of -196°C . In their natural environments, these trees are often exposed to, and survive, temperatures of -40°C to -50°C (Thomashow 1990).

A number of biochemical changes occur in plants during cold acclimation. These include alterations in lipid composition, the appearance of new isoenzymes and isoforms of proteins and increased sugar and soluble protein content (Levitt 1980a, Thomashow 1990). Also, the accumulation of osmotically active, low molecular weight, non-toxic compounds (osmoprotectants) is reported to take place in response to freezing stress. The role of betaine, for example, in tolerance to low temperature has been demonstrated in both bacteria and higher plants (Kishitani et al. 1994). Betaine accumulated in cold stressed wheat seedlings (Naidu et al. 1991) and accumulation was shown to be induced in response to cold stress in barley (Kishitani et al. 1994). An increase in the concentration of the total amino acids in plant tissues exposed to low temperatures has also been reported (Draper 1972). Proline is a major constituent of this amino acid pool (Benko 1968, Draper 1972). The accumulation of proline at low temperature has been connected with plant resistance to frost injury (Benko 1968, Duncan and Widholm 1987). The biochemical, biophysical, and physiological changes that occur in plant cells during cold acclimation could be brought about by preexisting macromolecules and structures - enzymes, structural proteins, lipids, membranes - that undergo changes in their physical properties at low temperatures (Thomashow 1990).

'Expansion induced lysis' is one form of cell and membrane injury that occurs in response to relatively high freezing temperatures around -3 to -7°C (Steponkus 1984). As temperatures drop below 0°C , the extracellular water of the plant begins to freeze, resulting in a lowered water activity and an increased solute concentration in the extracellular spaces. In response to these changes in chemical and osmotic

potentials, water moves out of the cells causing severe dehydration and cell shrinkage. When the extracellular ice melts, the cells rehydrate and expand. If the cells are to survive, their plasma membranes must be able to withstand the efflux and influx of water. This occurs in cells that can frost harden, but non-acclimated cells lyse (Thomashow 1990). The differences in the biophysical properties of plasma membranes from cold-acclimated and nonacclimated plants suggests that the biochemical composition of the plasma membrane is altered during cold hardening. Plasma membranes from acclimated and non acclimated rye seedlings had different protein profiles, with acclimated plants having significantly higher phospholipid-to-protein ratios (Steponkus 1984).

1.3.4 Stress Inducible Proteins

The transcription of genes encoding late-embryogenesis-abundant (LEA) proteins occurs during the maturation and desiccation phases of seed development and also in vegetative tissues during periods of water deficit (including drought, low temperatures and high salinity). LEA proteins have been classified into three major groups, based on their common amino acid sequence domains (Dure et al. 1989). It has been postulated that they protect cells and other proteins against dehydration by maintaining protein and membrane structure, sequestering ions, binding water and acting as molecular chaperones (Dune et al. 1993). In response to water deficit there is an increase in endogenous ABA levels which rapidly limits water loss through transpiration. Increasing ABA is responsible also for the induction of many genes which protect the cell against water deficit, including the LEA protein genes (Skriver & Mundy 1990). Expression of a LEA protein gene (*HVA1*) in Barley occurs during cold acclimation and in seedlings in response to ABA and water deficit (Hong et al. 1992).

1.3.5 Scavenging of Reactive Intermediates

Salt, freezing and drought stress are accompanied by the formation of reactive intermediates, also known as reactive oxygen species (ROS). These toxic molecules damage membranes, membrane-bound structures and macromolecules, especially in mitochondria and chloroplasts (as these are the source of oxygen radical production) resulting in oxidative stress (Ishizaki-Nishizawa et al. 1996). Production of ROS

leads to singlet oxygen, superoxide, hydrogen peroxide and hydroxyl radicals. Many tolerant plants have antioxidant defences consisting of enzymes, such as superoxide dismutases (SODS), peroxidases, catalases and glutathione reductases which can scavenge oxygen radicals. When removing superoxide radicals, SOD and peroxidases/catalases often work in concert. Immediately following stress, ROS concentrations increase and there is upregulation of mRNA transcript and protein levels, as well as accelerated turnover of detoxification systems components (McKersie et al. 1996, Van Camp et al. 1996, Nocter and Foyer 1998).

1.4 Forest-Tree Biotechnology

Forests are very important to the world economy and for preserving and maintaining our ecosystem. The global need for wood is not expected to decrease in the near future, in fact, the demand for several wood products (especially paper, pulp and energy) is expected to increase. Plantation forestry, with optimised and increased forest productivity is likely to become the major source for wood products, and so accelerated tree improvement programs, combining traditional- and molecular-breeding techniques, along with large scale clonal propagation of superior clones are key elements for the successful reforestation and management of future commercial forests.

In contrast to other agronomic and horticultural species, the long generation time of forest trees has been the main obstacle to breeding. Other obstacles include limited knowledge of the genetic maps of most forest trees and also the problem of identification of suitable parents and the technical difficulties involved in their controlled mating. Forest trees have undergone relatively little domestication, consequently biotechnology could potentially have a greater impact on forestry products than it has had on agronomic crops. Biotechnological approaches, such as *in vitro* propagation, gene transfer and marker-assisted breeding, have done much to bring about the genetic improvement of forest trees.

1.4.1 Micropropagation

There have been major advances over the past 25 years in the development of operational vegetative propagation for woody species. Micropropagation allows for

the production of a large number of plants from small pieces of a stock plant in relatively short periods of time. Depending on the species, the original tissue explant may be taken from shoot tip, leaf, lateral bud, stem or root tissue. The three major procedures are rooted cuttings, adventitious shoot formation and embryogenesis.

1.4.1.1 Rooted cuttings

Rooted cuttings are one of the most effective propagation techniques, 65 million conifer cuttings are produced annually (Grossnickle et al. 1996). Production of rooted cuttings is a 2-step process: the production of cutting-donor plants and the production of rooted cuttings. Rooted cuttings of forest tree species are most successfully produced from juvenile portions of donor plants because these portions of a plants will provide cuttings with the potential for good initiation of root primordia (Hackett 1988). Cuttings are placed in a rooting environment (high humidity and soil moisture, warm soils and moderate light levels), allowed to develop roots, then treated as rising 1-year-old seedlings.

1.4.1.2 Adventitious shoot formation

Adventitious shoot formation (ASF) is a tissue culture system that relies on the *de novo* formation of shoot meristems formation or induced meristematic primordia (Sharp et al. 1983). These primordia are rapidly dividing groups of cells in which there is presumably a degree of organisation conducive to differentiation. After organ initiation, several additional steps, including shoot elongation, rooting and acclimation, are usually required before suitable propagules for planting are produced.

1.4.1.3 Embryogenesis

Somatic embryogenesis (SE) is regarded as the *in vitro* system of choice with the best potential for the propagation of superior and genetically engineered forest tree genotypes. Embryogenesis studies have highlighted the development of the embryo, which passes through the globular, oblong, heart, torpedo and cotyledonary stages, and eventually to the mature embryo. Embryogenesis can arise from gametic or somatic cells either naturally or by induction (de Vries et al. 1988). The zygote is

intrinsically embryogenic, however SE requires the induction of embryogenic competence in cells that are not naturally embryogenic.

The classical method of somatic embryogenesis (SE) through the development of proembryonic masses from somatic cells (as seen in carrot and other members of the umbelliferae) is now very well understood. However, this method of embryogenesis is rarely seen in forest trees (*Q.suber* seems to be the exception). In both angiosperms and gymnosperms, somatic embryos are routinely developed from pre-existing immature embryos. In angiosperms, the embryos arise as secondary embryos from embryogenically-determined cells on the surface of pre-existing immature embryos. In gymnosperms, embryos proliferate by cleavage polyembryony, a commonly observed natural phenomenon in seeds of gymnosperms. At early stages, it may be possible to see several Embryonal Suspensor Masses (ESMs), all originating from one point. Only one survives because of constraints of space and nutrients in the developing seed. It is very difficult to form embryos from somatic cells in the gymnosperms.

The term somatic refers to embryos developing asexually from vegetative (somatic) tissue. Embryogenesis can be either (i) direct, where somatic embryos originate directly from an explant without a callus phase or (ii) indirect, where somatic embryos originate after a proliferation of callus tissue. SE proceeds, directly or indirectly, after the exposure of a responsive explant to critical concentrations of exogenously supplied hormones during the initial culture phase. Subsequent development of the immature somatic embryos into mature embryos proceeds after transfer to a secondary culture media with increased ABA levels (Hakman and Von Arnold 1985).

Zygotic embryos are those formed by the fertilised egg, whereas somatic embryos are defined as those formed by sporophytic cells either *in vitro* or *in vivo*. Somatic embryogenesis is analogous to zygotic embryogenesis, however in the case of somatic embryogenesis a single cell in a small group of vegetative cells is the precursor of the embryo.

1.4.1.3.1 Somatic embryogenesis in conifers

SE has been successful for the angiosperms for about 40 years, but developed much later for the gymnosperms. The type of SE known in conifers is described as cleavage polyembryony, where immature embryos are cleaved from the seed and placed in tissue culture systems with the appropriate nutrients and hormones. The first successful reports of somatic embryogenesis in the conifers were in 1985 with European Larch (*Larix decidua* Mill.) (Nagmani and Bonga 1985) and Norway spruce (*Picea abies* L. Karst) (Hakman and Von Arnold 1985). Since then, cultured explants of at least 18 different coniferous species (including the genera *Abies*, *Larix*, *Picea*, *Pinus*, *Pseudotsuga* and *Sequoia*) have been induced to produce somatic embryos. Explants of conifers that have been induced to form somatic embryos include female gametophyte, immature and mature embryos and cotyledons, however older explants do not readily become embryogenic (Attree et al. 1991, Taurus 1991). The development of SE of conifers can be divided into 4 steps: 1. Initiation of embryogenesis 2. Proliferation of the embryos 3. Maturation of the embryos 4. Regeneration of plants.

In 1988 a numbering system was devised which was used to describe the various stages of somatic embryo development (Dunstan et al. 1988). Stage 1 embryos were described as a repressed pro-embryo with translucent suspensor and semi translucent, densely staining embryo with an irregular outline. Stage 2 embryos were described as prominent and smooth in outline, opaque cream to pale yellow in colour and subtended by a suspensor. Stage 3 embryos have primordial cotyledons clustered around a central meristem and are cream and pale green in colour. Finally stage 4 embryos have distinct, partly elongated cotyledons clustered around a central meristem and are green in colour. Later stages in development included elongation of both cotyledons and hypocotyls and the development of a radicle occurring are referred to as germinating embryos.

1.4.1.3.2 Somatic embryogenesis in *Quercus*

In Oak, conventional propagation deals with a low rooting ability and seed storage is limited to two years due to high susceptibility to infections related to the high water contents of the acorns (Endemann and Wilhelm 1999). Therefore, somatic embryogenesis has become important for *in vitro* propagation. The type of SE is

known as embryogenic cluster formation, where cells on the surface and sub-surface of embryos are induced to form somatic embryos (secondary embryo formation), usually due to a high concentration of cytokinin. The initiation of somatic embryos in *Quercus* is greatly dependent on the type of explant used. Immature zygotic embryos are often used as they have been shown to be highly embryogenic. Again, somatic embryos can develop either from subcultured embryogenic tissue or directly from explants without involving any intermediate callus stage.

Embryogenic cultures of *Quercus robur* and *Quercus petraea* were originally initiated from immature zygotic embryos (Chapula 1985b, Chapula 1990, Chapula 1993), as were embryogenic cultures of *Quercus rubra* (Gingas and Lineberger 1989) and *Quercus serrata* (Sasamoto and Hasori 1992). A serious problem of Oak regeneration via somatic embryogenesis is either an absence, or a low frequency of somatic embryo conversion into plantlets. Even somatic embryos that appear normal often fail to complete germination and conversion into plantlets. More research is needed to improve the process of maturation and conversion of somatic embryos into plantlets.

The two techniques, cleavage polyembryony and secondary embryo formation, both also provide a means for the recovery of a whole, fertile plant from a single genetically transformed cell. Genetic transformation of cells also requires a means of transferring the gene to the cell and also a method to select for transformed cells.

1.4.2 Genetic engineering of woody species

Plant genetic transformation - the controlled introduction and expression of foreign genes in plants - has become a common technique both for basic research and for the introduction of novel traits into commercially important species. Early reports on the genetic transformation of forest trees focused on *Populus* species. Even today, *Populus* remains the principal genetically transformed tree species, both as a model species and for practical reasons. For conifers, reliable and efficient methods of *in vitro* regeneration and clonal propagation are still lacking, and this is a serious obstacle to their genetic engineering.

1.4.2.1 *Agrobacterium*-mediated transformation

Strategies for the production of transgenic plants using the soil bacteria *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* rely on their natural ability to form tumours and hairy root disease respectively. The *Agrobacterium* system is attractive due to the ease of the protocol, combined with low equipment costs. Also, transgenic plants obtained by this method often contain single gene inserts.

The presence of wounded plant cells triggers a cascade of molecular events mediated by the virulence (*vir*) region of the tumour inducing or Ti plasmid of *A. tumefaciens*. The *vir* region encodes proteins which sense and respond to the presence of phenolic compounds (such as acetosyringone) released by wounded plant cells. *vir* proteins are responsible for the presence of a single-stranded DNA, the T-DNA, and its transfer to the plant cell. During tumour formation the T-DNA is transferred to the plant cell and stably integrated into the plant's own nuclear genome.

The Ri plasmid found to be present in *Agrobacterium rhizogenes* is not used as frequently as the Ti plasmid in plant transformation experiments. Under the control of a virulent region, two separate T-DNA regions of the Ri plasmid are transferred to the plant genome (Hoffman et al. 1984). These regions are termed the Tl (left T-DNA) and the Tr (right T-DNA). The Tr T-DNA contains genes for opine production and strains have been found to be characterised by their particular opine genes (De Paolis et al. 1985).

The T-DNA regions in both *A. tumefaciens* and *A. rhizogenes* are flanked by 25 base pair direct repeats and the end points of integrated T-DNA in the plant genome are found to be close to these sequences (Draper et al. 1982). The T-DNA itself is found to contain 8-13 genes, including a set for the production of phytohormones which are responsible for the formation of the characteristic tumours when transferred to infected plants (onc genes). When *Agrobacterium* comes in contact with the compounds released from the wounded plant tissue, transcription of the genes in the Vir region of the Ti-plasmid occurs. One specific chemical highly active in this respect has been identified - acetosyringone (Stachel et al. 1986). The Vir region of the Ti plasmid codes for six genes that are responsible for the transfer of T-DNA to an infected plant, these are Vir A, Vir B, Vir G, Vir C and Vir E. Vir A

and Vir G are expressed in vegetatively growing bacteria. When *Agrobacterium* is exposed to a wounded plant cell, or simply pure acetosyringone, the Vir A gene product is thought to recognise and interact with it and transmit the extracellular signal intracellularly, resulting in the activation of the Vir G gene product. The altered Vir G protein then activates the rest of the virulence genes, as well as elevating transcription from the Vir G locus. This Vir G induction is followed by the appearance of single stranded nicks within the 25 bp border sequences which flank the T-DNA (Stachel et al. 1986, Albright et al. 1987), and the appearance of a single stranded linear molecule which corresponds to the T-DNA. The Vir D operon is thought to encode this specific endonuclease activity (Yanofsky et al. 1986). By a mechanism which remains unknown, but is thought to be analogous to bacterial conjugation, the T-DNA is transferred to the plant cell and stably inserted into the nuclear DNA (Stachel et al. 1986).

The properties of *Agrobacterium* as a natural genetic engineer make it highly suitable for use as a transformation vector. The main features that make it amenable to exploitation in the construction of plant transformation vectors are:

- 1) The onc genes are not required for the transfer of T-DNA to the plant cell and its integration within the nuclear genome
- 2) The Vir region of the Ti and Ri plasmids function in trans
- 3) DNA inserted between the 25 base pair border repeats of the T-DNA, whether the borders are natural or synthetic, is transferred to the plant cell
- 4) No apparent rearrangements of the DNA located between the T-DNA borders takes place during the transfer to the plant genome
- 5) The foreign DNA integrated into the plant genome can be stably inherited in a Mendelian manner (Walden 1988)

Using Ti plasmid vectors any foreign DNA which has been inserted or cloned can be transferred into the genome of a dicotyledonous plant cell. The foreign DNA which is to be transferred must be flanked by the T-DNA border sequence and stably maintained in an *Agrobacterium* harbouring a full complement of Vir genes either in cis or trans. The removal of the onc functions means that transformed tissue is no longer recognisable as neoplastic out-growths which can be selected by their ability

to grow on a medium lacking phytohormones, and some other means of selection of the transformed cells is therefore required.

Non oncogenic vectors can be divided into two types (1) cis acting (2) trans acting. This division is based on whether the T-DNA region flanked by 25 bp direct repeat sequences are carried on the same plasmid as the Vir genes, or on a separate plasmid. Cis-acting Vir genes are commonly referred to as co-integrative vectors, and they are based on wild-type Ti or Ri plasmids. Portions of the T-DNA have usually been removed or replaced by a novel sequence of DNA (often referred to as disarmed vectors). Often the region which has been removed encodes the onc function, and this allows for the regeneration of normal, non-tumourous plants using conventional procedures of tissue culture. Binary, or trans acting vectors are based on plasmids which are capable of replicating both in *E.coli* and strains of *Agrobacterium*, and they contain the T-DNA border sequence flanking multiple cloning sites, as well as markers that allow direct selection of the transformed plant cell. These binary vectors allow manipulation in *E. coli*, followed by transfer to *Agrobacterium* by conjugation in the presence of a helper plasmid (Hoekema et al. 1983).

Gymnosperm species are less amenable to *Agrobacterium* infection, and although transient and stable expression has been reported in several species, the regeneration of stably transgenic plants has been limited to European Larch (Huang et al. 1991, Shin et al. 1994), Hybrid Larch (Levee et al. 1997) and Norway spruce (Wenck et al. unpublished). Poplar was the first hardwood species to be transformed with a herbicide resistance gene (Fillatti et al. 1987). It is still one of the few examples of tree species which are readily transformable with *Agrobacterium*. However, the successful infection of conifer species with *Agrobacterium* and the stable transformation of *Picea* species, including the regeneration of transgenic plants has been demonstrated (Sederoff et al. 1986, Stopm et al. 1990).

1.4.2.2 Viral Vectors

Viral genomes have the potential of being engineered into vectors which, once inside the plant cell, can replicate to high copy number and provide the possibility of expressing DNA at high levels. Much initial interest in producing vectors based on viral genomes was paid to cauliflower mosaic virus (CaMV) (Walden and Schell 1990). The potential advantages of using CaMV as a vector were that it was well

characterised, comprises a single or double-stranded DNA genome which, once cloned, remains infectious by mechanical inoculation. The transforming capacities of the Ti-plasmid-derived and viral vectors have been combined to create a technique called *Agro*-infection. The introduction of maize streak virus (MSV) into maize and of the separate A and B components from tomato golden mosaic virus (TGMV) into petunia have been reported for this technique (Harrison 1985).

Viral vectors provide some advantages for the introduction of foreign genes into plants. These include ease of infection, a different host range to *Agrobacterium* and a high number/expression rate of inserted genes under the control of appropriate promoters. However, the maximum size of passenger DNA insertable without affecting viral infectivity may be limited. There is also a possibility of high error frequency during viral RNA synthesis, which may lead to incorrect expression of the inserted gene.

1.4.2.3 Microprojectile bombardment

One of the most significant developments in the area of cell transformation has been the introduction of 'particle gun' or high velocity microprojectile technology, Biolistics. In this system, DNA is carried through the cell wall and into the cytoplasm on the surface of small (0.5 to 5 μm) metal particles that have been accelerated to speeds of one to several hundred meters per second (Gasser and Fraley 1989). These particles are capable of penetrating through several layers allowing the transformation of cells within tissue explants.

The first report of microprojectile bombardment to deliver DNA to living cells was in 1987. DNA was delivered by discharging a 0.22 caliber cartridge to accelerate tungsten microprojectiles carrying DNA through an evacuated chamber and into the cells (Klein et al. 1987). The main advantage of this system for DNA delivery is that, unlike *Agrobacterium*, it is suitable for a variety of monocots such as Barley (Wan and Lemeux 1994). Transient gene expression in pollen of Norway spruce (*Picea abies*) was achieved by particle acceleration (Martinussen et al. 1994). Stable genetic transformation of *Picea mariana* (Black spruce) was achieved via particle bombardment of cotyledonary somatic embryos and suspensions from embryonal masses (Charest et al 1996). Stable genetic transformation and regeneration

of White spruce was also achieved through microprojectile bombardment (Bommineni et al. 1993).

1.4.2.4 Microinjection

This technique is associated more with infection of animal cells, and plant cells are much more difficult to infect than animal cells for two reasons. Firstly, plant cells have a cell wall composing of relatively thick layers of pectin, hemicellulose and cellulose that are difficult for a glass micro-needle to penetrate. Secondly, the vacuole contains many hydrolases and toxic compounds. If the vacuolar contents are emptied into the cytoplasm, the cell will usually die. There are many reports however demonstrating that protoplasts can survive microinjection (Griesbach 1985, Lawrence and Davies 1985, Mowikawa and Yamada 1985, Reich et al. 1986).

1.4.2.5 Protoplasts and Electroporation

Protoplasts are ideal cells for DNA delivery, and for selection of transgenic plants. The most commonly used procedure for direct DNA delivery into protoplasts involves treatment with polyethylene glycol (PEG) to alter plasma membrane properties by causing reversible permeabilisation that enables exogenous macromolecules to enter the cytoplasm. The first reports of direct DNA delivery and stable transformation involved transfer and expression of *Agrobacterium tumefaciens* T-DNA genes into tobacco protoplasts via PEG treatment (Draper et al. 1982), and the first example of transgenic plants was reported by Paszkowski et al. 1984. Electroporation involve subjecting protoplasts to electrical pulses of high field strength to cause reversible permeabilization of the plasma membrane enabling macromolecule delivery. Stable transformation of tobacco plants regenerated from electroporated protoplasts involved combinations of PEG and electroporation treatments (Negrutiu et al. 1987). The primary advantages of electroporation over PEG or other chemical treatments are reproducibility, high frequency DNA delivery and simplicity of the technique (Jones et al. 1987). Electroporation is the use of high field strength electrical pulses to make plant cell membranes permeable in a reversible manner to facilitate transfer of DNA directly into cells (Fromm et al. 1986). There are three important factors affecting the process:

- 1) the strength of the electric field

2) the diameter of the protoplasm

3) the pulse decay time

The first reports describing DNA delivery to intact electroporated tissue were by Abdul-Baki et al. 1990 and Mathews et al. 1990 using tobacco pollen.

1.4.2.6 Electrophoresis

DNA was first introduced into intact embryos by designing an electrophoretic chamber in which the cathode was connected to a pipette containing DNA (Ahokas 1989). After electrophoresis, radioactive labeled DNA could be observed throughout the apical meristem and transient gene expression was observed. Electrophoretic-mediated DNA delivery is influenced by various physical factors associated with plant tissue. These factors include the frictional coefficient (f) which is a physical property of the cell wall and plasma membrane. Within an electrical field, DNA migrates through the cellulose fibers of the cell wall (Dekeyser et al. 1990, Lu and Ferl 1992) and pass through the plasma membrane.

1.4.2.7 Silicon carbide whisker transformation

DNA delivery into plant cells using silicon carbide whiskers involves vortexing a mixture of plasmid DNA encoding screenable and selectable markers, silicon carbide fibers and plant cells to be transformed (Kaeppeler et al. 1990). Silicon carbide fibers mediate DNA delivery because of their shape, size, strength and chemical composition. The fibers are single crystals with an average diameter of 0.6 μm and length ranging from 10 - 80 μm . Silicon carbide fiber DNA delivery requires very little preparation prior to DNA delivery. The procedure is rapid and inexpensive and may be used on most cell types provided that some prior investigation to optimize DNA delivery parameters is conducted. The advantage of this process is its simplicity and flexibility. The primary disadvantage however relates to its similarity to asbestos and the presumed health risk potential (Songstad et al. 1995). The production of fertile transgenic maize plants by silicon carbide mediated transformation has been reported (Frame et al. 1994), and it has also been found that materials with similar characteristics to silicon carbide whiskers, such as silicon nitride whiskers, can also deliver DNA into plant cells (Wang et al. 1995).

1.4.2.8 Selection and regeneration of transgenic plants

Regardless of transformation strategy, genetic markers set the criteria by which a plant cell is judged to be transformed, by providing novel nucleic acid sequences that can be detected by Southern analysis and unique assayable enzymatic activities. An increasing number of genetic markers are becoming available and include dominant selectable markers for the direct selection of transgenic tissue, screenable markers which allow the detailed analysis of gene expression and markers which allow for negative selection. Generally, the genetic markers that have been developed for use in plant cells were derived from either bacterial or plant sources, although their expression is directed by plant-specific promoters.

Genes affording resistance to antibiotics are the most commonly used marker genes. One of the most extensively used markers, neomycin phosphotransferase II (NPT II), conferring resistance to aminoglycoside antibiotics such as kanamycin, can be used as dominant marker with protoplasts, callus, tissue explants and whole plants. However, there is increased public concern over the use of antibiotic resistance genes as selectors of transformation. It may be possible to use other traits such as the ability to grow on 200 mM NaCl, or the ability to grow at 4°C. These traits are possible in plants genetically engineered to overproduce osmoprotectants such as proline, thus eliminating the need for antibiotic resistance genes.

Several reporter genes have been frequently used in studies of expression in higher plants. The two most useful reporter genes up to 1987 were the bacterial genes chloramphenicol acetyl transferase (CAT) and the neomycin phosphotransferase (NPT II) which encode enzymes with specificities not normally found in plant tissue (Fraley et al. 1983, Herrera-Estrella et al. 1983a and 1983b). However both CAT and NPT II are relatively difficult, tedious and expensive to assay. The firefly luciferase gene has also been used as a marker in transgenic plants (Ow et al. 1986). This enzyme, however, is labile and difficult to assay accurately but has been used as a marker for the transformation by particle acceleration of Norway spruce cultures (Martinussen et al. 1994).

By far the most widely used reporter gene since its discovery (Jefferson et al. 1987) has been the *E. coli* β -glucuronidase (GUS) gene. It has been the choice of reporter gene for a number of woody plant transformations (Roest and Evers 1991, Bommineni et al. 1993, Li et al. 1994, Frame et al. 1994, Charest et al. 1996, Drake

et al. 1997, Santarem et al. 1998). Many plants assayed to date lack detectable β -glucuronidase activity, providing a null background in which to assay gene expression. Also, β -glucuronidase is easily, sensitively and cheaply assayed *in vitro* and can also be assayed histochemically to localise GUS activity in cells and tissues or quantitatively using a fluorometric MUG assay. β -glucuronidase therefore fits most the criteria for a successful reported gene, it is encoded by the *UidA* locus and it is a hydrolase that catalyses the cleavage of a wide variety of glucuronidases. The Green Fluorescent Protein (GFP) gene is now becoming the reporter system of choice as it requires no substrates, just excitation with UV or blue light; therefore it can be assayed for non-destructively (Chalfie et al. 1994).

1.5 Genetic Engineering of Stress Tolerance in Plants

1.5.1 Osmoprotective Compounds

1.5.1.1 Proline

The role of proline as an osmoprotectant was demonstrated in *Salmonella oranienburg* (Christian 1955), where it was reported that exogenous proline could alleviate the inhibition of growth of *S. oranienburg* that was due to osmotic stress. It was reported subsequently that a wide variety of osmotically stressed bacteria accumulate proline (Measures 1975). Moreover, a mutation (*proB74*) in *E. coli* that resulted in the overproduction of proline also endowed a resistance to osmotic stress (Csonka et al. 1988). These observations indicated that proline can act as an osmoprotectant.

Eubacteria, protozoa, marine invertebrates and many plants (e.g. halophytes, spinach, potato, *Arabidopsis*, Alfalfa, Barley and Soybean) can all accumulate proline (McCue and Hanson 1990, Delauney and Verma 1993). Therefore, among compatible organic solutes it is possible that proline is the most widely distributed osmolyte. Genes encoding P5CS (Hu et al. 1992, Savoure et al. 1995, Yoshiba et al. 1995, Igarashi et al. 1997 and Strizhov et al. 1997) and P5CR (Delauney and Verma 1993 and Verbruggen et al. 1993) have been cloned from several plants and their expression studied under various abiotic stresses. The P5CS gene was cloned from *Vigna aconitifolia* under the CaMV35S promoter for genetic transformation of *Nicotiana tabacum* cv. Xanthi (Kishor et al. 1995). Some transgenic plants expressed a high level of P5CS protein and produced 10 to 18-fold more proline than the

control plants, resulting in enhanced biomass production under salt stress. Introduction of P5CS into rice (*Oryza sativa* L.), where gene expression was under the control of a stress-inducible promoter, led to stress-induced overproduction of P5CS and proline (Zhu et al. 1998). The elevated expression of an *AtP5CS* gene encoding the P5CS protein in *Arabidopsis* also preceded proline accumulation in response to water deficit (Yoshida et al. 1995). Where anti-sense *AtP5CS* cDNA was used, plants showed susceptibility to osmotic stress, were deficient in proline accumulation, wilted rapidly and exhibited morphological abnormalities. This is a critical finding suggesting a very important role for proline accumulation in osmotic stress tolerance. The application of exogenous L-proline (but not D-proline) suppressed each of these phenotypes (Nanjo et al. 1999). Physiological and morphological investigations have led to the conclusion that proline acts as a constituent of the major proteins that play a key role in osmotolerance as well as morphogenesis. These proteins are specifically the matrix proteins localised in the cell wall, and any defect in proline biosynthesis affects their biosynthesis. Therefore it can be seen that proline also has a unique function in morphogenesis as a major constituent of cell wall structural proteins in plants.

1.5.1.2 Glycine Betaine

Glycine betaine synthesis has been engineered into plants by introducing choline oxidising enzymes from bacterial or plant sources. In particular, the *betA* gene encoding choline dehydrogenase from *E.coli* has been introduced into tobacco, yielding salt tolerant phenotypes (Lilius et al. 1996). Stabilisation of cellular structures was proposed as the mode of action, as the concentration of glycine betaine was not thought to be sufficiently high (<1 mM) to facilitate balancing of the osmotic potential. Introduction of a choline oxidase gene (*codA*) from *Arthrobacter globiformis* into *Arabidopsis* created salt and freezing tolerant phenotypes. Protein production was targeted to the chloroplast using a transit peptide and higher glycine betaine concentrations were achieved, up to 50 mM, suggesting an osmoprotective role (Hayashi et al. 1997). At 300 mM NaCl the seeds of the wild type plants did not germinate, whereas all seeds of the transformed lines germinated, indicating that the *codA* transformants had increased tolerance for salt stress. Also, the transformed plants grew slowly at 200 mM NaCl, while none of the wild type plants grew. After

incubation at 400 mM NaCl for two days, the Photosystem II activity of control plants was inactive whereas the transformed plants had maintained more than 50 % activity. Accumulation of glycine betaine in any cell puts a demand on choline resources (as a glycine betaine precursor). Choline is usually directed almost exclusively to phosphatidyl-choline synthesis, making it difficult to divert choline to glycine betaine (Nuccio et al. 1998). Thus, the choline synthesis pathway must be more active in glycine betaine accumulators, with more choline being directed to glycine betaine synthesis.

1.5.1.3 Trehalose

In tobacco transformed with a construct containing the gene encoding the trehalose-6-phosphate synthase subunit (TPSI) of yeast trehalose synthase, trehalose was detected in the leaves, flower buds and roots of the TPSI positive transformants, although only at low and osmotically insignificant levels (Romero et al. 1997). Transformed tobacco contained 0.17 mg trehalose per gram fresh weight, compared to negligible amounts in control plants; the average tissue concentration of trehalose (<0.5 mM) was 5 to 10 times lower than in plants engineered for either mannitol (Tarczynski et al. 1993) or proline overaccumulation (Kishor et al. 1995) and appears too low to play a normal osmoprotective role. When present at such low concentrations, trehalose may function in a non-osmotic fashion for example by radical oxygen scavenging. A high proportion of transformants showed morphological changes (such as stunted growth, loss of apical dominance and sterility) and the alleviation of wilting symptoms in transgenic plants correlated with the extent of morphological changes. In transformants there was also a deviation from the normal carbohydrate profile suggesting changes in basic biochemical pathways (Romero et al. 1997). Similar studies by Holmström et al. (1996) yielded conflicting results whereby no phenotypic alterations in tobacco transformed with TPSI were observed but an osmoprotective effect of trehalose production was reported. In this case trehalose production was associated with a 30-50 % reduction in growth rate under conditions optimal for control plants.

1.5.1.4 Mannitol

Transgenic tobacco plants that synthesised and accumulated mannitol were produced by introducing an *E.coli mtlD* gene encoding mannitol-1-phosphate dehydrogenase (Tarczynski et al. 1993). The mannitol-overproducing tobacco exhibited an increased ability to tolerate high salinity in terms of maintenance of leaf and root growth after 30 days exposure. No differences were detected between control plants and mannitol producing plants in the absence of NaCl. Bohnert and co-workers have now produced transgenic tobacco, in which *mtlD* expression is targeted to the chloroplast (Shen et al. 1997a). Mannitol at concentrations less than 100 mM in chloroplasts specifically reduces damage by hydroxyl radicals, suggesting that mannitol could act as an antioxidant by scavenging hydroxyl radicals (Smirnoff and Cumbes 1989, Orthen et al. 1994). Formation of toxic hydroxyl radicals is linked to salt, freezing and drought stress and a reduction in the levels of these radicals may alleviate symptoms of water deficit stress (Shen et al. 1997a, b). It has also shown that mannitol was active specifically against hydroxyl radicals, and not against hydrogen peroxide or radical oxygen (Shen et al. 1997a, b). This is important as normal chloroplast detoxification systems exist that can deal with hydrogen peroxide and reactive oxygen, while there is no enzyme system that could deal with highly reactive hydroxyl radicals (Shen et al. 1997a, b). Targeting mannitol biosynthesis to tobacco chloroplasts enhanced the hydroxyl radical-scavenging capacity of the plants and increased their resistance to oxidative stress. It is therefore suggested that, in addition to its role in osmotic adjustment, the protective effect of mannitol in stressed plants may be related to its ability to scavenge hydroxyl radicals (Tarczynski et al. 1993, Kishor et al. 1995).

1.5.2 Cold Acclimation

One of the first signs of abiotic stress is often injury to specific membranes. Chilling and freezing both affect the membrane fluidity and disturb membrane-bound processes. Differences between the biophysical properties of plasma membranes from cold-acclimated and nonacclimated plants suggest that the biochemical composition of the plasma membrane is altered during cold hardening (Nishida and Murata 1996). There is a strong correlation between chilling sensitivity and the degree of unsaturated fatty acids in plastid membranes of various higher plants (Nishida and Murata 1996). The presence of *cis*-double bonds in the membrane lipid

lowers the chilling temperature. *Cis*-double bonds in saturated or mono-saturated fatty acids can be formed by introducing an enzyme capable of catalysing their formation. A broad specificity $\Delta 9$ -desaturase gene (*Des9*) was introduced into tobacco, which induces *cis*-double bonds in specific saturated fatty acids in membrane lipids (Ishizaki-Nishizawa et al. 1996). Levels of $\Delta 9$ -monosaturated fatty acids in the transgenic plants were significantly higher. Also when transgenic and wild type plants were held at 1 °C for 11 days, only the wild type plants developed chlorosis.

1.5.3 Stress Inducible Proteins

Rice plants transformed to express *HVA1* (a LEA protein) from barley displayed improved osmotic stress tolerance, as indicated by delayed development of damage symptoms (wilting, dying of old leaves, necrosis of young leaves) and improved recovery after stress. They also exhibited higher growth rates under water deficit and salt stress. These plants were found to express a large amount of HVA1 protein, up to 2.5 % of the total soluble proteins (no production in non-transformed), correlating to the level of water stress tolerance of the transformants (Xu et al. 1996). When the stress conditions were removed, the transgenic plants showed better recovery than did the control plants. Plants accumulating the highest levels of HVA1 protein performed better under stress conditions than the lower producers (<1 % of soluble proteins), and also showed better recovery. These results strongly support the hypothesised role of LEA protein in water-stress protection.

1.5.4 Scavenging of Reactive Intermediates

The transgenic enhancement of ROS scavenging components has been shown to positively affect plant performance during stress in some (McKersie et al. 1996, Van Camp et al. 1996, Roxas et al. 1997) but not all (Nocter and Foyer 1998) experiments. The Mn-SOD cDNA from *Nicotiana plumbaginifolia* has been introduced into alfalfa, being expressed either in the chloroplasts or mitochondria. In field trials yield levels and survival rates were significantly higher in the transgenic plants than in wild-types. It has been suggested that introduced SOD may function to simply lower the titer of free radicals in frozen tissues, or to stop the spread of oxidative stress after freezing. Alternatively, higher SOD activity may increase the

steady-state levels of hydrogen peroxide, which is proposed to function in some manner in promoting responses to stress. It is proposed therefore that, rather than representing a direct effect of the SOD on the plant cells, the results indicated that the overall stress-defence system in the plants was enhanced by the antioxidant produced. (McKersie et al. 1996).

1.6 Environmental problems and public concern over genetically modified plants

Transformation systems are now available in a wide variety of plant species and examples of transgenes for pest, viral, fungal, herbicide and environmental stress tolerance exist. With these new developments however comes the problem of gene escapes. The fear that genes inserted will spread to wild populations, or in the case of bacteria spread giving them an advantage or disadvantage in their natural environment, has become a real issue.

1.6.1 Antibiotic resistance

The selectable marker most frequently used in transgenic plants is Npt II, which confers kanamycin antibiotic resistance. The kanamycin gene continues to express in the regenerated plants, and would be present in any transgenic crop variety derived from them. Risk assessments of selectable marker genes have to date focused largely on the Npt II gene due to its presence in the Calgene Flavr SAVR® tomatoes (Flavell et al. 1992). In the health and safety arena one of the major apprehensions with the commercialisation of transgenic products has been the concern that selectable marker genes or their products may be toxic or allergenic when consumed. Additionally, when selectable markers for antibiotics which have clinical or veterinary applications are used, the concern has been raised that the marker gene could be transferred into micro-organisms and increase the number of resistant pathogenic micro-organisms in the human or animal gut (Yoder et al. 1994). The FDA evaluated these issues and found that there is no known mechanism by which genes could be transferred from a plant chromosome to a micro-organism (transgenic plants were fed to mice and the coliform bacteria isolated were examined for the presence of antibiotic resistance genes: none could be detected, and also transformation of gut bacteria was not

detected and naturally occurring horizontal gene transfer has not been demonstrated (Syvanen 1999). If such gene transfer were ever to occur, several approaches are available to eliminate antibiotic-resistance genes from transgenic plants:

1. Excision of the selectable marker gene
2. Use of herbicide resistance or hormones (cytokinins) as alternative selectable markers
3. Co-transformation of plants with two vectors, one carrying the marker gene and the other carrying the gene of interest

1.6.2 Foreign gene escape

Another common environmental concern is the escape of a foreign gene by pollen or seed dispersal, so creating super weeds or causing genetic pollution among other crops. Escape of foreign genes to wild relatives occurs predominately via the dispersal of viable pollen. However, it is possible, under exceptional circumstances, for the GM crop (engineered, for example, for herbicide resistance) to be fertilized by pollen from wild relatives and then serve as a female parent for a hybrid seed. If this happens the hybrid seed can germinate and establish a resistant population. However, for this to happen, the herbicide-resistant crop that acts as the female parent must escape harvesting, and the hybrid seeds must survive to germinate, grow and reproduce. Alternatively, dispersal of seeds from transgenic plants can occur among weedy relatives during harvest, transportation, planting and harvest. This can give rise to mixed populations or super weeds. Two of the most successful methods for containing gene pollution are male sterility and maternal inheritance. The prevalent method of plastid inheritance found in the majority of angiosperms is uniparental maternal, and chloroplast genomes are maternally inherited for most of the crops (Daniell et al. 1998). Engineering foreign genes through chloroplast genomes is a practical solution to this problem. Recently, maternal inheritance of a herbicide-resistant gene through chloroplast genetic engineering and prevention of escape via pollen has been demonstrated successfully (Daniell et al. 1998, Scott and Wilkinson 1999). Expression of genes, such as RNase, can prevent pollen formation and produce male sterile plants. However, male sterility is only possible in those crops which are cross-fertilised.

1.6.3 Restricting gene flow

Transgenic technology raises the issue of environmental biosafety and possible gene leakage from transgenic cultivars to related wild-type species. Trees are very close to their wild relatives (unlike many herbaceous plants) and are also planted in large, uncontrolled areas where they can exchange genes more easily with other plants. Gene flow from transgenic to wild-type plants is especially problematic in trees because they produce large amounts of pollen and seeds, which are easily dispersed over relatively long distances. The use of developmental-stage-dependent promoters, co-engineering for reproductive sterility, naturally sterile or low-fertility tree hybrids (e.g. triploid Hybrid Poplars), limiting the use of transgenic trees to nurseries and harvesting age should facilitate the commercial approval of such trees. Also, blocking the reproduction pathway might re-direct energy resources to vegetative growth, leading to accelerated growth and yield. For example, the average growth rate of Douglas fir is approximately 16% lower when the cones are being produced (Eis et al. 1965).

1.7 Introduction to Experimental Work

The experimental work undertaken in this thesis serves to add to the knowledge available in the literature in a number of ways. A number of studies have shown the effect of exogenous proline on cold and salt treated plant material, such as *Solanum* (Van Swaaij et al 1985) and maize (Duncan and Widholm 1987). In this experimental work, the effect of cold, saline and freezing stress on the growth and ion leakage (indicative of membrane damage) of Larch (*Larix X leptoeuropaea*), Sitka spruce (*Picea sitchensis* (Bong.) Carr.) and Oak (*Quercus robur* L.), as well as the effect of exogenously added proline will be examined. Both Sitka spruce and Larch have poor soil salt tolerance, a high water demand and are also damaged by spring frosts. Oaks require highly fertile soil, which is high in moisture in which to grow to timber size. An attempt will be made in the present work to demonstrate that exogenously applied proline could help alleviate the negative effects observed under the stated environmental constraints to a significant degree. If exogenously applied proline can be shown to protect the cells to a significant degree, it is proposed that genetically engineering proline overproducing cell lines of Larch, Sitka spruce and

Oak would provide these species with protection against cold, saline and freezing stress. Two proline overproduction genes will be obtained (Prof. DPS Verma, University of Ohio, OH, USA) and mated into *Agrobacterium tumefaciens*. Transformation experiments will then be carried out in an attempt to introduce the proline overproduction genes into Larch, Sitka spruce and Oak. Transgenic lines will be examined for cold, salt and freezing stress as well as proline production. Genetic analysis will also be carried out on the transgenic lines in the form of PCR reactions and Southern Blotting to determine their molecular characteristics, and to possibly link these to their proline producing capabilities.

Chapter 2

Materials and Methods

2.1 Plant Material, Culture Conditions and Media

2.1.1 Growth conditions

All material was grown at $24^{\circ}\text{C} \pm 2^{\circ}\text{C}$ in a constant temperature growth room, under warm white fluorescent light using a 16 hour photoperiod with approximately 20 $\mu\text{moles}/\text{m}^2/\text{s}$ of photosynthetically active radiation. Embryogenic suspension cultures were maintained on an orbital shaker at 100 rpm.

2.1.2 Larch (*Larix X leptoeuropaea*)

Embryogenic suspensor masses (ESMs) of Hybrid Larch were kindly donated by Dr. MA Lelu, Institut National de la Recherche Agronomique (INRA), Orléans, France. Controlled crosses were performed at INRA: *Larix leptolepsis* was crossed with pollen collected from *Larix decidua* (Mill.), the hybrid obtained being *Larix X leptoeuropaea*. ESMs were then induced from immature zygotic embryos of the Hybrid Larch (Lelu et al. 1994). One cell line (69.18) was maintained for this work. Suspension cultures were maintained in liquid MSG 0 medium (Becwar et al. 1990) (Table 2.1). Suspensions (50 ml) were maintained in sterile 250 ml Erlenmeyer flasks and subcultured every 10 days. Sub-culturing involved the transfer of 50 ml of fresh medium into a flask of cells and conditioned medium. The resulting 100 ml was then split into 2 X 50 ml quantities in 2 flasks. Transgenic cell suspensions (2 ml) were maintained in 10 ml Erlenmyer flasks and sub-cultured every 10 days. Callus material was maintained on solidified MSG in two ways. Firstly, as pieces of embryonal callus (approximately 8 per petri dish) which were routinely subcultured every 10-12 days by dividing the calli into smaller pieces and replacing on fresh medium. Secondly, pieces of embryonal callus were transferred into a sterile universal with liquid maintenance medium (MSG 0) and dissociated into a suspension. Then, embryonal masses were poured in a thin layer on Whatman number 2 filter paper (70 mm diameter), filtered under vacuum and subsequently the filters were placed on solidified proliferation medium. Cell mass on the filters was set at approximately 100 mg fresh weight cells/filter. Phytigel at $2 \text{ g}/\text{L}^{-1}$ was used as the solidifying agent.

Table 2.1 *Larix X leptoeuropaea* embryo maintenance medium (MSG 0 Medium)

Component	Quantity (gL⁻¹)
KNO ₃	1.0
CaCl ₂ .2H ₂ O	4.4
MgSO ₄ .7H ₂ O	3.7
KH ₂ PO ₄	1.7
KCl	7.4
FeNaEDTA	0.4
BAP (filter sterilise)	0.4
L-Glutamine (filter sterilise)	1.5
KI	8.3 mg
MSG Micro (100X)	100 ml
MSG Vitamins (100X)	100ml

Table 2.1a MSG Micro elements

Component	Quantity (mgL⁻¹)
H ₃ BO ₃	620
MnSO ₄ .H ₂ O	1690
ZnSO ₄ .7H ₂ O	860
Na ₂ MoO ₄ .2H ₂ O	25
CuSO ₄ .5H ₂ O	2.5
CoCl ₂ .6H ₂ O	2.5

Table 2.1b MSG vitamins

Component	Quantity (mgL⁻¹)
Inositol	10,000
Nicotinic Acid	50
Pyridoxine HCl	50
Thiamine HCl	10

2.1.3 Sitka spruce (*Picea sitchensis* (Bong.) Carr.)

Sitka spruce ESMs were kindly donated by Dr D Thompson, Coillte Laboratories, Co. Wicklow, Ireland. They had been raised from immature embryos of Sitka spruce clones and one cell line (574F) was maintained for this work. Suspensor masses were maintained in Sitka spruce embryo initiation medium (Gupta and Durzan 1986) (Table 2.2). Suspensions were maintained and subcultured as outlined for Larch. Embryogenic callus was maintained on solidified embryo initiation medium, in both manners as outlined for Larch (except at a concentration of approximately 150 mg fresh weight cells/filter). Phytigel at 4 gL⁻¹ was used as the solidifying agent.

Table 2.2 *Picea sitchensis* (Bong.) Carr. embryo initiation medium

Component	Quantity (mgL ⁻¹)
KNO ₃	2034
CaCl ₂	220
CoCl ₂ .6H ₂ O	0.025
FeNaEDTA	36.7
KI	8
Na ₂ Mo.O ₄ .2H ₂ O	0.25
Myoinositol	1000
2,4-D	1.1
NH ₄ NO ₃	206
KH ₂ PO ₄	85
MgSO ₄	185
CaSO ₄ .5H ₂ O	0.025
H ₃ BO ₃	6.2
MnSO ₄ .H ₂ O	17
ZnSO ₄ .7H ₂ O	86
Kinetin	0.4
Sucrose	30000
Glutamine	450
Casamino acids	500
BAP	0.4
H-Vitamins	10mL ⁻¹

Table 2.2a H-Vitamin stock solution

Component	Quantity (mg100ml⁻¹)
Nicotinic acid	50
Thiamine	5
Pyridoxine-HCl	5
Glycine	20

2.1.4 Oak (*Quercus robur L.*)

Oak embryogenic cells were again kindly donated by Dr D Thompson, Coillte Laboratories, Co. Wicklow, Ireland. A single embryogenic cell line, CEF, was initiated from immature zygotic embryos of pedunculate Oak. Embryogenic cell suspension cultures were maintained on modified Murashige and Skoog medium (MS) (Murashige and Skoog 1962) (Table 2.3). Suspensions were maintained and subcultured as outlined for Larch. Callus material was maintained only on solidified medium as 6 embryonal pieces per petri dish, and subcultured every 14 days. Phytagel at 5 gL⁻¹ was used as the solidifying agent.

Table 2.3 *Quercus robur L.* embryo maintenance medium

Component	Quantity (mgL⁻¹)
Murashige and Skoog basal salt mixture	4400
Myoinositol	100
Sucrose	3000
Glutamine (filter sterilise)	200
BAP (filter sterilise)	1000
H-Vitamins	10 mL ⁻¹

2.1.4a Production of Oak (*Quercus robur L.*) single cells

Oak embryogenic suspension cultures were filtered through a 100 micron mesh and the resulting retentate was transferred to MS suspension medium containing a reduced cytokinin level (0.001 mg/L BAP). Culture aggregation was related to cytokinin concentration and under conditions of low cytokinin, the embryogenic callus became friable and single cells dissociated from them. These were sub-cultured as outlined previously, continuing the low cytokinin level, unless it was desired to produce embryogenic aggregations from the single cells, in which case the cytokinin level was returned to its original concentration.

2.2 Maturation and Regeneration

2.2.1 Larch (*Larix X leptoeuropaea*) maturation

Larch ESMs (on Whatman No. 2 filter paper, approximately 100mg cells/filter) were matured on Larch pre-maturation medium for one week. Following this, the filter containing ESMs was transferred to Larch maturation medium for 3 weeks. Mature embryos were then transferred to Larch germination medium, where they were maintained from this point onwards (Lelu et al. 1994a and 1994b).

Transformed cell lines were matured and germinated as per normal, except maintaining a ticarcillin/clavulanic acid concentration of 500 mgL⁻¹, cefotaxime at 250 mgL⁻¹ and kanamycin at 25 and after 12 weeks, 50 mgL⁻¹.

Table 2.4 *Larix X leptoeuropaea* maturation and germination media

Compounds	Pre-Maturation mgL ⁻¹	Maturation mgL ⁻¹	Germination mgL ⁻¹
MSG 0 (ml, 5X)	200	200	200
Sucrose	34,000	68,400	30,000
Activated Charcoal	10,000	-	-
Gelrite	4000	4000	4000
Glutamine (filter sterilise)	1500	1500	1500
ABA (μM, filter sterilise)	-	60	-
IBA (μM, filter sterilise)	-	1	-

2.2.2 Sitka spruce (*Picea sitchensis* (Bong.) Carr.) maturation

Sitka spruce ESMs were matured on Sitka spruce embryo development medium (Table 2.6). ESMs were cultured on embryo development medium in the dark for one week, and then for 7 weeks in the light. Following this, mature embryos were transferred to Sitka spruce embryo germination medium (Table 2.7) (Gupta and Pullman, 1991), where embryos germinated and produced roots and shoots.

Transformed cell lines were matured and germinated as per normal, except maintaining a ticarcillin/clavulanic acid concentration of 500 mgL⁻¹, cefotaxime at 250 mgL⁻¹ and kanamycin at 50 mgL⁻¹.

Table 2.5 *Picea sitchensis* (Bong.) Carr. embryo development medium

Component	Quantity (mgL ⁻¹)
As for embryo initiation except	
KNO ₃	1170
Arginine	40
Asparagine	100
Sucrose	30000
Activated charcoal	1025

The autoclaved medium was placed in a water bath at 42°C, ABA (50 mgL⁻¹) which had been dissolved in a few drops of NaOH was filter sterilised into the medium. The medium was incubated at this temperature for a further 10 min with swirling every 2-3 min.

Table 2.6 *Picea sitchensis* (Bong.) Carr. embryo germination medium

Component	Quantity (mgL ⁻¹)
NH ₄ NO ₃	360
Ca(NO ₃) ₂ .4H ₂ O	709
KNO ₃	506
KH ₂ PO ₄	272
MgSO ₄ .7H ₂ O	493
KCl	149
Phytigel	7000
Sucrose	30000

2.2.3 Oak (*Quercus robur* L.) maturation

Oak embryos were maintained on P24 maintenance medium (solidified with 8% Daishin agar) for four weeks prior to maturation. Small (<1 mm) white, translucent embryo clusters were transferred to P24 maturation medium (Table 2.3, Wilhelm E., Austrian Research Centre, per comm) for 5-7 weeks under fluorescent lighting. Once the cotyledons started to green they were transferred to a desiccation chamber with water at 27°C for approximately 5 days. Following desiccation, embryos were transferred to P24 germination medium, where they were maintained while shoots and roots formed.

Table 2.7 P24 Medium

Component	Maintenance	Maturation	Germination
	Volume/weightL ⁻¹	Volume/weightL ⁻¹	Volume/weightL ⁻¹
Sucrose	30000 mg	30000 mg	30000 mg
P24 macro nutrients (5X)	200 ml	200 ml	200 ml
P24 micro nutrients (100X)	10 ml	10 ml	10 ml
MS Vitamins	25 ml	25 ml	25 ml
MS FeEDTA	10 ml	10 ml	10 ml
Arginine-HCl	500 mg	500 mg	500 mg
Daishin Agar	8000 mg	10000 mg	8000 mg
BAP	0.2 mg	-	0.2 mg
IBA	-	-	0.4 mg

Table 2.7a P24 macro nutrients stock (5X)

Component	Quantity (mgL ⁻¹)
Ca(NO ₃) ₂ .4H ₂ O	6610
Mg(NO ₃) ₂ .6H ₂ O	1920
KH ₂ PO ₄	4080
K ₂ SO ₄	4360
NH ₄ H ₂ PO ₄	1440
KNO ₃	2530
NaCl	58
Store + 4°C	

Table 2.7b P24 micro nutrients stock (100X)

Component	Quantity (ml/100ml)
KI (60mM)	0.6
CuSO ₄ .5H ₂ O (4mM)	25
ZnSO ₄ .7H ₂ O (20mM)	20
CoCl ₂ .6H ₂ O (4mM)	0.5
Na ₂ MoO ₄ .2H ₂ O (4mM)	0.5
NiCl ₂ .6H ₂ O (1mM)	2
MnSO ₄ .H ₂ O (20mM)	10
H ₃ BO ₃ (50mM)	24
dH ₂ O to 100ml	
Store + 4°C	

Table 2.7c MS Vitamins

Component	Quantity (mgL ⁻¹)
Inositol	4000
Nicotinic Acid	20
Pyridoxine HCl	20
Thiamine HCl	4

Table 2.7d MS FeEDTA

Component	Quantity (mgL ⁻¹)
Fe EDTA	3760

2.3 Growth Methods

2.3.1 Settled Cell Volume (SCV) measurements of Larch (*Larix X leptoeuropaea*) and Sitka spruce (*Picea sitchensis* (Bong.) Carr.) embryogenic suspension cultures and Oak (*Quercus robur* L.) somatic embryo suspension cultures

Growth of Larch and Sitka spruce embryogenic suspension cultures and Oak somatic embryo cultures were assessed by recording the settled cell volume of cells in the flask (Gilissen et al. 1983). SCV's were recorded by placing flasks on a stand at a 45° angle (see Plate 2.1). Flasks were left to settle for 15 min, the chord length of settled cells from one end of the base of the flask to the other was measured in mm. Settled cell volume in 250 ml flasks was utilised for all exogenous proline experiments. For transgenic lines, 10 ml flasks were used due to difficulties experienced in obtaining sufficient quantities for experimentation in 250 ml flasks. SCV in 250 ml flasks was converted to ml using the following equation:

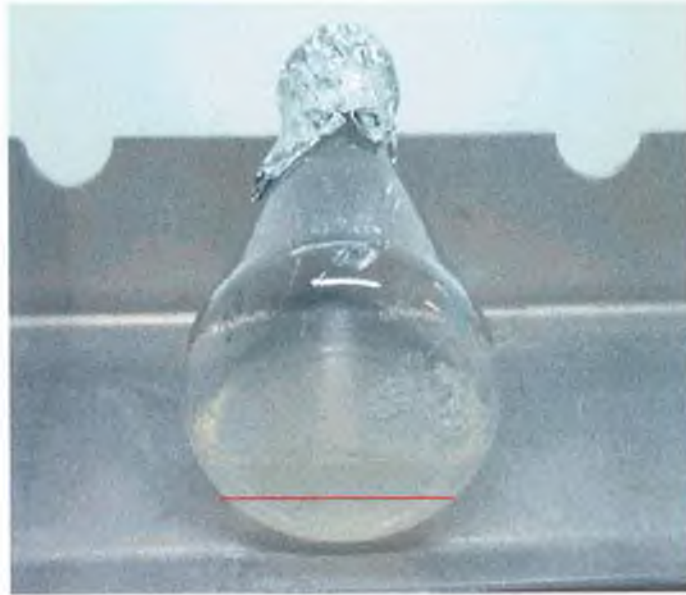
$$\text{SCV (ml)} = 10^{((x*0.0378)-1.3489)}$$

and in 10 ml flasks using the following equation:

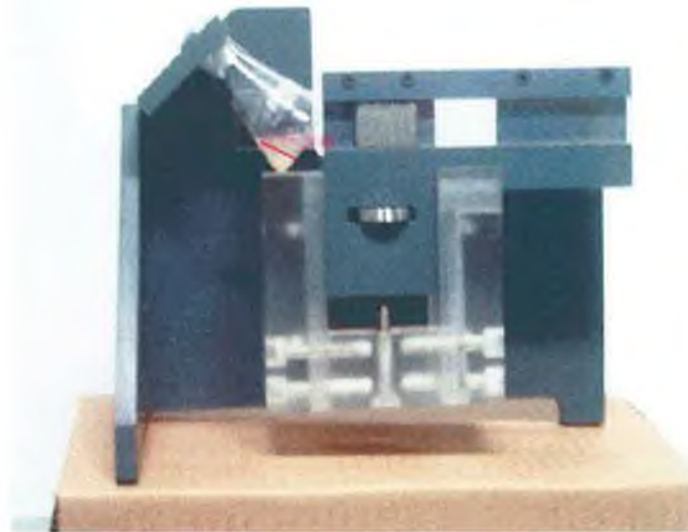
$$\text{SCV (ml)} = 0.0088 * 10^{(0.2665 * x)}$$

where x = chord length (mm)

Chord length was recorded along the underside of the 250 ml flasks and along the side of the 10 ml flasks. 250 ml flasks containing 50 ml cells plus medium were used in all stress experiments involving untransformed cells. 10 ml flasks containing 2 ml cells plus medium were used in all stress experiments involving transformed cells (due to restricted quantity of transgenic material available). Apparatus used for both measurements are shown in Plate 2.1.



(a)



(b)

Plate 2.1

Settled Cell Volume Measurement (a) in 250 ml flasks containing 50 ml cells plus medium and (b) in 10 ml flasks containing 2 ml cells plus medium. Measurement areas shown in red.

2.3.2 Growth of Larch (*Larix X leptoeuropaea*) and Sitka spruce (*Picea sitchensis* (Bong.) Carr.) embryogenic suspension cultures and Oak (*Quercus robur* L.) somatic embryo suspension cultures at 4°C and 24°C, with and without L-proline

Replicate embryogenic suspension cultures of Larch and Sitka spruce and somatic embryo suspension cultures of Oak were incubated at 4°C and 24°C with 0, 1, 10 or 100 mM proline. Proline was added at time of subculture and filter sterilised through a 0.2 µm filter. Flasks were maintained for 14 days and settled cell volume (SCV) was measured every 2 days (Gilissen et al. 1989).

2.3.3 Growth of Larch (*Larix X leptoeuropaea*) and Sitka spruce (*Picea sitchensis* (Bong.) Carr.) embryogenic suspension cultures and Oak (*Quercus robur* L.) somatic embryo suspension cultures under saline conditions, with and without L-proline

Replicate cultures of Larch, Sitka spruce and Oak were maintained at 24°C with 0, 50, 100, 150 or 200 mM NaCl. Proline was added to each salt concentration to give a final concentration of 0, 1, 10 or 100 mM. Cultures were maintained for 14 days, and SCV was recorded every 2 days.

2.4 Analytical Methods

2.4.1 Cold tolerance (electrolyte leakage)

Proline was added to replicate 5 day old cultures of Larch, Sitka spruce and Oak (untransformed only) to give a final concentration of 0, 1, 10 or 100 mM. After 48 h, cultures were sieved using a 200 µm nylon mesh and washed three times with distilled water. 500 mg samples were taken for each temperature (24, 0, -5, -10, -20 and -30°C). Tissue samples were placed in 15 ml polypropylene test tubes and transferred to an alcohol bath. The temperature (checked with an alcohol thermometer) was started at 0°C and held for 90 min. After this time replicate samples were removed. Thereafter the temperature was lowered in 5°C or 10°C intervals, maintained each time for 90 min, to -30°C. The cooling rate was 0.5°C per

minute. After 90 min at each temperature interval replicate tubes were removed, 10 ml of ice-cold distilled water was added to each and all tubes placed in the refrigerator overnight. The following day leakage of potassium (K^+) was measured from each of the samples using a Perkin Elmer 3100 atomic absorption spectrometer (AAS) fitted with a 10 cm single slot burner head, with an air-acetylene flame. For the 24°C sample, water was added at room temperature and K^+ analysis carried out as normal. K^+ concentrations were determined by reference to appropriate standard solutions.

K_I (initial potassium) was measured, the samples were then autoclaved for 5 min at 121°C and potassium levels were re-measured (K_F). Percent K^+ release was calculated as:

$$\% K^+ \text{ Release} = (K_I / K_F) * 100$$

2.4.2 Proline assay

Free proline was estimated using a modified ninhydrin method (Bates et al. 1973). 200 mg of cells were sampled in each case, all analysed in triplicate. Purified proline was used to standardise the procedure for quantifying sample values. Acid-ninhydrin was prepared by warming 1.25 g ninhydrin in 30 ml glacial acetic acid and 20 ml 6 M phosphoric acid, with agitation, until dissolved. Kept cool (4°C) the reagent remained stable for 24 hour. 200 mg plant material was ground in liquid nitrogen and then homogenised in 10 ml of 3% sulfosalicylic acid (w/v). The homogenate was centrifuged for 10 min at 13,000 x g. 2 ml of the supernatant was reacted with 2 ml of acid-ninhydrin and 2 ml of glacial acetic acid in a polypropylene test tube for 1 hour at 100°C, and the reaction was terminated in an ice bath. The reaction mixture was extracted with 4 ml toluene and mixed vigorously. The chromophore containing toluene was aspirated from the aqueous phase, warmed to room temperature and the absorbance read at 520nm. The proline concentration was determined from a standard curve and calculated on a fresh weight basis as follows:

μmoles proline/g fresh weight =

$$[(\mu\text{g proline/ml} \times \text{ml toluene}) / 115.5 \mu\text{g}/\mu\text{mole}] / (\text{g sample}/5)$$

2.4.3 Histochemical assay of β -glucuronidase activity

Cells were weighed and fixed in 1 ml of formaldehyde solution (0.3% formaldehyde, 0.3 M mannitol, 10 mM MES in 30 ml dH₂O) for 1 hr (Jefferson, 1987). To 1 ml of 50 mM NaPO₄ (pH 7.0), 1 ml X-gluc (5-bromo-4-chloro-3-indoyl β -D-glucuronic acid) and 10 μ l dimethylformamide was added. This solution was applied to a sample of plant material previously fixed in formaldehyde solution. Each sample was incubated overnight at 37°C, samples were then analysed for densely blue stained areas, staining indicated β -glucuronidase activity.

2.5 Transformation Methods

2.5.1 Triparental mating technique

Most manipulations of binary plasmid DNA for use in plant transformations are carried out in *E.coli*. Prior to plant transformation, it was necessary to transfer this plasmid to an appropriate strain of *Agrobacterium tumefaciens*, using an appropriate helper strain of *E.coli*. The transformation experiments carried out in this work utilised three plasmid vectors, one a variant of the pBin19 *Agrobacterium* binary vector, **pBI121** (kindly donated by Dr. John Draper, University of Leicester, UK), the second a variant of pBI121, **pBI-P5CS**, and the third a variant of pBI-P5CS, **pBI-P5CSF129A** (both kindly donated by Dr. DPS Verma, Ohio State University, Columbus, Ohio, USA). Triparental mating was carried out using *E.coli* HB101 helper strain. Three *Agrobacterium* strains were successfully created: LAB4404::pBI121, LAB4404::pBI-P5CS and LAB4404::pBI-P5CSF129A.

2.5.1.1 Strains used to create *A.tumefaciens* LAB4404::pBI121

E.coli donor strain Jm83::pBI121. This strain is kanamycin resistant and contains the cloning vector.

E.coli helper strain HB101::pROK2013. This strain facilitates mobilisation of the cloning vector.

A.tumefaciens recipient strain LBA4404. This strain is resistant to rifampicin.

2.5.1.2 Strains used to create *A.tumefaciens* LAB4404::pBI-P5CS and LAB4404::pBI-F129A

As for LAB4404::pBI121 except:

E.coli donor strain 1289::pBI-P5CS. This strain is kanamycin resistant and contains the cloning vector containing a pyrroline-5-carboxylate synthetase gene from a mutant *V.aconitifolia* species (for the overproduction of proline).

E.coli donor strain 1341::pBI-P5CS-F129A. This strain is kanamycin resistant and contains the cloning vector containing a site-directed mutagenesis of the P5CS gene for proline over production. Codon TTT at nucleotide positions 421 to 423 of the *V.aconitifolia* P5CS cDNA was changed to GCC by site-directed mutagenesis so that Phe (F) at amino acid position 129 of the P5CS polypeptide is replaced by Ala (A). The mutant enzyme retained similar kinetic characteristics as the wild-type P5CS, except that its allosteric regulation by proline is reduced.

2.5.1.3 Culture of strains

E.coli Jm83::pBIN19, 1289::pBI-P5CS and 1289::pBI-P5CSF129A strains were cultured on nutrient agar plates at 30°C containing 50 mgL⁻¹ kanamycin sulphate. Overnight cultures were prepared in nutrient broth supplemented with 50 mgL⁻¹ kanamycin sulphate.

E.coli HB101::pROK2013 was cultured on nutrient agar plates at 30°C. Overnight cultures were prepared in nutrient broth.

A.tumefaciens strain LAB4404 was cultured on nutrient agar plates at 25°C containing 100 mgL⁻¹ rifampicin. Overnight cultures were prepared in nutrient broth supplemented with 100 mgL⁻¹ rifampicin.

2.5.1.4 Triparental mating protocol

Note: all centrifugations carried out in microfuge tubes were performed in a Heraeus Biofuge 13, unless otherwise stated

In this protocol the *E.coli* strains Jm83::pBI121 and 1289::pBI-P5CS were used as the recipient in the first mating with and *E.coli* strain harbouring pRK2013, a helper plasmid acting as a donor strain. In this mating pRK2013 was transferred to the strain harbouring pBIN19 or PBI-P5CS. The second mating involved mobilising pBI121 or

PBI-P5CS by the pRK2013 helper plasmid to *Agrobacterium* LBA4404 which is the final recipient in the mating.

From overnight cultures, 0.7 ml of donor (pRK2013) and of recipient (Jm83::pBI121 or pBI-P5CS) were mixed together in sterile microfuge tube and centrifuged at 13,000 rpm for 5 min. The pellet was resuspended in 50 μ l of nutrient broth. This was then transferred to the surface of a sterile filter that had been placed on the surface of a nutrient agar plate. This was cultured at 30°C overnight. The filter paper was removed, place in a sterile microfuge tube with 1 ml sterile ringers solution. This culture then acted as the donor for the next stage of the mating. 0.7 ml of this donor was mixed with 0.7 ml of overnight recipient (LBA4404) in a sterile microfuge tube and centrifuged at 13,000 rpm for 5 min. The procedure was repeated as above, and when the mating was complete dilutions were plated on nutrient agar containing 50 mgL^{-1} kanamycin sulphate and 100 mgL^{-1} rifampicin. The plates were then cultured at 25°C overnight and colonies selected and restreaked the following day.

2.5.2 *Agrobacterium*-mediated transformation

2.5.2.1 Growth of *Agrobacterium* strains

Agrobacterium tumefaciens LBA4404::pBI121, LBA4404::pBI-P5CS and LBA4404::pBI-P5CSF129A were cultured on nutrient agar plates and nutrient broth suspensions (50 mgL^{-1} kanamycin sulphate, 100 mgL^{-1} rifampicin) at 25°C.

Agrobacterium tumefaciens LAB4404 was cultured on nutrient agar plates and nutrient broth suspensions (100 mgL^{-1} rifampicin) at 25°C.

2.5.2.2 Larch (*Larix X leptoeuropaea*)/Sitka spruce (*Picea sitchensis* (Bong.) Carr.) transformation protocol

Seven days prior to transformation, Larch or Sitka spruce ESMs were prepared for experimentation. 100 mg cell mass was plated onto Whatman number 2 filter papers and placed on MSG plates. These plates would yield approximately 300 mg seven days later. *Agrobacterium* LBA4404::pBI121, LBA4404::pBI-P5CS, LBA4404::pBI-P5CSF129A and LAB4404 streak plates were prepared 2 days prior to transformation.

The *Agrobacterium* was taken from the plates and resuspended in Larch or Sitka spruce liquid maintenance medium (without hormone addition) to obtain an OD of 0.3. This was then spread over the embryonal suspensor masses (until they were completely covered), and both cell types were pre-cultured for 6 hours. Following pre-culture, the filters were removed and blotted dry on sterile paper. The filters were then placed on fresh MSG plates, where the cells were incubated at 25°C for 2 days. Following incubation, the cells were removed from the filter papers and placed into Larch or Sitka spruce liquid maintenance medium (again without hormone addition). At this time, the cells were washed 3 times in liquid maintenance medium (10 minutes each wash), and another 3 times in liquid maintenance medium containing 500 mgL⁻¹ ticarcillin/clavulanic acid and 250 mgL⁻¹ cefotaxime. Following washings, the cells were filtered onto 100 micron nylon mesh (or Whatman No.2 filter paper), approximately 150 mg per filter. The filters were then placed onto solidified maintenance medium containing 500 mgL⁻¹ ticarcillin/clavulanic acid and 250 mgL⁻¹ cefotaxime. Cells were left on this medium until they had recommenced growth, and cell weight had doubled. This took approximately 10-12 days. Once cell weight had at least doubled, selection was applied. Kanamycin at 25 mgL⁻¹ was added to maintenance medium to select for Larch and 50 mgL⁻¹ for Sitka transformants (for cultures transformed with LBA4404::pBI121, LBA4404::pBI-P5CS and LBA4404::pBI-P5CSF129A). Cells were assayed for the continued presence of the *gus* gene periodically. Cells transformed with LBA4404 were grown on medium containing ticarcillin/clavulanic acid 500 mgL⁻¹ and cefotaxime 250 mgL⁻¹ only (negative control).

2.5.2.3 Oak (*Quercus robur* L.) transformation protocol

A 10 ml overnight culture of *Agrobacterium* was prepared in nutrient broth (containing 50 mgL⁻¹ kanamycin if required). On recording an OD of 0.8-1.0 the following day, the cells were sedimented and resuspended in 1ml Oak embryo maintenance medium. Oak single cells had previously been subcultured 7 days previously (50:50). The *Agrobacterium* was added to the Oak single cells, and they were incubated for 3 hours, 25°C at 100 rpm. After incubation, the cells were washed 3 times with Oak embryo maintenance medium. The cells were then co-cultivated with residual *Agrobacterium* for 2 days. Following co-cultivation, the cells were

washed in medium containing 500 mgL⁻¹ ticarcillin to remove the *Agrobacterium*. After 3 washes, cells were resuspended in medium containing 500 mgL⁻¹ ticarcillin/clavulanic acid. Cells were allowed to grow for 10-14 days, during which time growth was monitored. Once cells had resumed growth, kanamycin was added at 10 mgL⁻¹ (selective agent). Cells were then monitored and subcultured every 2 weeks in selective medium. Following a period of 2 months, where transformed cells were growing in selective medium, cells were placed on solid embryo maintenance medium containing 1 mgL⁻¹ BAP and 15 mgL⁻¹ kanamycin.

2.6 Molecular Methods

2.6.1 Solutions and Buffers

Table 2.8 TE Buffer

Component	Concentration (mM)
Tris-HCl	10
Na ₂ -EDTA	1
PH	8

Table 2.9 50X Tris Acetate EDTA (TAE) buffer*

Component	Quantity (ml)
EDTA (0.5M)	100
Glacial acetic acid	57.1
Tris	242g

*Dilute to 1X with dH₂O before use

Table 2.10 Solution 1 (Plasmid Prep.)

Component	Concentration
Lysozyme	4 mgml ⁻¹
Glucose	50 mM
EDTA	10 mM
Tris-HCl	25 mM

Table 2.11 Solution 2 (Plasmid Prep.)

Component	Concentration
SDS	1%
NaOH	0.2M

Table 2.12 Depurination Solution (Southern Blotting)

Component	Concentration (M)
HCl	0.2

Table 2.13 Denaturation Solution (Southern Blotting)

Component	Concentration (M)
NaCl	1.5
NaOH	0.5

Table 2.14 Neutralising Solution (Southern Blotting)

Component	Concentration (M)
Tris (pH 7.4)	1.0
NaCl	1.5

Table 2.15 Neutral Southern Transfer Solution

Component	Concentration
SSC	20X

Table 2.16 Prehybridisation Solution

Component	Concentration
SSC	5X
Denhardt's reagent	5X
SDS	0.5%
Salmon testes DNA	100 μ gml ⁻¹

Table 2.17 Hybridisation Solution

Component	Concentration
SSC	5X
Denhardt's reagent	5X
SDS	0.5%
Salmon testes DNA	100 μgml^{-1}
DIG-labelled probe	0.5 μgml^{-1}

Table 2.18 Wash Buffers (Post Hybridisation)

Buffer	Components
Wash Buffer 1	0.1%SDS, 2X SSC
Wash Buffer 2	0.1%SDS, 0.2X SSC
Wash Buffer 3	0.1% SDS, 0.16X SSC

Table 2.19 Blocking Buffer (Post Hybridisation Washes)

Component	Concentration
Tris (pH 7.5)	0.1M
NaCl	0.15M
Acetylated BSA	3% (w/v)

Table 2.20 Detection Buffer (DIG detection)*

Component	Concentration (M)
Tris-HCl	0.1
NaCl	0.1
MgCl ₂	0.05

* pH to 9.5

Table 2.21 Colour Substrate Solution

Component	Quantity
Detection buffer	10 ml
NBT/BCIP stock (10X)	200 µl

2.6.2 Agrobacterial Plasmid DNA isolation

1 ml of an overnight culture of each *Agrobacterium* strain to be examined was centrifuged, the supernatant discarded and the cells resuspended in 100 µl of ice-cold Solution 1. The solution was incubated at room temperature for 10 min. 200 µl of freshly prepared Solution 2 was then added, and the mixture incubated for a further 10 min at room temperature. 30 µl of phenol equilibrated with two volumes of Solution 2 was added and the mixture vortexed for approximately 10s. 150 µl of 3M sodium acetate (pH 4.8) was added, and the mixture was vortexed briefly. The solution was incubated at -20°C for 15 min, and then centrifuged for 3 min. The supernatant was placed in a fresh microfuge tube, ice-cold 95% ethanol was added, the tube was mixed by inversion and stored at -80°C for 15 min. The solution was then centrifuged for 3 min, the supernatant discarded and 500 µl 0.3M sodium acetate (pH 7.0) was added to resuspend the pellet. The tube was again filled with ice-cold 95% ethanol, mixed by inversion and the tube placed at -80°C for 15 min. Following

incubation, the solution was centrifuged for 3 min, the supernatant discarded and the tube inverted to drain liquid. The pellet was resuspended in 50 μ l TE buffer and stored at 4°C for further analysis.

2.6.3 Plant DNA Isolation

Plant DNA was isolated using the Qiagen DNeasy procedure (Qiagen UK, West Sussex, UK). In this procedure plant material is first mechanically disrupted and then lysed by addition of a lysis buffer at incubation at 65°C. RNase in the lysis buffer digests the RNA in the sample. After lysis, proteins and polysaccharides and salt are precipitated. Cell debris and precipitates are removed in a single step by a brief spin through QIAshredder™ (filtration and homogenisation unit). The cleared lysate is then transferred to a new tube and binding buffer and ethanol are added to promote binding of the DNA to the DNeasy membrane. The sample is then applied to the membrane while contaminants such as proteins and polysaccharides are efficiently removed by two wash steps. Pure DNA is eluted in a small volume of low-salt buffer.

2.6.4 Agarose gel electrophoresis

DNA was analysed by running on an agarose gels in a horizontal gel apparatus. Gels were prepared by dissolving agarose in 1 X TAE buffer to the required concentration (typically 0.7-1.2 %) and boiling until a clear solution was obtained. The 1 X TAE buffer was also used as the running buffer. A tracker dye was incorporated into DNA samples (2 μ l to 7 μ l sample) to facilitate loading of samples. Mini-gels were run at 100 Volts for 1-2 hour, or until the tracker dye reached the base of the gel. Gels were stained by immersing in a bath of ethidium bromide for 30 min, and then de-staining for 10 min in a water bath. Gels were then visualised on a UV transilluminator and images captured.

*All agarose gels shown in this thesis are 1.2% unless otherwise stated. A standard 1 Kb ladder was run on all gels to facilitate estimation of DNA size

2.6.4.1 Preparation of ethidium bromide

One 100 mg tablet of ethidium bromide was added to 1 ml dH₂O. The container was covered in aluminium foil and stored at 4°C. A 100 µl aliquot of this solution was added to 1 L of dH₂O for staining agarose gels.

2.6.5 Polymerase Chain Reaction (PCR)

Table 2.22 Standard PCR reaction mixture

Component	Quantity (µl)
MgCl ₂	3
Thermo-buffer (10X, MgCl ₂ free)	5
dNTP (100 mM)	2
Primers (100pmol/µl)	1 (of each)
Template DNA	3-5
Sterile dH ₂ O	34-32
Red-Taq polymerase (5u/µl)	1

The reaction was then over-layered with 50 µl of sterile mineral oil. *E.coli* or *Agrobacterium* plasmid DNA was used as positive/negative controls for PCR, as outlined in results. Total genomic plant DNA isolated using the Qiagen DNeasy kit was used as a source of template for PCR reactions to investigate transformation of plant material.

2.6.5.1 Standard PCR Program Cycle

Stage 1: **Step 1:** 95°C for 10 min

Stage 2: **Step 1:** 95°C for 1 min

Step 2: Annealing temperature for 30 sec

Step 3: 72°C for 1 min for every 1Kb to be synthesised

Cycle Stage 2: 30 times

Stage 3: **Step 1:** 72°C for 10 min

Table 2.23 Primer Sequences

Primer name	Primer Sequence								Conc. (nmol)	T _m (°C)	
	5'							3'			
VirE2F	ATG	GAT	CTT	TCT	GGC	AAT	GAG			66.7	60.1
VirE2R	TCA	AAA	GCT	GTT	GAC	GCT	TTG	G		84.2	64.2
RBCSF	ATG	GCT	TCC	TCT	ATC	ATG	GC			112.2	57.3
RBCSR	TTA	ATT	GTA	TTC	AGG	CTT	GTG	G		90.5	54.7
PSRBCLF	ATG	TCA	CCA	AAA	ACA	GAG	ACT			85.0	54.0
PSRBCLR	TCA	CAA	GGT	ATC	CAT	TGC	CT			155.9	55.3
NPTIIXF	ATG	GGG	ATT	GAA	CAA	GAT	GGA			42.1	55.9
NPTIIXR	TCA	GAA	GAA	CTC	GTC	AAG	AAG			61.6	55.9
GUSXF	GGT	GGG	AAA	GCG	CGT	TAC	AAG			44.8	61.8
GUSXR	GTT	TAC	GCG	TTG	CTT	CCG	CCA			146.9	61.8
CAP5CSF1	AAT	ATC	AAA	GAT	ACA	GTC	TCA	GAA G		37.7	56.4
CAP5CSR1	AAT	AAG	CTT	TGA	ATG	AGG	GTC	ACT T		49.4	58.1

2.6.6 Southern Blotting

2.6.6.1 Preparation and electrophoresis of DNA

Genomic DNA from untransformed (LN) and transgenic lines (L51 and LF7) of Larch was purified using the Qiagen DNA DNeasy kit. DNA concentration was determined by recording absorbance at 260nm. All genomic DNA was concentrated by freeze drying to achieve 10 µg DNA per 20µl digest. Genomic DNA was digested

with HindIII and resolved on a 0.6% agarose gel (approximately 10 µg DNA was used per digest).

2.6.6.2 Transfer of DNA from the gel to nitrocellulose

The gel was firstly depurinated and then submerged in denaturation solution for 2 x 15 min at room temperature. This denatured the DNA, making it single stranded and therefore accessible for the probe in later stages. The gel was rinsed in dH₂O, and then submerged in neutralisation solution for 2 x 15 min at room temperature. The DNA was then blotted from the gel by capillary transfer to the membrane using 20X SSC buffer (Neutral Southern Transfer Solution). Blotting was continued overnight to ensure efficient transfer of the DNA. The membrane was then baked at 80°C and stored at room temperature until hybridisation.

2.6.6.3 Hybridisation procedure

The probe was prepared by performing a PCR reaction using a CAP5CS forward primer that was labelled with DIG (digoxigenin). Asymmetric PCR was performed under conditions outlined for normal PCR (except using only one primer). The probe was purified and analysed by agarose gel electrophoresis prior to use as a hybridisation probe. The nitrocellulose DNA blot was soaked in 2X SSC until uniformly hydrated, then it was placed in a hybridisation container with 100 µl of prehybridisation solution per cm² of membrane surface area. Prehybridisation was performed at 42°C for 3 hours. The probe was diluted in hybridisation solution and the membrane was hybridised with the solution containing the probe at 62°C overnight. After hybridisation, the membrane was washed twice each in wash buffers 1 and 2, and once in wash buffer 3 (at room temperature). The membrane was then washed in blocking buffer, also at room temperature.

2.6.6.4 Detection of the DIG labelled probe

The membrane was incubated for 1 hour at room temperature in blocking buffer (200 µl per cm² of membrane). The anti-DIG-AP conjugate was diluted in blocking buffer

to 150 units per ml, and the membrane was incubated with this solution for 30 min. The membrane was washed in wash buffer 3 for 2 x 15 min, and then equilibrated in detection buffer for 2 min. The membrane was then incubated with 20 ml freshly prepared colour-substrate solution, sealed in a plastic bag and left overnight in the dark. The following day, the membrane was washed in deionised water to stop the reaction and the membrane was allowed to dry before being copied.

2.7 Kinetic Analysis

Kinetic analysis of data was carried out in a number of ways. Firstly, it was noted that when growth rate was plotted as a function of proline concentration, hyperbolic curves were obtained. These hyperbolas were typical of that normally associated with Michaelis-Menton kinetics. The data was then analysed in a number of ways to obtain estimates for V_{max} , the maximum growth rate and K_m , the concentration of proline that gives half maximal growth rate. In all cases, the data used was that obtained for either 200 mM NaCl or 4°C (both with no proline addition). These were the conditions that most closely resembled enzyme kinetics when no substrate is present.

2.7.1 Direct Linear Plot

The growth rate (v) was plotted onto a V_{max} vertical axis and the corresponding negative proline concentration (s) value was plotted onto a K_m horizontal axis. The two points were joined and the line was extrapolated into the ' V_{max} , K_m parameter space'. The process was repeated for all the (v,s) values. All lines intersected at the co-ordinates of the best fit V_{max} and K_m values.

2.7.2 Lineweaver-Burk Plot

In the Lineweaver-Burk plot, $1/v$ was plotted on the vertical axis and $1/s$ on the horizontal axis. A straight line was obtained from which the y-axis intercept equated to $-1/V_{max}$ and the x-axis intercept equated to $-1/K_m$. From this, both V_{max} and K_m could be calculated.

2.7.3 Hanes Plot

In the Hanes plot, s/v was plotted on the vertical axis and s on the horizontal axis. A straight line was obtained and the x-axis intercept equated to $-K_m$ and the y-axis intercept equated to K_m/V_{max} . From this, both V_{max} and K_m could be calculated.

2.7.4 Eadie-Hofstee Plot

In the Eadie-Hofstee plot, v was plotted on the vertical axis and v/s on the vertical axis. A straight line was obtained and the x-axis intercept equated to V_m/K_m and the y-axis intercept equated to V_{max} . From this, both V_{max} and K_m could be calculated.

2.7.5 EnzFitter™

EnzFitter™ is an iterative, non-linear, least squares fitting computer based method for estimating V_m and K_{max} .

2.8 Statistical Analysis

All statistical analysis was carried out using SPSS™ for Windows Version 8.0. Where more than two treatments were applied simultaneously, factorial analysis of variance (ANOVA) was used. Where there were interactions between the two treatments, the analysis was broken down for each level of treatment using one-way ANOVA. Comparison of different levels of a treatment was carried out using Post-Hoc testing by Tukeys' honestly significant difference.

Chapter 3

Growth of Larch (*Larix X leptoeuropaea*) and Sitka spruce (*Picea sitchensis* (Bong.) Carr.) embryonal suspensor masses and Oak (*Quercus robur* L.) somatic embryos in suspension supplemented with exogenous L-proline and subjected to environmental stress.

3.1 Introduction

During its life cycle, a forest tree must adapt to seasonal climatic changes and to a wide range of pests and abiotic stresses. Cold, drought, salinity and heavy metal toxicity are the main abiotic stresses specifically affecting trees (Tzfira et al. 1998). Cold tolerance in trees would enable the use of cold-sensitive species in northern areas, as well as providing better protection of native plants from chilling damage. Drought and salinity tolerance is particularly important for forestation in arid and semiarid areas.

The amino acid proline is thought to play an important role as an osmoregulatory solute in plants exposed to high levels of salt or drought (Delauney and Verma 1993, Guerrier 1997/8, Hare and Cress 1998). The accumulation of proline is also associated with plant responses to chilling (Chu et al. 1974, Duncan and Widholm 1987, Xin and Li 1993). Plants often overproduce proline in response to these abiotic stresses. For example, tobacco cells adapted to NaCl accumulate proline to 80-fold higher levels, and this is accounted for by increased synthesis (Rhodes and Handa 1989). Possible roles suggested for proline are: osmoregulation, protection of cellular membranes and enzymes and conservation of energy and amino groups for post stress growth (Aspinall and Paleg 1981).

A number of studies have shown the effect of exogenous proline on cold tolerance of species such as *Solanum* (van Swaaij et al. 1985) and Maize (Duncan and Widholm 1987) and also on osmotolerance (Santos et al. 1996). In the present work the effect of cold and salinity on the growth and membrane integrity of three forest species: *Larix X leptoeuropaea* (Larch), *Quercus robur* L. (Oak) and *Picea sitchensis* (Bong.) Carr. (Sitka spruce), and also the effect of exogenously added proline are examined. Oaks provide fine hardwood valued not only because of its great strength and durability, but also for its beauty (Gingas and Lineberger 1989). Sitka spruce produces a highly versatile softwood timber and very good paper pulp (John et al 1995). Foresters have long been familiar with the autumn frost damage on young Sitka spruce trees, which kills many plants each year. It has been shown that the growth and physiological condition of Oak was affected by low NaCl concentrations, when grown in culture (Alaoui-Sousse

1998). We therefore investigated the role of proline in environmental stress resistance in the three forest species previously mentioned.

3.2 Results

3.2.1 Growth of Larch (*Larix X leptoeuropaea*) and Sitka spruce (*Picea sitchensis* (Bong.) Carr.) embryonal suspensor masses and Oak (*Quercus robur* L.) somatic embryo cultures

Larch (*Larix X leptoeuropaea*) and Sitka spruce (*Picea sitchensis* (Bong.) Carr.) embryonal suspensor masses and Oak (*Quercus robur* L.) somatic embryo cultures were maintained at all times for the purposes of this research. Magnified cells are shown in Plate 3.1.

50 ml replicate cultures of Larch, Sitka spruce and Oak were maintained in 250 ml flasks at 24°C and at a rotating speed of 100 rpm. Settled cell volume (SCV) was recorded every 2 days and growth curves established (Figure 3.1)

Callus material was maintained on solidified maintenance medium in two ways. Firstly, as pieces of embryonal callus (approximately 10 per petri dish) and secondly as a thin layer on Whatman number 2 filter paper (70 mm diameter) which was subsequently placed on solidified proliferation medium. Cell mass on the filters was set at approximately 100 mg fresh weight cells/filter (Plate 3.2)



(a)



(b)



(c)

Plate 3.1

Embryogenic cells of (a) Larch (*Larix X leptoeuropaea*) and (b) Sitka spruce (*Picea sitchensis* (Bong.) Carr.), cell cluster of (c) Oak (*Quercus robur* L.). Images were taken using the OPTIMAS 6 image analysis programme under 10X magnification (Larch and Sitka spruce, bar = 40 μ m) and 4X magnification (Oak, bar = 100 μ m)

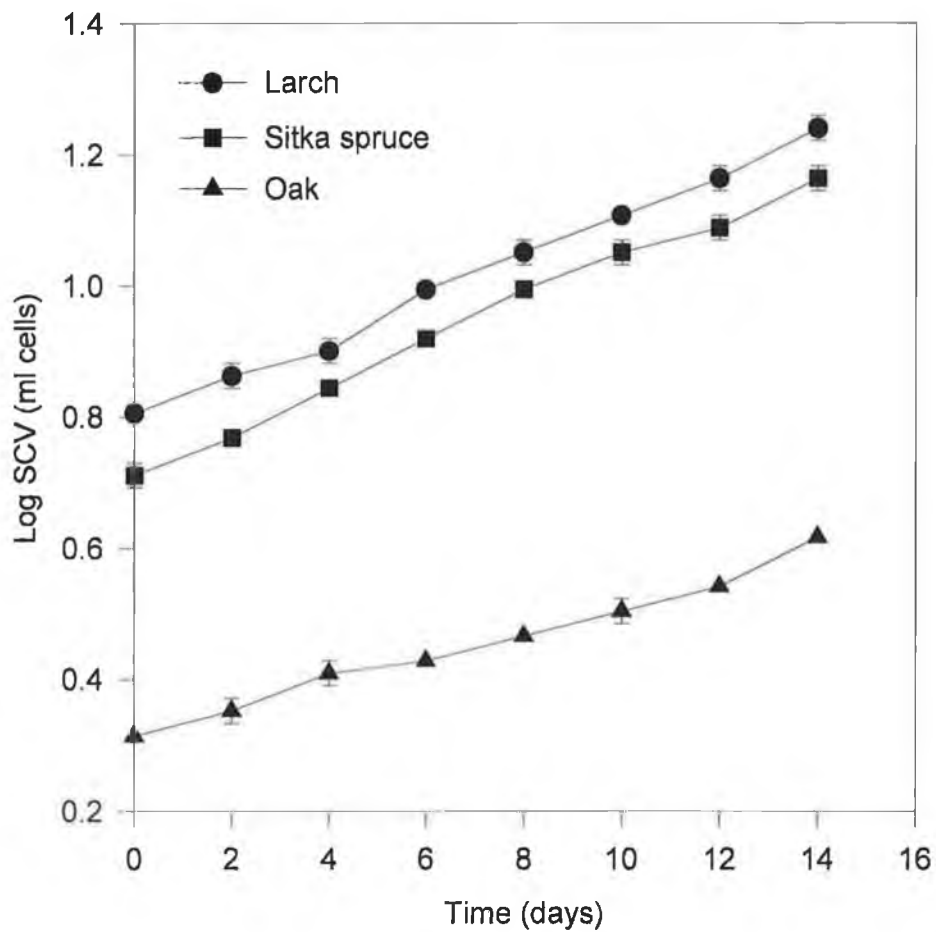
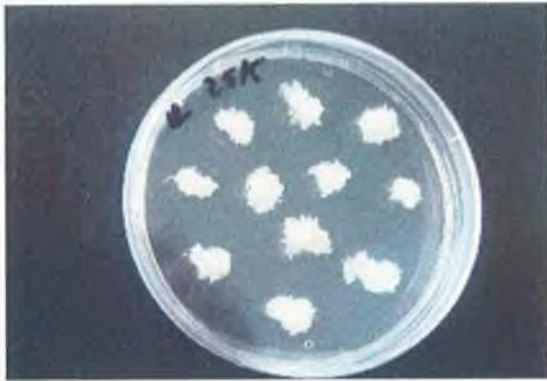
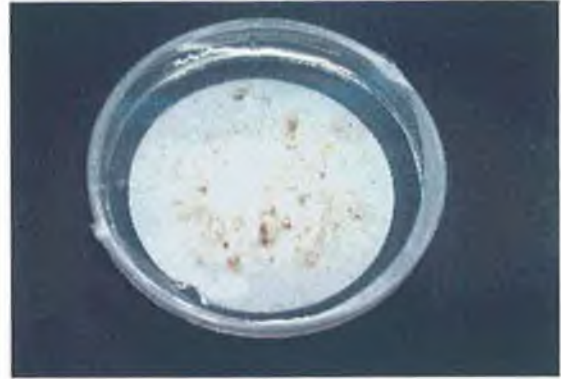
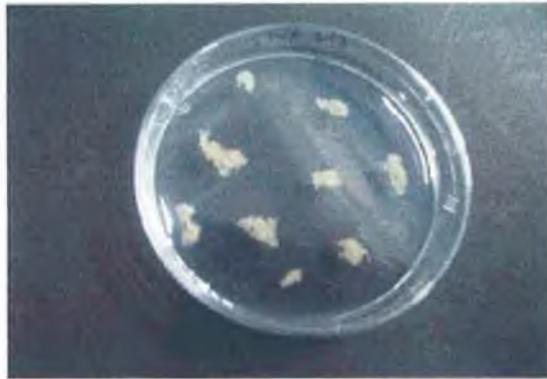


Figure 3.1

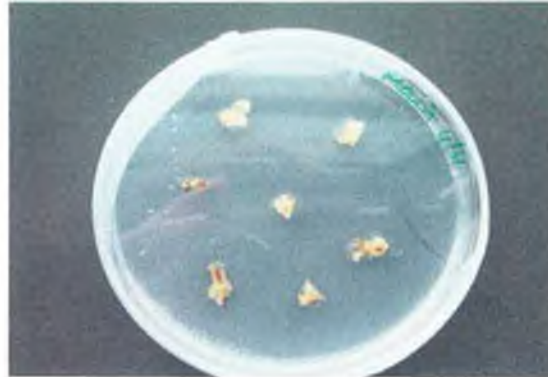
Growth of Larch (*Larix X leptoeuropaea*) and Sitka spruce (*Picea sitchensis* (Bong.) Carr.) embryonal suspensor masses and Oak (*Quercus robur* L.) somatic embryo cultures in suspension. Mean values \pm SEM are shown where these exceed the dimensions of the symbols.



(a)



(b)



(c)

Plate 3.2

Embryogenic cells (in 9 cm petri-dishes) of (a) Larch (*Larix X leptoeuropaea*) and (b) Sitka spruce (*Picea sitchensis* (Bong.) Carr.) maintained as callus and on filter paper. (c) Oak (*Quercus robur* L.) maintained as solid somatic material.

3.2.2 Influence of proline on the growth rate of Larch (*Larix X leptoeuropaea*), Sitka spruce (*Picea sitchensis* (Bong.) Carr.) and Oak (*Quercus robur* L.) embryonal suspensor masses at 4°C and 24°C

Replicate cultures of Larch, Sitka spruce and Oak were incubated at 4°C and 24°C with 0, 1, 10 or 100 mM proline. Proline was added through a 0.2 micron filter at time of subculture, flasks were maintained for 14 days and settled cell volume (SCV) was measured every two days (Gilissen et al. 1983).

Figure 3.2 shows the growth rates of Larch, Sitka spruce and Oak grown at 4°C and 24°C. Growth rates were determined over a 14-day period.

A temperature of 4°C, with no proline supplementation completely inhibited growth of all three species. Proline, even at a low concentration (1 mM), significantly increased the growth rate of all cultures at 4°C. For all three species, there was a very highly significant effect of proline and temperature, and of the interaction between them ($p < 0.0005$), with a very highly significant effect of proline at each temperature. Growth was most affected by the addition of 1 mM proline and further addition of proline to 100 mM did not result in a similar increase in growth rate. At 24°C, proline slightly increased the growth rate. Larch and Sitka spruce were more affected by the 4°C temperature than Oak. With 1 mM proline addition, Larch and Sitka spruce growth rate was approximately 33% that of normal growth, while Oak recorded a growth rate that was approximately 50% that of the normal Oak growth rate. At 4°C and 100 mM proline the growth of Oak cells almost reached that of normal cell growth, while for both Larch and Sitka spruce with 100 mM proline at 4°C growth only reached 50% of normal growth.

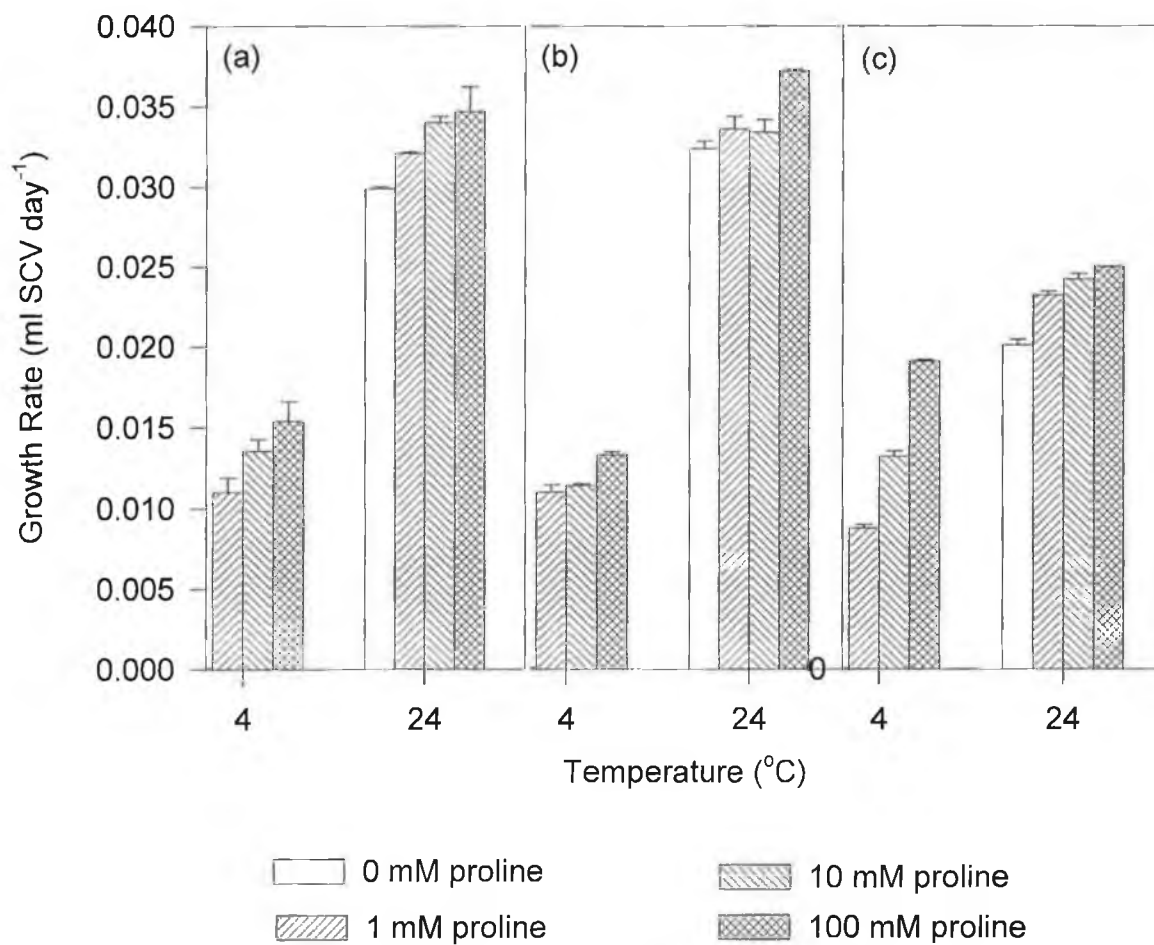


Figure 3.2

Influence of proline on the growth rate of (a) Larch (*Larix X leptoeuropaea*) and (b) Sitka spruce (*Picea sitchensis* (Bong.) Carr.) embryonal suspensor masses and (c) Oak (*Quercus robur* L.) somatic embryos at 4 °C and 24 °C. Mean values \pm SEM are shown where these exceed the dimensions of the symbols.

3.2.3 Influence of proline on the growth rate of Larch (*Larix X leptoeuropaea*), Sitka spruce (*Picea sitchensis* (Bong.) Carr.) and Oak (*Quercus robur* L.) embryonal suspensor masses in varying concentrations of NaCl

Replicate cultures were maintained at 24°C with 0, 50, 100, 150, 200 mM NaCl (Sigma-Aldrich). After addition of NaCl, pH was adjusted to 5.7 using molar KOH, and media then autoclaved. 0, 1, 10 or 100 mM proline was added to each salt concentration. Double concentrate of NaCl and proline was used in each case to provide the correct concentration after subculture (50% as outlined previously). Cultures were maintained for 14 days, SCV was recorded every 2 days.

Figures 3.3, 3.4 and 3.5 show the growth rates of Larch, Sitka spruce and Oak grown in varying concentrations of NaCl. Growth rates were determined over a 14 day period.

There was a significant effect on all three cultures of increasing NaCl concentration ($p < 0.0005$) and NaCl caused a progressive decline in the growth rate of all three cultures. Proline significantly improved cell growth at every concentration of NaCl ($p < 0.0005$). At 200 mM NaCl, and no proline addition, the growth rate was recorded at 0 ml SCV day⁻¹ for each of the species. The growth rate of Larch, Sitka spruce and Oak cultures decreased as the concentration of NaCl was increased for all concentrations examined, with complete inhibition of growth at 200 mM NaCl in the absence of proline. With 200 mM NaCl, 1 mM proline addition had a profound effect on growth rate. For Larch, 100 mM proline and 200 mM NaCl resulted in 50% of the normal growth rate. For Sitka spruce and Oak, 100 mM proline and 200 mM NaCl resulted in growth rates approaching that of normal cell growth.

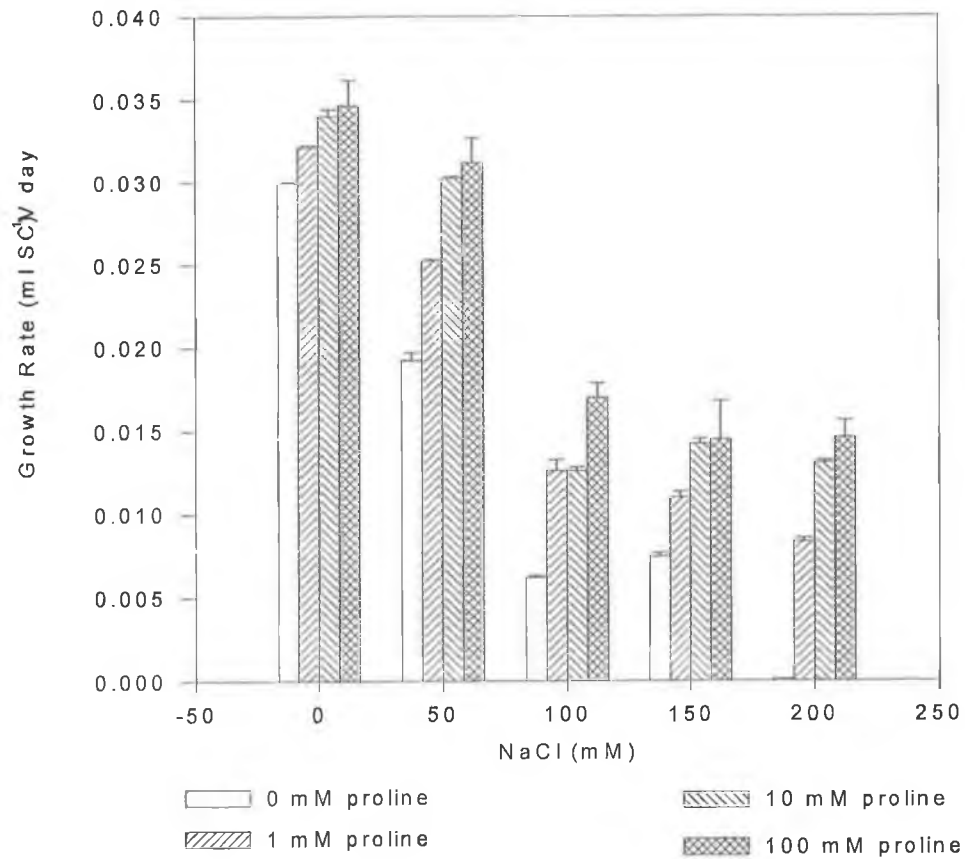


Figure 3.3

Influence of proline on the growth rate of Larch (*Larix X leptoeuropaea*) embryonal suspensor masses in varying concentrations of NaCl. Mean values \pm SEM are shown where these exceed the dimensions of the symbols.

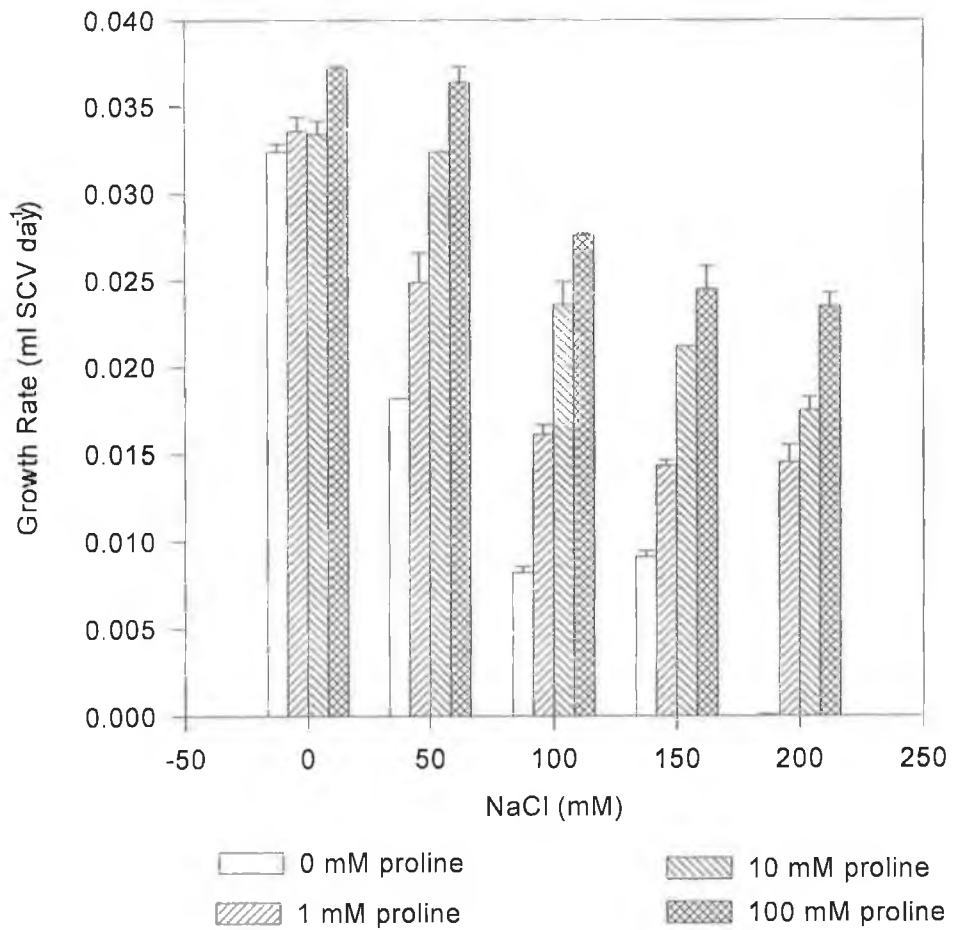


Figure 3.4

Influence of proline on the growth rate of Sitka spruce (*Picea sitchensis* (Bong.) Carr.) embryonal suspensor masses in varying concentrations of NaCl. Mean values \pm SEM are shown where these exceed the dimensions of the symbols.

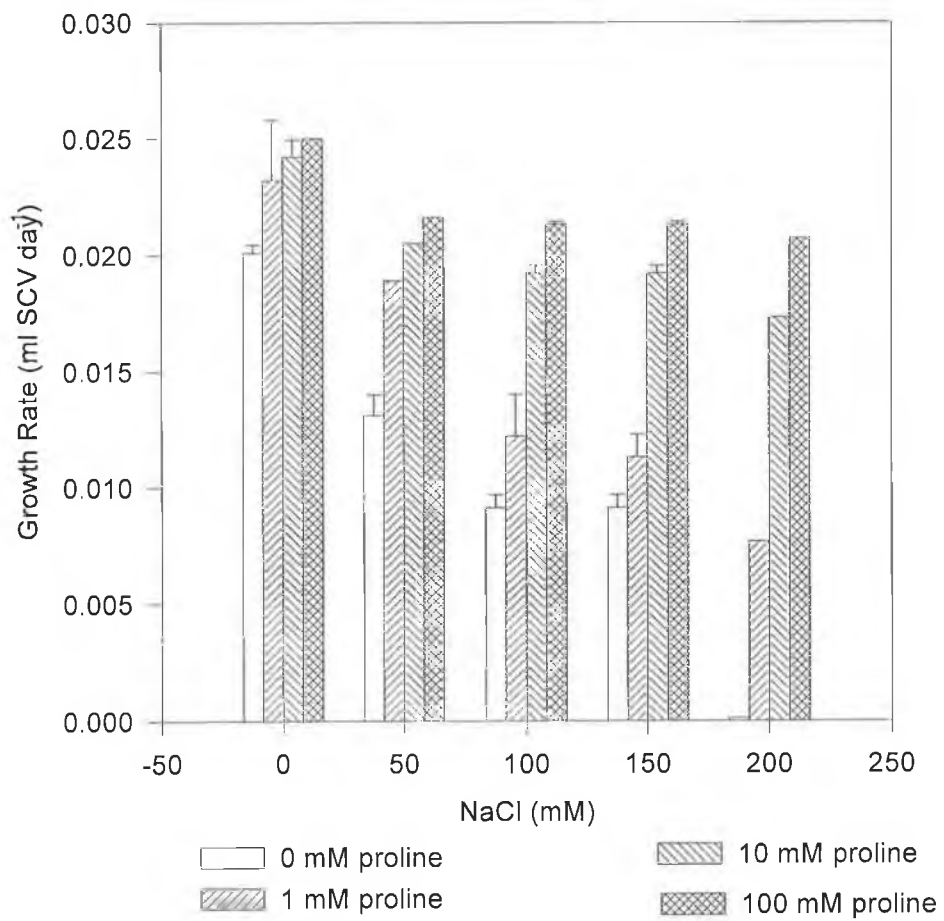


Figure 3.5

Influence of proline on the growth rate of Oak (*Quercus robur* L.) somatic embryo cultures in varying concentrations of NaCl. Mean values \pm SEM are shown where these exceed the dimensions of the symbols.

3.2.4 Potassium Leakage from Larch (*Larix X leptoeuropaea*), Sitka spruce (*Picea sitchensis* (Bong.) Carr.) and Oak (*Quercus robur* L.) embryonal suspensor masses subjected to below freezing temperatures

Replicate 5 day old cultures were maintained for 48 hours with 0, 1, 10 or 100 mM proline. Cells were then subjected to a decrease in temperature to -30°C , with samples taken at appropriate time intervals. Potassium leakage was then recorded using a Perkin Elmer 3100 atomic absorption spectrometer (AAS). K^{+} concentrations were determined by reference to appropriate standard solutions.

Figures 3.6, 3.7 and 3.8 show the effects of proline on K^{+} release for Larch, Sitka spruce and Oak subjected to freezing temperatures

Potassium leakage was found to be inversely proportional to temperature. At -30°C (0 mM proline) Oak released over 90% of its cellular K^{+} . Addition of proline reduced the amount of potassium released, to a minimum of 63% with 100 mM proline. Increasing the proline concentration resulted in less K^{+} leakage at each temperature (see Fig. 3.8). The relative protective effect of proline on K^{+} release was particularly pronounced at temperatures close to 0°C (freezing of the cells does not take place at 0°C due to ions present in the cells). At 0°C there was leakage of K^{+} (approx. 60%, compared to 18% at 24°C) with no proline. Addition of proline, even at 1 mM, decreased leakage to 50 %. Sitka spruce followed this trend, and released approximately 80% of its cellular K^{+} at -30°C (0 mM proline). Addition of proline at this temperature decreased leakage to 62%. Larch released approximately 90% of its cellular potassium at -30°C with no proline addition. Addition of proline, even at 1mM reduced this figure to approximately 82%. Proline at 100mM reduced leakage to 60% (-30°C), which is comparable to leakage at -10°C for which no proline has been added.

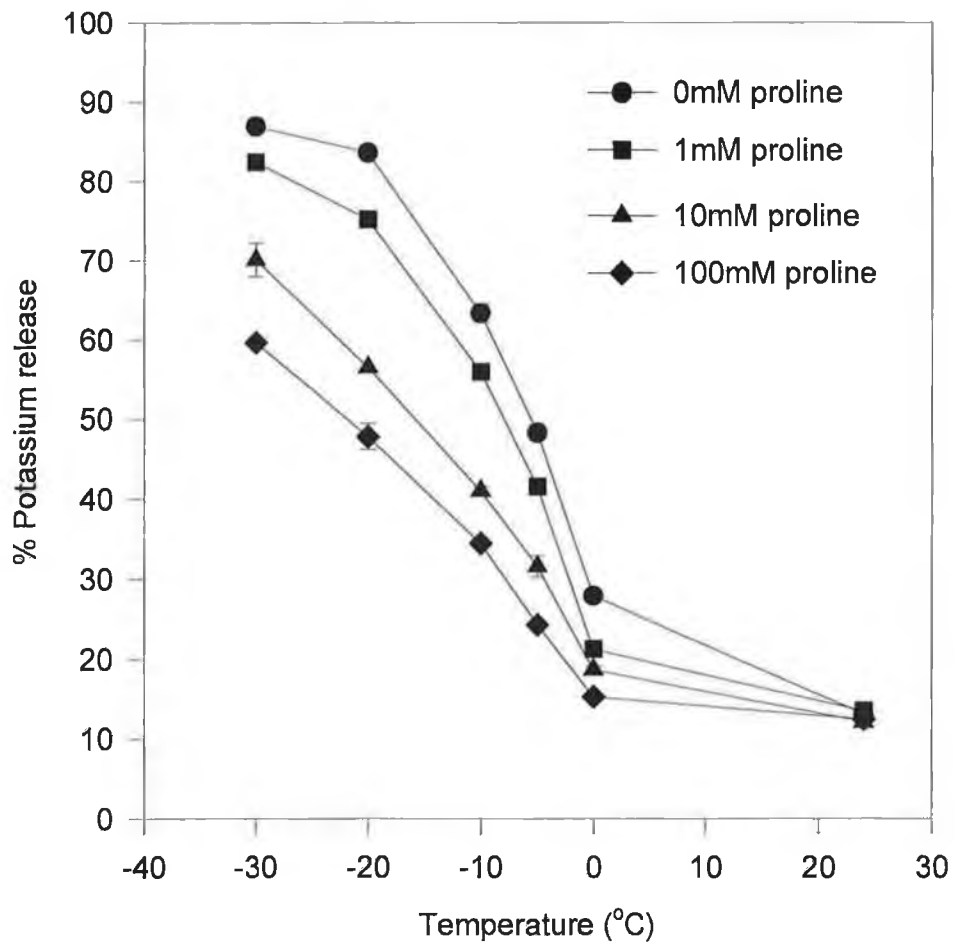


Figure 3.6

Influence of proline on potassium leakage from Larch (*Larix X leptoeuropaea*) at below freezing temperatures. Mean values \pm SEM are shown where these exceed the dimensions of the symbols.

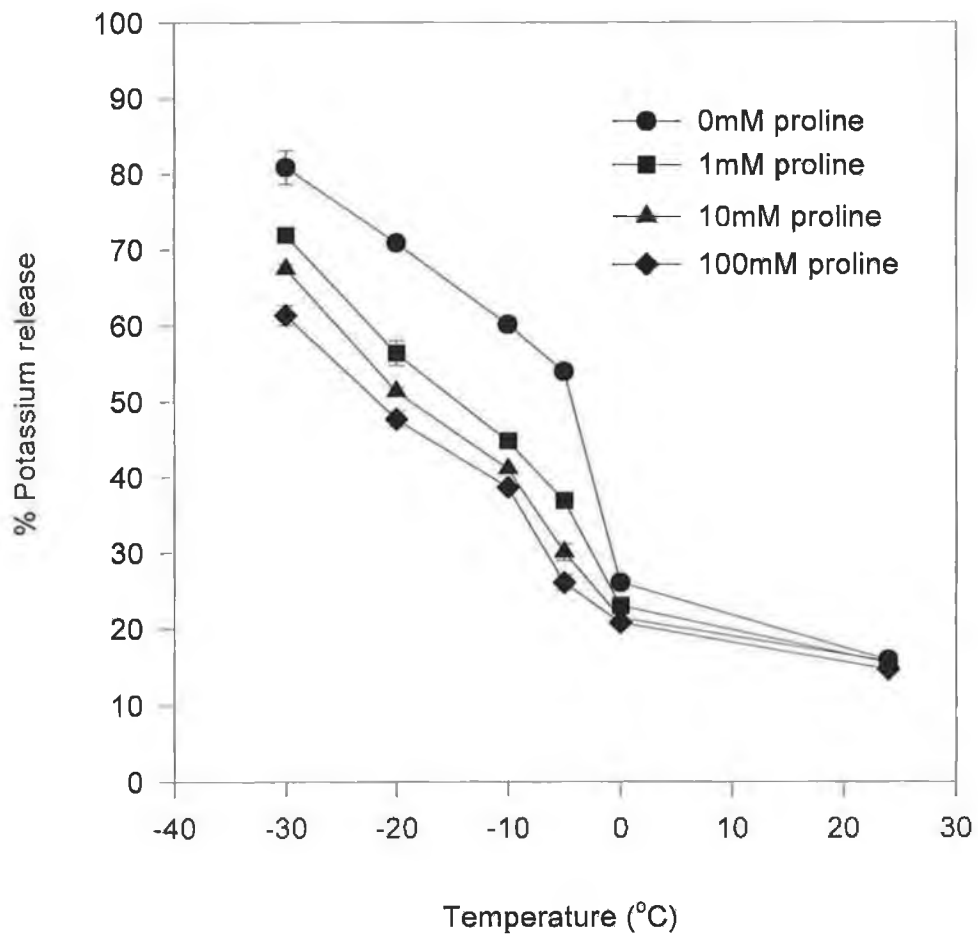


Figure 3.7

Influence of proline on potassium leakage from Sitka spruce (*Picea sitchensis* (Bong.)

Carr. at below freezing temperatures. Mean values \pm SEM are shown where these exceed the dimensions of the symbols.

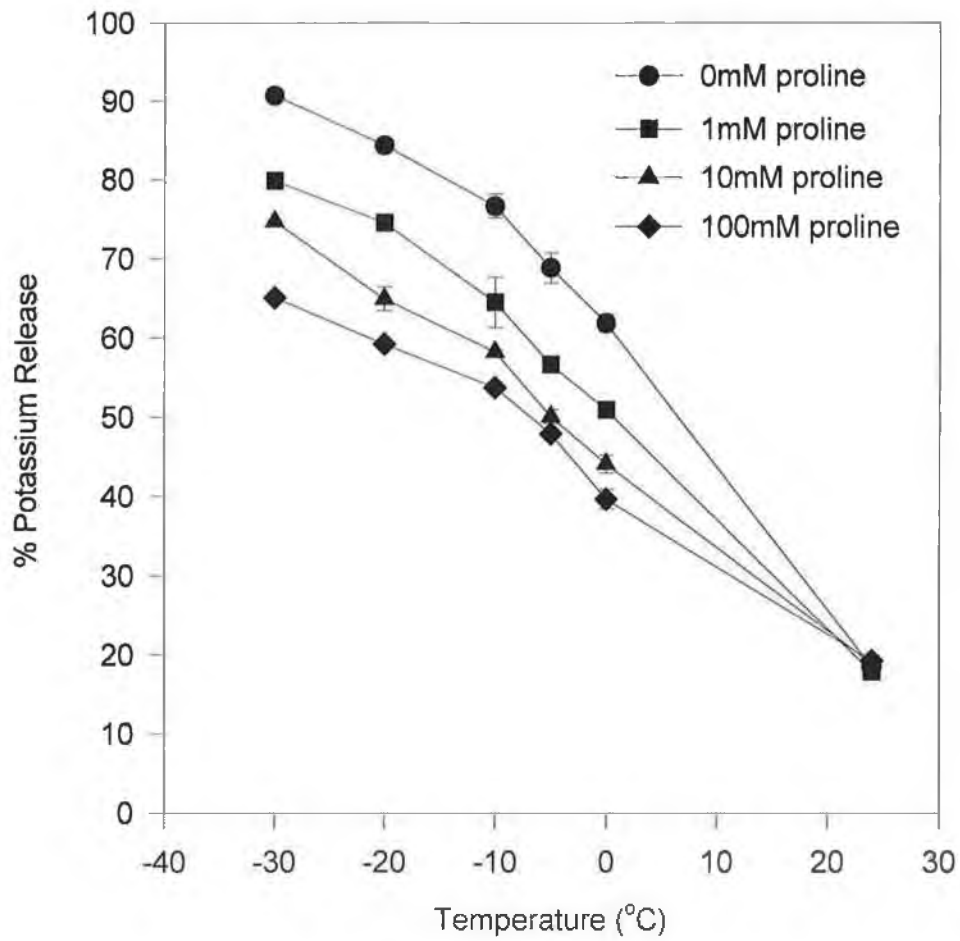


Figure 3.8

Influence of proline on potassium leakage from Oak (*Quercus robur* L.) at below freezing temperatures. Mean values \pm SEM are shown where these exceed the dimensions of the symbols.

3.2.5 Measurement of intracellular proline concentration in embryonal suspensor masses of Larch (*Larix X leptoeuropaea*), Sitka spruce (*Picea sitchensis* (Bong) Carr.) and Oak (*Quercus robur* L.) subjected to below freezing temperatures

Samples were removed from cell cultures maintained for 2 days with 0, 1, 10 or 100 mM proline prior to frost tolerance assay. Intracellular proline was then measured using a modified ninhydrin assay and expressed as μ moles proline per g fresh weight (Table 3.1). It can be seen that with no proline addition, intracellular proline levels are correspondingly low, and that intracellular proline levels recorded correspond approximately to the amount of proline added to each culture.

Proline added to cultures (mM)	Larch intracellular proline	Sitka spruce intracellular proline	Oak intracellular proline
0	0.0424 \pm 0.006	0.0402 \pm 0.023	0.0283 \pm 0.009
1	1.708 \pm 0.029	1.621 \pm 0.036	0.834 \pm 0.017
10	9.829 \pm 0.002	9.632 \pm 0.021	7.8107 \pm 0.014
100	77.938 \pm 0.012	76.572 \pm 0.065	51.816 \pm 0.091

Table 3.1

Intracellular proline concentration 48 hours after addition of exogenous proline. Results are mean of multiple samples \pm SEM (μ moles proline per g fresh weight). Mean values \pm SEM are shown.

3.2.6 Kinetic analysis

The data showing the effect of proline on growth rate with varying concentrations of NaCl was re-plotted (from Fig. 3.3) as shown in Figure 3.9

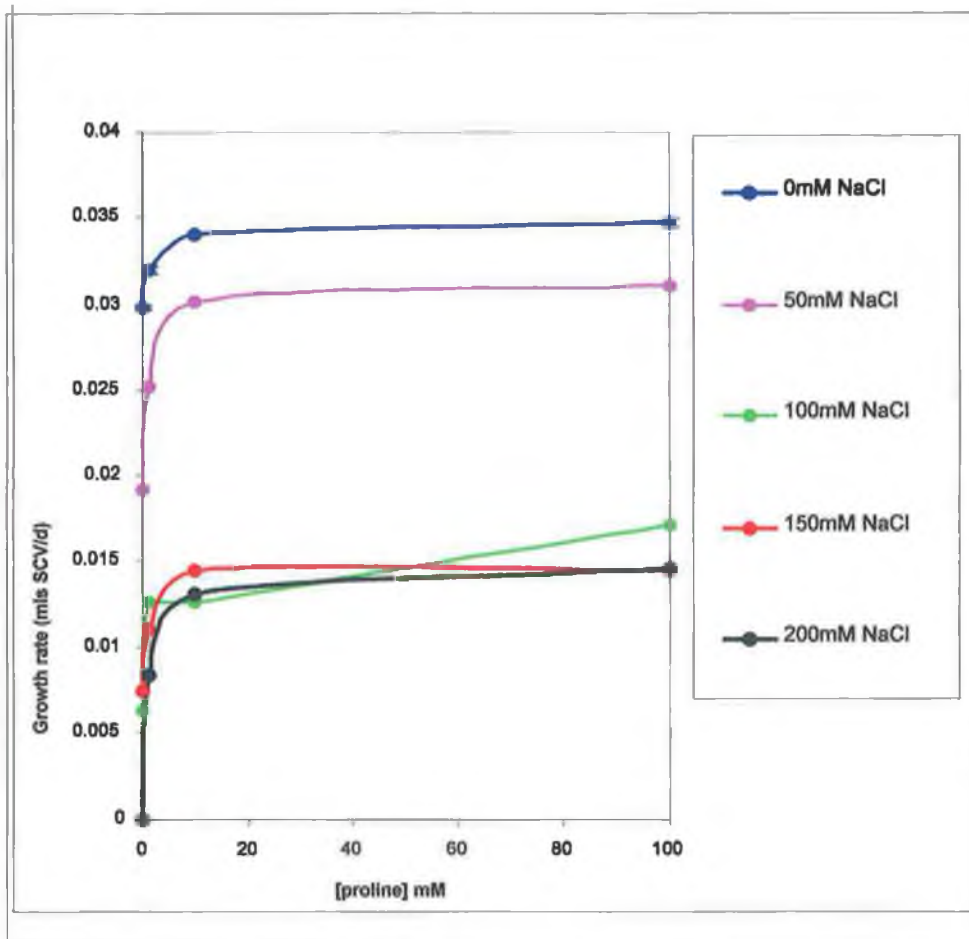


Figure 3.9

Estimation of proline effects on the growth of Larch ESMs exposed to salt

The hyperbolas produced are typical of that normally associated with Michaelis-Menton kinetics. It is therefore possible to re-examine the data to check that it is a good fit for Michaelis-Menton kinetics, and to estimate V_{\max} , the maximum growth rate, and K_m , the concentration of proline that gives half maximum growth.

The data for Larch ESMs grown with 200mM NaCl have been replotted in the 3 forms traditionally associated with the analysis of kinetic data (Lineweaver-Burke, Hanes, and Eadie-Hofstee plots), and V_{\max} and K_m calculated from these plots. V_{\max} and K_m were also calculated by the Direct Linear method, and an iterative non-linear least squares fitting method, EnzFitter™.

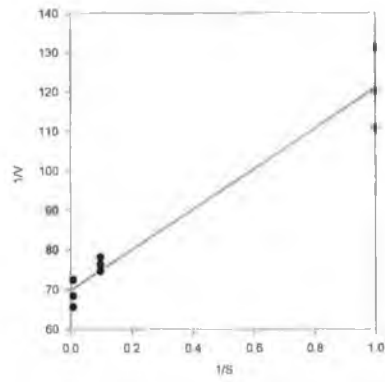


Figure 3.10 Lineweaver-Burk plot

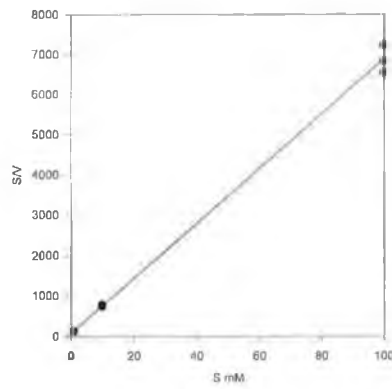


Figure 3.11 Hanes plot

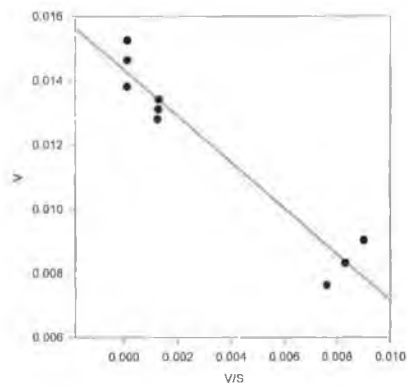


Figure 3.12 Eadie-Hofstee plot

It can be seen that for each of these plots, there is an excellent linear relationship, thus there appears to be a good fit of the data to Michaelis-Menton kinetics.

The values for V_{\max} and K_m are given for each of the methods in Table 3.2

	Direct Linear	EnzFitter	Lineweaver/ Burke	Eadie/ Hofstee	Hanes
K_m	0.850 ± 0.062	0.748 ± 0.088	0.784	1 – 1.3	0.87 – 1.02
V_{\max}	0.014520 ± 0.000162	0.01442 ± 0.00029	0.0144	0.0187	0.015

Table 3.2 V_m and K_{\max} values for Larch ESMs grown with 200 mM NaCl, and calculated using 5 different methods

Where appropriate values are expressed with the standard error of the mean.

It can be seen that each of the methods gives comparable results for both K_m and V_{\max} , and there is little difference between the values recorded for the Direct Linear Method, and by EnzFitter. These two methods are the most robust way to measure K_m and V_{\max} and both give an estimate of the variability associated with the measurement. It is less time consuming to use EnzFitter than the Direct Linear Method, accordingly, EnzFitter was used to calculate K_m and V_{\max} for all further data.

The values for the 3 species for growth with 200mM NaCl are given in Table 3.3

	Larch	Sitka Spruce	Oak
K_m (mM)	0.748 ± 0.088	0.524 ± 0.182	1.789 ± 0.071
V_{max} (ml SCV days ⁻¹)	0.01442 ± 0.00029	0.02129 ± 0.00112	0.02083 ± 0.00016

Table 3.3 V_m and K_{max} for each of Larch, Sitka spruce and Oak under salt stress

For all 3 species, the K_m is very low at around mM.

These procedures were repeated for the effect of cold on the growth of embryo cultures with essentially similar results.

	Larch	Sitka Spruce	Oak
K_m (mM)	0.329 ± 0.068	0.149 ± 0.059	1.208 ± 0.376
V_{max} (ml SCV days ⁻¹)	0.01443 ± 0.00036	0.01253 ± 0.00035	0.01748 ± 0.00010

Table 3.4 V_m and K_{max} for each of Larch, Sitka spruce and Oak under cold stress

3.3 Discussion

Water deficit elicits a complex series of events within a cell, and occurs as a result of drought, salt or low temperature. The set of responses depends upon the severity and duration of the stress, plant genotype, developmental stage and environmental factors providing the stress.

Reducing the temperature of the surrounding environment to near freezing results in a slowing of plant growth. In the present work the effect of such a lowering of temperature was investigated, along with the positive effect of added proline. Researchers have shown a positive effect of exogenous proline with cold stressed cultures of maize and potato. They did so, however, by applying the stress for a certain length of time (e.g. 2 weeks), and then transferring cultures to 28°C and determining cold tolerance via recovery from stress (Songstad et al. 1990, van Swaaij et al. 1985). Such studies indicate that proline can reduce cold-induced injury. This is consistent with our own investigations where browning and necrosis were evident at low temperatures without proline. Our studies however show that not only can cultures survive low temperatures, but are capable of active growth. Addition of proline, at 1 mM, influenced the growth of the cultures to such an extent that they did not brown and grew at a growth rate of 0.009-0.012 ml SCV days⁻¹, which is approximately one third the growth rate at 24°C with 1 mM proline for Larch and Sitka spruce (approximately 0.031 ml SCV days⁻¹ for Larch and Sitka spruce, 0.025 ml SCV days⁻¹ for Oak). Increasing the proline concentration increased the growth rate for each species at both 4°C and 24°C.

The common opinion is that the primary injuries by salts involve specific toxic effects, (a) directly on the external plasma membrane or (b) after penetration through the membrane into the protoplast. Injury is increased by salt uptake (Hazem Kalaji and Pietkiewicz 1993). Salt stress usually results in loss of cell turgor, shrinkage of the cell, and plasmolysis with subsequent cell death. In order to balance loss of cell turgor and to permit rehydration of the cell there must be an increase in cell solute content. The increase must be sufficient to lower osmotic potential below that of the aqueous environment. This process is called osmoregulation (Levitt 1980a). An accumulation of

free amino acids, including proline has been found in many plants in response to increased salinity.

This formed the basis for investigating the effect of exogenous proline on the growth of Larch, Sitka spruce and Oak embryogenic cultures. Previous researchers have shown that salt has a negative effect on cell growth (Lone et al. 1996, Gangopadhyay et al. 1997, Bajji et al. 1998). Our investigations have also shown the negative effect of salt (NaCl) on the growth of Larch, Sitka spruce and Oak cultures. Proline was added to cultures at various concentrations and shown to partially alleviate the negative effects caused by increasing the salt concentration. 1 mM amounts were shown to be sufficient to have an effect, increasing the proline concentration above this did not result in a similar increase in growth rate. 200 mM NaCl inhibited growth completely, unless proline was applied. Proline at 100 mM increased growth rate to approximately 50%, 75% and 95% that of normal unsalted cells grown without proline. This effect has been shown by previous researchers working with other species. The addition of NaCl caused a progressive decline in elongation of shoots of *Hordeum vulgare* L. cv. Maris Mink (cultured Barley) as well as a decrease in tissue fresh weight. Addition of proline at 10 mM was reported to reduce the inhibition of growth caused by the addition of NaCl and also reduce Na⁺ and Cl⁻ ion accumulation in tissues and increasing proline concentration over 10 mM produced no additional protection (Lone et al. 1996), this is in line with our own results.

As temperatures drop below 0°C ice typically forms in the extra-cytoplasmic space within the plant tissue, where solute concentration is lowest. This accumulation of ice can potentially result in the physical disruption of cells and tissues, caused in part by the formation of adhesions between the intercellular ice and the cell walls and membranes (Levitt 1980b). The lower vapour pressure of the ice in the extra-cytoplasmic space also draws water from the cytoplasm desiccating the cells and shrinking the cellular membranes. Most of the cellular injury results from the severe cellular dehydration that occurs with freezing (Levitt 1980b, Steponkus and Webb 1992). As the temperature drops below -10°C, ice formation can penetrate the cytoplasm, coincident with the fracture of the cell membrane and destruction of subcellular organisation. Ice

formation within the cytoplasm is lethal. Frost-hardy species produce cryoprotectants such as proline which reduce damage by desiccation. Positive correlations have been found between leaf proline content and frost tolerance in a wide range of species: in young apple shoots (Benko 1968), alfalfa (Paquin 1977) and winter rape and wheat (Stefl et al. 1978). It is reported that proline may help to stabilise the cell membrane and thereby help the cell to survive harsh environmental conditions, such as frost damage (Csonka 1989, van Rensburg 1993, Holmberg and Bulow 1998). To test this hypothesis with Larch, Sitka spruce and Oak the leakage of ions from cultures at temperatures below 0°C was examined. Electrical conductivity is usually used to determine the leakage of ions from cell cultures, however the low conductivity levels recorded led to high variability. It was therefore decided to measure leakage of the potassium ion, which proved to be much more reliable and reproducible. K⁺ is maintained at high levels in all cells and cell damage is indicated by a high level of K⁺ leakage from cells. Many researchers have used ion leakage as an indicator of freezing injury in various plant varieties (Sulc et al. 1991, Dix et al. 1994, Boorse et al. 1998).

It can be seen that exogenous proline reduces K⁺ leakage from Larch, Sitka spruce and Oak, and that the lower the temperature, the more proline benefits the cells. Although 100 mM proline provides for minimum release of K⁺ at each temperature, it can be seen that mM amounts are sufficient to reduce the K⁺ leakage from the cells at each temperature examined. At 24°C there is movement of K⁺ ions between the external aqueous environment and the cells, and this is recorded as a low (10-15%) level of K⁺ leakage. At 0°C there is little change in the level of K⁺ released as no freezing of the cells has taken place due to the presence of salts within the cells. Once the temperature drops below 0°C, a greater increase in K⁺ leakage can be seen. It is at this time that the membranes and organelles start to freeze and therefore when defrosted the membranes are damaged and leak more potassium than normal. However, it can be seen for each species at all below freezing temperatures examined that proline helps reduce K⁺ leakage from the cells. It is possible that proline is protecting the membranes during the freezing and defrosting process.

The effect of proline concentration on growth rate under conditions where growth ceases in control cultures follows typical Michaelis-Menton kinetics. Half-maximal growth rate (K_m) was determined to be at sub-millimolar concentrations for ESMs of the two conifer species, and less than 2 mM for Oak. It will be shown that in transgenic plantlets engineered to over-express proline that sufficient proline will be produced endogenously to provide for half maximal growth under stress conditions.

In the case of the three species examined it was found that exogenously applied proline protected the cells from the effects of the salt, cold and freezing stresses applied. Both 4°C and 200 mM NaCl completely inhibited cell growth in the three species, proline at a concentration of 1 mM was able to help the cells overcome the growth restraints applied, and at every salt concentration examined proline had a positive effect. Potassium leakage from all cells was found to be reduced if the cells were exposed to extracellular proline for a time before the stress was applied. The cells took up the proline, which we postulate helped protect the membranes against the freezing stress. This provided the basis for an experiment to genetically transform the species examined here with a gene allowing them to overproduce proline, and therefore provide them with a mechanism to overcome cold, salt and freezing stresses without the need for exogenous proline addition.

Chapter 4

**Transformation and analysis of both transformed
and untransformed cell lines of Larch (*Larix X
leptoeuropaea*), Sitka spruce (*Picea sitchensis* (Bong)
Carr.) and Oak (*Quercus robur* L.)**

4.1 Introduction

The amino acid proline has been proven to play an important role as an osmoregulatory solute in plants exposed to high levels of salt, drought (Delauney and Verma 1993, Guerrier 1997/8 and Hare and Cress 1998) and is also associated with plant responses to chilling (Chu et al. 1974, Duncan and Widholm 1987 and Xin and Li 1993). We have shown the positive effect proline has on salt treated embryogenic callus of Larch, Sitka spruce and Oak, as well as the positive effect this amino acid has on cold and frost tolerance on these cultures.

Under environmental stress, proline is produced via the glutamic acid pathway, as outlined in Figure 1.1 (Introduction) (Delauney and Verma 1993, Kishor et al. 1995). P5CS is the rate limiting enzyme in the production of proline from glutamate. Genes encoding P5CS have been cloned from several plants (Hu et al. 1992, Savoure et al. 1995, Yoshida et al. 1995, Igarashi et al. 1997 and Strizhov et al. 1997) and their expression studied under various abiotic stresses. The P5CS gene was cloned from *Vigna aconitifolia* under the CaMV-35S promoter for genetic transformation of *Nicotiana tabacum* cv Xanthi (Kishor et al. 1995), and some transgenic plants expressed a high level of P5CS protein and produced 10- to 18-fold more proline than the control plants, resulting in enhanced biomass production.

A plasmid (pBI-P5CS) containing *Vigna aconitifolia* P5CS cDNA (Hu et al. 1992) under the control of the CaMV 35S promoter in vector pBI121 was introduced into *Agrobacterium tumefaciens*. The P5CS cDNA was placed between the CaMV 35S promoter and the Nos 3' regions and the resulting construct was inserted into the EcoRI site of vector pBI121. The vector also contains the NPTII and uid A (GUS) coding regions that were used for selection of transgenic plants on kanamycin and as a reporter of transformation, respectively (Figure 4.1).

Embryogenic cultures of Larch, Sitka spruce and Oak were transformed with *A.tumefaciens* containing pBI-P5CS or pBI121 (control) plasmid. All transformants were selected on kanamycin and screened for GUS activity as well as for proline production. Following selection of transformed lines, regeneration on kanamycin was performed and regenerated transformants were screened for GUS activity.

A second series of transformations were then carried out using a plasmid pBI-P5CSF129A, which contains mutagenised *Vigna aconitifolia* cDNA (Hong et al. 2000) under the control of the CaMV 35S promoter. This mutated P5CS gene

contains a mutation at nucleotide positions 421- to 423 of the *Vigna aconitifolia* cDNA. Codon TTT was changed to GCC by site-directed mutagenesis so that Phe at amino acid position 129 of the P5CS peptide is replaced by Ala generating pBI-P5CSF129A (Figure 4.2). The mutant enzyme retains similar kinetic characteristics as the wild-type P5CS, except that its allosteric regulation by proline is reduced (Hong et al. 2000). Again, transformants were selected on kanamycin and transformants were screened for GUS activity.

Once GUS positive clones had been identified these were subjected to further analysis by PCR. Primers were designed for the NPTII, GUS and P5CS genes, and once DNA had been isolated, PCR procedures were carried out, using *Agrobacterium* strains as positive and negative controls. Once PCR positive clones were identified, these lines were placed on regeneration medium, and also used in cold, salt, freezing and proline analysis.

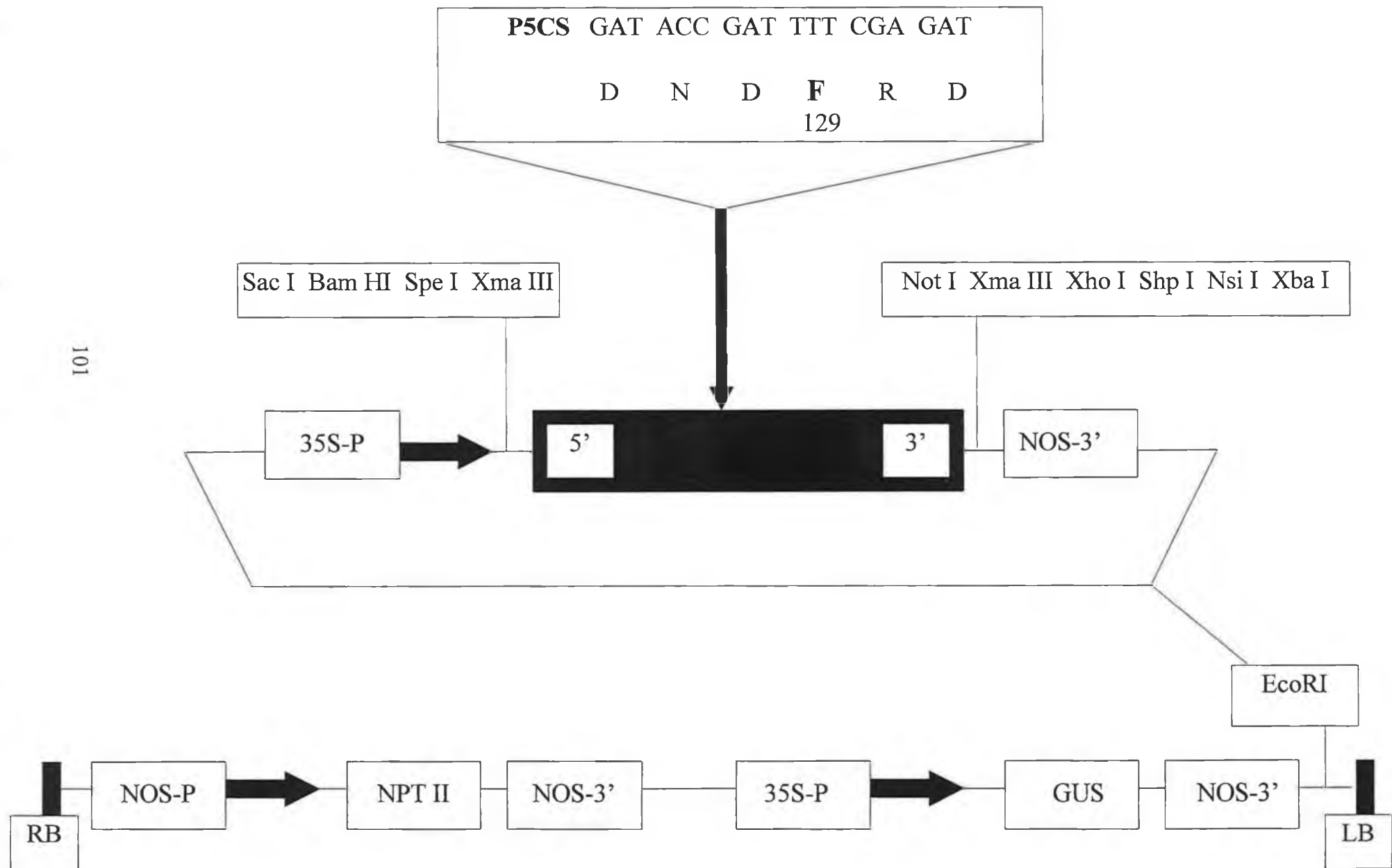


Figure 4.1

Plasmid map of pBI-P5CS

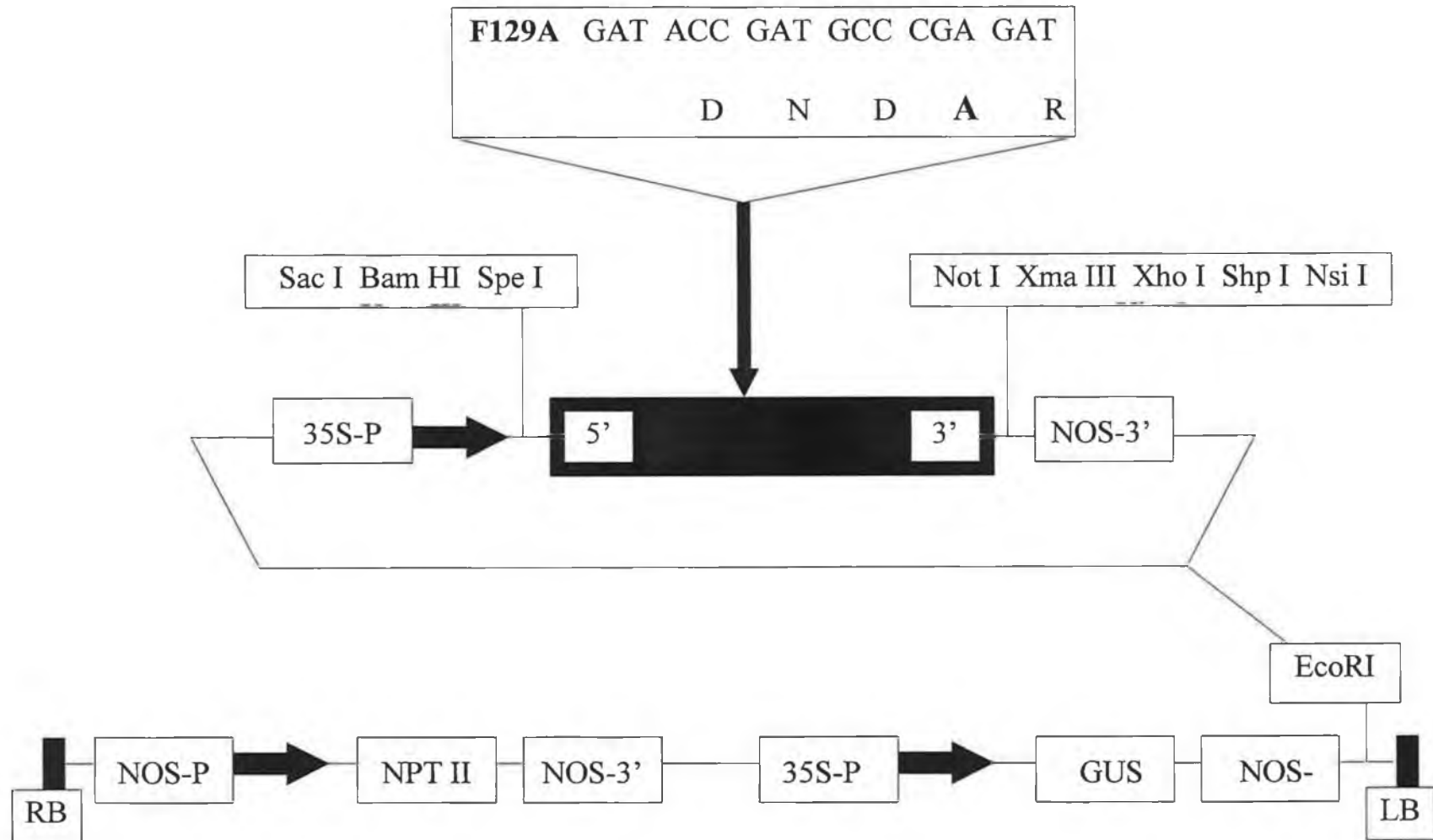


Figure 4.2

Plasmid map of pBI-P5CSF129A

4.2 Results

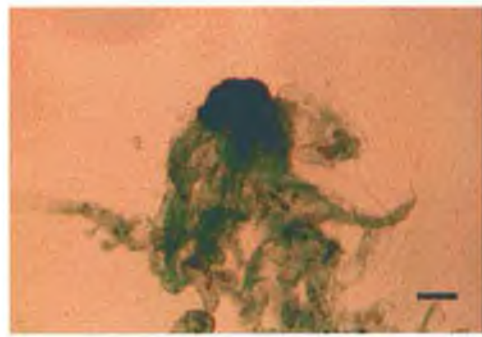
4.2.1 Transformations and GUS Analysis

4.2.1.1 Transformation of Larch (*Larix X leptoeuropaea*) with pBI121, pBI-P5CS and pBI-P5CSF129A

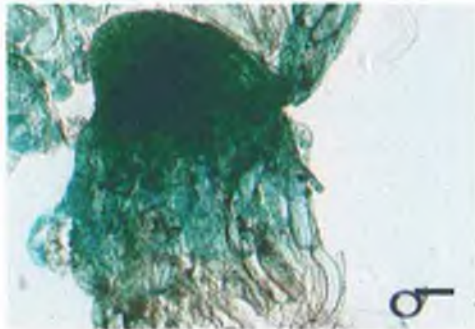
A number of transformation experiments were carried out using the above plasmids. Transformants were selected over a 6 month period on 25-50 mgL⁻¹ kanamycin. Following this selection procedure, transformed calli were screened for GUS activity, which was observed in transformed cells, as shown below. The cells shown in Plate 4.1 were assayed 6 months after transformation, just prior to molecular analysis and regeneration. Gus positive clones were then identified and screened for proline production. GUS activity shown in Plate 4.1



(a)



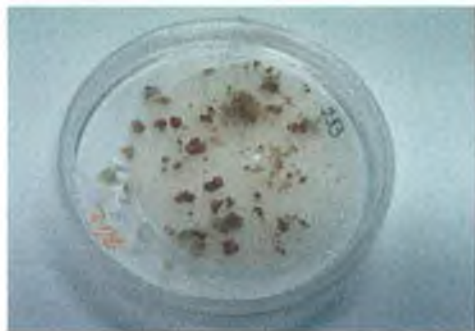
(b)



(c)



(d)



(e)

Plate 4.1

GUS activity observed in transformed cells of Larch (*Larix X leptoeuropaea*) (a) untransformed cells, (b) pBI121, (c) pBI-P5CS (L51) (d) pBI-P5CS (LF7). Images were taken using the OPTIMAS 6 image analysis programme under 10X magnification (bar = 40 μ m) and (e) transformed Larch on selection media in a 9 cm petri-dish. Plasmid pBI-P5CSF129A resulted only in transient expression of the GUS gene (results not show) and therefore these cell lines were not selected for further analysis.

4.2.1.2 Transformation of Sitka spruce (*Picea sitchensis* (Bong.) Carr) with pBI121, pBI-P5CS and pBI-P5CSF129A

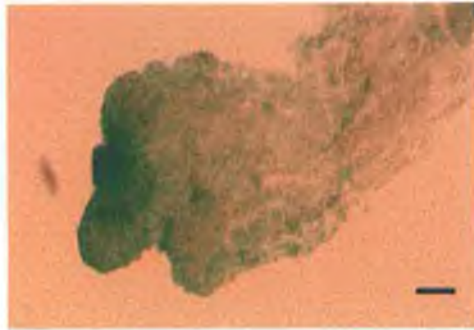
A number of transformation experiments were carried out using the above plasmids. Transformants were selected over a 6 month period on 50 mgL⁻¹ kanamycin. Following this selection procedure, transformed calli were screened for GUS activity, which was observed in transformed cells, as shown below (Plate 4.2). The cells shown in Plate 4.2 were GUS assayed 2 months after transformation. These cell lines did not exhibit a blue colour at later stages and therefore transformation was deemed to be transient. No stable transgenic cell lines of Sitka spruce were isolated, therefore GUS activity shown is transient.



(a)



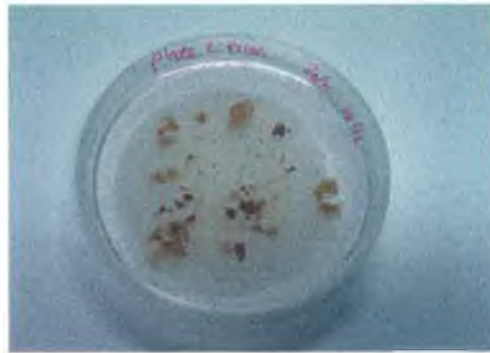
(b)



(c)



(d)



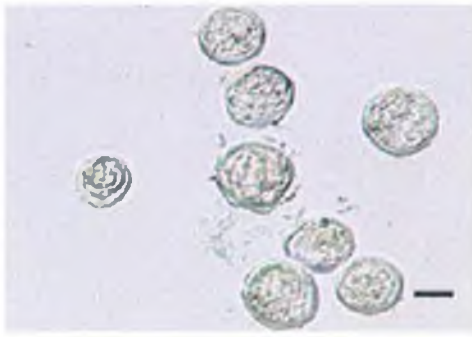
(e)

Plate 4.2

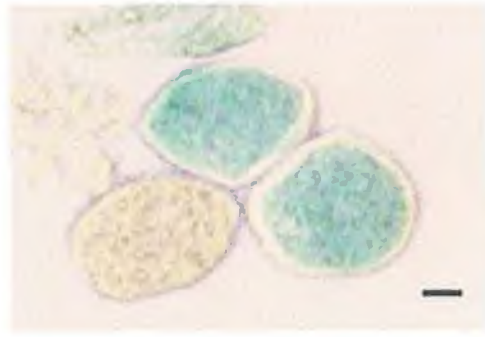
GUS activity observed in transiently transformed cells of Sitka spruce (*Picea sitchensis* (Bong.) Carr (a) untransformed cells, (b) pBI121, (c) pBI-P5CS (d) pBI-P5CSF129A. Images were taken using the OPTIMAS 6 image analysis programme under 10X magnification (bar = 40 μ m) and (e) Sitka spruce on selection media in a 9 cm petri-dish.

4.2.1.3 Transformation of Oak (*Quercus robur* L.) with pBI121, pBI-P5CS and pBI-P5CSF129A

A number of transformation experiments were carried out using the above plasmids. Transformants were selected over a twelve to sixteen week period in 10mgL^{-1} kanamycin. Following this selection procedure, cells were plated on solid media containing 15mgL^{-1} . Transformed cells were screened for GUS activity, which was observed in transformed cells, as shown below. GUS activity is shown in Plate 4.3. No stable transformed cells lines were achieved with Oak (transient only) due to difficulties in converting transformed single cells into transformed cell clusters and then to transformed cell lines. Cells shown in Plate 4.3 were GUS assayed 1 month after transformation.



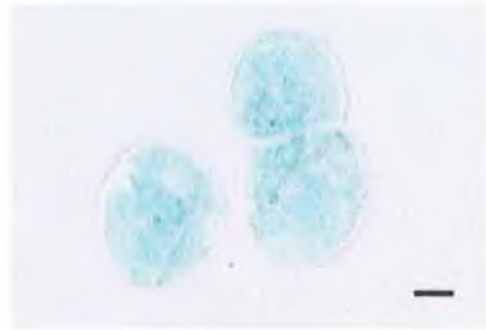
(a)



(b)



(c)



(d)

Plate 4.3

GUS activity observed in transiently transformed cells of Oak (*Quercus robur* L.) (a) untransformed cells, (b) pBI121, (c) pBI-P5CS and (d) pBI-P5CSF129A. Images were taken using the OPTIMAS 6 image analysis programme under 40X magnification (bar = 4 μm), except (a), which was taken under 20X magnification (bar = 20 μm)

4.2.2 PCR analysis

Three stable transgenic cell lines of Larch were selected for further analysis (one transformed with pBI121: line L121, and two with pBI-P5CS: lines L51 and LF7). Total genomic DNA was isolated from all *Agrobacterium* strains and from Larch both untransformed and transformed, as well as Sitka spruce untransformed cells, and two possibly transformed lines (transformed with pBI-P5CS: lines Pex and Pey, determined after PCR to be untransformed). This DNA was then quantified by measuring absorbance at 260nm, and an appropriate quantity was selected for PCR reactions. All PCR reactions were optimised by altering the annealing temperature of the primers. Primers were designed by appropriate reference to the gene sequences in question. In the case of the NPTII, GUS and P5CS genes, *Agrobacterium* (either pBI121, pBI-P5CS or pBI-P5CSF129A) was used as a positive control and untransformed Larch or Sitka spruce were used as negative controls (as well as dH₂O). PCR was carried out on all samples using *vir* gene sequences to determine that the transformed cell lines were not expressing *Agrobacterium* genes. Primers were also designed to the ribulose-1,5-bisphosphate carboxylase small sub-unit genes in both Larch and Sitka spruce. These determined that the quality of the DNA was sufficient for the procedures, and that negative PCR results for NPTII or GUS in suspected transgenic lines was not due to the quality of the DNA.

4.2.2.1 PCR analysis using primers for the *Agrobacterium vir* gene sequence

The following primers were designed for the VirE2 coding region of the *Agrobacterium* Ti plasmid after reference to the coding sequence (shown in Figure 4.3)

VIRE2FOR: 5' ATG GAT CTT TCT GGC AAT GAG 3'

VIRE2REV: 5' TCA AAA GCT GTT GAC GCT TTG G 3'

```
ttgtccatcgggtgcccgcctcccaggagacaatcttgctggtcaga      32385
RBS          VirE2 start →
aggagtgagacgatggatctttctggcaatgagaaatccaggcct      32430
          Forward primer →
tggaagaagg cgaatgtcag ttccagcaccatctccgatattc
-----
-----
-----

ttcggccagagccggcgtcgcgtccaatcagtgacagccgcagga
          ← Vir E2 stop
tatacgaagtcgaccgcgtagccaaagcgtcaacagcttttgac      34000
          ← Reverse primer
gttccttctgccgcgtcaatgaggaagctcgttgacccgggtttg
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Figure 4.3

The *Agrobacterium tumefaciens* VirE2 sequence (Accession number X06826) outlining the ribosome binding site (RBS), the start and stop codons, as well as the primed region. The product obtained should be approximately 1.6kb

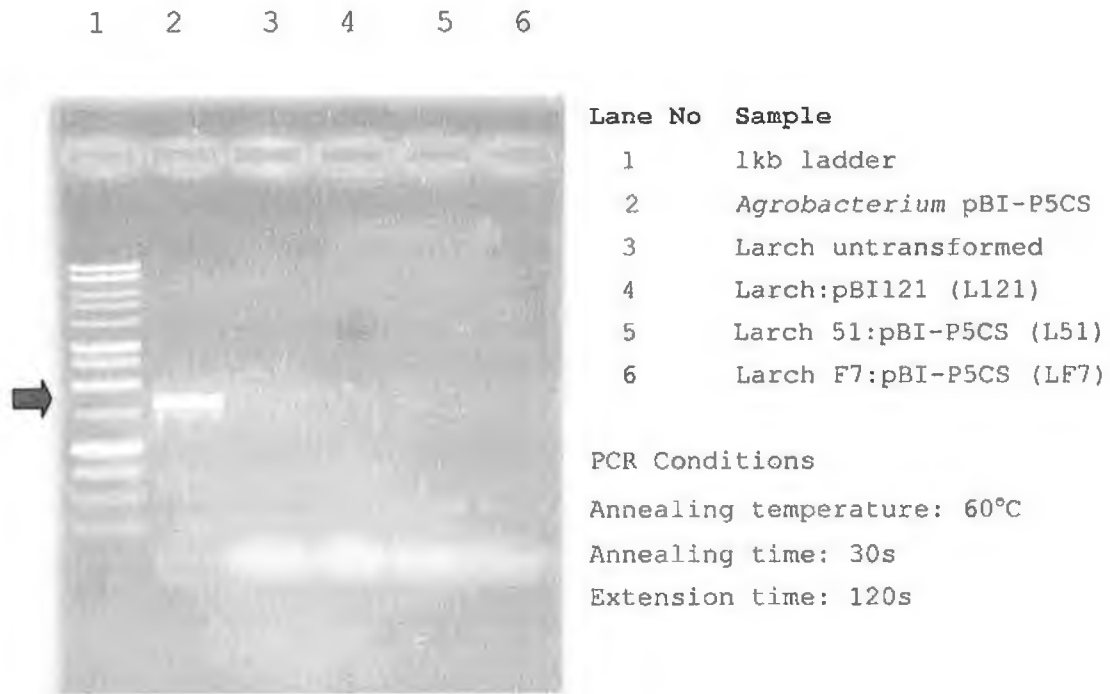


Plate 4.4

1.2 % agarose gel showing a 1.6 kb VirE3 fragment (situated above the 1.5 kb band on the ladder) primed from *Agrobacterium* pBI-P5CS (lane 2) and no band present in untransformed Larch (lane 3) or transformed Larch (lanes 4, 5 and 6). *Agrobacterium* pBI121 and pBI-P5CSF129A also show 1.2 kb bands when run on a 1.2 % agarose gel (results not shown). The position on the DNA ladder corresponding to 1.2 kb is arrowed.

4.2.2.2 PCR analysis using primers for the Larch RBCS gene sequence

The following primers were designed for the Larch RBCS coding region after reference to the coding sequence (shown in Figure 4.4)

RBCSFOR: 5' ATG GCT TCC TCT ATC ATG GC 3'

RBCSREV: 5' TTA ATT GTA TTC AGG CTT GTG G 3'

```
tgtgtagagccgctggaaggtaaatacagcgagaatttctttgtat      1612
  RBCS start →
cagatggcttcctctatcatggctctgtcctccacagctgcagtg      1657
  Forward primer →
gcagcggtagccgcgccctccaagacaggcaacagcaatgtggtg
-----
-----
-----
agtgtgcaaggcataccccaacgccttcacccgcgtcatcggat
tcgacaacgtccgccaagtgcagtgcacatctccttcacgtccaca
  ← RBCS stop
agcctgaatacaattaaacttgacagctcaatctatttctctgcgt      2415
  ←Reverse primer
ctcttcttcttcttccatcatgatttgggtacaacagaggcgttg
-----
-----
```

Figure 4.4

The *Larix laricina* RBCS sequence (Accession number X16039) outlining the start and stop codons, as well as the primed region. The product obtained should be approximately 800 bp

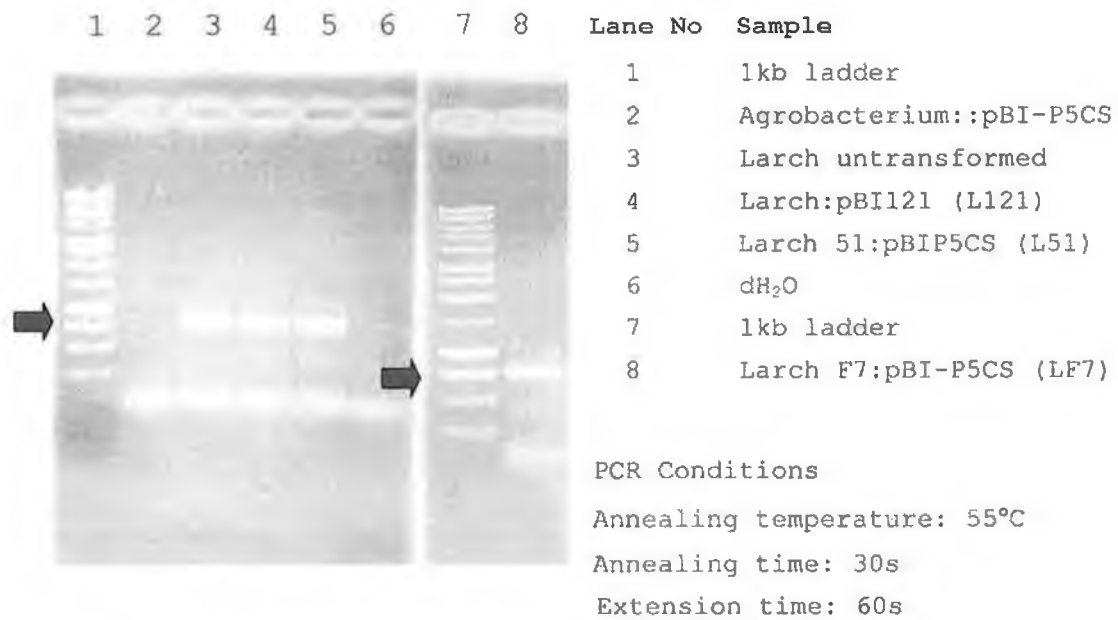


Plate 4.5

1.2 % agarose gel showing an 800bp Larch RBCS fragment (situated just at the 750bp band on the ladder) primed from untransformed Larch (lane 3) and transformed Larch (lanes 4, 5 and 8). *Agrobacterium* pBIP5CS and Sitka spruce show no bands (lanes 2 and 6 respectively). *Agrobacterium* pBI121 and pBI-P5CSF129A also do not show any band when run on a 1.2 % agarose gel (results not shown). The position on the DNA ladder corresponding to 800 bp is arrowed.

4.2.2.3 PCR analysis using primers for the Sitka spruce RBCL gene sequence

The following primers were designed for the Sitka spruce RBCL coding region after reference to the coding sequence (shown in Figure 4.5)

RBCLFOR: 5' ATG TCA CCA AAA ACA GAG ACT 3'

RBCLREV: 5' TCA CAA GGT ATC CAT TGC CT 3'

```

                                RBS      RBCL start →
gagagataattattaataaaaggaggacttatgtcaccaaaaaca      45
                                Forward primer →
gagactaaagctagtgctcggatttaaagctggtgttaaagattac      90
agattaacttattatactcctgaatatcagaccaagatacggat
-----
-----
tggagtcctgaactagctgctgcttgtgaaatatggaaggagatc
                                ← RBCL stop
aaatttgaatttgaggcaatggataccttgtgatccagtgacttt      1470
                                ← Reverse primer
cgttctaccaatcggactcggccaatcttttaccgctaccacta
agatt
```

Figure 4.5

The *Picea sitchensis* RBCL sequence (Accession number X63660) outlining the start and stop codons, as well as the primed region. The product obtained should be approximately 1.4kb

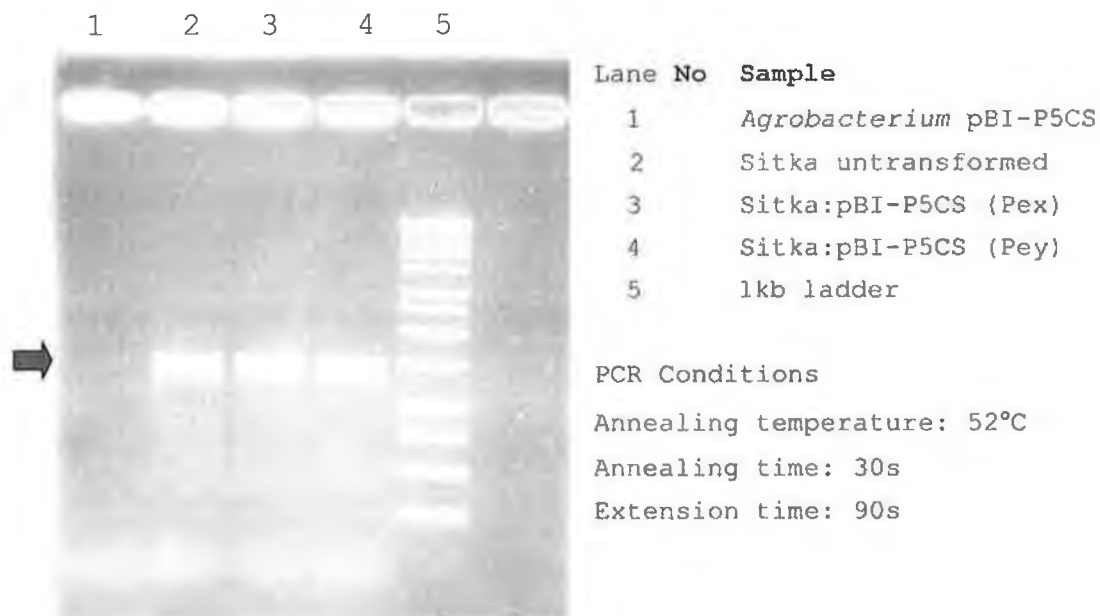


Plate 4.6

1.2 % agarose gel showing a 1.4kb Sitka spruce RBCL fragment (situated just below the 1.5 kb band on the ladder) primed from untransformed Sitka (lane 2) and possibly transformed Sitka, Pex and Pey (lanes 3 and 4). *Agrobacterium* pBIP5CS shows no bands (lane 1). *Agrobacterium* pBI121 and pBI-P5CSF129A also do not show any band when run on a 1.2 % agarose gel (results not shown). The position on the DNA ladder corresponding to 1.4 kb is arrowed.

4.2.2.4 PCR analysis using primers for the NPTII gene sequence

The following primers were designed for the NPTII coding region after reference to the coding sequence (shown in Figure 4.6)

NPTIIFOR: 5' ATG GGG ATT GAA CAA GAT GGA 3'

NPTIIREV: 5' TCA GAA GAA CTC GTC AAG AAG 3'

```
→ NPTII start
atggggattgaacaagatggattgcacgcaggttctccggccgct      45
  → Forward primer
tgggtggagaggctattcggctatgactgggcacaacagacaatc
-----
-----
-----
ggctgaccgcttctcgtgctttacggtatcgccgctcccgattc
                                     ← NPTII stop
gcagcgcacatgccttctatcgcttcttgacgagttcttctga      798
                                     ← Reverse primer
```

Figure 4.6

The NPTII sequence (Accession number AF234314) outlining the start and stop codons, as well as the primed region. The product obtained should be approximately 800bp

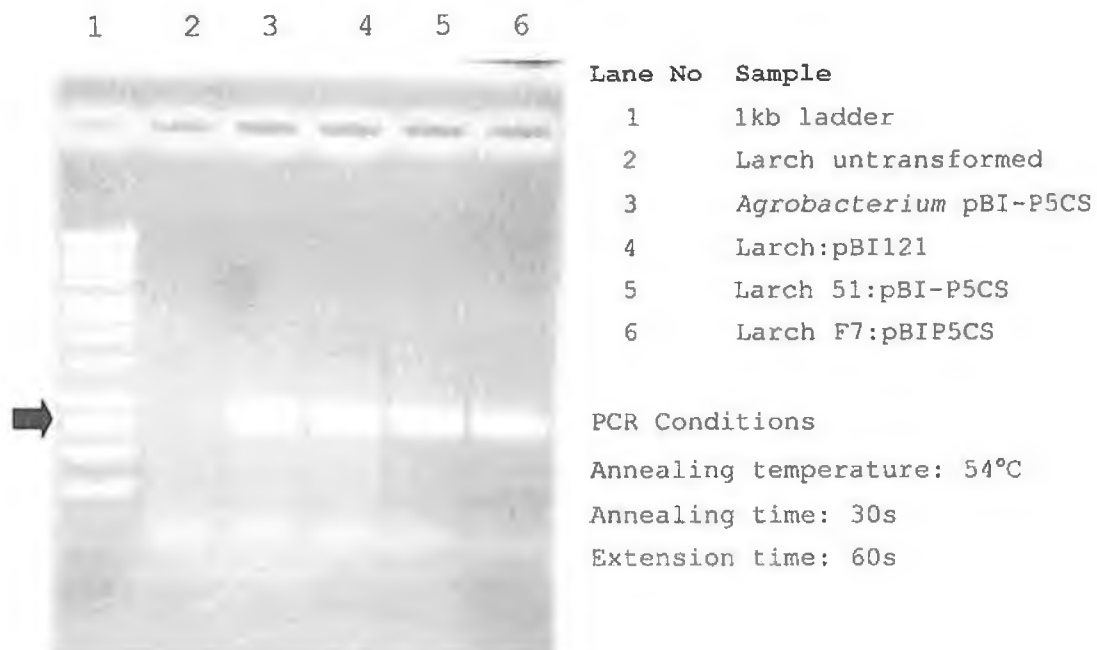


Plate 4.7

1.2 % agarose gel showing a 800bp NPTII fragment (situated above the 750 bp band on the ladder) primed from *Agrobacterium* (lane 3) and from transgenic Larch (lanes 4, 5 and 6). *Agrobacterium* pBI121 and pBI-P5CSF129A also show an 800bp band when run on a 1.2 % agarose gel (results not shown). The position on the DNA ladder corresponding to 800 bp is arrowed.

4.2.2.5 PCR analysis using primers for the GUS gene sequence

The following primers were designed for the GUS coding region after reference to the coding sequence (shown in Figure 4.7)

GUSFOR: 5' GGT GGG AAA GCG CGT TAC AAG 3'

GUSREV: 5' GTT TAC GCG TTG CTT CCG CCA 3'

```

                GUS start →
aaactaaactatcgatgggtcagtcaccttatgttacgtcctgtag           45
aaacccaaccctgaaatcaaaaaactcgacggcctgtgggcat           90
                Forward primer →
tgtggaattgatcagcgttggtgggaaagcgcggttacaagaaagc       135
cgggcaattgctgtgccaggcagtt-----                         170
-----
-----
ttgccaacgaaccggatacccgctccgcaagtgcacgggaatattt
                ← Reverse primer
cgccactggcgggaagcaacgcgtaaaactcgacccgacgcgtccga       1347
-----
cgctggactggcatgaacttcggtgaaaaaccgcagcagggaggc
                ← GUS stop
aaacaatgaatcaacaactctcctggcgcaccatcgtcggctaca       1873
-----
-----
```

Figure 4.7

The GUS sequence (Accession number 4191253) outlining the start and stop codons, as well as the primed region. The product obtained should be approximately 1.2 kb.

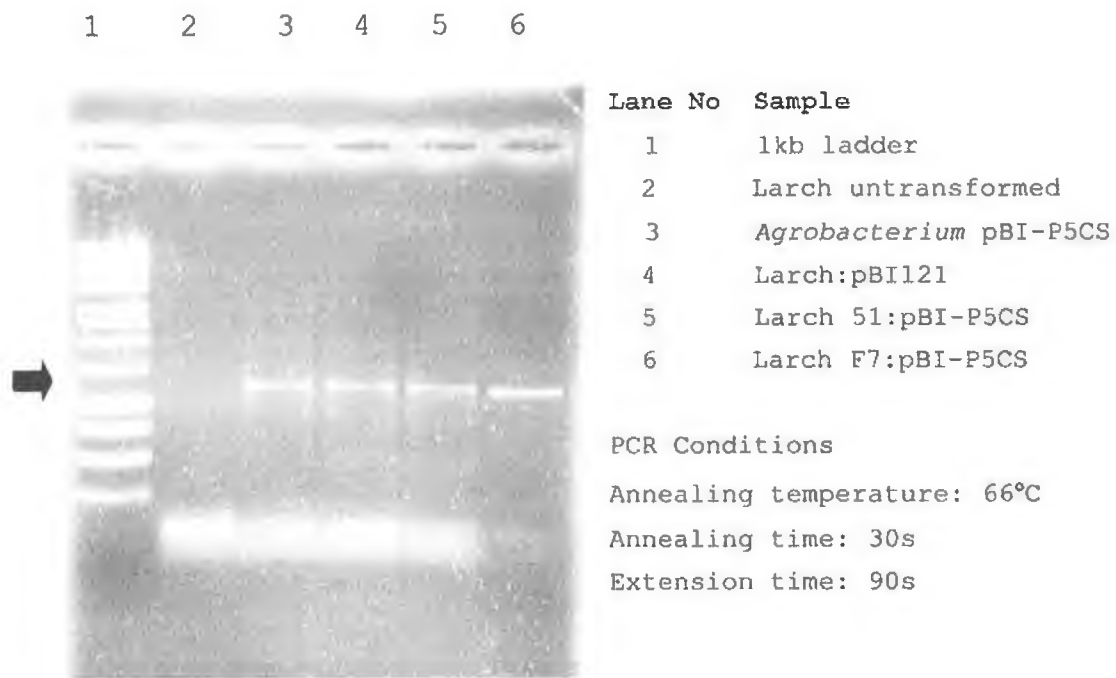


Plate 4.8

1.2 % agarose gel showing a 1.2 kb GUS fragment (situated between the 1kb and the 1.5 kb bands on the ladder) primed from *Agrobacterium* (lane 3) and from transgenic Larch (lanes 4 and 5). *Agrobacterium* pBI121 and pBI-P5CSF129A also show 1.2 kb bands when run on a 1.2 % agarose gel (results not shown). The position on the DNA ladder corresponding to 1.2 kb is arrowed.

4.2.2.6 PCR analysis using primers for the proline gene sequence

The following primers were designed for the CaMV 35S promoter and P5CS coding regions after reference to the coding sequence (shown in Figure 4.8). Referring to Figure 4.1, it can be seen that the proline gene (P5CS) is preceded by a 35S promoter. In order to prime a region that would only be present in transformed cell lines, primers were not designed solely for the coding sequence of the P5CS gene (present in untransformed DNA, results not shown), but for a region comprising the promoter region (35S) and the P5CS gene (present only in transformed cell lines).

CAP5CSF: 5' AAT ATC AAA GAT ACA GTC TCA GAA G 3'

CAP5CSR: 5' AAT AAG CTT TGA ATG AGG GTC ACT T 3'

(35S DNA sequence →)

35S start →

atggtggagcagcagactct----- 20
-----tgataacatggtggagcagcagactctcgtctac

forward primer →

tccaagaatatcaaagatacagtctcagaagaccaaagggctatt 400
gagacttttcaacaaagggtaatatcgggaaacctcctcggattc

-----ttggagaggacacgctgaaatcaccagtctctctctac

← 35S stop

aaatctatctct 781

(P5CS DNA sequence →)

P5CS start →

tgctctaaaggctattgctcgtatcagtgctcagccatggagagc 45
gcggtggatccttctcgggggttcatgaaggacgtgaagcgtgtg

← reverse primer

ccctccaagtgacocctcattcaaagcttatttatacatataacaa 695
agaaaaacatcagaatgaaattacttttggcgacaagtctagagt

← P5CS stop

caatttcaatttgagttaatttttatatgtaaaaaaaaaaaaaaaa 2417

Figure 4.8

The 35S promoter and P5CS gene sequences (Accession numbers AF234316 and M92276, respectively) outlining the start and stop codons of each gene, the polycloning site in the centre, as well as the primed region. The product obtained should be approximately 1.15 kb.

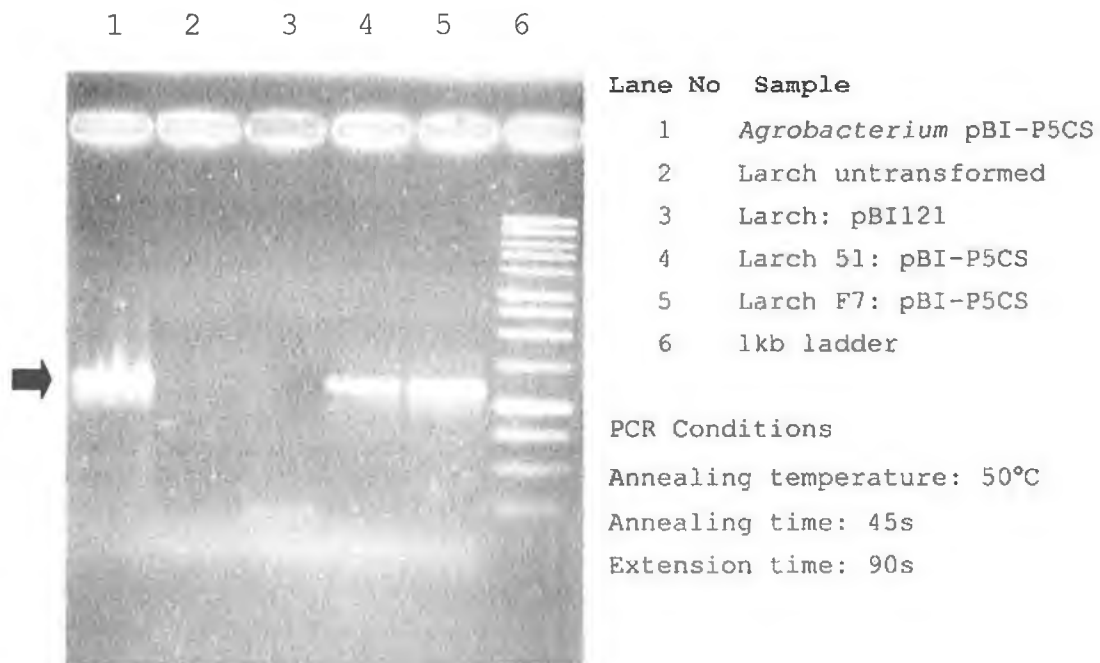


Plate 4.9

1.2 % agarose gel showing approximately a 1.15 kb fragment (situated just above the 1kb band on the ladder) primed from *Agrobacterium* (lane 1) and from Larch transformed with pBI-P5CS (lanes 4 and 5), but not from Larch transformed with pBI121 (lane 3). *Agrobacterium* pBI-P5CSF129A also shows 1.15kb band when run on a 1.2 % agarose gel (results not shown). The position on the DNA ladder corresponding to 1.15 kb is arrowed.

4.2.3 Southern Blot Analysis

Genomic DNA was isolated as previously described (from untransformed and transformed Larch). DNA (10 µg) was digested with *HindIII*, and then resolved on a 0.6% agarose gel. The probe was prepared by incorporating the DIG label into the primer sequences for the CAP5CS primers. PCR was carried out as stated in section 4.2.2.6. The agarose gel was blotted onto nitrocellulose paper, and probed with the DIG labelled DNA.

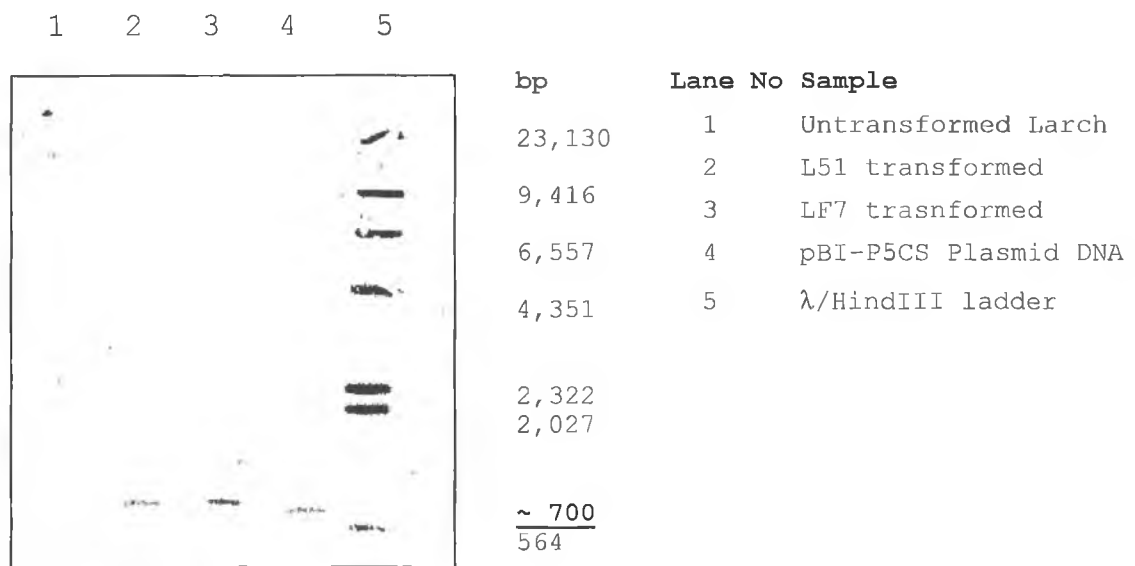


Plate 4.10

Genomic southern blot of two lines of Larch transformed with pBI-P5CS. Untransformed Larch DNA was used as a negative control, pBI-P5CS plasmid DNA as a positive control. The blot was probed with a DIG labelled proline gene asymmetric PCR product (primer CAP5CSF (DIG label incorporated) as outlined in Section 4.2.2.6). Figure 4.9 (p124) shows a restriction map of the plasmid pBI-P5CS. From this, it can be seen that cutting with *HindIII* yields approximately a 700 bp fragment between the multi-cloning site in front of the 35S promoter and a *HindIII* site at position 673 within the P5CS gene. This gives the bands shown in the Plate above.

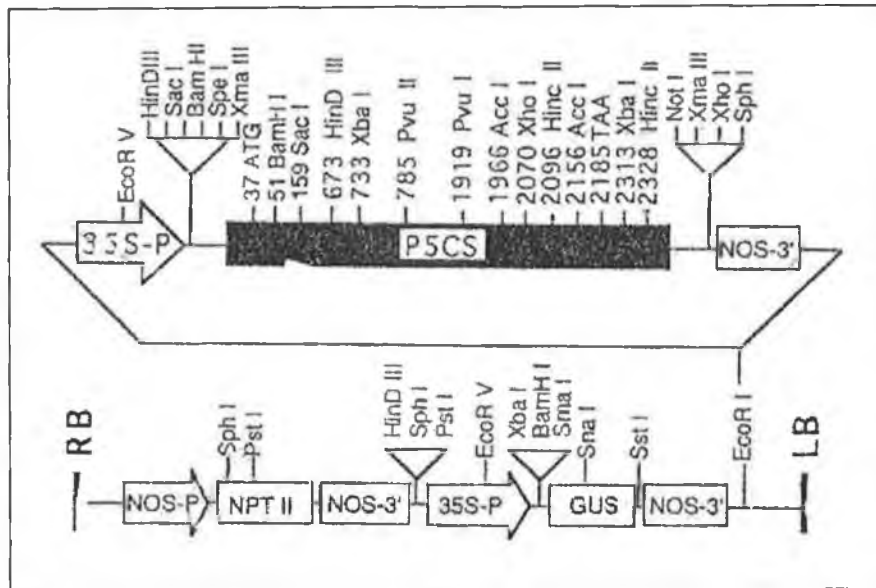


Figure 4.9

Restriction map of the plasmid pBI-P5CS used for the producing transgenic plants. The vector contains the CaMV 35S promoter, the NOS 3' region, NPTII, uidA and P5CS coding regions. The positions of the restriction enzyme sites of the *Vigna* P5CS cDNA are indicated on the map. Note the position of the HindDIII sites within the vector.

4.2.4 Cold tolerance of transgenic lines

Replicate cultures of Larch (untransformed) and L51, LF7 (transgenic lines) were incubated at 4°C and 24°C in 10 ml conical flasks containing no exogenous proline. Settled cell volume (SCV) was recorded every 2 days for 14 days, and growth rate was determined. A growth rate of approximately 0 was recorded at 4°C for the untransformed (normal) Larch cultures, while growth rates of approximately 0.01 were recorded for the transgenic lines. This compares well to results recorded for the addition of 1 mM proline in earlier experiments.

Figure 4.10 shows the growth rate of Larch (untransformed and transgenic) grown at 4°C and 24°C.

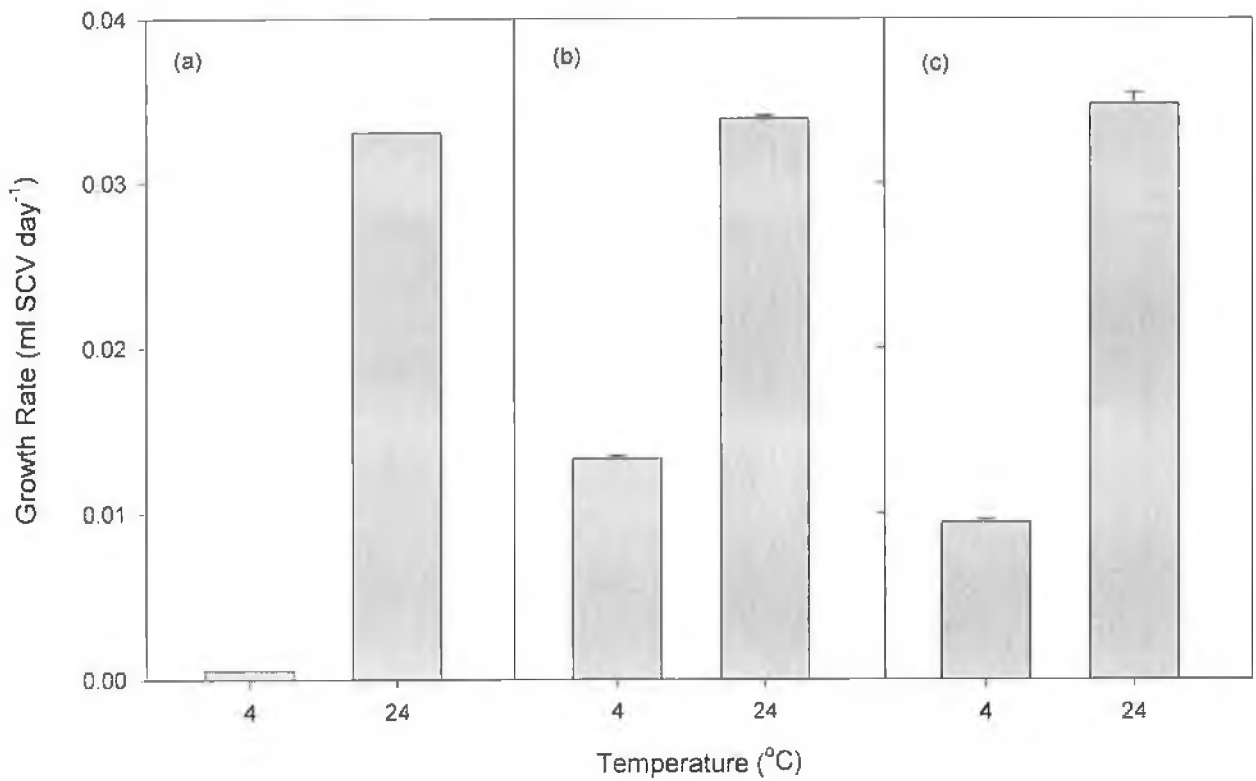


Figure 4.10

Growth rates of (a) untransformed Larch, (b) L51 transgenic line and (c) LF7 transgenic line at 4°C and 24°C. Mean values \pm SEM are shown where these exceed the dimensions of the symbols.

4.2.5 Salt tolerance of transgenic lines

Replicate cultures of Larch (untransformed) and L51, LF7 (transgenic lines) were incubated at 24°C in 10 ml conical flasks with 0, 50, 100 150 or 200 mM NaCl. Settled cell volume (SCV) was recorded every 2 days for 14 days, and growth rate was determined. A growth rate of approximately 0 was recorded at 200 mM NaCl for the untransformed (normal) Larch cultures, while low growth rates were recorded for the transgenic lines. At each salt concentration, the transgenic lines outperformed untransformed Larch.

Figure 4.11 shows the growth rate of Larch (untransformed and transgenic) grown with varying concentrations of NaCl.

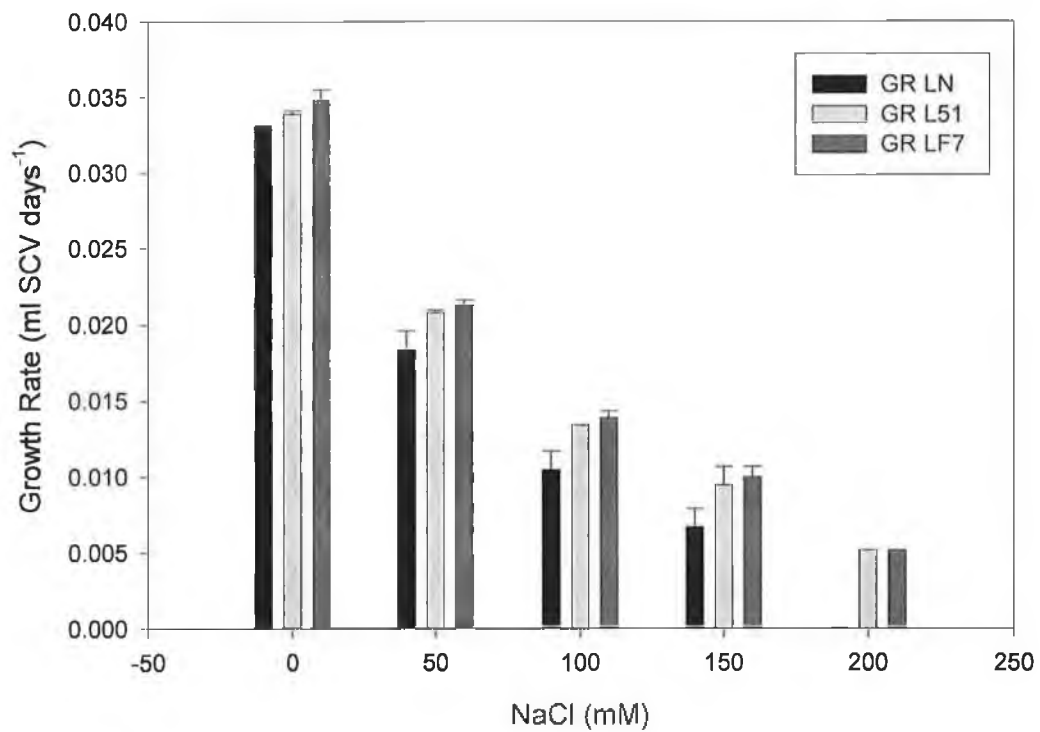


Figure 4.11

Growth rates of untransformed Larch (LN), L51 transgenic line and LF7 transgenic line at 24°C in varying concentrations of NaCl. Mean values \pm SEM are shown where these exceed the dimensions of the symbols.

4.2.6 Freezing tolerance of transgenic lines

Replicate cultures were chosen and samples removed which were then subjected to a decrease in temperature to -30°C , with samples taken at appropriate time intervals. Potassium leakage was then recorded using a Perkin Elmer 3100 atomic absorption spectrometer (AAS). K^{+} concentrations were determined by reference to appropriate standard solutions.

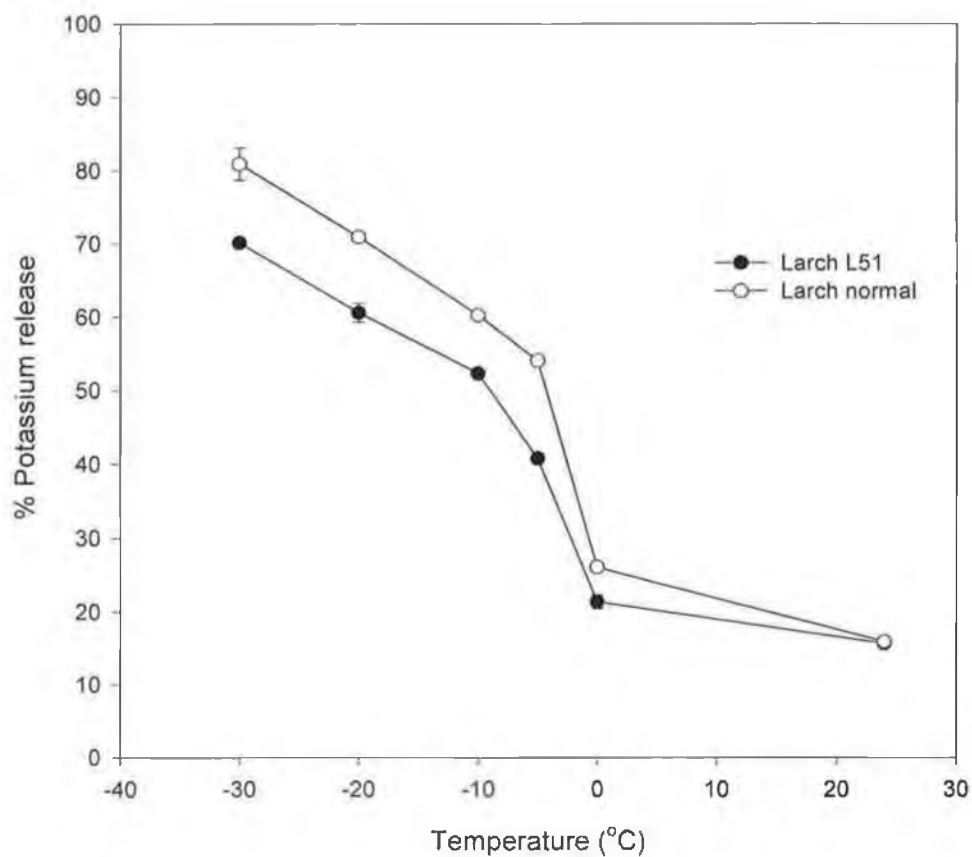


Figure 4.12

Freezing tolerance of transgenic Larch line L51 compared to normal Larch cultures, as indicated by potassium release. Mean values \pm SEM are shown where these exceed the dimensions of the symbols.

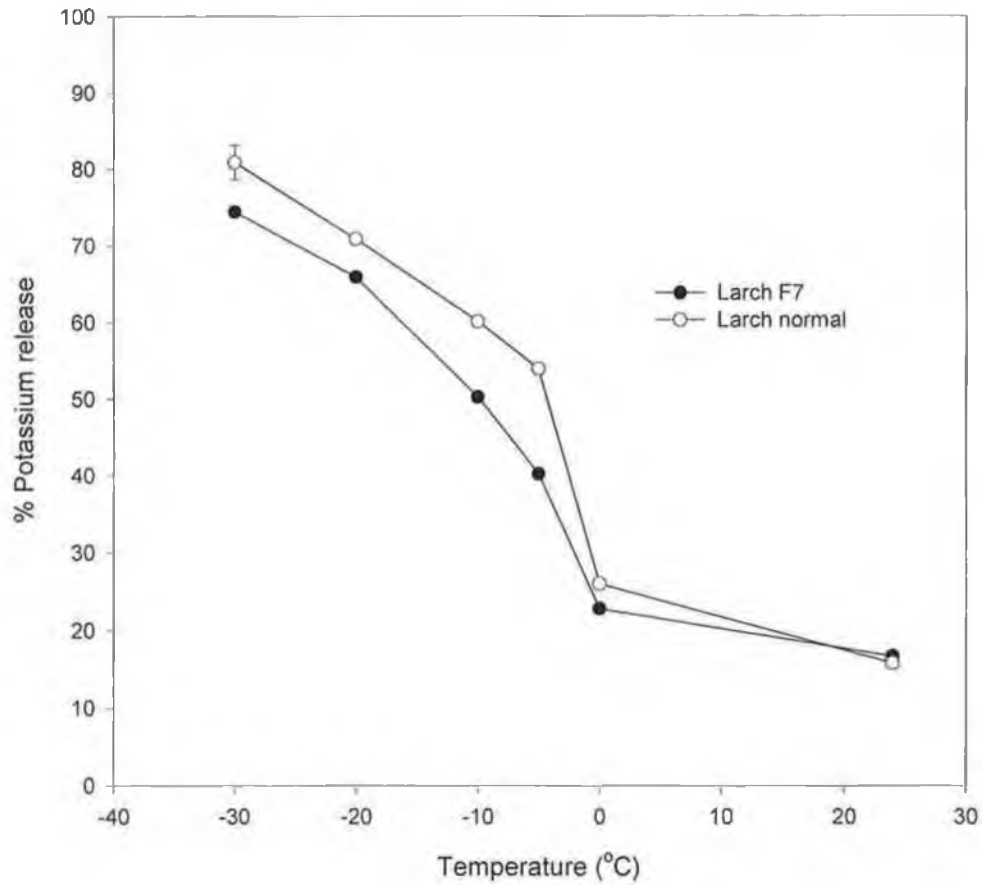


Figure 4.13

Freezing tolerance of transgenic Larch line LF7 compared to normal Larch cultures, as indicated by potassium release. Mean values \pm SEM are shown where these exceed the dimensions of the symbols.

4.2.7 Measurement of intracellular proline in transgenic lines of Larch

Samples were removed from both transgenic (L51 and LF7) and untransformed Larch and proline was measured using a modified ninhydrin assay and expressed as μ moles proline per g fresh weight.

Sample	Intracellular proline
Larch (normal)	0.0424 \pm 0.006
Larch: L51	1.361 \pm 0.0004
Larch: LF7	1.161 \pm .0348

Table 4.1

Intracellular proline concentration in transgenic and untransformed Larch. Results are the mean of multiple samples \pm SEM (μ moles proline per g fresh weight \equiv mM).

4.3 Discussion

All three plasmids, pBI121, pBI-P5CS and pBI-P5CSF129A, were introduced into *Agrobacterium tumefaciens* LAB4404 using *E.coli* HB101 as a helper plasmid. Many transformations were carried out in Larch, Sitka spruce and Oak. For Larch and Sitka spruce, it was necessary to prepare the cells seven days in advance by plating out 100mg (150mg for Sitka spruce) of embryogenic cells on filter papers. This allowed for growth over the following seven days, leading to the presence of 300mg cells per filter for transformation. *Agrobacterium* was streaked two days prior to transformation on 50 mgL⁻¹ kanamycin and grown at 25°C. Transformation and washing of cells are generally stressful procedures for the plant cells, and so sufficient time is allowed for the cells to recover before selection is applied. Following selection, transgenic cell clusters grew and pieces were screened for GUS activity. Positive clones were removed and labelled. These calli were then subcultured frequently to allow enough material for proline assay and for the regeneration of transformed plantlets. Selection and GUS assay were applied for an 8 month period before cell lines were deemed 'possibly transgenic', and subjected to molecular analysis.

No stable transgenic lines of Sitka spruce were obtained and for both pBI-P5CS and pBI-P5CSF129A only transient expression was recorded. Therefore, although PCR analysis was carried out with two suspected Sitka spruce transformants (suspected as they were growing on kanamycin 8 months after transformation), all PCR results were negative. No further molecular, biochemical or growth analysis was therefore carried out. Stable transgenic lines of Oak were also not obtained. Transient expression in single cells was routinely achieved, however difficulties arose in the selection of these transgenic single cells, and their conversion to globular embryos. Even in untransformed cells, it was difficult and time consuming to obtain globular embryos from single cells, this process taking a number of months. With the transgenic single cells it is possible that the process involving selection put extra pressure on the cells during the time of conversion to globular embryos. This process requires further investigations, and it is possible that this initial work will lead to a method of stably obtaining transgenic Oak plantlets in the near future.

Two stable transgenic lines of Larch were finally isolated, L51 and LF7. Both of these cell lines were subjected firstly to molecular analysis. PCR was carried out

on each cell line for each of the genes, UIDa, NPTII and P5CS. Both L51 and LF7 were shown to contain the GUS, NPTII and proline coding regions, as shown in the results section (Plate 4.7, 4.8 and 4.9). Each was also proven not to contain *Agrobacterium* DNA and therefore the gene sequences could be attributed to transformation of the cells rather than expression due to the presence of *Agrobacterium* DNA. This was further validated by Southern blot analysis, where a band of approximately 700 bp was achieved for the control DNA (from *Agrobacterium* pBI-P5CS) and for the transgenic lines L51 and LF7. This common band was detected in each transgenic line because the *HinD* III enzyme releases a 5' terminal fragment from the *Vigna* P5CS DNA. No larger bands were detected indicating that multiple insertions did not take place (see Restriction map Figure 4.9 and Plate 4.10).

Following from the molecular analysis, each cell line was analysed for its cold, salt and freezing tolerance as well as the concentration of proline it produces. Both transgenic cell lines responded better to cold than untransformed Larch. L51 recorded a growth rate of 0.0133 ml SCV day⁻¹ and LF7 0.00943 ml SCV day⁻¹, which is approximately 1/3 that of normal (24°C) growth for both of these cell lines, and compares favourably with the lack of growth realised in non-transgenic Larch. Again, both transgenic lines grew with 200 mM NaCl, which had completely inhibited the growth of untransformed Larch. In the freezing test, transgenic Larch at each temperature performed better than non-transformed Larch, for example, at -30°C, normal Larch released over 80% of its cellular potassium, whereas L51 released 70% and LF7 74%. Each of these cell lines were also examined for proline production, which was shown to be approximately 15 times higher than that recorded for non-transformed Larch.

In the light of the above results it can be seen that endogenous proline production does confer a level of tolerance to environmental stress, in particular cold, salt and freezing. Only mM amounts of proline are required for protection against water stress, as predicted in the exogenous proline experiments. When proline was added exogenously, levels of approximately 1 mM were predicted by Enzfitter™ as being the optimal level of proline required for half maximal growth. In the transgenic lines we managed to produce above 1 mM proline, which is sufficient to provide protection as predicted by earlier experimentation.

Chapter 5

Maturation and regeneration of both untransformed and transformed embryogenic cell lines of Larch (*Larix X leptoeuropaea*) and untransformed cell lines of Sitka spruce (*Picea sitchensis* (Bong) Carr.) and Oak (*Quercus robur* L.)

5.1 Introduction

Somatic embryogenesis allows the production of an unlimited number of propagules from a competent explant (Tautorus et al. 1991) and mimics zygotic embryogenesis, producing somatic embryos similar in morphology and development (Tautorus et al. 1991). An ability to convert somatic embryos to mature plantlets would shorten the time required to obtain a sufficient number of clonal plants for reforestation programmes.

The maturation of Larch and Sitka spruce beyond stage 1 embryos requires changes in the initiation and maintenance medium (Lelu et al 1994a,b, John et al. 1995). Somatic embryos are first removed to cytokinin free medium containing ABA (often a pre-maturation step is included where embryos are placed on medium containing activated charcoal for a limited time to remove hormones already present). This results in embryo development, resulting in yellow masses of globular somatic embryos with emerging cotyledons (John et al. 1995, Lelu et al. 1994a,b). In the absence of ABA somatic embryos are poorly developed and exhibit abnormal morphology, asynchronous development and precocious germination (Lelu et al. 1994a). ABA in the maturation medium promotes the development of high quality somatic embryos in large quantities. Under appropriate conditions, these somatic embryos germinate and develop into plantlets at high frequency (Lelu et al. 1994b). ABA prevents precocious germination and stimulates the accumulation of storage reserves such as storage proteins, triglycerides and lipids (Attree et al. 1992). Several workers have established optimal concentrations of ABA for both Larch and Sitka spruce (Klimaszewska 1989, Lelu et al. 1994, John et al. 1995), ranging from 40-60 μM . Germination of matured embryos takes place on hormone free and ABA free medium. During this stage the cotyledons start to elongate and develop. Tissues are firstly placed in the dark for a short period of time, this is when germination occurs. Following this, the germinating embryos are removed to the light, whereupon root and shoot growth begins.

Plant regeneration from Oak somatic embryos has been achieved for *Quercus rubra* (Gingas and Lineberger 1989) and *Quercus robur* (Chapula 1990), however at low frequency. The absence (*Q. suber* (El Maataoui and Espagnac 1987), *Q. ilex* (Feraund-Keller and Espagnac 1989)) or low frequency (*Q. robur* L., *Q. suber*) conversion of

somatic embryos into plantlets poses a serious problem for Oak regeneration. The development of somatic embryos is often blocked after the formation of cotyledons. The maturation and conversion of embryos involves a sequence of medium changes and the correct choice of embryogenic material (Chapula 1995). Somatic embryos are removed to maturation medium with no hormones. Again, this allows for the development of the embryos. Following this step, Oak embryos are subjected to a short period of desiccation in a desiccation chamber at 27°C. After desiccation, embryos are removed to germination medium containing BAP and IBA, which allows for the development of roots and shoots.

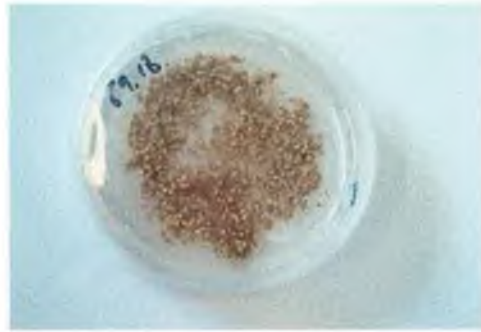
5.2 Results

5.2.1 Maturation and regeneration of untransformed and transformed cell lines of Larch (*Larix X leptoeuropaea*)

Hybrid Larch was maintained on Whatman No. 2 filter paper at a concentration of 100 mg cells per filter. Every 10 days these cells were subcultured. For the purposes of maturation, a filter (containing cells that were 7 days old) was transferred to a pre-maturation medium (containing activated charcoal and no growth regulators) and incubated for 7 days. Following this the filter was removed to maturation medium (containing an elevated sucrose concentration, ABA and IBA. Embryos were then allowed to mature for 3 weeks, after which time matured embryos were removed to germination medium (no phytohormones) to allow for germination of the embryo into a plantlet. For the transgenic lines, L121 (pBI121), L51 (pBI-P5CS) and LF7 (pBI-P5CS), kanamycin at 50 mgL⁻¹ was incorporated into all media and all transgenic plantlets were analysed for GUS expression after 4 weeks on germination medium.



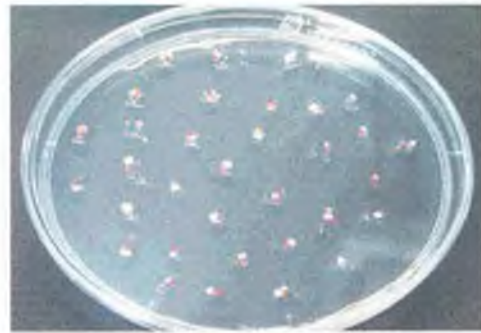
(a)



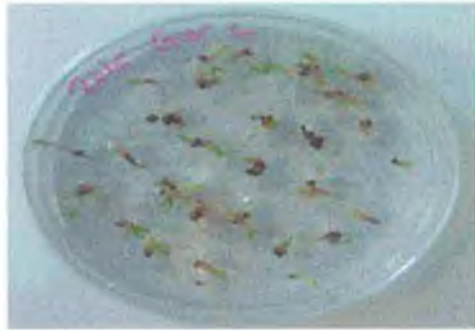
(b)



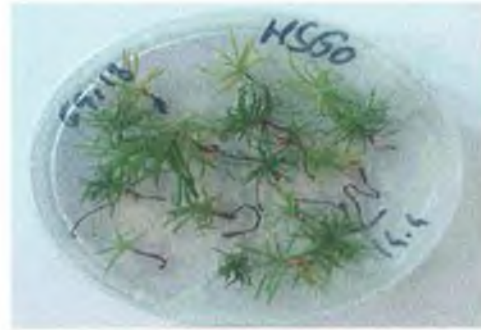
(c)



(d)



(e)



(f)

Plate 5.1

Maturation and regeneration of Larch (*Larix X leptoeuropaea*) in 9 cm petri-dishes (a) embryos on maintenance medium (b) embryos on maturation 2 medium (c) close up of maturing embryos (zoom = 250%) (d) embryos on germination medium: 2 weeks (e) embryos on germination medium 4 weeks (f) plantlets after 2 months



(a)



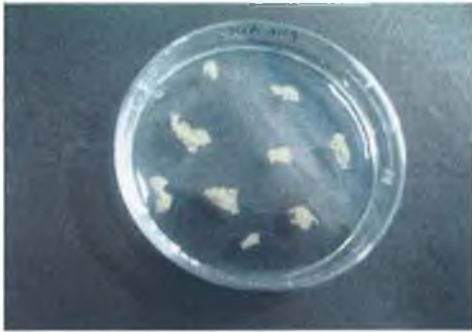
(b)

Plate 5.2

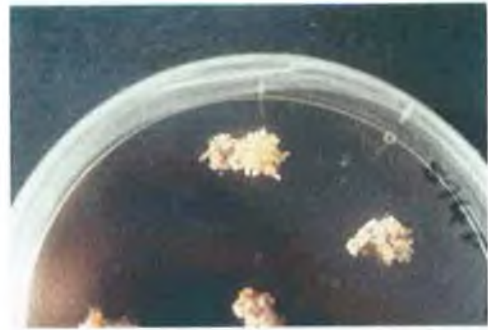
Maturation and regeneration of Larch (*Larix X leptoeuropaea*) transgenic cell lines (L51 and LF7). (a) GUS assay of transgenic embryos that were matured on pre-maturation & maturation media with 50 mgL^{-1} kanamycin. Blue colouration can be observed within the embryos (b) GUS assay of transgenic plantlets, again blue colouration can be seen within the needles and shoot in the embryo on the left, compared to no colouration in the untransformed plantlet on the right.

5.2.2 Maturation and regeneration of untransformed and transformed cell lines of Sitka spruce (*Picea sitchensis* (Bong) Carr.)

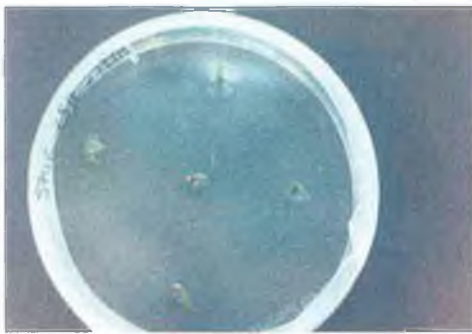
Sitka spruce embryos were maintained on embryo initiation medium for 3-4 sub-cultures prior to maturation. Following this period, ESMs were transferred to embryo development medium (containing activated charcoal and ABA) for a period of 6-8 weeks in the dark. Developing somatic embryos were seen on the ESMs after 6-8 weeks and were heart-shaped and yellow in appearance. Each developing embryo was transferred to embryo germination medium (containing no phytohormones). Each of these was cultured for 5-7 days in the dark and then light for 5-7 weeks. The individual plantlets were separated out as they developed and placed on fresh germination medium every 6-8 weeks.



(a)



(b)



(c)

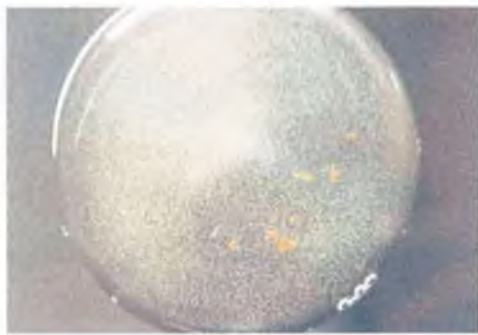


(d)

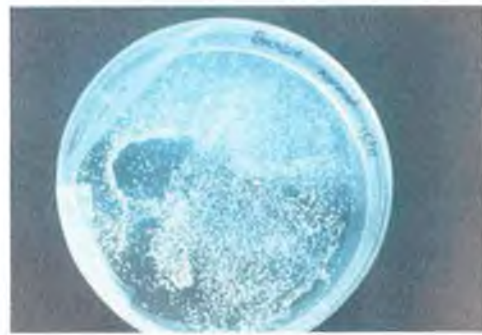
Plate 5.3

Maturation and regeneration of Sitka spruce (*Picea sitchensis* (Bong) Carr.) in 9 cm petri-dishes (a) embryos on maintenance medium (b) embryos on maturation medium, 8 weeks (c) embryos on germination medium: 1 week (d) embryos on germination medium, 4 weeks

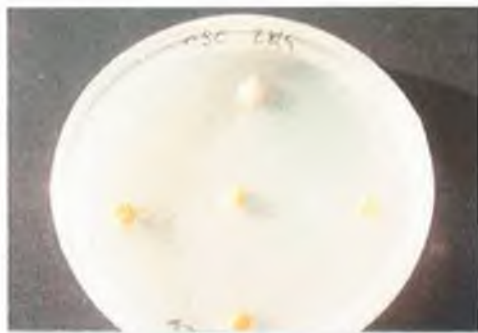
5.2.3 Conversion of Oak single cells to globular embryos



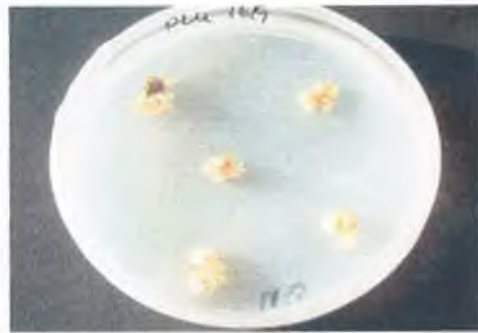
(a)



(b)



(c)

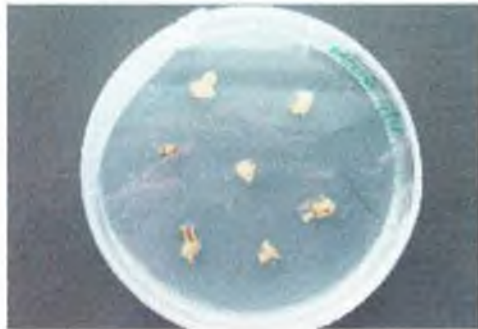


(d)

Plate 5.4

(a) Oak single cells grown in liquid medium with a reduced cytokinin concentration ($10 \mu\text{l L}^{-1}$ BAP) (b) Oak single cells plated onto solid MS medium with elevated cytokinin concentration (1ml L^{-1}) (c) Oak single cells forming large aggregates (d) Oak single cells enter the globular stage, at which time they could be transferred to a maturation and regeneration process. (c) and (d) are growing in 9 cm petri-dishes.

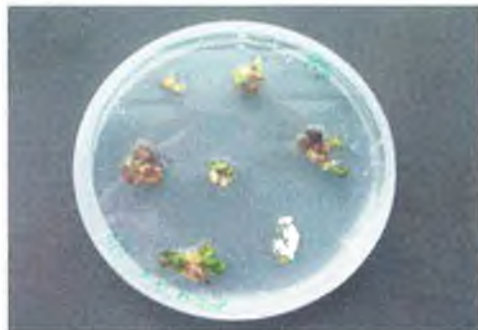
5.2.4 Regeneration of untransformed cell lines of Oak (*Quercus robur* L.)



(a)



(b)



(c)



(d)

Plate 5.5

Maturation of Oak embryos in 9 cm petri-dishes (a) Oak embryos on P24 maintenance medium (b) Close-up of Oak embryo transferred to P24 maturation medium, after approximately 4 weeks these embryos are transferred to dessication chambers (c) Embryos on germination medium, after 1 week in dessication chamber (d) Regenerated plantlet, showing root and shoot

5.3 Discussion

It is necessary when carrying out genetic transformation experiments to determine that it is possible to regenerate the relevant cell lines via a maturation and germination process. In this case all cell lines used were regenerated as non-transformed plantlets in order to determine the ability of the cells used to be regenerated. Plant regeneration is affected by many factors, including abscisic acid, increased osmoticum, duration of maturation of somatic embryos and general hormone levels, and previously investigated maturation and regeneration processes were utilised within this work to determine the ability of the cell lines in question to regeneration.

The most important factor influencing the germination and plantlet growth in Hybrid Larch was the time that the somatic embryos were left on the maturation medium (Lelu et al. 1994b). 3 weeks on maturation was recommended as optimal as those embryos left an extra week, although morphologically similar, resulted in lower germination frequency, plantlet conversion frequency and slower epicotyl development. It is also reported that for Hybrid Larch there was no significant effect abscisic acid concentration on either the number of somatic embryos germinated or the number of plantlets obtained (Lelu et al. 1994b). Sucrose at 0.2 M applied during maturation was significantly more beneficial in attaining high germination rates (compared to lower sucrose concentrations). The optimal plantlet recovery procedure therefore called for 3 weeks on maturation medium containing 40-60 μM ABA with 0.2 M sucrose, and this allowed for the germination of the maximal number of embryos, as utilised in this work. Transgenic Larch lines (L51 and LF7) were matured and germinated in exactly the same manner as normal, except for the inclusion of antibiotics ticarcillin (500 mgL^{-1}) and kanamycin (50 mgL^{-1}). Both transgenic lines produced similar germination numbers to normal Larch and both also when screened for GUS activity were positive.

Maturation of Sitka spruce ESMS was greatly affected by the presence of exogenous ABA. It was reported that the use of ABA in the maturation medium resulted in synchronised maturation of Sitka spruce somatic embryos (Krogstrup 1988). The best maturation in terms of morphological characters reported was obtained on medium with ABA as the sole growth regulator. Embryo development (containing charcoal) was

supplemented with 50 mgL⁻¹ ABA, and at this concentration yielded maximal embryo conversion (Patterson 1999).

The production of Oak single cells was investigated due to the difficulties in transformation of globular Oak embryos. Single cells were produced by reducing the BAP concentration 100-fold, and after approximately 10 days removing the liquid and continuing subculture with the reduced cytokinin level. This produced an almost homogenous suspension of single cells and small cell aggregates. It was then attempted to re-produce globular embryos from the single cells by placing them once again with elevated BAP levels. We were able to produce globular embryos again, however difficulties were encountered in the production of plantlets from any of the single cell originating embryos. Further investigations need to be carried out in the area of the formation of globular embryos from single cells, and their subsequent regeneration to Oak plantlets. It was possible to transiently transform the single cell suspensions, however we did not obtain any transgenic plantlets due to the difficulties in conversion of single cell originating globular embryos to matured plantlets.

Maturation and regeneration of Oak was a more involved process than that for either Larch or Sitka spruce, and was performed using P24 medium. It was found that the frequency of root and shoot proliferation was greatly improved by the use of agar as an osmoticum and previous culture on P24 medium containing BAP (Eva Wilhelm, per comm). An increase in germination rates was also obtained by the use of a desiccation step after maturation. Within the germination medium, both BAP and IBA are recommended, while culture in the light was beneficial for cotyledon formation and for greening (Wilhelm, per comm).

It has been shown within this work that each of Larch, Sitka spruce and Oak can be regenerated from somatic embryo cultures given the correct treatment. Only Larch yielded transgenic cell lines, from which we were also able to successfully regenerate without changing the protocol beyond the inclusion of antibiotics for the exclusion of *Agrobacterium* and for selection of the transgene.

Chapter 6

Discussion

6.1 Discussion

Forestry in Ireland is fast becoming one of the major issues facing agriculture; Ireland is one of the least forested countries in the EU and urgently needs to address this problem (ENFO). Breeding programmes have been introduced gradually by the state forestry agency, Coillte, who are also now developing new breeds of trees both through classical breeding and genetic engineering. Although there is increased experimentation into the production of transgenic trees, the application of classical breeding techniques to tree species has been limited by their long regeneration times. Other obstacles include limited knowledge of the genetic maps of most forest trees and difficulties involved in controlled mating. Genetic engineering of somatic embryos is potentially useful in forestry as specific genetic changes could be made in a relatively short period of time. One of the major problems facing genetic engineering of somatic embryo cultures is the low frequency of transformation and low conversion of embryos to plantlets.

Trees are the largest living organisms in the world and greatly influence food chains, air quality, industry and many forms of landscape. However they also face many problems in survival including insects, environmental stresses, air pollution problems and soil fertility. Cold, drought, salinity and freezing are the main abiotic stresses specifically affecting trees, and it is possible that genetic engineering could help address these problems. Forestry productivity has been improved by a number of methods such as the biochemical modification of wood characteristics (lignin content (Halpin et al. 1994), tree form and performance (Gaudin et al. 1994)), resistances (insect resistances (Klopfenstein et al. 1993, Shin et al. 1994, Shah et al. 1995), herbicide resistance (Shin et al. 1994, Fillalti et al. 1995)) and abiotic stresses (antifreeze genes (Georges et al. 1990), lipid composition (Murata et al. 1992)).

The production of compatible solutes is a general plant response to an environmental stress such as salt, cold and freezing (Bohnert and Jensen 1996, Holmström et al. 1996, Hare et al. 1998). These compatible solutes are usually produced in order to provide turgor, to protect proteins and membranes or to alleviate oxidative damage. One such solute is proline, which is a non-essential amino acid produced under environmental stress. It has been shown by many researchers that exogenous proline

helps alleviate environmental stress (Duncan and Widholm 1987, Van Swaij et al. 1995, Santos et al 1996). Using three forest species, Larch (*Larix X leptoeuropaea*), Sitka spruce (*Picea sitchensis* (Bong.) Carr.) and Oak (*Quercus robur* L.), the effect of exogenous proline on growth under salt, cold and freezing stress was examined.

Embryogenic cultures of each of the three species were used in the investigations, which showed that growth was limited in the presence of cold temperature (4°C) and increasing salt concentration (0-200 mM NaCl). Reducing the temperature of the surrounding environment close to freezing resulted in cessation of growth under normal conditions and browning and necrosis of cultures was evident. Addition of proline at 1 mM influenced the growth of the Larch and Sitka spruce cultures to such an extent that they did not brown and grew at approximately 1/3 the normal growth rate. Increasing the proline concentration above this level continued to result in an increase in growth rate. Queiroz et al. (1998) show that exposure of Coffee to low temperatures causes growth inhibition, changes in metabolic rates and membrane alterations. Duncan and Widholm (1989) also show that proline added exogenously, as well as conditions that induce proline accumulation (ABA and mannitol application), increase the cold tolerance of Maize.

By carrying out kinetic analysis of the cold stress data, it was possible to show that in order to achieve 50% of the maximum growth rate, an intracellular proline concentration of 0.329 μ moles/g fresh weight (Larch) (given that plant cells are >90% water this is equivalent to mM), 0.149 mM (Sitka spruce) or 1.208 mM (Oak) is required. Under normal conditions these species produce approximately 0.04 mM, and again under normal conditions but with the addition of 1 mM proline they accumulate 1.708 mM (Larch), 1.621 mM (Sitka spruce) and 0.834 mM (Oak). According to the kinetic analysis, these amounts are easily sufficient to help alleviate cold stress. Genetic engineering may make it possible to produce trees that over accumulate to give the desired proline concentration to give half maximal growth.

Previous researchers have shown that salt has a negative effect on cell growth (Lone et al. 1996, Gangopadhyay et al. 1997 and Bajji et al. 1998). In this work it has been shown that salt has a negative effect on the growth of embryogenic suspensions of

Larch, Sitka spruce and Oak. As the salt concentration was increased, the growth rate decreased in every case. Addition of proline, again at 1 mM, increased the growth rate at each salt concentration examined. Gadallah (1999) show that both proline and glycine betaine application (8.7 mM) results in lower membrane injury and increases in growth under salt stress in Bean (*Vicia faba* L.cv. Calvor). Okuma et al. (2000) show also that 10 mM proline promotes growth of tobacco suspensions subjected to osmotic stress and also an increase in intercellular proline. However, they suggest that the levels of proline in the cells is not sufficient to act as an osmoprotectant, but they report that they are unclear as to the exact role that proline is playing in providing osmotic tolerance. Analysing the kinetic data, it can be seen that the proline concentration required to achieve half maximal growth is 0.748 mM (Larch), 0.524 mM (Sitka spruce) and 1.789 mM (Oak). Again, genetic transformation with proline overproduction genes may have the same effect as exogenously adding proline.

Lone et al. (1999) also show that the addition of exogenous proline to cultured Barley (*Hordeum vulgare* L.cv. Maris Mink) embryos increased shoot elongation under saline conditions. Inhibition of shoot elongation by NaCl was relieved by proline when plantlets were grown in deep crystallizing dishes but not in petri dishes where shoots come into direct contact with the medium. The effect of proline could therefore be related to a decrease in shoot Cl⁻ and Na⁺ accumulation which was only observed in plantlets grown in crystallizing dishes. This suggests that proline may be interacting with NaCl uptake in the roots and that the transgenic plantlets obtained in the present work may have a different tolerance to salinity than shown here, as all the work was carried out with embryos submerged completely in salt, while if only roots of the transgenic plants were exposed, they may show increased ion selectivity due to endogenous proline production and therefore have increased tolerance.

In many species cells are adversely affected by freezing temperatures. Ice forms in the cytoplasm and extra cytoplasmic spaces fracturing the membranes and destroying subcellular organisation. It has been shown for many species that proline provides protection against damage by freezing temperatures: apple (Benko 1968), alfalfa (Paquin 1977), wheat (Stefl et al. 1978) and it has also been shown that proline may stabilise cell

membranes during such damage (Csonka 1989, Van Rensburg 1993, Holmberg 1998). In the case of Larch, Sitka spruce and Oak it was found that exogenously applied proline protected the cells to a certain extent from frost damage, as indicated by a reduced potassium leakage with the addition of proline. At every temperature examined, potassium leakage was lowered by the addition of proline from 1-100 mM. Van Swaij et al. (1985) positively correlated frost tolerance to leaf proline content in *Solanum*. They show that an increase in frost tolerance accompanied by proline accumulation could be achieved by drought (wilting) and cold hardening, also that an increase in frost tolerance can be achieved by exogenously adding proline. At low levels accumulation of proline was accompanied by a large increase in frost tolerance, but that at high levels frost tolerance hardly changed with increasing proline concentration. This is in agreement with all of our findings with Larch, Sitka spruce and Oak in relation to increasing proline concentration.

The positive effect proline had on each of the three species has been shown. We hypothesised that the genetic engineering of a proline overproduction gene into the species in question would allow the cells to over produce proline and thereby provide themselves with protection against the environmental stresses mentioned. It is reported that proline production is regulated by the P5CS gene and in order to determine this Nanjo et al. (1999) generated anti-sense transgenic *Arabidopsis* plants with a P5CS cDNA. They found that the transgenic lines accumulated proline at a significantly lower level than wild-type plants. This work provides a direct evidence for the key role of P5CS in proline production and thus this was the gene used in transformation experiments in the present work.

There are many methods available for the genetic transformation of tree species. *Agrobacterium* is capable of infecting a number of conifer (Sederoff et al 1986, Ellis et al 1989, Loopstra et al. 1990, Stomp et al. 1990, Tzfira et al. 1997) and *Quercus* species (Evers et al. 1988, Roest et al. 1991) and it was chosen as the method of choice in the present work. It was shown previously in the laboratory (Patterson 1999) that up to 500 mgL⁻¹ cefotaxime eliminated *Agrobacterium* and did not adversely affect the growth of the cells (Roest and Evers 1991, Sarma et al. 1995). Lelu (per comm) recommended also

using 500 mgL⁻¹ ticarcillin/clavulanic acid, which also helped to eliminate *Agrobacterium* growth and did not adversely affect the growth of the embryogenic cells (Nauerby et al. 1997, Cheng et al. 1998). These antibiotics were incorporated into the medium two days after transformation, and their use was continued right through to the regeneration procedures to ensure continued elimination of the *Agrobacterium*.

The selectable marker present on the plasmids used was NPTII which confers resistance to a number of amino glycoside antibiotics and has been routinely used in a number of transformations for the selection of transgenic plant material (Loopstra et al. 1990, Roest and Evers 1991, Bommineni et al. 1993, 1994, Shin et al. 1994, Howe et al. 1994, Charest et al. 1996, Drake et al. 1997, Levee et al. 1997, 1998, Lelu 1999). Kanamycin was previously shown to be selective at a level of 25 mgL⁻¹ for Larch (Lelu, per comm), 50 mgL⁻¹ for Sitka spruce (results not shown), and 10 mgL⁻¹ for Oak single cells (results not shown). For Larch and Sitka spruce, 10 days after transformation, cells were placed on medium containing kanamycin. This time period allowed the cells sufficient time to recover from the transformation and washing procedures and to incorporate the desired genes into their genome. Once kanamycin was applied, the cells were subcultured every 10 days, and every 5 days liquid medium was dispensed over the cells to ensure continued selective pressure. The selective agent was incorporated into the medium right through to the regeneration stage to ensure continued presence and expression of the desired genes.

The use of β -glucuronidase as a reporter gene proved successful for the initial identification of possible transgenic cell lines. The major disadvantage however was the destruction of valuable transformed material at regular (3-5 days) intervals to carry out histochemical assays. All possible transgenic lines identified originally via GUS assay were subjected to PCR analysis for the presence of the gene prior to any further manipulations. The green fluorescent protein (GFP) gene is proving to be very useful as a reported gene because it requires no additional substrate to assay, merely excitation with near ultra violet (UV) or blue light and perhaps where transgenic material is precious would prove a better reporter of transformation (Chalfie et al. 1994).

The solitary report on the transformation of embryogenic cell lines of Sitka spruce is described by Drake et al. (1997), however they only report transient expression of the GUS gene, and fail to report successful regeneration of transgenic cell lines. Lelu (per comm) has identified a procedure for the transformation of Larch, for which she has reported the regeneration of transgenic lines (Lelu 1999). Throughout the present work, many methods of *Agrobacterium* transformation were investigated, liquid v solid culture, change in pre-culture and co-cultivation times, different *Agrobacterium* concentrations (methods and results not described), however only the method outlined in detail in Chapter 2 proved to be successful. Obtaining transient expression was relatively straightforward using any of the methods, however GUS activity generally rapidly deteriorated, ultimately resulting in no stably transformed cell lines. The successful transformation procedure outlined here required many manipulations that needed to be performed at the correct time and under the correct conditions. The cells had to be prepared for transformation 7 days in advance and experience proved the best method of knowing if the condition of the cells was sufficiently good to allow for successful transformation. In general the cells had to be well hydrated and lacking in necrotic or blackened sections, as reported by F. Harrington (Coillte) and M.A. Lelu (INRA) (per comm). After transformation and co-cultivation, the cells required washing to remove the *Agrobacterium*. These washings proved critical, and medium was required rather than water. Antibiotics were applied in the final three washings to ensure that all of the cells were immersed in antibiotic solution to eliminate *Agrobacterium*. Once the washings were complete, the cells were filtered onto nylon membrane. The filtration more successfully removed the *Agrobacterium* than any other method attempted, due in part to the amount of liquid drawn off the cells. The cells were then allowed 10 days to recover from all the manipulations before kanamycin selection was applied. This timing was critical and proved successful for Larch but not for Sitka spruce. Selection was applied in total for 8 months before genetic analysis and regeneration took place. After this time, 12 possible transgenic lines of Larch and 8 possible transgenic lines of Sitka spruce were still growing on kanamycin, however only 2 of the Larch lines were still expressing GUS. In any case, all these lines were analysed by PCR for NPTII, GUS and

Agrobacterium genes before any further manipulations took place. No Sitka spruce lines were positive for NPTII, GUS or *Agrobacterium*, and the two GUS positive lines of Larch were the only 2 positive NPTII and GUS lines identified by PCR. The GUS expression obtained was generally weak and we report a possible problem in using GUS with conifer species. None of the lines were positive for *Agrobacterium*, proving that the genes being identified were contained in the plant DNA

Transformation of woody species has traditionally been slow and difficult and attempts to use *Agrobacterium* for genetic transformation of conifers followed by subsequent regeneration of whole plants has largely been unsuccessful with the exception of Larch: *Larix deciduas* (Huang et al. 1991) and *Larix leptoeuropaea* (Lelu 1999). Many methods for transient transformation of forest species have been described (Ellis et al. 1989, Roest and Evers 1991, Drake et al. 1997, Patterson 1999, Wenck et al. 1999) Recently, methods have been developed to produce transgenic forest species including Hybrid poplar (*Populus alba* X *P. grandidentata* cv. 'Crandon') (Howe et al. 1994), *Populus tremula* (Tzfira et al. 1997) and Japanese white birch (*Betula platyphylla* var. *japonica*) (Mohri et al. 1997). The methods described here are similar to those described else where in the literature and proved successful as reported by Lelu 1999 and in the present work.

Oak single cell transformation was performed using a procedure investigated within the Plant Cell Culture Laboratory. No previous reports of transformations involving Oak single cells could be located in the literature, therefore procedures had to be investigated. Again, as was the case for Larch and Sitka spruce, many transformation methods were investigated (methods and results not shown) but the most successful was the one outlined in the present work. Transient expression was again identified, however as mentioned previously we experienced difficulties in regeneration from these cells and in fact we had difficulties in obtaining globular embryos from the transformed single cells. The time frame involved in this experiment was quite long, as it takes months to convert from single cells to globular embryos and together with applying selection this may have hindered the development of globular embryos from transformed single cells.

There wasn't enough material available to perform any PCR analysis on these lines and only transient expression of GUS in single cells was observed.

Together with the NPTII and GUS genes, a gene for the overproduction of proline (P5CS gene) was present within the gene construct to be transferred to the plant genome. This DNA it was hoped would provide the cells with a method of producing enough proline themselves to provide protection without the application of exogenous proline. Once 2 cell lines (Larch) proved to be PCR positive for NPTII and GUS, the presence of the P5CS gene was analysed by PCR. In order to detect the P5CS sequence inserted (and not any P5CS gene that may be present already in the plant), the PCR primers were designed around the promoter region and the P5CS gene sequence. We identified a set of primers encompassing a section of the 35S promoter sequence (forward primer; upstream from the start codon) and a section of the P5CS sequence (reverse primer). These primers resulted in a 1.15 kb fragment to be isolated from *Agrobacterium* carrying the P5CS plasmid, and from the two transgenic lines. No band was visible for normal Larch, indicating that the P5CS gene had been successfully incorporated into the plant DNA. Following these results, further genetic analysis was carried out in the form of a Southern blot. Plant genomic DNA was digested with Hind III and probed with DIG labelled P5CS DNA from an asymmetric PCR. The Southern blot shows that only one copy of the gene has been inserted into each of the genomes, one 0.7 kb band. This common band was detected in each transgenic line, because the Hind III enzyme releases a 5' terminal fragment from the *Vigna* P5CS DNA (see Restriction map. Figure 4.9). No larger bands were detected indicating that multiple insertions did not take place.

Once insertion of the P5CS gene was confirmed by genetic analysis, the level of cold, salt and freezing tolerance, as well as proline levels needed to be examined. It was found that both transgenic lines responded better to the cold than untransformed Larch, actually recording a growth rate compared to none with untransformed Larch. Also, each transgenic cell line out performed untransformed Larch at each salt concentration examined. In the freezing tolerance test, transgenic Larch at each temperature released less cellular potassium indicating it was surviving better than untransformed Larch.

Results found for transgenic Larch were compatible in all three tests (cold, salt and freezing) to those results obtained when 1 mM proline was added exogenously to untransformed Larch. Levels of cellular proline were much higher in the transgenic lines: 1.36 mM for L51 and 1.16 mM for LF7, compared to 0.04 mM in untransformed Larch. Our transformants were obtained using the pBI-P5CS plasmid, and not the further mutated pBI-P5CSF129A version. It is thought that had we obtained any transformants using this plasmid that they would have produced more proline and had a higher level of tolerance than the transgenic cell lines obtained through this work. Hong et al. (2000) report transgenic tobacco lines transformed with the F129A version that produced more than double the proline achieved through P5CS transformation. They also report that this level of proline resulted in a reduction of free radical levels in response to osmotic stress, an area we did not examine but that could provide useful in the future.

It was possible to regenerate plantlets from all untransformed cell lines used in this work. In order to carry out any manipulations with somatic embryo cultures, it is important to carry out regeneration experiments, in order that any transgenic lines obtained may also be regenerated. Both Larch and Sitka spruce could be regenerated with ease by following established protocols. The regeneration of Oak proved much more difficult, with much expertise required in order to select the correct embryos to put through the regeneration protocol. Once it was established that these lines could be regenerated, it was known that regeneration of transgenic lines would be feasible. Both transgenic Larch lines were regenerated using the established protocol and incorporating cefotaxime, ticarcillin/clavulanic acid and kanamycin. GUS stained matured embryos and plantlets can be seen in Chapter 5, Plate 5.2. No further analysis of regenerated lines was carried out, as all analysis was performed on the embryogenic cell lines and it was only required to be established that the transgenic lines could indeed be regenerated and that they would still be transgenic (GUS positive). The genetic analysis of the embryogenic cell lines prove that the integration of the genes in question is stable rather than transient and that therefore the regenerated plantlets would still carry this DNA.

In order to obtain the two transgenic lines of Larch, many transformation experiments were performed, which ideally should have resulted in many more

transformation events. With Sitka spruce no transgenic lines were obtained, despite again many transformation experiments. Reasons for such low levels of transformation are unclear, however for Sitka spruce it is thought that the procedure needs more modification as it is essentially tailored for Larch. Patterson (1999) carried out much research into optimization of Sitka spruce transformation in liquid culture (reporting only transient transformation), and it is possible that the same level of work is required for the procedure as described in the present work. Perhaps Sitka spruce requires a longer co-cultivation time, or a higher/lower concentration of *Agrobacterium*, however as much manipulations had previously been carried out with other Sitka spruce transformation protocols, time did not allow for further modification of the most successful procedure. Although two transgenic lines of Larch were obtained, the rate of transformation was much lower than expected and the reasons are unclear. It may be due to the nature of the plasmid or the *Agrobacterium* being used in this case, as in previous transformation carried out by Lelu using different plasmids and strains of *Agrobacterium* a higher frequency of transformation was obtained (per comm). It is possible in general that other factors relating to integration and structure of the transgene DNA, such as number of transgene copies, position in the genome and methylation, may greatly influence expression of transgenes. Transgene silencing is the inactivation of transgene expression despite the presence of an unchanged but possibly methylated transgene sequence in the plant genome. Transgene silencing is associated with specific changes in the transgene integration pattern. The presence of multiple copies at the integration locus as reported to be a causative factor in transgene silencing (Matzke and Matzke 1997). This phenomenon of transgene silencing is the most likely reason for the loss of transgenes in transiently transformed embryogenic cells. It is possible that some of the procedures also have been too optimized for transient expression and therefore multiple insertions are taking place and thus resulting in transgene silencing.

Proline overproduction has long been associated with plant tolerance to a range of environmental stresses. The exact role played by proline is as yet unclear, however researchers propose many modes of action. It is possible to relate the levels of proline produced in some cases to the mechanism of resistance. In the case of the present work,

we have produced transgenic cell lines of Larch that accumulate mM levels of proline. There are a number of possible modes of action, including

1. Proline is acting as an osmoprotectant, and partitioning itself into the cytoplasm in order to do this. As plant cells are very vacuolate, unless the proline is partitioned into the cytoplasm the concentration produced here is probably not high enough to act purely as an osmoprotectant
2. Proline is acting as an anti-oxidant defence mechanism, helping the cells to avoid injury due to ROS. Most ROS are produced in the chloroplast, and the cells examined here are not actively photosynthesizing and therefore the cells will not be producing light-activated ROS, but may be producing ROS due to the stress applied
3. Proline is acting as a membrane protector, stabilizing proteins and membrane structures
4. Proline is acting as a cryoprotectant (cold stress), redox sink, energy store

It is difficult to determine from the present work to what extent the above modes of action of proline are responsible for providing protection from environmental stress. Much work still needs to be carried out to investigate the exact role of proline in stress tolerance, and in the current work it may be possible to investigate this further by carrying out analysis of the transgenic plantlets rather than the embryogenic cell lines. However, the work completed here shows that introduction of a gene for proline overproduction into forest species does indeed lead to an increased environmental stress tolerance. However, there are modifications that could be performed to increase the value of the work. These include removal of the NPTII gene prior to transformation and using perhaps growth at 200 mM NaCl or 4°C as a selective agent. This would remove fears associated with the use of antibiotic resistance genes in the environment. Also GFP could be used as a reported gene instead of GUS allowing for the determination of transformation without the loss of so much transgenic material. It would also have been useful to have obtained some F129A transformants which could have been compared to the two lines already achieved in terms of environmental tolerance and proline

production. Reasons for low level of transformation events also needs to be examined. Inclusion of a stress inducible promoter instead of the CaMV promoter in front of the proline gene would also have meant that the transgenic plants would not constitutively over produce proline whether under stress or not, but that they would only overproduce when stress was present.

References

Abdul-Baki AA, Saunders JA, Matthews BF and Piltarelli GW(1990). DNA uptake during electroporation of germinating pollen grains. *Plant Science* 70: 181-190

Ahokas H (1989) Transfection of fermenting barley seed electrophoretically with exogenous DNA. *Theor. Appl. Genet.* 77: 469-472

Alaoui-Sosse B., L. Sehmer, P. Barnola and P. Dizengremel. 1998. Effect of NaCl salinity on growth and mineral partitioning in *Quercus robur* L., a rhythmically growing species. *Trees* 12:424-430

Albright LM, Yanofsky MF, Lereux RM and Nester EW (1987) Processing of the T-DNA of the *Agrobacterium tumefaciens* generates border nicks and linear single stranded T-DNA. *J. Bacteriol.* 169: 1046-1055

Alia, Pardha Saradhi P and Mohanty P (1991) Proline enhances primary photochemical activities in isolated thylakoid membranes of *Brassica juncea* by arresting photo-inhibitory damage. *Biochem Biophys Res Commun* 181:1238-1244

Asada K (1994) Production and action of active oxygen species in photosynthetic tissues. In: Kyle DJ, Osmond CB and Arntzen CJ (Eds.). *Photoinhibition*. Elsevier, Amsterdam, pp227-287

Aspinall D. and L.G. Paleg. 1981. Proline accumulation: physiological aspects. In: Paleg LG and Aspinall D (Eds.). *The Physiology and Biochemistry of Drought Resistance in Plants*. Academic Press, Sydney, pp206-241

Atkinson DE (1977) *Cellular energy metabolism and its regulation*. New York. Academic Press

Attree SM, Dunstan DI and Fowke LC (1991) White spruce (*Picea glauca* (Moench) Voss) and Black spruce (*Picea mariana* (Mill) B.S.P.). In: Bajaj YPS (Ed.). *Trees III. Biotechnology in Agriculture and Forestry*, Vol. 116. Springer-Verlag. Berlin

Baertlein DA, Lindow SE, Panopoulos NJ, Lee SP, Mindrinos MN and Chen THH (1992) Expression of a bacterial ice nucleation gene in plants. *Plant Physiol.* 100: 1730-1736

Bajji M, Kinet J-M and Lutts S (1998) Salt stress effects on roots and leaves of *Atriplex halimus* L. and their corresponding callus cultures. *Plant Science* 137: 131-142

Bartels D and Nelson DE (1994) Approaches to improve stress tolerance using molecular genetics. *Plant Cell and Environment* 17: 659-667

Bates LS, Waldren RP and Teare ID (1972) Rapid determination of free proline for water-stress studies. *Plant and Soil* 39: 205-207

Beatriz Garcia A, de Almeida Engler J, Iyer S, Gerats T, Van Montagu M and Caplan AB (1997) Effects of osmoprotectants upon NaCl stress in rice. *Plant Physiol.* 115: 159-169

Becwar MR, Nagmani R and Wann SR (1990) Initiation of embryogenic cultures and somatic embryo development in Loblolly pine (*Pinus taeda*). *Can. J. For. Res.* 20: 810-817

Benko B (1968) The content of some amino acids in young apple shoots in relation to frost resistance. *Biol. Plant.* 11: 334-337

Biermann CJ (1993) *Essentials of Pulping and Papermaking*, Academic Press

Bohnert HJ and Jensen RG (1996) Metabolic engineering for increased salt tolerance-the next step. *Aust J Plant Physiol* 23: 661-667

Bohnert HJ, Nelson DE and Jensen RG (1995) Adaptations to environmental stresses. *Plant Cell* 7: 1099-1111

Bohnert HJ and Shen B (1999) Transformation and compatible solutes. *Scientia Horticulturae* 78: 237-260

Bohnert HJ and Sheveleva E (1998) Plant stress adaptations-making metabolism move. *Current Opinion in Plant Biol* 1: 267-274

Bommineni VR, Chibbar RN, Dalta RSS and Tsang EWT (1993) Transformation of White spruce (*Picea glauca*) somatic embryos by microprojectile bombardment. *Plant Cell Reports* 13: 17-23

Boorse GC, Bosma TL, Meyer A-C, Ewers FW and Davis SD (1998) Comparative methods of estimating freezing temperatures and freezing injury in leaves of Chaparral shrubs. *Int. J. Plant Sci.* 159(3): 513-521

Boudet AM, Lapierre C and Grima-Pettenati J (1995) Tansley. Review No. 80. Biochemistry and molecular biology of lignification. *New Phytol.* 129: 203-236

Bray EA (1993) Molecular responses to water deficit. *Plant Physiol.* 101: 1035-1040

Brown AD and Simpson JR (1972) Water relations of sugar-tolerant yeasts: the role of intracellular polyols. *J. Gen. Micro.* 72: 589-591

Chalfie M, Yuan T, Euskirchen G, Ward WW and Prasher DD (1994) Green fluorescent protein as a marker for gene expression. *Science* 263: 802-805

Chapula V (1985) *In vitro* propagation of *Larix*, *Picea*, *Pinus*, *Quercus*, *Fagus* and other species using adenine-type cytokinins and thidiazuron. *Communi. Inst. For. Cech.* 14: 65-90

Chapula V (1990) Plant regeneration by somatic embryogenesis from cultured immature embryos of Oak (*Quercus robur* L.) and Linden (*Tilia cordata* Mill.). *Plant Cell Reports* 9: 398-401

Chapula V (1993) Vegetative propagation of Oak (*Quercus robur* and *Quercus petraea*) by cutting and tissue culture. *Annals of Scientific Forestry* 50, suppl. 1: 295-307)

Chapula V (1995) Somatic embryogenesis in Oak (*Quercus* spp.). In: Jain S, Gupta P and Newton R (Eds.), *Somatic embryogenesis in Woody Plants*. 2: 67-87. Kluwer Academic Publishers, The Netherlands

Charest PJ, Devantier Y and Lachance D (1996) Stable genetic transformation of *Picea mariana* (Black spruce) via particle bombardment. *In Vitro Cell. and Dev. Biol.- Plant* 32: 91-99

Charest PJ, DeVerno LL, Klimaszewska KK, Lelu MA and Ward C (1992) Advanced biotechnology in the Genus *Larix*: potential integration into tree improvement programs. *In Proceedings of the Symposium on Ecology and Management of Larix forests: a look ahead*. Whitefish, Mont. 5-9 Oct 1992. In press

Cheliak WM and Rogers DL (1990) Integrating biotechnology into tree improvement programs. *Can. J. For. Res.* 20: 452-463

Cheng ZM, Schnurr JA and Kapaun JA (1998) Timentin as an alternative antibiotic for suppression of *Agrobacterium tumefaciens* in genetic transformation. *Plant Cell Reports* 17: 646-649

Chiang VL, Puumsls RG and Takeuchi H (1988) Comparison of softwood and hardwood kraft pulping. *Tappi. J.* 71: 173-176

Christian JHB (1954) The influence of nutrition on the water relations of *Salmonella oranienburg*. *Aust. J. Biol. Sci.* 8: 75-82

Chu TM, Aspinall D and Paleg LG. 1974. Stress metabolism VI* temperature stress and the accumulation of proline in barley and radish. *Aust. J. Plant Physiology* 1:87-97

Conn EE, Stumpf PK, Bruening G and Doi R (1987) The metabolism of ammonia and nitrogen-containing compounds. In: Outlines of biochemistry, 5th Edition. John Wiley and Sons, New York, p530

Coughlan SJ and Heber U (1982) The role of glycine betaine in the protection of spinach thylakoids against freezing stress. *Planta* 156: 62-69

Csonka LN (1988) Regulation of cytoplasmic proline levels in *Salmonella typhimurium*: effect of osmotic stress on synthesis, degradation and cellular retention of proline. *J. Bacteriol.* 170: 2374-2378

Csonka LN (1989) Physiological and genetic responses of bacteria to osmotic stress. *Microbiological Reviews* 53(1): 121-147

Csonka LN and Baich A (1983) Proline biosynthesis. In Herrmann KM and Somerville RK (Eds.). *Amino acids: biosynthesis and genetic regulation*. Addison-Wesley, Reading, Mass.

Csonka LN and Hanson AD (1991) Prokaryotic osmoregulation: genetics and physiology. *Annu. Rev. Microbiol.* 45: 569-606

Dandekar AM and Uratsu SL (1988) A single base pair change in proline biosynthesis genes causes osmotic stress tolerance. *J. Bacteriol.* 170(12): 5943-5945

Daniell H (1998) Containment of herbicide resistance through genetic engineering of the chloroplast genome. *Nat. Biotechnol.* 16: 345-348

De Block M (1990) Factors influencing the tissue culture and the *Agrobacterium tumefaciens*-mediated transformation of Hybrid Aspen and Poplar clones. *Plant Physiol.* 93: 1110-1116

De Paolis A, Mauro ML, Pomponi M, Cardarelli M, Sparo L and Costantino P (1985) Localisation of agropine-synthesizing functions in the Tr region of the root inducing plasmid of *Agrobacterium rhizogenes* 1855. *Plasmid* 13: 1-7

De Vries SC (1998) Making embryos in plants. *Trends in Plant Sci.* 3(12): 451-452

Dekeyser RA, Claes B, Riet MU, Rycke D, Habets ME, Van Montagu MC and Caplan AB (1990) Transient gene expression in intact and organised Rice tissues. *The Plant Cell* 2: 591-602

Delauney AJ and Verma DPS (1993) Proline biosynthesis and osmoregulation in plants. *The Plant Journal* 4 (2) : 215-223

Deutch AH, Rushlow KE and Smith CJ (1984) Analysis of the *E. coli* proBA locus by DNA and protein sequencing. *Nucleic Acids Research* 12(15): 6337-6355

Deutch AH, Smith CJ, Rushlow KE and Kretschmer PJ (1982) *E. coli* Δ^1 -pyrroline-5-carboxylate reductase: gene sequence, protein overproduction and purification. *Nucleic Acids Research* 10 (23): 7701-7714

Dhariwal HS, Kawai M and Uchimiya H (1998) Genetic engineering for abiotic stress tolerance in plants. *Plant Biotechnology* 15: 1-10

Dix PJ, Finch I and Burke JI (1994) Genotypic differences in cold tolerance are masked by high sucrose and cytokinin in shoot cultures of Sugarbeet. *Plant Cell, Tissue and Organ Culture* 36: 285-290

Drake PMW, John A, Power JB and Davey MR (1997) Expression of the gus A gene in embryogenic cell lines of Sitka spruce following *Agrobacterium*-mediated transformation. *J. Exper. Botany* 48 (306): 151-155

Draper SR (1972) Amino acid changes associated with low temperature treatment of *Lolium perenne*. *Phytochemistry* 11: 639-641

Draper J, Davey MR, Freeman JP, Cocking EC and Cox BJ (1982) Ti plasmid homologous sequences present in tissues from *Agrobacterium* plasmid transformed *Petunia* protoplasts. *Plant Cell Physiol.* 23: 451-458

Duncan DR and Widholm JM (1987) Proline accumulation and its implication in cold tolerance of regenerated maize callus. *Plant Physiol.* 83:703-708

Dune L III, Couch M, Harada J, Mo T-HD, Mundy J, Quantrano RS, Thomas T and Sung ZR (1989) *Plant Mol Biol* 12: 475-486

Dunstan DL, Bekkaoui F, Pilon M, Fowke LC and Abrams SR (1988) Effects of abscisic acid and analogues on the maturation of white spruce (*Picea glauca*) somatic embryos. *Plant Science* 58: 77-84

Dure L (1993) Structural motifs in Lea proteins, in *Plant Responses to Cellular Dehydration during Environmental Stress*. In: Close TJ and Bray EA (Eds.). *Current Topics in Plant Physiology*, Vol. 10. American Society of Plant Physiologists, Rockville, MD

Ellis D, Roberts D, Sutton B, Lazaroff W, Webb D and Flinn B (1989) Transformation of white spruce and other conifer species by *Agrobacterium tumefaciens*. *Plant Cell Reports* 8: 16-20

Elthon TE and Stewart CR (1981) Submitochondrial location and electron transport characteristics of enzymes involved in proline oxidation. *Plant Physiol.* 67: 780-784

Elthon TE and Stewart CR (1982) Proline oxidation in corn mitochondria. *Plant Physiol.* 70: 567-572

Endemann M and Wilhelm E (1999) Factors influencing the induction and viability of somatic embryos of *Quercus robur* L. *Biologia Plantarum* 42(4): 499-504

- Evers PE and Hanish Ten Cate (1988) Preliminary experiments on transformation of Oak (*Quercus robur* L.). Abstract poster IURFO Workshop, Ames, USA
- Faulkner R (1987) Genetics and breeding of Sitka spruce. Proc. Roy. Soc. Edin. 93B: 41-50
- Feraund-Keller C and Espagnac H (1989) Condition d'apparition d'une embryogenese somatique sur des calissus de la culture de tissus foliaires du chene vert (*Quercus ilex*). Can. J. Bot. 67: 1066-1070
- Fillatti JJ, Sellmer J, McCown B, Haissing B and Comai L (1987) *Agrobacterium*-mediated transformation and regeneration of Populus. Mol. Gen. Genet. 206: 192-199
- Flavell RB, Dart E, Fuchs RL and Fraley RT (1992) Selectable marker genes: Safe for plants. Bio/Tech 10: 141-144
- Flowers TJ, Troke PF and Yeo AR (1977) The mechanism of salt tolerance in halophytes. Ann. Rev. Plant Physiol. 28: 89-121
- Fraley RT, Rogers SG, Horsch RB, Sanders PR, Flick JS, Adams SP, Bittner ML, Brand LA, Fink CL, Fry JS, Galluppi GR, Goldberg SB, Hoffmann NL and Woo SC (1983). Expression of bacterial genes in plant cells. Proc. Natl. Acad. Sci. USA 80: 4803-4807
- Frame BR, Drayton PR, Bagnell SV, Lewnau CJ, Bullock WP, Wilson HM, Dunwell JM, Thompson JA and Wang K (1994) Production of fertile transgenic Maize plants by silicon carbide whisker-mediated transformation. The Plant Journal 6: 941-948
- Fromm ME, Taylor LP and Walbot V (1986) Stable transformation of Maize after gene transfer by electroporation. Nature 319: 791-793
- Gadallah MAA (1999) Effects of proline and glycine betaine on *Vicia faba* responses to salt stress. Biologia Plantarum 42(2):249-257

Gangopadhyay G, Basu S and Gupta S (1997) *In vitro* selection and physiological characterization of NaCl and mannitol-adapted callus lines in *Brassica juncea*. Plant Cell, Tissue and Organ Culture 50: 161-169

Gasser CS and Fraley RT (1989) Genetically engineering plants for crop improvement. Science 244: 1293-1298

Gaudin V, Vrain T and Jouanin L (1994) Bacterial genes modifying hormone balances in plants. Plant Physiol. Biochem 32: 11-29

Georges F, Saleem M and Culter AJ (1990) Design and cloning of a synthetic gene for the flounder anti-freeze protein and its expression in plant cells. Gene 91: 159-165

Gilissen, L.J.W., C.H. Hanisch-Ten Cate and B. Keen (1983) A rapid method of determining growth characteristics of plant cell populations in batch suspension culture. Plant Cell Reports 2:232-235

Gingas, V.M. and R.D. Lineberger. 1989. Asexual embryogenesis and plant regeneration in *Quercus*. Plant Cell, Tissue and Organ Culture 17:191-203

Giraudat J, Parcy F, Bertauche N, Gosti F, Leung J, Morris P-C, Bouvier-Durand M and Vartanian N (1994) Current advances in abscisic acid action and signaling. Plant Mol Biol 26: 1557-1577

Griesbach RJ (1985) Advances in microinjection of higher plant cells. Biotechniques 3: 348-350

Grossnickle SC, Cyr D and Polonenko DR (1996) Somatic embryogenesis tissue culture for the propagation of conifer seedlings: a technology comes of age. Tree Planters Notes 47 (2)

Guerrier G. 1997/98. Proline accumulation in leaves of NaCl-sensitive and NaCl-tolerant tomatoes. *Biologia Plantarum* 40(4): 623-628

Gupta, P.K. and D.J. Durzan. 1986. Plantlet production via somatic embryogenesis from subcultured callus of mature embryos of *Picea abies* (Norway spruce). *In Vitro Cellular and Developmental Biology* 22:685-688

Gupta PK and Pullman GS (1991) Method for producing coniferous plants by somatic embryogenesis using abscisic acid and osmotic potential variation. US Patent No. 5, 036, 007

Hackett WP (1988) Donor plant maturation and adventitious shoot formation. In: Davis TD, Haissig BE and Sankhia N (Eds.) *Advances in Plant Sciences Series. Volume 2, Adventitious root formation in cuttings* (Dudley TR, General editor). Portland, OR: Dioscorides Press 11-28

Hakman I and Von Arnold S (1985) Plantlet regeneration through somatic embryogenesis in *Picea abies* (Norway spruce). *J. Plant Physiol.* 121: 149-153

Halpin C, Knight ME, Foxon GA, Campbell MM, Boudet AM, Boon JJ, Chabbert B, Tollier M and Schuch W (1994) Manipulation of lignin quality by down regulation of cinnamyl alcohol dehydrogenase. *Plant J.* 6: 339-350

Handa S, Handa AK, Hasegawa PM and Bressan RA (1986) Proline accumulation and the adaptation of cultured plant cells to salinity stress. *Plant Physiol* 80: 938-945

Hare PD and Cress WA (1997) Metabolic implications of stress-induced proline accumulation in plants. *Plant Growth Regulation* 21: 79-102

Hare PD, Cress WA and Van Staden J (1998) Dissecting the roles of osmolyte accumulation during stress. *Plant, Cell and Environment* 21: 535-553

Harper CP and O'Reilly C (2000/1999) Dept. Crop Science, Horticulture and Forestry, UCD

Harrison BD (1985) Advances in gemini virus research. *Ann. Rev. Phytopathology* 23: 52-82

Hayashi H, Alia, Mustardy L, Deshnum, Ida M and Murata N (1997) Transformation of *Arabidopsis thaliana* with the *codA* gene for choline oxidase, accumulation of glycine betaine and enhanced tolerance to salt and cold stress. *Plant Journal* 12: 133-142

Hayzer DJ and Leisinger T (1980) The gene-enzyme relationships of proline biosynthesis in *E.coli*. *J. Gen. Micro.* 118: 287-293

Hayzer DJ and Leisinger T (1981) Proline biosynthesis in *E.coli*: stoichiometry and end-product identification of the reaction catalysed by glutamate semialdehyde dehydrogenase. *Biochem. J.* 197: 269-274

Hayzer DJ and Leisinger T (1983) Sub-cloning of the wild-type proBA region of the *E.coli* genome. *J. Gen. Micro.* 129: 3215-3225

Hazem Kalaji M and Pietkiewicz (1993) Salinity effects on plant growth and other physiological processes. *Acta. Physiologiae Plantarum* 15 (2): 89-124

Herrera-Esterella L, De Block MN, Messens E, Hernalsteens JP, Van Montagu M and Schell JM (1983a) Chimeric genes as dominant selectable markers in plant cells. *EMBO J.*: 987-995

Herrera-Esterella L, Depicker A, Van Montagu M and Schell J (1983b) Expression of chimeric genes transferred into plants using a Ti plasmid-derived vector. *Nature* 303: 209-213

Hoekema A, Hirsch PR, Hooykaas PJ and Schilperout RA (1983) A binary plant vector strategy based on the separation of the Vir and T-regions of *Agrobacteria*. Nature 303: 179-181

Hoffmann GA, White FF, Gordon MP and Nester EW (1984) Hairy root inducing plasmid; physical map and homology to tumour inducing plasmids. J Bacteriology 157: 269-276

Holmberg N and Bulow L (1998) Improving stress tolerance in plants by gene transfer. Trends in Plant Science 3(2): 61-66

Holmstrom HO, Mantyla E, Welin B, Mandal A, Palva ET, Tunnela OE and Londesborough J (1996) Drought tolerance in tobacco. Nature 379: 683-684

Hong B, Barg R and Ho T-HD (1992) Developmental and organ-specific expression of an ABA- and stress-induced protein in barley. Plant Mol. Biol. 18: 663-674

Hong Z, Lakkineni K, Zhang Z and Verma DPS (2000) Removal of feedback inhibition of Δ^1 -pyrroline-5-carboxylate synthetase results in increased proline accumulation and protection of plants from osmotic stress. Plant Physiology 122: 1129-1136

Howe GT, Goldfarb B and Strauss SH (1994) *Agrobacterium*-mediated transformation of hybrid poplar suspension cultures and regeneration of transformed plants. Plant Cell, Tissue and Organ Culture 36: 59-71

Hu CA-A, Delauney AJ and Verma DPS (1992) A bifunctional enzyme (Δ^1 -pyrroline-5-carboxylate synthetase) catalyses the first two steps in proline biosynthesis in plants. Proc Natl Acad Sci USA 89: 9354-9358

Huang Y, Diner AM and Karnosky DF (1991) *Agrobacterium rhizogenes*-mediated genetic transformation and regeneration of a conifer: *Larix deciduas*. In Vitro Cell Dev Biol 27: 201-207

- Igarashi Y, Yoshiba Y, Sanada Y, Yamaguchi-Shinozaki K, Wada K and Shinozaki K (1997) Characterisation of the gene for Δ^1 -pyrroline-5-carboxylate synthetase and correlation between the expression of the gene and salt tolerance in *Oryza sativa* L. *Plant Molecular Biology* 33: 857-865
- Ingram J and Bartels D (1996) The molecular basis of dehydration tolerance in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 47: 377-403
- Ishizaki-Nishizawa O, Fujii T, Azuma M, Sekiguchi K, Murata N, Ohtani T and Yoguri T (1996) Low-temperature resistance of higher plants is significantly enhanced by a non-specific cyanobacterial desaturase. *Nature Biotechnology* 14: 1003-1006
- Iwahashi M, Obuchi K, Fujii S and Komatsu Y (1995) The correlative evidence suggesting that trehalose stabilises membrane structure in the yeast *Saccharomyces cerevisiae*. *Cell Mol. Biol.* 41: 763-769
- Jefferson RA, Kavanagh RA and Bevan MW (1987) GUS fusions: β -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* 6: 3901-3907
- John, A., P. Drake and C. Selby .1995. Somatic embryogenesis in sitka spruce (*Picea sitchensis* (Bong.) Carr). In: Mohn-Jain S, Gupta PK and Newton RJ (Eds.). *Somatic embryogenesis in woody plants*. Kluwer Publishers, Netherlands, pp126-143
- Jones H, Tempelaar MJ and Jones MGK (1987) Recent advances in plant electroporation. *Oxford Surveys of Plant Molecular and Cell Biology.* 4: 347-357
- Joyce PM, Huss J, McCarthy R, Pfeiter A and Hendrick E (1998) Growing broadleaves - silvi-cultural guidelines for Ash, Sycamore, Wild cherry and oak in Ireland. COFORD, Dublin

- Kaepllar HF, Gu W, Somers DA, Rines HW and Cockburn AF (1990) Silicon carbide fiber-mediated DNA delivery into plant cells. *Plant Cell Reports* 8: 415-418
- Keegan CE, Blatner KA and Wichman DP (1992) Use and value of Western Larch as a commercial timber species. Symposium on Ecology and Management of *Larix* forests: A look ahead.
- Kemble AR and MacPherson HT (1954) Liberation of amino acids in perennial Rye grass during wilting. *Biochem J.* 58: 46-59
- Kishitani S, Watanabe K, Yasuda S, Arakawa K and Takabe T (1994) Accumulation of glycinebetaine during cold acclimate and freezing tolerance in leaves of winter and spring barley plants. *Plant, Cell and Environment* 17: 89-95
- Kishor PB, Hong Z, Miao G-H, Hu C-A A and Verma DPS (1995) Overexpression of Δ^1 -pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. *Plant Physiol* 108: 1387-1394
- Klein TM, Wolf ED, Wu R and Sanford JC (1987) High velocity microprojectiles for delivering nucleic acids into living cells. *Nature* 327: 70-73
- Klimaszewska K (1989) Recovery of somatic embryos and plantlets from protoplast cultures of *Larix X eurolepis*. *Plant Cell Reports* 8: 440-444
- Klopfenstein NB, McNabb Jr., Hart ER, Heuchelin SA, Allen KK, Shi N-Q and Thornburg RW (1993) Transformation of *Populus* hybrids to study and improve pest resistance. *Silvae Genetica* 42: 2-3
- Krogstrup P, Eriksen EN, Moller JD and Rouland H (1988) Somatic embryogenesis in Sitka spruce (*Picea sitchensis* (Bong.) Carr.). *Plant cell Reports* 7: 594-597
- Lawrence WA and Davies DR (1985) A method for the microinjection and culture of protoplasts at very low densities. *Plant Cell Reports* 4:33-35

Lelu MA (1999) Transgenic in *Larix*. In: Jain SM and Minocha SC (Eds.). Molecular Biology in Woody Plants, Vol. 2. Kluwer Academic Publishers, Netherlands

Lelu MA, Bastien C, Klimaszewska K, Ward C and Charest PJ (1994a) An improved method for somatic plantlet production in hybrid larch (*Larix X leptoeuropaea*):Part 1. Somatic embryo maturation. Plant Cell, Tissue and Organ Culture 36: 107-115.

Lelu MA, Bastien C, Klimaszewska K, Ward C and Charest PJ (1994b) An improved method for somatic plantlet production in hybrid larch (*Larix X leptoeuropaea*): Part 2. Control of germination and plantlet development. Plant Cell, Tissue and Organ Culture 36: 117-127

Lelu MA, Klimaszewska K and Charest PJ (1994c) Somatic embryogenesis from immature and mature zygotic embryos and from cotyledons and needles of somatic plantlets of Larch. Can. J. For. Res. 24: 100-106

Levee V, Lelu M-A, Jouanin L, Cornu D and Pilate G (1997) *Agrobacterium tumefaciens*-mediated transformation of Hybrid Larch (*Larix kaempferi* x *Larix deciduas*) and transgenic plant regeneration. Plant Cell Reports 16: 680-685

Levitt J (1980a) Responses of Plants to Environmental Stress: Water, radiation, salt and other stresses. 2nd Edition, Academic Press, New York

Levitt J (1980b) Response of Plants to Environmental Stress: Chilling, freezing and high temperature stresses. 2nd Edition, Academic Press, New York

Li YH, Tremblay FM and Seguin A (1994) Transient transformation of pollen and embryogenic tissues of White spruce (*Picea glauca* (Moenck) Voss) resulting from microprojectile bombardment. Plant Cell Reports 13: 661-665

Lichtentaler HK (1995) Vegetation stress: an introduction to the stress concept in plants. J Plant Physiol 148: 4-14

Lilius G, Holmberg N and Bulow L (1996) Enhanced NaCl stress tolerance in transgenic tobacco expressing bacterial choline dehydrogenase. *Bio\Technology* 14: 177-180

Lone MI, Huch JSH, Wyn Jones RG and Bright SWJ (1987) Influence of proline and glycine betaine on salt tolerance of cultured barley embryos. *J. Exp. Bot.* 38 (188) 479-490

Loopstra CA, Stomp AM and Sederoff RR (1990) *Agrobacterium*-mediated DNA transfer in Sugar pine. *Plant Mol. Biol.* 15: 1-9

Lu G and Ferl RJ (1992) Site-specific oligodeoxynucleotide binding to Maize Adh 1 gene promoter represses Adh 1-GUS gene expression *in vivo*. *Plant Mol. Biol.* 19: 715-723

Mackenzie KF, Singh KK and Brown AD (1988) Water stress plating hypersensitivity of yeast: protective role of trehalose in *Saccharomyces cerevisiae*. *J Gen Microbiol* 134: 1661-1666

Martinussen I, Juntilla O and Twell D (1994) Optimisation of transient gene expression in pollen of Norway spruce (*Picea abies*) by particle acceleration. *Physiologia Plantarum* 92: 412-416

Mathews BF, Abdul-Baki AA and Saunders JA (1990) Expression of a foreign gene in electroporated pollen grains of tobacco. *Sex. Plant Reprod.* 3: 147-151

Matzke MA and Matzke AJM (1995) How and why do plants inactivate homologous (trans) genes? *Plant Physiology* 107: 679-685

McCue KF and Hanson AD (1990) Drought and salt tolerance: towards understanding and application. *Biotechnology* 8: 458-362

McKersie BD, Bowley SR, Harjanto E and Leprince O (1996) Water-deficit tolerance and field performance of transgenic alfalfa overexpressing superoxide dismutase. *Plant Physiol* 107: 737-750

Measures JC (1975) Role of amino acids in osmoregulation of nonhalophilic bacteria. *Nature* 257: 398-400

Mohri T, Mukai Y and Shinohara K (1997) *Agrobacterium tumefaciens*-mediated transformation of Japanese white birch (*Betula platyphylla* var. *japonica*). *Plant Science* 127: 53-60

Morgan JM (1984) Osmoregulation and water stress in higher plants. *Ann. Rev. Plant Physiol.* 35: 299-319

Mowikawa H and Yamada Y (1985) Capillary microinjection into protoplasts and intranuclear localization of injected materials. *Plant Cell Physiology* 26:229-236

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plant* 52:375-379

Murata N, Ishizaki-Nishizawa O, Higashi S, Hayashi H, Tasaka Y and Nishida I (1992) Genetically engineered alteration in the chilling sensitivity of plants. *Nature* 356: 710-713

Nagmani R and Bonga JM (1985) Embryogenesis in subcultured callus of *Larix decidua*. *Can. J. For. Res.* 15: 1988-1091

Naidu BP, Paleg LG, Aspinall D, Jennings AC and Jones GP (1991) Amino acid and glycine betaine accumulation in cold stressed wheat seedlings. *Phytochemistry* 30(2): 407-409)

Nanjo T, Kobayashi M, Yoshiba Y, Sanada Y, Wada K, Tsukaya H, Kakubari Y, Yamaguchi-Shinozaki K and Shinozaki K (1999) Biological functions of proline in morphogenesis and osmotolerance revealed in antisense transgenic *Arabidopsis thaliana*. *The Plant Journal* 18(2): 185-193

Nauerby B, Katauzyna B and Wyndaela R (1997) Influence of the antibiotic timentin on plant regeneration compared to carbencillin and cefotaxime in concentrations suitable for the elimination of *Agrobacterium tumefaciens*. *Plant Science* 123: 169-177

Negrutiu L, Shillito R, Potrykus I, Biasini G and Sala F (1987) Hybrid genes in the analysis of transformation conditions. I. Setting up a simple method for direct gene transfer in plant protoplasts. *Plant Molecular Biology* 8: 363-367

Nieman RH, Clark RA, Pap D, Ogata G and Maas EV (1988) Effects of salt stress on adenine and uridine nucleotide pools, sugar and acid-soluble phosphate in shoots of pepper and safflower. *J. Exp. Botany* 39 (200): 301-309

Nishida I and Murata N (1996) Chilling sensitivity in plants and cyanobacteria: The crucial contribution of membrane lipids. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 47: 541-568

Noctor G and Foyer CH (1998) Ascorbate and glutathione: keeping active oxygen under control. *Annu Rev Plant Physiol Plant Mol Biol* 49:13-19

Nuccio ML, Russell BL, Nolte HD, Rathinasabapathi B, Gage DA and Hanson AD (1998) The endogenous choline supply limits glycine betaine synthesis in transgenic tobacco expressing choline monooxygenase. *Plant Journal* 16: 487-496

O'Carroll (1992) *The Forests of Ireland: History, Distribution and Silviculture* (pp39-127). The Society of Irish Foresters, Turoe Press, Ireland.

Okuma E, Soeda K, Tada M and Murata Y (2000) Exogenous proline mitigates the inhibition of growth of *Nicotiana tabacum* cultured cells under saline conditions. *Soil Science and Plant Nutrition* 46(1): 257-263

Orthen B, Popp M and Smirnoff N (1994) Hydroxyl radical scavenging properties of cycitols. *Proc Royal Soc Edin* 102B: 260-272

Ow DN, Wood KV, De Luc AM, De Wet JR, Helinski DR and Howell SH (1986) Transient and stable expression of the firefly luciferase gene in plant cells and transgenic plants. *Science* 234: 856-859

Paquin R (1977) Effet des basses temperatures sur la resistance au gel de la Luzerne (*Medicago media* pers.) et son contenu en proline libre. *Physiol. Veg.* 15: 657-665

Paszkowski J, Shillito RD, Saul M, Mandak V, Hohn T, Hohn B and Potrykus I (1984) Direct gene transfer to plants. *EMBO Journal* 3: 2717-2722

Patterson V (1999) Optimisation of transformation in embryogenic cultures of Oak (*Quercus robur* L.) and Sitka spruce (*Picea sitchensis* (Bong.) Carr.) Ph.D. Thesis, Dublin City University, Ireland

Queiroz CGS, Alonso A, Mares-Guia M and Magalhaes AC (1998) Chilling-induced changes in membrane flexibility and antioxidant enzyme activities in *Coffea arabica* L. roots. *Biologia Plantarum* 41(3): 403-413

Rajashekar CB, Zhou H, Marcum KB and Prakash O (1999) Glycine betaine accumulation and induction of cold tolerance in strawberry (*Fragaria x ananassa* Duch.) plants. *Plant Science* 148: 175-183

Rayapati PJ and Stewart CR (1991) Solubilisation of a proline dehydrogenase from Maize (*Zea mays* L.) mitochondria. *Plant Physiol.* 95: 787-791

Reich TJ, Iyer VN, Scobie B and Miki BL (1986) A detailed procedure for the intranuclear microinjection of plant protoplasts. *Canadian Journal of Botany* 64: 1255-1258

Rhodes, D. and S. Handa. 1989. Amino acid metabolism in relation to osmotic adjustment in plant cells. In: Cherry JH (Ed.). *Biochemical and Physiological Mechanisms Associated with Environmental Stress Tolerance*. Springer-Verlag, Berlin, pp41-62

Rhodes D and Hanson AD (1993) Quaternary ammonium and tertiary sulfonium compounds in higher plants. *Ann Rev Plant Physiol Plant Mol Biol* 44: 357-384

Roest S and Evers PW (1991) *Agrobacterium*-mediated transformation of Oak (*Quercus robur* L.). *Acta Hort.* 289: 259-260

Romero C, Bellees JM, Vaya JL, Serrano R and Culianez-Marcia FA (1997) Expression of the yeast trehalose-6-phosphate synthase gene in transgenic tobacco plants: pleiotropic phenotypes include drought tolerance. *Planta* 201: 293-297

Roxas VP, Smith RK JR and Allen RD (1997) Overexpression of glutathione s-transferase/glutathione peroxidase enhances the growth of transgenic tobacco seedlings during stress. *Nature Biotech* 15: 988-991

Santarem ER, Trick HN, Essig JS and Finer (1998) Sonication-assisted *Agrobacterium*-mediated transformation of Soybean immature cotyledons: optimization of transient expression. *Plant Cell Reports* 7: 752-759

Santos, M.A., T. Camara, P. Rodriguez, I. Claparols and J.M. Torne. 1996. Influence of exogenous proline on embryogenic and organogenic maize callus subjected to salt stress. *Plant Cell, Tissue and Organ Culture* 47:59-65

Sarma KS, Evans C and Selby C (1995) Effect of carbencillin and cefotaxime on somatic embryogenesis of Sitka spruce (*Picea sitchensis* (Bong.) Carr.). J. Exp. Bot. 46: 1779-1781

Sasamoto H and Hosoi Y (1992) Callus proliferation from protoplasts of embryogenic cells of *Quercus serrata*. Plant Cell, Tissue and Organ Culture 29: 241-245

Savoure A, Joana S, Hua X-J, Ardiles W, van Montagu M and Verbruggen N (1995) Isolation, characterisation and chromosomal location of a gene encoding the Δ^1 -pyrroline-5-carboxylate synthetase in *Arabidopsis thaliana*. FEBS Letters 372: 13-19

Schobert B and Tschesche H (1978) Unusual solution properties of proline and its interactions with proteins. Biochem Biophys Acta 541: 270-277

Scott SE and Wilkinson MJ (1999) Low probability of chloroplast movement from oilseed rape (*Brassica napus*) into wild *Brassica rapa*. Nat. Biotechnol. 17: 390-392

Sederoff R, Stomp AM, Chilton WS and Moore LW (1986) Gene transfer into Loblolly pine by *Agrobacterium tumefaciens*. Bio/Tech 4: 647-649

Sharp WR, Evans DA, Flick CE and Sommer HE (1983) Strategies and specifications for management of *in vitro* plant propagation. In: Meudt WJ (Ed.). Strategies of plant reproduction. Beltsville Symp. Agric. Res. 6: 287-303

Shen B, Jensen RG and Bonhert HJ (1997a) Increased resistance to oxidative stress in transgenic plants by targeting mannitol biosynthesis to chloroplasts. Plant Physiol 113: 1177-1183

Shen B, Jensen RG and Bonhert HJ (1997b) Mannitol protects against oxidation by hydroxyl radicals. Plant Physiol. 115(2): 527-532

- Shin DI, Podila GK, Huang Y and Karnosky DF (1994) Transgenic Larch expressing genes for herbicide and insect resistance. *Can. J. Forest Research* 24: 2059-2067
- Skriver K and Mundy J (1990) Gene expression in response to abscisic acid and osmotic stress. *Plant Cell* 2: 503-512
- Smirnoff N (1993) The role of active oxygen in the response of plants to water deficit and desiccation. *New Phytol.* 125: 27-58
- Smirnoff N and Cumbes QJ (1989) Hydroxyl radical scavenging activity of compatible solutes. *Phytochemistry* 28: 1057-1060
- Smith LT (1985) Characterisation of a γ -glutamyl kinase from *Escherichia coli* that confers proline overproduction and osmotic tolerance. *J. Bacteriol.* 164: 1088-1093
- Smith CJ, Deutch AH and Rushlow KE (1984) Purification and characteristics of a γ -glutamyl kinase involved in *E. coli* proline biosynthesis. *J. Bact.* 157(2): 545-551
- Songstad DD, Somers DA and Griesbach RT (1995) Advances in alternative DNA delivery techniques. *Plant Cell, Tissue and Organ Culture* 40: 1-15
- Stachel SE, Timmerman B and Zambryskii P (1986) Generation of single stranded T-DNA molecules during the initial stages of T-DNA transfer from *Agrobacterium tumefaciens* to plant cells. *Nature* 322: 706-712
- Stefl M, Trcka I and Vratny P (1978) Proline biosynthesis in winterplants due to exposure to low temperatures. *Biol. Plant.* 20: 119-128
- Steponkus PL (1984) Role of the plasma membrane in freezing injury and cold accumulation. *Ann. Rev. Plant Physiol.* 35: 543-584

Steponkus PL and Webb MS (1992) Freeze-induced dehydration and membrane destabilization in plants. In: Somero G and Osmond B (Eds.). Water and Life: Comparative analysis of water relationships at the organismic, cellular and molecular level. Springer-Verlag, Berlin

Stomp AM, Loopstra C, Chilton WS, Sederoff R and Moore LW (1990) Extended host range of *Agrobacterium tumefaciens* in the genus *Pinus*. *Plant Physiol.* 92: 1226-1232

Stoop JMH, Williamson JD and Pharr DH (1996) Mannitol metabolism in plants: a method for coping with stress. *Trends in Plant Science* 1: 139

Strecker HJ (1960) The inter-conversion of glutamic acid and proline II. The preparation and properties of Δ^1 -pyrroline-5-carboxylic acid dehydrogenase. *J. Biol. Chem.* 235: 3218-3223

Strizhov N, Abraham E, Ökrész L, Blickling S, Zilberstein A, Schell J, Koncz C and Szabados L (1997) Differential expression of two Δ^1 -pyrroline-5-carboxylate synthetase genes controlling proline accumulation during salt-stress requires ABA and is regulated by ABA1, ABI1 and AXR2 in *Arabidopsis*. *The Plant Journal* 12: 557-569

Syvanen M (1999) In search of horizontal gene transfer. *Nature Biotechnol* 17: 833

Tarzynski MC, Jensen RG and Bohnert HJ (1993) Stress protection of transgenic tobacco by production of the osmolyte mannitol. *Science* 259: 508-510

Tautorius TE, Fowke LC and Dunstan DI (1991) Somatic embryogenesis in conifers. *Can. J. Bot.* 69: 1873-1899

Thomashow MF (1990) Molecular genetics of cold acclimation in higher plants. *Advances in Genetics* 28: 99-131

Tuominen H, Sitbon F, Jacobsson C, Sandberg G, Olsson O and Sundberg B (1995) Altered growth and wood characteristics in indoleacetic acid-biosynthesis genes. *Plant Physiol* 109: 1179-1189

Tzfira T, Jensen CS, Wang W, Zuker A, Vinocur B, Altman A and Vainstein A (1997) Transgenic *Populus tremula*: a step-by-step protocol for its *Agrobacterium*-mediated transformation. *Plant Molecular Biology Reporter* 15: 219-235

Tzfira, T., A. Zuker and A. Altman. 1998. Forest tree biotechnology: genetic transformation and its application to future forests. *Trends in Biotechnology* 16(10):439-446

Van Camp W, Capiou K, Montagu M, Inze D and Stooten L (1996) Enhancement of oxidative stress tolerance in transgenic tobacco plants overproducing Fe-superoxide dismutase in chloroplasts. *Plant Physiol.* 112: 1703-1714

Van Rensburg L, Kruger GHJ and Kruger H (1993) Proline accumulation as drought-tolerance selection criterion: its relationship to membrane integrity and chloroplast ultrastructure in *Nicotiana tabacum* L. *J. Plant Physiol.* **141**: 188-194

Van Swaaij, C., E. Jacobsen and W.J. Feensta. 1985. Effect of cold hardening, wilting and exogenously applied proline on leaf proline content and frost tolerance of several genotypes of *Solanum*. *Physiol. Plant* 64:230-236

Verbruggen N, Villarrod R and Van Montagu M (1993) Osmoregulation of a Δ^1 -pyrroline-5-carboxylate reductase gene in *Arabidopsis thaliana*. *Plant Physiol* 103: 771-781

Walden R (1988) Genetic transformation in plants. Open University Press, Biotechnology Series.

Walden R and Schell J (1990) Techniques in plant molecular biology-progress and problems. *Eur. J. Biochem.* 192: 563-576

Wan Y and Lemeux PG (1994) Generation of large numbers of independently transformed fertile Barley plants. *Plant Physiol.* 104: 37-48

Wang Y, Huang MR, Wei ZM, Sun YR, Chen DM, Xu ZH, Zhang LM and Xu N (1995) Regeneration of Simon poplar (*Populus simonii*) from protoplast culture. *Plant Cell Reports* 14: 442-445

Wenck AR, Quinn M, Whetten RW, Pullman G and Sederoff R (1999) High-efficiency *Agrobacterium*-mediated transformation of Norway spruce (*Picea abies*) and loblolly pine (*Pinus taeda*). *Plant Molecular Biology* 39: 407-416

Werner JE and Finkelstein RR (1995) *Arabidopsis* mutants with reduced response to NaCl and osmotic stress. *Physiol Plant* 93: 659-666

Wyn-Jones RG and Storey R (1981) Betaines. In: Paleg LG and Aspinall D (Eds.) *The Physiology and Biochemistry of Drought Resistance in Plants*. Academic Press, Sydney, pp171-204

Xin Z and Li PH (1993) Relationship between proline and abscisic acid in the induction of chilling tolerance in maize suspension-cultured cells. *Plant Physiol.* 103:607-613

Xu D, Duan X, Wang B, Ho THD and Wu R (1996) Expression of a late embryogenesis abundant protein gene, *HVA1*, from barley confers tolerance to water deficits and salt. *Plant Physiology* 110: 249-257

Yanofsky MF, Pomer SC, Young C, Albright LM, Gordon MP and Nester EW (1986) The *VirD* operon of *Agrobacterium tumefaciens* encodes a site specific endonuclease. *Cell* 47: 471-475

Yoder JL and Gold Briugh AP (1994) Transformation systems for generating marker free transgenic plants. *Bio/Tech* 12: 263-267

Yoshihara Y, Kiyosue T, Katagiri T, Ueda H, Mizoguchi T, Yamaguchi-Shinozaki K, Wada K, Harada Y and Shinozaki K (1995) Correlation between the induction of a gene for Δ^1 -pyrroline-5-carboxylate synthetase and the accumulation of proline in *Arabidopsis thaliana* under osmotic stress. *The Plant Journal* 7(5): 751-760

Zhang SY, Gosselin R and Chauret G eds. (1997) Timber management toward wood quality and end-product value, Forintek Canada, Sainte-Foy, Quebec, Canada

Zhao Y, Aspinall D and Paleg LG (1992) Protection of membrane integrity in *Medicago sativa* L. by glycine betaine against the effects of freezing. *J Plant Physiol* 140: 541-543

Zhu B, Su J, Chang M, Verma DPS, Fan Y and Wu R (1998) Overexpression of a Δ^1 P5CS gene and analysis of tolerance to water and salt-stress in transgenic rice. *Plant Science* 139: 41-48

Zobel B (1984) The changing quality of the world wood supply. *Wood Sci. Technol.* 18: 1-17