Cytokinins and phase change in *Pinus radiata*:

Morphological, physiological and molecular studies

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy in Plant Biology

at Massey University

Palmerston North

New Zealand

HUAIBI ZHANG

1998

Abstract

Phase change in higher plants is a developmental process during which changes occur at morphological, physiological and molecular levels. In *Pinus radiata*, buds of juvenile trees produce photosynthetically functional primary needles while buds from mature trees do not produce such primary needles. Cytokinin, however, causes production of primary needles from mature buds *in vitro* (Horgan, 1987). Pursuing this observation, morphological and anatomical examinations of the buds were carried out using light microscopy. The results showed that the cytokinin-induced transition from mature to juvenile bud morphology may be through resetting the fate of fascicle meristems and/or foliar primordia.

To determine if a correlation existed between the endogenous cytokinin content and the maturation status of the buds, buds from the juvenile and mature *P. radiata* were analysed using a range of modern techniques, including column complex purification, immunoaffinity purification, normal and reverse HPLC, radioimmunoassay and electrospray tandem mass spectrometry. A wide spectrum of endogenous cytokinins were detected in the bud tissues, including five novel forms discovered in this work. Quantitative analyses revealed a general trend with seedling buds > juvenile (J4) buds > mature (M4) buds > mature (M8) buds for the combined concentration of free base and riboside cytokinins. High concentrations of phosphorylated cytokinins were found in the mature buds but not the juvenile buds. Novel cytokinin glucosides were the most abundant forms in the buds, with zeatin-9-(glucopyranosyl-1,3-ribosyl) and dihydrozeatin-9-(glucopyranosyl-1,3-ribosyl) being higher in the mature buds and isopentenyladenine-9-(glucopyranosyl-1,3-ribosyl) being higher in the juvenile buds. Overall, particular patterns of cytokinins in the field buds reflected the maturation status of the buds.

Extensive metabolism of 6-benzylaminopurine occurred, including the production of the novel forms, 6-benzylaminopurine-9-(glucopyranosyl-1,3-ribosyl) and phosphorylated 6-benzylaminopurine-9-(glucopyranosyl-1,3-ribosyl), during the *in vitro* 'rejuvenation' of mature buds to the juvenile phenotype. Among the metabolites, the abundance of 6-benzylaminopurine, 6-benzylaminopurine riboside and 6-benzylaminopurine-9-

(glucopyranosyl-1,3-ribosyl) was high while phosphorylated forms were very low over the duration of the experiment. The patterns of metabolites reflected the patterns of endogenous cytokinins observed in juvenile buds. The results also indicated that 6-benzylaminopurine did not regulate phase-specific traits by increasing endogenous cytokinins.

Molecular tools were used to clone cytokinin-responsive genes which may also be involved in the regulation of phase change. A cDNA sequence (*Prcr5*) was cloned using a modified mRNA differential display technique. Northern analyses showed that cytokinin promoted and maintained the expression of *Prcr5* at a high level during rejuvenation of the mature buds *in vitro*. The deduced PrCR5 protein sequence displays homology to Ginseng RNases and PR-10. A possible function of the *Prcr5* gene in the regulation of phase change is discussed.

A cDNA sequence (*Prcab*) coding for a chlorophyll a/b binding protein was also cloned. Although expression of the *cab* gene has been reported to be associated with phase change in other species, no such change was observed in *P. radiata*.

Acknowledgements

I would like to express my gratitude to my supervisors Professor Paula Jameson (Department of Plant Biology and Biotechnology*), Dr Paul Reynolds (HortResearch, Palmerston North, now Ministry of Science and Technology) and Ms Kathy Horgan (NZFRI, Rotorua) for their encouragement, advice and guidance throughout the course of this work.

I gratefully acknowledge the assistance generously provided by Ms Astrid Coker during the early stages of the PhD programme.

I wish to express my appreciation for the financial support provided by New Zealand Forest Research Institute and Massey University. Also, I would like to thank the nursery of NZFRI for allowing me to use their *Pinus radiata* trees.

I am very grateful to Dr Gill Norris of the Biochemistry Department*, Massey University, for the provision of mass spectrometry expertise and helpful comments.

I appreciate the help I received from Associate Professor John Tweedie of the Biochemistry Department*, Massey University, in helping me use the GCG software.

I would like to thank all those academic, technical and administrative staff of Department of Plant Biology and Biotechnology*, Massey University, who helped me in various occasions. Special thanks to Dr Michael McManus and Dr Bruce Veit for their kindness whenever I came to them with questions, Ms Lyn Watson for her willing help in the lab, Ms Liz Nickless and Mrs Geraldine Wood for their assistance with various matters.

My thanks to the members of Dr Paul Reynolds' laboratory for their help. Especially to Mr Chris Kirk for his assistance in manual DNA sequencing.

Thanks also to the members of Plant Tissue Culture and Propagation laboratory, NZFRI for their assistance during my tissue culture work there.

My appreciation to all those people working in the Physiological and Molecular Plant Biology Lab, Massey University, for creating a pleasant atmosphere and making the lab a most enjoyable place to work in. My special thanks to fellow students Marian McKenzie, Michelle Davey, Sean Clarke, Nigel Gapper, Don Hunter, Dan Jeffares, Sang Dong Yoo, Simoene Downs, Trish Murray, Celia Zhang and Xiouyin Zhou for their friendship, advice and assistance on various aspects during the years .

Finally, a special thank you to my wife Xiaochun and my son Dian for their support.

*Now part of the Institute of Molecular Biosciences.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AMP	adenosine-5′-monophosphate
amp1	*altered meristem programming 1* mutation
amu	atomic mass units
AP1	*APETALA1* gene
API	atmospheric pressure ionisation
BA	6-benzylaminopurine
BA3G	6-benzylaminopurine-3-glucoside
BA7G	6-benzylaminopurine-7-glucoside
BA9G	6-benzylaminopurine-9-glucoside
BANT	6-benzylaminopurine nucleotide (6-benzylaminopurine riboside-5′-monophosphate)
BAR	6-benzylaminopurine riboside
BAR-G	6-benzylaminopurine-9-(glucopyranosyl-1,3-ribosyl)
BAR-G-P	6-benzylaminopurine-9-9glucopyranosyl-1,3-ribosyl)-phosphate
BSA	bovine serum albumin
BV	column volume
cab	chlorophyll a/b binding protein gene
Cg	*Corngrass* gene
CHS	chalcone synthase
CK	cytokinin
cpm	counts per minute
CPPU	N-(2-chloro-4-pyridyl)-N′-phenylurea
CTAB	cetyltrimethylammonium bromide
cyr1	cytokinin-resistant mutant
*c*Z	*cis*-zeatin
*c*ZR	*cis*-zeatin riboside
Δ^2-iPP	Δ^2-isopentenyl pyrophosphate
DE52	DEAE cellulose 52
DEPC	diethyl pyrocarbonate
DFR	dihydroflavonol reductase
DMSO	dimethylsulphoxide
DZ	dihydrozeatin
DZ9A	dihydrozeatin-9-alanine (dihydrolupinic acid)
DZ9G	dihydrozeatin-9-glucoside
DZNT	dihydrozeatin nucleotide
DZOG	dihydrozeatin-*O*-glucoside
DZR	dihydrozeatin riboside
DZR-G	dihydrozeatin-9-(glucopyranosyl-1,3-ribosyl)
DZR-G-P	dihydrozeatin-9-(glucopyranosyl-1,3-ribosyl)-phosphate
DZROG	dihydrozeatin riboside-*O*-glucoside
EDTA	ethylenedinitrilotetraacetic acid
ELISA	enzyme-linked immunosorbant assay
EMF1	*EMBRYONIC FLOWERING* gene 1
EMF2	*EMBRYONIC FLOWERING* gene 2
ES MS/MS	electrospray tandem mass spectrometry
FAA	formalin-acetic acid-alcohol fixative

FPF1	flowering promoting fator 1
FW	fresh weight
GA	gibberellin
GA_3	gibberellic acid
GC-MS	gas chromatography mass spectrometry
GL15	*GLOSSY15* gene
HPLC	high performance liquid chromatography
iP	isopentenyladenine
iP9G	isopentenyladenine-9-glucoside
iPA	isopentenyladenosine
iPA-G	isopentenyladenine-9-(glucopyranosyl-1,3-ribosyl)
iPA-G-P	isopentenyladenine-9-(glucopyranosyl-1,3-ribosyl)-phosphate
iPATA	isopentenyladenosine trialcohol
iPNT	isopentenyladenosine nucleotide (isopentenyladenosine-5′-monophosphate)
ipt	isopentenyl transferase gene
J4	juvenile-looking buds from 4-year-old trees
LC-MS	liquid chromatography-linked mass spectrometry
lec	*leafy cotyledon* mutation
LFY	*LEAFY* gene
LP5	LP medium conaining 5.0 mg/L BA
LPCH	LP medium without CK
m/z	mass/ion charge ratio
M4	mature-looking buds from 4-year-old trees
M8	mature buds from 8-year-old trees
MOPS	4-morpholinepropanesulphonic acid
mT	meta-topolin
mT9G	meta-topolin-9-glucoside
mT9RG	meta-topolin-9-(glucopyranosyl-1,3-ribosyl)
mTOG	meta-topolin-*O*-glucoside
mTR	meta-topolin riboside
mTR5′P	meta-topolin riboside-5′-monophosphate
mTROG	meta-topolin riboside-*O*-glucoside
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PR-10	pathogenesis-related protein 10
Prcab	*Pinus radiata* chlorophyll a/b binding protein gene
Prcr5	*Pinus radiata* cytokinin-responsive gene
psd	*paused* mutation
PVP	polyvinylpyrrolidone
PVPP	polyvinylpolypyrrolidone
RIA	radioimmunoassay
RNase	ribonuclease
S	seedling buds
SDS	sodium dodecyl sulphate
TAE	Tris-acetic acid-EDTA buffer
TBA	tertialbutylalcohol
TBE	Tris-boric acid-EDTA buffer
TEA	triethylammonium acetate

TLC	thin layer chromatography
Tp	*Teopod* gene
Tris	tris(hydroxylmethyl)aminomethane
tZ	*trans*-zeatin
tZR	*trans*-zeatin riboside
UTR	untranslated region
UV	ultraviolet
v/v	volume/volume
vp8	*viviparous8* mutation
xtc1	*extra cotyledon 1* mutation
xtc2	*extra cotyledon 2* mutation
Z	zeatin
Z7G	zeatin-7-glucoside
Z9A	zeatin-9-alanine (lupinic acid)
Z9G	zeatin-9-glucoside
ZNT	zeatin nucleotide (zeatin riboside-5′-monophosphate)
ZOG	zeatin-*O*-glucoside
ZR	zeatin riboside
ZR-G	zeatin-9-(glucopyranosyl-1,3-ribosyl)
ZR-G-P	zeatin-9-(glucopyranosyl-1,3-ribosyl)-phosphate
ZROG	zeatin riboside-*O*-glucoside
ZRTA	zeatin riboside trialcohol

Chapter 1 Introduction

Pinus radiata was introduced into New Zealand from California, USA at least 130 years ago (Weston, 1957). Today it is the major plantation forestry species in New Zealand and makes a significant contribution to the New Zealand economy. Although pine forests occupy only 4.5 % of the land area of New Zealand, they accounted for most of the domestic demand for forest products and provided 13.5% of New Zealand's total export eamings (NZ$2.6 billion) in the year to March 1994 (Walter and Smith, 1995).

Tree improvement practices, along with a favourable climate and innovative management, have contributed to the success of *P. radiata* plantation forestry in New Zealand. In fact, *P. radiata* has been incorporated in breeding projects for more than 40 years in New Zealand (Walter and Smith, 1995). However, the consequences of tree maturation (i.e. phase change) have been a major obstacle in the deployment of selected clones (Chaperon, 1989; Libby and Ahuja, 1993). For instance, clones of *P. radiata* with commercially desirable genotypes can normally only be identified as "superior" after they have grown for around eight years and have reached their mature phase. Not until this age can clones be reliably ranked according to their economic traits such as long-term growth, straightness, and wood properties. Mass propagation is then required of the proven superior trees. Unfortunately, cuttings taken from these trees exhibit mature characteristics. They are more difficult to root, and have a slower diameter growth rate than seedlings or cuttings from juvenile trees. As a result of these maturation effects, the genetic gain made by selecting the proven trees is lost (Horgan et al., 1997). In 1969, for example, personel from NZFP planted more than a quarter of a million cuttings of selected clones of *P. radiata* in the nursery in Tokoroa. Unfortunately, little was gained from these efforts, because of a lack of understanding of, or inability to control, maturation effects (Carter, 1989).

Consequently, understanding the maturation phenomenon in *P. radiata* may be the key to clonal forestry. If phase change or maturation processes could be controlled, one could overcome the maturation problem by either prolonging the juvenile phase of superior clones or rejuvenating mature clones once they are proven to be superior. It is

well accepted that plant growth and development are regulated by a range of plant hormones of which the cytokinins are one type (Kamínek, 1992). In *P. radiata*, it has been observed that cytokinin applied to mature buds during micropropagation leads to an apparent "rejuvenation" of the plantlets (Horgan, 1987). Instead of the very small brown bud scales characteristic of the mature phase, primary needles are formed which is a morphology associated with the juvenile phase. In the absence of exogenous cytokinin, mature buds in culture maintain the growth form typical of the mature phase.

Juvenile and mature *P. radiata* buds, *in vivo* and *in vitro*, provide a unique system by which to investigate the role of the cytokinins in the regulation of phase change phenomena. The key aims of this project were:

1. To undertake qualitative and quantitative analyses of endogenous cytokinins in buds from juvenile and mature *P. radiata*.
2. To undertake qualitative and quantitative analyses of cytokinins in micropropagated juvenile and mature *P. radiata* in the presence and absence of the cytokinin 6-benzylaminopurine (BA).
3. To develop a strategy, using molecular biology techniques, to study the roles of the cytokinins in the regulation of gene expression during phase change.
4. To isolate and monitor the expression of cytokinin-responsive genes which may be involved in phase change.

Such information obtained in this thesis is vital to furthering our understanding of the mechanisms of phase change and cytokinin-induced rejuvenation in *P. radiata*.

1.1 Phase change

1.1.1 Morphological, physiological, biochemical and molecular features

Higher plants pass through distinct phases during their growth and development which lead to the production of shoots with unique morphological and physiological attributes (Kester, 1976; Hackett, 1985; Meier-Dinkel and Kleinschmid, 1990; Greenwood and Hutchison, 1993; Poethig, 1990; Lawson and Poethig, 1995). Those characteristics that appear early in development are referred to as juvenile and those that appear late in development are referred to as mature or adult characteristics (Poethig, 1990; Hackett

and Murray, 1996). The transition from juvenile to mature characteristics is referred to variously as phase change (Brink, 1962), maturation (Wareing, 1959) or ontogenetic aging (Fortanier and Jonkers, 1976).

Morphological comparisons show that, in many species, the most obvious differences which distinguish post-embyonic developmental phases are leaf shape, phyllotaxy, branch architecture, epidermal characters and the capacity for reproductive development. The best documented differences in shoot traits between juvenile and mature phases have been recorded in English ivy (*Hedera helix* L.) and maize (*Zea mays* L.) (Table 1.1). In all species, leaf traits are the most important markers in studying phase change. For example, cotyledons are specialised leaves formed during embryogenesis for storage purpose, true leaves are formed during the vegetative phase for photosynthetic purpose and flower organs are specialised leaves for reproductive purpose.

In the past, attainment and maintenance of the ability to flower have been the main criteria for assessing the begining of the mature phase in higher plants (Hackett, 1985) and this is probably the only consistent trait which can be used in evaluating the mature phase in all higher plant species. However, although flowering occurs only after 25 - 40 years in some woody species such as *Quercus*, *Fagus*, *Abies* or *Picea*, this does not mean that the trees are completely juvenile before the onset of the first flower. In fact, other vegetative features such as reduction in the ability to form adventitious roots change much earlier (Meier-Dinkel and Kleinschmid, 1990). Therefore, it must be emphasised that various characteristics may change at different rates during phase change in a given species (Greenwood et al., 1989; Steele et al., 1989; Hackett and Murray, 1996) and that it is very important to recognise individual characteristics in studying phase change. In fact, most of the more recent work has been focused on individual traits associated with leaf morphology and physiology, rather than the onset of flowering, as experimental markers in elucidation of phase change mechanisms in woody and herbaceous species (Greenwood, 1992; Moose and Sisco, 1994; Evans and Poethig, 1995; Telfer et al., 1997; Poethig, 1997; Van Lijsebettens and Clarke 1998).

Table 1.1 Morphological and physiological features that distinguish the juvenile and mature phases of a woody species - English ivy (*Hedera helix* L.) and a herbaceous species - maize (*Zea mays*) (Poethig, 1990; Hackett Murray, 1996)

Features	Juvenile	Mature
	Hedera helix	
Shoot apex width	Ca 140 μm	Ca 200 μm
Leaf shape	5-lobed, palmate	Entire, ovate
Leaf thickness	230 μm	330 μm
Phyllotaxis	Distichous (1,2)	Spiral (2,3)
Growth habit	Plagiotropic	Orthotropic
Internode length	Long	Short
Stem anthocyanin	Present	Absent
Photosynthetic capability	Low	High
Stem aerial roots	Present	Absent
Rooting ability	Good	Poor
Ability to flower	Absent	Present
	Zea mays	
Adventitious roots	Present	Absent
Lateral buds	Tiller-like	Ears or absent
Leaf shape	Narrow	Broad
Internode length	Compressed	Extended
Anthracnose resistance	Poor	Good
Epidermal traits		
Cuticle thickness	< 1 μm	> 3 μm
Epidermal cell shape	Circular	Rectangular
Epicuticular wax	Present	Absent
Epidermal hair	Absent	Present
Bulliform cells	Absent	Present

Studies on the transitions between the phases have indicated that the apical and subapical meristem regions of the shoot apex are the sites of origin of phase change (Hackett et al., 1987; Marc and Hackett, 1991, 1992; Greenwood, 1995). For example, when apices from mature plants of *Citrus* or *Sequoia* having only the apical meristem and one or two leaf primordia were grafted onto juvenile shoots they grew out into plants which had mature traits (Navarro et al., 1975; Monteuuis, 1991). In English ivy, it has been found that the apical and subapical meristems of juvenile and mature plants are very different in size, configuration and cell division activity, and these differences in meristem characteristics are related to several phase-dependent, morphological characteristics such as differences in phyllotaxis (Stein and Fosket, 1969; Hackett and Murray, 1996). Differences in the morphology of apical meristems between juvenile and mature trees have also been observed in *Pinus* species (Browne, 1995; Hackett, personal communication). These changes in the characteristics of the apical meristem and its flanking primordia during phase change are logically consistent with the fact that the plant shoot system is formed through the activity of apical meristems.

Further evidence for the apical meristem being the origin of maturation-related differences in physiological, anatomical and morphological features is that the developmental behaviour of axillary buds located in various positions along the primary shoot actually depends on the maturation state of the shoot apical meristem at the time they were initiated (Hackett, 1985; Poethig, 1990). This means that the activity of the axillary buds from the juvenile portion of a shoot results in an expression of juvenile characteristics while those from the mature portion of the shoot produces mature phase traits.

Physiological investigations revealed that the net photosynthetic capability of the mature foliage of larch (*Larix laricina*) was significantly greater than that of juvenile foliage and photosynthesis was positively correlated with chlorophyll content in foliage (Greenwood et al., 1989; Hutchison et al., 1990). In contrast, Hutchison et al. (1990) showed that the mature foliage of red spruce (*Picea rubens*) scions grafted onto juvenile shoots exhibited significantly less net photosynthesis than juvenile foliage, which was associated with decreased stomatal conductance (Rebbeck et al., 1993). Futhermore, it

has been found that photosynthetic pathways can change during phase change. In *Mesembryanthemum crystallinum*, the photosynthetic pathway switches from C_3 metabolism in the juvenile phase to crassulacean acid metabolism (CAM) in the mature phase (Adams et al., 1998).

Biochemical examinations have shown that the abilities to accumulate certain biochemical products can be very different between the juvenile and mature phases. In the model woody plant, *Hedera helix* L., the collenchyma cells of the hypodermis of juvenile stems and leaf petioles accumulate anthocyanins while apparently identical collenchyma cells in mature stems and petioles do not (Murray, 1988). Therefore, anthocyanin accumulation can be used as a reliable marker for phase change in English ivy. In addition, changes in protein composition associated with phase change have been observed in several species (Bon, 1988; Bon et al., 1994; Amo-Marco et al., 1993; Snowball et al., 1991; Bestford et al., 1996; Hand et al., 1996). For example, Bon (1988) detected a 16 kDa membrane-associated protein in shoot apices of juvenile *Sequoiadendron giganteum* while this protein was not present in apices of mature trees.

Molecular studies have demonstrated that differences in gene expression also exist between the juvenile and mature phases. It has been found in both larch and English ivy that the expression of genes for the chlorophyll a/b binding protein (*cab*) decreases with maturation (Hutchison et al., 1990; Woo et al., 1994). Further, in English ivy, Woo et al. (1994) has observed that a prolin-rich protein (PRP) is expressed more strongly in mature petioles than in juvenile petioles. The increased expression of the PRP gene may inhibit root meristem activity (Murray et al., 1994). In addition, Murray et al. (1994) have demonstrated that the inability of mature lamina tissues in English ivy to synthesise anthocyanins is due to the lack of transcription of the gene encoding the enzyme dihydroflavonol reductase (DFR). In contrast, in response to the signals that induce DFR in juvenile tissues both phases transcribe chalcone synthase (CHS) and accumulate CHS mRNA, which is involved in upstream DFR in anthocyanin biosynthesis.

1.1.2. Molecular genetic studies of phase change

Although genetic makeup is important in determining the length of the juvenile phase and the transition from juvenile to mature (Hackett, 1985), little progress could be made in identifying and isolating the genes involved in regulating phase change until the development of molecular genetic techniques. *Arabidopsis* and maize are the main plant species which have been used to study the genetic mechanisms of phase change due to their short life cycles, well studied genetics, and ease of manipulation with molecular genetic tools. By making use of mutants, researchers have identified a number of genes which are involved in the transition from juvenile to mature or from vegetative to reproductive phases.

The isolation of early- and late-flowering mutants in *Arabidopsis* has led to the identification of genes that control the time of the transition from the vegetative to the reproductive phase (Zagotta et al., 1992, 1996; Haughn et al., 1995; Martinez-Zapater et al., 1994; Hicks et al., 1996). Several genes have been identified that cause early flowering (Haughn et al., 1995; Amasino, 1996). The most extreme examples are the two *EMBYONIC FLOWER* genes, *EMF1* and *EMF2* (Sung et al., 1992). Mutants impaired in *EMF* genes produce an inflorescence upon germination. Anatomical studies indicate that these mutants form a flower-forming meristem immediately on germination, bypassing vegetative development altogether (Bai and Sung, 1995; Yang et al., 1995). In addition, *emf* mutants exhibit an enlarged and domed shoot apical meristem, elongated internodes and petioleless cotyledons (Sung et al., 1992). One way to explain the function of *EMF* genes in wild-type *Arabidopsis* is that a gradual decrease in *EMF* gene activity promotes phase change during shoot development. When *EMF* activity drops below a threshold, the shoot apical meristem transforms from being a structure producing vegetative primordia to one producing reproductive primordia (Yang et al., 1995). This hypothesis is consistent with the concept that phase change originates from the changing fate of the developing primordia. Therefore, the activity of *EMF* gene products is to maintain the juvenile state of development.

Several *Arabidopsis* mutants exhibiting delayed flowering have also been isolated (Haughn et al., 1994; Amasino, 1996). Because these mutants postpone the transition

from the vegetative phase to the reproductive phase, the gene products would have a promotive effect on reproductive development (Haughn et al., 1995). For example, *FWA* and *FT* genes, that have been identified by late-flowering mutations, can function redundantly with *LFY* in positively controlling downstream genes such as *AP1* and other flower initiation genes while *FVE* and *FPA* genes (also identified by late-flowering mutations) promote the transition from the vegetative to the reproductive phase through *LFY* or *FT* and *FWA* functions (Ruiz-Garcia et al., 1997).

Genetic and molecular analyses have also led to the identification and isolation from *Arabidopsis* of other genes which are involved in the change from the vegetative phase to the reproductive state (Haughn et al., 1995). Two of these genes are *LEAFY* (*LFY*) and *APETALA1* (*AP1*), whose mutations severely impair the transition from a vegetative to a reproductive meristem (Irish and Sussex, 1990; Schultz and Haughn, 1991; Huala and Sussex, 1992; Mandel et al., 1992; Weigel et al., 1992; Bowman et al., 1993; Weigel and Meyerowitz,1993). *LFY* and *AP1* are homeotic genes (Schultz and Haughn, 1991; Mandel et al., 1992) which encode transcriptional factors. In *Arabidopsis*, overexpression of both genes led to earlier flowering and the formation of terminal flowers (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995), indicating that *LFY* and *AP1* not only are necessary but are sufficient to promote the initiation of flowers at the shoot meristems. More excitingly, *LFY* overexpression reduced flowering time in transgenic aspen from many years to a few months (Weigel and Nilsson, 1995). Moreover, increasing the copy number of endogenous *LFY* genes reduced the number of leaves produced before the first flower was formed (Blazquez et al., 1997). These results strongly demonstrate that *LFY* controls the transition time from vegetative to reproductive development. This is in agreement with the conclusion obtained from expression studies with *LFY* during floral determination in *Arabidopsis*. Here it was shown that the time point of the transition from the vegetative to the reproductive development was at least partially controlled by the levels of *LFY* activity in *Arabidopsis* (Blazquez et al., 1997; Hempel et al., 1997).

Mutants have been used successfully in the search for genes that control the transition from the juvenile to the mature phase. Around 10 genes, which are involved in

regulating vegetative phase change, have been identified from *Arabidopsis* (Meinke, 1992; Meinke et al., 1994; West et al., 1994; Conway and Poethig, 1997; Telfer et al., 1997) and maize (Poethig, 1988a, 1988b; Dudley and Poethig, 1993; Evans et al., 1994; Moose and Sisco, 1994; Evans and Poethig, 1997). However, the majority of the genes, with the exception of *GLOSSY15,* have not been isolated (Moose and Sisco, 1996).

All the genes that have been identified in *Arabidopsis* so far are involved in the regulation of early juvenile events. Meinke (1992) has identified a recessive mutant, *leafy cotyledon* (*lec*), whose cotyledons produce trichomes characteristic of the distribution pattern of juvenile leaves and exhibit a vascular pattern intermediate between that of leaves and cotyledons. Therefore, *lec* cotyledons are partially transformed into leaves, implying that postgerminative juvenile phase development is initiated precociously and that embyonic and postgerminative juvenile programmes operate simultaneously in mutant embryos (Meinke et al., 1994; West et al, 1994). Therefore, the wild-type gene *LEC* may play a fundamental role in timing the initiation of the expression of the juvenile leaf traits.

By contrast to the *lec* mutant, Conway and Poethig (1997) have identified mutations of three genes in *Arabidopsis thaliana* - *extra cotyledon1* (*xtc1*), *extra cotyledon2* (*xtc2*) and *altered meristem programming1* (*amp1*) - that led to juvenile leaves developing as cotyledons. In all three of these mutations, the development of the first two juvenile leaves as cotyledons is associated with their time of emergence. Leaves that emerged earlier compared to wild-type leaves (three days after imbibition of seeds) were completely devoid of trichomes and had a simple venation pattern which is a feature of cotyledons; leaves that emerged at the same time as wild-type leaves (five days after imbibition) were completely normal (Conway and Poethig, 1997). The phenotypes of these mutants are very similar to precociously germinating *Brassica napus* (oilseed rape) embryos that produce extra cotyledons or chimeric organs with sectors of cotyledon and leaf tissue (Finkelstein and Crouch, 1984; Bisgrove et al., 1995; Fernandez, 1997). The treatment inducing premature germination apparently causes leaves to develop as cotyledons because their primordia are forced to develop in the context of the embyonic phase of development. All of these results demonstrate that a

change in the relative timing of shoot and embryo development is responsible for the development of leaves as cotyledons.

More recently, Evans and Poethig (1997) have focused on the postgerminative effects of a mutation, *viviparous8* (*vp8*) (Robertson, 1955), which increases the number of juvenile leaves and decreases the number of mature leaves but does not change the flowering time. Thus, the wild-type gene Vp8 is involved in repressing the rate of leaf production early in shoot development and promoting vegetative maturation but does not impact on the timing of reproductive development. These results support the proposal that phase-specific characteristics are not regulated by the size of the plant but by the rate of organ initiation and by changing the time at which a phasic programme begins or ends (Evans and Poethig, 1997; Telfer et al., 1997)

Consistent with these results, Telfer et al. (1997) isolated a mutation in *Arabidopsis* called *paused* (*psd*). Seedlings homozygous for this gene produce cotyledons at the normal time during embryogenesis, but fail to make the first true leaf primordia for several days after germination. The leaves that are eventually produced in this position by *psd* seedlings phenotypically resemble leaves that are normally being initiated at the same time by wild-type siblings, rather than having the characteristic morphology of the first two true leaves (Lawson and Poethig, 1995; Telfer et al., 1997). Telfer et al. (1997), therefore, concluded that the phenotypes that leaves develop is regulated by the age, rather than the size of the plant.

In studying maize mutations, Poethig (1988a, 1988b) identified four genes, *Teopod1*, *2* and *3* (*Tp1*, *Tp2* and *Tp3*) and *Corngrass* (*Cg*) that all significantly prolong the expression of juvenile traits such as epicuticular wax production but do not affect the onset of vegetative mature characteristics such as macrohair production (Evans et al., 1994). Furthermore, although *Teopod* mutants have profound effects on the morphology of reproductive structures, the reproductive phase is initiated at the same time as in wild-type plants, despite the extended juvenile development in these mutants (Bassiri et al., 1992). A logical explanation of these results is that *Teopod* mutations prolong the juvenile developmental programme while leaving vegetative mature and

reproductive developmental programmes relatively unaffected (Lawson and Poethig, 1995).

In contrast to *Tp* mutations, Evans et al. (1994) and Moose and Sisco (1994) have identified a recessive mutant, *glossy15* (*gl15*), which shortens the juvenile phase of maize and promotes the expression of mature characteristics, indicating that the function of the wild-type *GLOSSY15* (*GL15*) is both to promote juvenile epidermal traits and to repress a mature phase programme of epidermal development in maize (Poethig, 1997). Recently, the *GL15* gene cDNA was cloned using transposon-tagging and, of interest, sequence analysis revealed that *GL15* encoded a transcriptional factor with significant sequence similarity to the *Arabidopsis* regulatory gene *APETALA2* (Moose and Sisco, 1996), which acts primarily to regulate reproductive development (Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994). This finding indicates that there may exist a similar regulatory mechanism between the vegetative and reproductive developmental programs, which is consistent with the proposal made by Goethe (1790) more than 200 years ago that all leaf-like organs, including the parts of a flower, derive from transformations of a single type of organ - a leaf. Double mutant and molecular analyses have demonstrated that *GL15* is required for the prolonged expression of the juvenile epidermal traits displayed in *Tp*, *Cg* and *vp8* mutants (Evans et al., 1994; Moose and Sisco, 1994; Moose and Sisco, 1996; Evans and Poethig, 1997), meaning that there are at least two levels of regulation of phase-specific characteristics in maize (Hackett and Murray, 1996).

1.1.3. Phase change and plant hormones

For many years, investigators have been trying to identify the factors that regulate phase change. Studies using grafting (Hackett, 1985; Huang et al., 1992, 1995, 1996), rooting (Irish and Nelson, 1988) and genetic mutants (Poethig, 1988a, 1988b; Dudley and Poethig, 1993) have all pointed to the involvement of transmissible factor(s) performing a key role in phase change. Logically, the transmissible factor(s) has been considered to be a plant hormone. For many years the gibberellins have been implicated in phase change (Robbins, 1957; Rogler and Hackett, 1975; Hackett, 1985; Zimmerman et al.,

1985) but, depending on the species and the conditions, gibberellins may either promote or inhibit phase change.

In *Arabidopsis*, application of GA_3 promotes the transition from vegetative to reproductive development (Langridge, 1957; Zeevaart, 1983). Furthermore, GA-deficient and GA-insensitive mutants of *Arabidopsis* have provided additional evidence that GA promotes flowering in this species (Wilson et al., 1992). More recently, Kania et al. (1997) has cloned a gene cDNA, termed flowering promoting factor1 (FPF1), which is expressed in apical meristems immediately after the photoperiodic induction of flowering in the long-day plants mustard and *Arabidopsis*. Constitutive expression of the gene in *Arabidopsis* under the control of the cauliflower mosaic virus 35S promoter resulted in a dominant heritable trait of early flowering under both short- and long-day conditions. Treatments with gibberellin and paclobutrazol (a GA biosynthesis inhibitor) as well as crosses with GA-deficient mutants indicate that FPF1 is involved in a GA-dependent signaling pathway and modulates a GA response in apical meristems during the transition to flowering.

During phase change, one of the first known responses in apical meristems after the induction of flowering is the increase in the activity of the expression of *LEAFY* (*LFY*) (Weigel et al., 1992). Blazquez et al. (1997) has demonstrated that application of GA_3, which hastens flowering in short days, enhances *LEAFY* expression in *Arabidopsis* in short days. Furthermore, in *Arabidopsis*, a heterochronic switch from flower to shoot development can be induced in plants heterozygous for *leafy*. This transformation from flower to shoot meristems is suppressed by *spindly*, a mutation that activates basal gibberellin signal transduction in a hormone independent manner, or by the exogenous application of GA_3 and GA_{4+7}.

In addition to promoting flowering, GAs also promote trichome production in *Arabidopsis* (Chien and Sussex, 1996; Telfer et al., 1997). Trichomes have been used as markers for mature phase development in *Arabidopsis* (Telfer et al., 1997).

The effects of GA on phase change in *Arabidopsis* are consistent with the effects of GA on phase-specific characteristics in other herbaceous species. In maize, earlier studies (Olson, 1954; Stein, 1955) had demonstrated that the GA-deficient *dwarf1* (*d1*) mutant produced more leaves than the wild-type plant before flowering, indicating that GA promotes flowering in this species. Indeed, applications of GA_3 accelerate reproductive development (i.e. reduce leaf number before tassel development) in this maize mutant (Poethig, 1985). More recently, Evans and Poethig (1995) have also demonstrated that GA_3 promotes both vegetative phase change and reproductive maturity in maize. By analysing GA-deficient and juvenile-prolonging mutants, double mutants and exogenous application of GA_3 to these mutants, they concluded that GAs act in conjunction with several other factors to promote both vegetative and reproductive maturation in maize. Very similar results have also been observed in herbaceous perennials (Gianfagna and Merritt, 1998). *Aquilegia × hybrida* Sim plants treated at the 7-leaf stage with GA_{4+7} bolted and flowered 20 days earlier than untreated plants. Gibberellin treatment did not increase the growth rate (leaves/day), but induced flowering after the production of fewer leaves.

GAs are also considered as important regulators of phase change in woody species. The most striking examples of GAs on flower promotion can be found in conifers. These plants usually require at least about five to 20 years of vegetative development before flowering (Hackett, 1985). However, exogenous GAs can induce flowering in less than one year old plants (Pharis and Morf, 1968; Pharis and King, 1985). For example, spraying three-month-old western red ceder seedlings with GA_3 can induce the plant to produce visible flowers at the top of the plant at four months (Pharis and Owens, 1966). Many studies has demonstrated that polar GAs such as GA_3 are effective in members of Cuprasaceae and Taxodiaceae families while less polar GAs such as GA_{4+7} and GA_9 are necessary for induction of precocious flowering in Pinaceae species (Zimmerman et al., 1985; Pharis and Ross, 1986). For instance, GA_{4+7} effectively induced flowering in *Pinus radiata* (Sweet, 1979), Douglas-fir (McMullan, 1980; Owens et al., 1985) and *Larix lariccina* (Eysteinsson and Greenwood, 1990, 1995).

In contrast with conifers, exogenous GAs act to repress the transition to flowering in woody angiosperm plants such as apple, peach, birch and mango (see review: Pharis and King, 1985; Zimmerman et al., 1985) and *Metrosideros collina* (Clemens et al, 1995), indicating a positive relationship between certain GA forms and maintenance of the juvenile phase. In English ivy, for example, exogenous application of GA_3 causes rejuvenation of mature ivy plants (Rogler and Hackett, 1975; Horrell et al., 1990). Consistent with these results is the observation that GA-like substances were higher in 1-cm-long juvenile apices than in mature apices, and were moderately high in roots of juvenile plants (Frydman and Wareing, 1973a, 1973b). If it is true that high levels of endogenous GAs indeed contribute to the maintenance of the juvenile phase, one would predict that reduction in endogenous GAs in juvenile plants would lead to a transition to the mature phase. However, attempts using inhibitors of GA biosynthesis to promote maturation in juvenile ivy have been unsuccessful (Frydmand and Wareing, 1974; Horrell et al., 1990).

In some species such as *Pharbitis nil* (King et al., 1987) and *Pisum sativum* (Reid et al., 1977), GA application can either promote or inhibit flowering, depending on the time of treatment, day length and genotype, suggesting that GAs are involved in part but not all of a phase change programme. Indeed, even in conifers, the effects of GAs on the transition to reproductive development is often strongly enhanced by timing of treatments, root pruning, nutrition, heat and water stress (Zimmerman et al., 1985; Pharis and Ross, 1986, Owen and Simpson, 1988; Ross, 1988; Owen et al., 1992; Eysteinsson and Greenwood, 1995; Smith and Greenwood, 1995). However, GA applications by themselves often do not induce flowering when other environmental conditions are unfavorable. Although one may argue that the promoting effects of these treatments on flowering is in part through effects on GA metabolism (Moritz et al., 1989; Moritz and Oden, 1990; Chalupka et al., 1982; Dunberg et al., 1983; Pharis et al., 1989), the fact that these treatments frequently interact synergistically with GA application indicate that GAs do act in conjunction with other factors.

Although most physiological analyses reported to date have focused on gibberellins, there have been some investigations showing that cytokinins may also play a part during phase change in a variety of species (reviewed by Bernier, 1988; Kinet et al., 1993)

Exogenous cytokinin applications have been demonstrated to enhance giberellin-induced flowering in Douglas fir (Ross and Pharis, 1976) and in *Picea sitchensis* (Tompsett, 1977). However, these results do not necessarily mean that cytokinins themselves promote the transition from vegetative to reproductive development in these species. Through studying endogenous variations and exogenous applications of cytokinins in Douglas fir, Imbault et al. (1988) pointed out that the effect of certain cytokinins is possibly to increase the level of flowering which was normally possible, but not definitively to induce flowering on a normally nonflowering tree. This is consistent with the finding that cytokinin is an absolute requirement for flowering of explants from mature *Passiflora* plants, but juvenile explants do not flower even in its presence (Scorza and Janick, 1980).

As mentioned earlier, cultural conditions such as root pruning, drought and heat stress significantly stimulate the transition of members of the *Pinaceae* from the vegetative to the reproductive development. Because root systems are believed to be a key site of cytokinin synthesis in plants (Letham, 1994), the stimulatory effects of these treatments may be through decreasing the production of cytokinins in roots. Indeed, in various species, the inhibitory effect of the roots on flowering can be mimicked by cytokinins, although a few studies have shown that the effect of exogenous cytokinins is strongly dependent on other factors such as the applied concentration, the environmental conditions and the time of application (Kinet et al., 1993).

By analysing endogenous cytokinin concentrations, Zaerr and Bonnet-Masimbert (1987) have found that a reduction in endogenous cytokinins in the shoot of Douglas fir plants is associated with those treatments that promote the transition from vegetative to reproductive development. High concentrations of cytokinins have been observed to be associated with the vegetative state and low concentrations with reproductive development. In black spruce (*Picea mariana*), it has been observed that exogenous

cytokinin applications reduce the stimulatory effects of GA_{4+7} and root pruning on reproductive bud production (Smith and Greenwood, 1995), which is in contrast to the finding of Ross and Pharis (1976).

In woody angiosperms, Galoch (1985) compared the content of plant hormones in buds and apical sections of shoots from juvenile to fully mature plants of birch (*Betula verrucoca* Ehrh). The results showed that the juvenile vegetative plants had the highest level of cytokinins while fully mature 15-year-old plants exhibited the lowest level. Recently, Day et al. (1995) analysed the cytokinin content in the leaves of juvenile, transitional and mature plants of *Elaeocarpus hookerianus*, a species endemic to New Zealand, under forest- and glasshouse-grown conditions. A trend of decreasing concentration of active (free base, riboside and nucleotide) cytokinins was found to exist between juvenile, transitional and mature forest-grown leaves. This trend was confirmed by investigating endogenous cytokinins in the leaves of juvenile and mature plants grown in a glasshouse. The high concentration of cytokinins in juvenile tissues found in these studies is supported by other investigations in citrus (Hendry et al., 1982), rubber-tree (*Hevea brasiliensis*) (Perrin et al., 1997) and *Sophora sp* (Carswell et al., 1996).

In *Xanthium*, a short-day plant, Podolnyi et al. (1988) compared the cytokinins in organs possessing either juvenile or mature features. The results showed that the juvenile features of cotyledons were linked with high levels of endogenous cytokinins. The plants which were treated with low concentrations of exogenous cytokinin also showed a significant decrease in the percentage of the plants developing flowers. Comparable with these results, other researchers had observed that cytokinin content was higher in vegetative *Xanthium* plants (Henson and Wareing, 1977a, 1977b). There was a marked decline of cytokinin activity in leaves, buds, and root exudates of the *Xanthium* plants if the plants were induced by one long night (flowering-inducing condition). Flowering and the decrease in cytokinins were both nullified by a night break, suggesting that they are closely related (Henson and Wareing, 1977a, 1977b).

In *Arabidopsis*, studies on genetic mutations have produced more direct evidence that cytokinins are involved in the regulation of phase change in higher plants. Chaudhury et al. (1993) have isolated a mutant that produces elevated levels of cytokinin, *altered meristem program 1* (*amp1*). The *amp1* light-grown seedlings had six times more zeatin riboside than wild type and similarly elevated zeatin and dihydrozeatin. Cytokinin levels were also elevated in dark-grown *amp1* seedlings (Chin-Atkins et al., 1996). Several aspects of the phenotype of the *amp1* indicate that cytokinins prolong the juvenile phase of the *amp1* mutant. For example, *amp1* produces multiple cotyledons and significantly more rosette leaves before flowering (an average of 20 leaves for *amp1* and seven for wild type). Using trichome distribution as a marker, Telfer et al. (1997) concluded that *amp1* prolongs the juvenile phase by increasing the rate of leaf initiation because the *amp1* mutation causes an increase in the number of leaves without abaxial (lower surface) trichomes (a mature feature). The product of the wild-type gene, *AMP1,* may be required for cytokinin degradation or it may be a negative regulator of cytokinin biosynthesis (Deikman, 1997).

Recently, Deikman and Ulrich (1995) have identified a novel cytokinin-resistant mutant (*cyr1*) of *Arabidopsis* which displays an abbreviated shoot development. The cotyledons and true leaves of the *cyr1* fail to expand. Only a few leaves are produced before a single flower is made. The roots of the mutant are resistant to cytokinins. All the features observed in the mutant are consistent with a disruption in cytokinin perception or signal transduction (Deikman, 1997). The shoot phenotype of *cyr1* is very similar to that of *emf* mutants (see earlier discussion in this chapter). These results indicate that a lack of response to cytokinin in *cyr1* is responsible for the very short juvenile phase of the *cyr1*. The implications from the *emf* mutant and the *cry1* mutant are that cytokinin is required for expression of the juvenile phase. This is in agreement with the conclusion that cytokinins prolong the juvenile phase in *amp1*. In addition, the findings that cytokinin represses expression of the genes important in flower formation would also support this conclusion (Estruch et al., 1993; Venglat and Sawhney, 1996).

1.1.4 Rejuvenation

The term rejuvenation implies a reversal of the maturation process (Hackett and Murray,

1993), i.e. the development of the plant reverts from the production of mature characteristics to that of juvenile ones. Higher plants undergo rejuvenation naturally through sexual reproduction processes during which gametes are produced from the somatic cells of the mature plant body. Rejuvenation by this route is associated with meiotic division of somatic cells. Another route through which rejuvenation occurs is via the production of adventitious embryos from somatic cells in the ovule, such as the adventitious embyos in *Citrus* (Lakshmanan and Ambeggaokar, 1984; Wakana and Uemoto, 1987,1988). The latter route indicates that either somatic cells in the ovule have become rejuvenated from mature somatic cells in the ovule or some of the cells never became mature (Zimmerman et al., 1985; Greenwood, 1995).

In practice, rejuvenation of higher plants can be manipulated with different approaches such as grafting mature scions onto juvenile rootstocks, *in vitro* propagation, and treatment with plant hormones (Hackett, 1985), although it is difficult to achieve complete rejuvenation by these procedures in many species. Many experiments have demonstrated that some phase-specific traits are more easily rejuvenated than others. The ease of rejuvenation of any one trait may also change during the course of development or in response to a particular treatment regime (Hackett, 1985; Hackett and Murray, 1993). In the literature, the term "rejuvenation" has been used quite loosely to refer either to the reversal of a collection of mature phase-related traits or to just one single mature character under certain experimental or cultural conditions.

There are numerous reports of regaining seedling or juvenile phase characteristics from plants in the mature phase (see reviews by Hackett, 1985, Zimmerman et al., 1985; Greenwood, 1987; Hackett and Murray, 1993). For example, Huang et al (1992) used rooting competence, stem elongation, lateral branching rates, adventitious shoot formation, callus development and protein patterns as criteria of phase reversal, when he was demonstrating a gradual rejuvenation of a woody species (*Sequoia sempervirens)* by repeated grafting of shoot tips from a mature tree onto fresh, rooted juvenile stem cuttings *in vitro.* In conifers, based on the assessment of foliar characteristics, growth rate, ease of grafting and the ability of cuttings to root, Franclet (1981) also observed a gradual rejuvenation of originally mature Douglas-fir scions by repeatedly regrafting the

scions onto juvenile rootstock. In English ivy, rejuvenation can be achieved after grafting only once (Doorenbos, 1954). Banks(1979) also demonstrated that English ivy plantlets regenerated from callus derived from juvenile or mature stems were juvenile in both cases. However, plantlet formation was through somatic embryogenesis for mature callus but through bud organogenesis for juvenile callus.

Rejuvenation of mature tissues *in vitro* usually involves application of a cytokinin (usually 6-benzylaminopurine, BA) at some point to promote outgrowth of lateral buds or to promote the formation of adventitious shoots (Greenwood, 1987). Examples of cytokinin-induced rejuvenation in various species can be found in the reviews by Hackett (1985), Zimmerman et al. (1985) and Greenwood (1987). In *Pinus sylvestris* L., application of cytokinin can induce the outgrowth of fascicular shoots *in vivo* which take on juvenile-like morphology (Kossuth, 1978). When placed in tissue culture, the fascicles of mature radiata and loblolly pine can be induced by exogenous cytokinins to produce shoots with juvenile morphology (ABO EL-NIL, 1982; Horgan, 1987).

The mechanism of rejuvenation is not yet known. The first question that needs to be answered is whether the rejuvenation originated from never-matured cells in the mature apex of the shoot or from the reversion of mature cells in the mature plants. Greenwood (1995) believes that the gradual rejuvenation phenomena observed is the evidence that rejuvenation results from conditions that favor more rapid multiplication of residual juvenile cells in the mature phase apex. However, there is no direct experimental evidence to support this explanation. One could also argue that the mature phase apex as a whole could be reprogrammed to take on juvenile developmental processes under conditions that promote rejuvenation.

1.1.5 Models of phase change

Based on woody species, Kester (1976) proposed a model (Fig. 1.1A) to depict many experimental findings (see review by Hackett, 1985) that juvenile characteristics usually occupy the lower part of the stem; at some point during plant development, the plant produces intermediate forms of structures and thereafter the plant forms mature traits which occupy the upper portion and the periphery of the plant body. This model implies

the coexistence of juvenile and mature characteristics during the growth and development of the main shoot axis and their branches. Using maize as an example of an herbaceous species, Poethig (1990) proposed an eventually similar model of phase change (Fig. 1.1B). Poethig suggested that the production of phase-related characteristics is specified by a series of independently regulated, overlapping programmes that modify the expression of a common set of processes required for shoot growth (Poethig, 1990). Evidence for this conclusion can be found in both herbaceous and woody species. For example, leaves and axillary buds produced during the transition from juvenile to mature phase development in maize express a combination of juvenile and mature traits (Poethig, 1990, Lawson and Poethig, 1995). In woody species such as *Eucalyptus* and English ivy, shoots also generate intermediate patterns that combine juvenile and mature characteristics during the juvenile-to-mature transition (Hackett and Murray, 1993). The key feature of Poethig's model is the proposal that shoot development of higher plants consists of discretely regulated phases, contrasting with the traditional view of shoot development: quantitative changes in the physiology of the plant as the basis of phase change (Lawson and Poethig, 1995).

More recently, Hackett and Murray (1996) have proposed some alternative models of phase change (Fig. 1.2). The main point of these models is that phase change is probably not a single 'master-switch' process as shown on the left of Fig. 1.2 but a composite of processes or programmes. The models emphasise the importance of studying the regulation of individual phase-specific characteristics to understanding the whole picture of phase change. These models can be used to explain the results obtained from genetic analyses of phase change in mutants that have either prolonged expression of the juvenile phase (Poethig, 1988a, 1988b; Bassiri et al., 1992) or accelerated appearance of the mature programme (Sung et al., 1992; Haughn et al., 1995; Amasino, 1996). These genetic analyses indicate that the pathways involved in phase change are likely to be complex with frequent branch points and many interacting pathways as proposed by Lawson and Poethig (1995). Physiological studies also show that the changes of individual phase-specific traits may not be closely linked temporally or mechanistically (Greenwood, 1992; Hackett et al., 1992) but display differential time

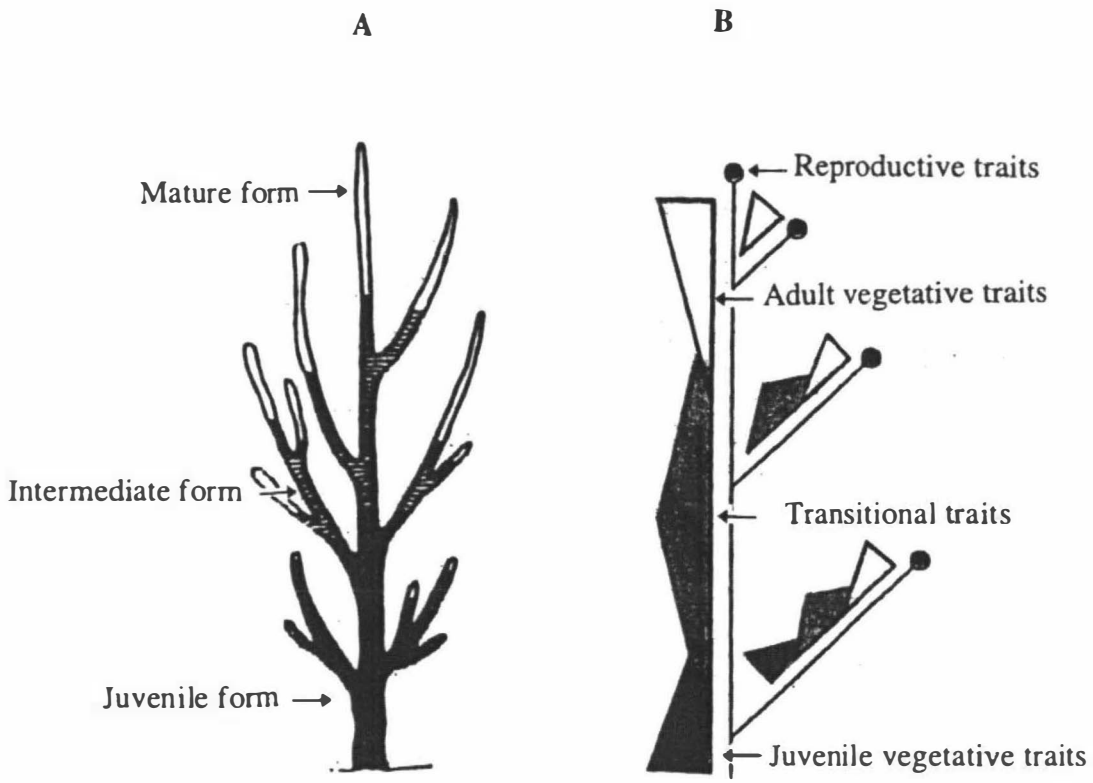


Figure 1.1 Kester's and Poethig's models of phase change. A: Schematic representation of the localisation of different maturation states on a mature, woody seedling-grown plant (Kester, 1976). B: Schematic representation of the expression of juvenile vegetative, adult vegetative, and reproductative traits in the shoot of an annual plant (Poethig, 1990)

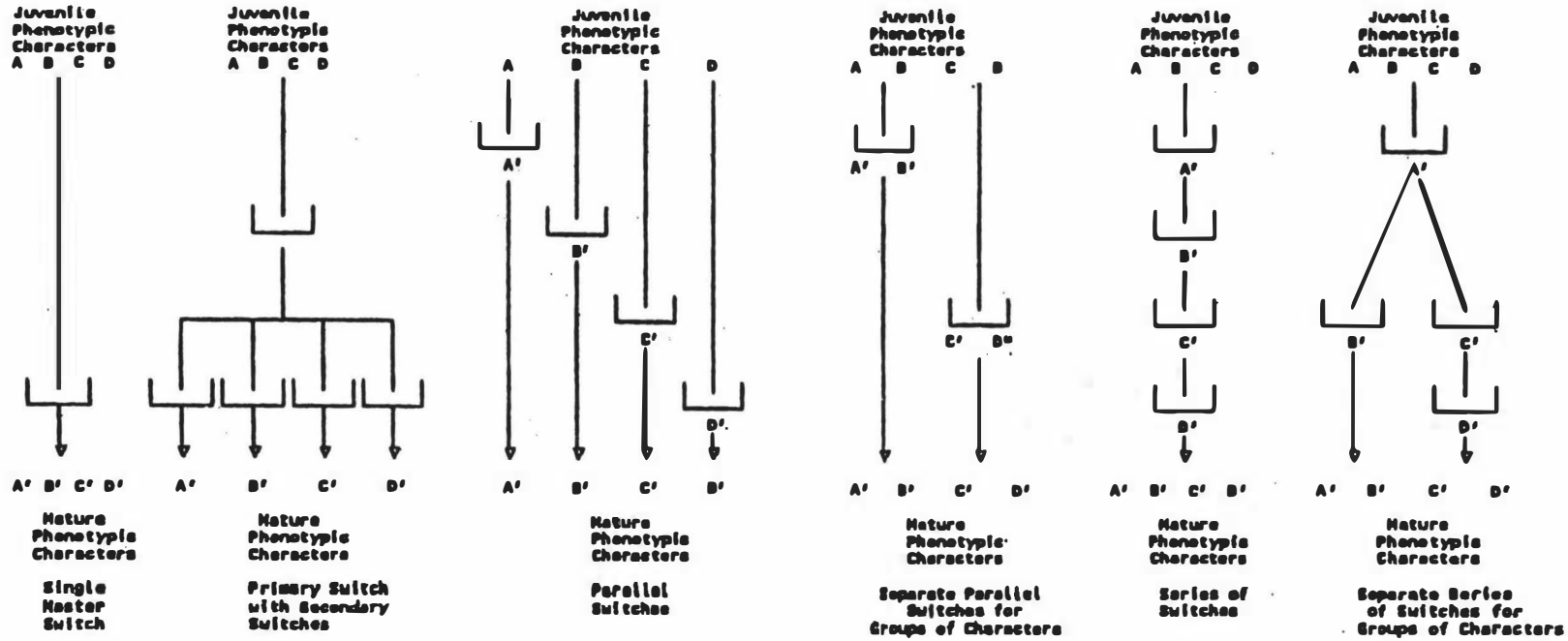


Figure 1.2 Alternate models of phase change (Hackett and Murray, 1996).

courses (Steele et al., 1989; Greenwood et al., 1989; Browne, 1995). Further supporting evidence comes from the differential sensitivity of various phase-specific traits of the same plant to rejuvenation treatments. For example, in English ivy, the sensitivity of different morphological and physiological features to rejuvenation treatment varies. During the reversion from the mature English ivy to the juvenile state in response to GA_3 treatment, induction of aerial rootlets and anthocyanin pigmentation (juvenile traits) was much more sensitive than the induction of changes in phyllotaxy and leaf shape (Rogler and Hackett, 1975).

Based on studies on conifers, Greenwood (1995) claims that models developed to explain phase change or maturation must be evaluated with five major criteria: (1) the onset of the mature state is usually gradual, but can be abrupt; (2) phase change affects a wide range of morphological, physiological and biochemical traits, but these traits appear to vary independently of one another; (3) maturation traits are often persistent and their maintenance is not always a function of tree size or proximity to roots; (4) the cells of the apical meristem itself become determined in some woody plants; (5) rejuvenation treatments bring about gradual reversion to the juvenile condition. According to these principles, Greenwood (1995) contends that

> "the switch (of maturation) could reside within individual cells in the apical meristem, and once activated would make that cell mature, so that over time the apex would become a mosaic with an ever increasing percentage of mature cells. Expression of mature characteristics would, therefore, be a function of the ratio of juvenile to mature cells in the apex at a given time".

Therefore, one of the most striking features unique to this model is that rejuvenation would come from those juvenile cells, not mature cells, in the apex by their more rapid multiplication under favorable conditions.

1.2. Cytokinins

As discussed in Sections 1.1.3 and 1.1.4, cytokinins may play a role in the regulation of phase change in higher plants. Therefore, understanding cytokinin biosynthesis, metabolism and action is important in elucidating its regulatory function during plant growth and development in phenomena such as phase change.

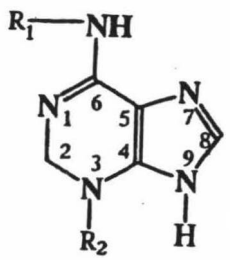
1.2.1 Cytokinin structures

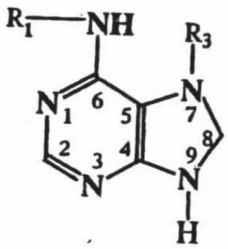
In the late 1940s and 1950s, investigations into the nutritional requirements of tissue cultures of tobacco stem pith, carried out by Folke Skoog and co-workers, led to the isolation and identification of a cell division promotor 6-furfurylaminopurine (kinetin) (Miller et al., 1955, 1956). Meanwhile, F.C. Steward and his co-workers isolated and identified another compound, 1,3-diphenylurea, from coconut milk which displayed growth promoting effects (Shantz and Steward, 1955). Unfortunately, kinetin was later found to be an artefactual rearrangement product of heated DNA and is not found in plant tissue (McGaw and Burch, 1995) and 1,3-diphenylurea was most likely to have been a contaminant from a urea-based herbicide (Jacobs, 1979). The first naturally occurring cytokinin was purified from immature kernels of *Zea mays* and obtained in a crystalline form by Letham in 1963 (Letham, 1963) and identified as 6-(4-hydroxy-3-methylbut-trans-2-enyl)aminopurine (more commonly known as zeatin) in the following year (Letham et al., 1964). A chemical synthesis immediately confirmed the structure of this compound (Shaw and Wilson, 1964; Shaw et al., 1966).

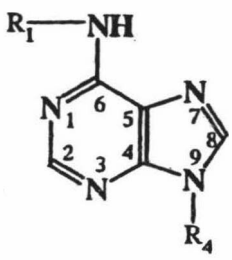
Cytokinins are defined as "substances which, in combination with auxin, stimulate cell division in plants and which interact with auxin in determining the direction which differentiation of cells takes" (Wareing and Phillips, 1978; Whitty and Hall, 1974). The term cytokinins is currently used to cover all purine derivatives with a branched five carbon N^6 (McGaw and Burch, 1995) or benzyl group N^6 substituent.
Figure 1.3 and 1.4 show the structures, common names and abbreviations of the major naturally occurring cytokinins. The basic unit of cytokinin molecules is adenine and the diverse forms are due to the modifications occurring either on the purine ring (Fig. 1.3) and/or on the side chain (Fig. 1.4).

1.2.2. Cytokinin biosynthesis

The actual pathway(s) of cytokinin biosynthesis within higher plants is still unknown even though considerable effort has been made to elucidate it (reviewed by Letham and Palni, 1983; Horgan, 1992; Binns,1994; Letham, 1994; McGaw and Burch, 1995; Chen, 1997; Kamínek et al., 1997; Prinsen et al., 1997). Two biosynthetic pathways have







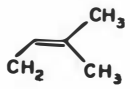
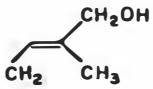
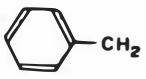
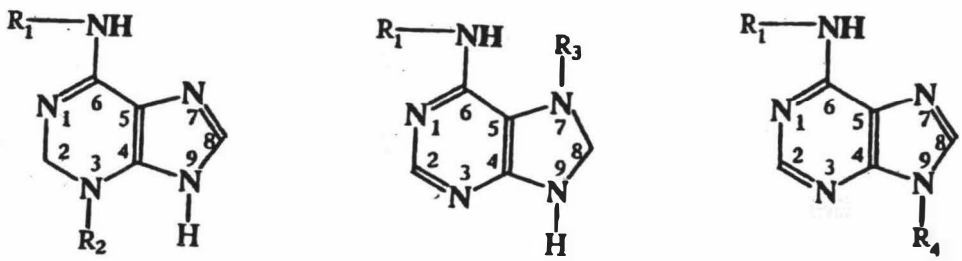
R1	R2	R3	R4	Common name	Abbreviation
			H	isopentenyladenine	iP
			ribosyl	isopentenyladenosine	iPA
			ribotide	isopentenyladenosine-5'-monophosphate	iPNT
			glucosyl	isopentenyladenine-9-glucoside	iP9G
		glucosyl		isopentenyladenine-7-glucoside	iP7G
			H	zeatin	Z
			ribosyl	zeatin-riboside	ZR
			ribotide	zeatin riboside-5'-monophosphate	ZNT
			glucosyl	zeatin-9-glucoside	Z9G
			alanyl	zeatin-9-alanine	Z9A
		glucosyl		zeatin-7-glucoside	Z7G
			H	6-benzylaminopurine	BA
			ribosyl	6-benzylaminopurine riboside	BAR
			ribotide	6-benzylaminopurine riboside-5'-monophosphate	BANT
			glucosyl	6-benzylaminopurine 9-glucoside	BA9G
			glucopyranosyl-1,3-ribosyl	6-benzylaminopurine 9-(glucopyranosyl-1,3-ribosyl)	BAR-G
		glucosyl		6-benzylaminopurine 7-glucoside	BA7G
	Glucosyl			6-benzylaminopurine 3-glucoside	BA3G

Figure 1.3 Cytokinin structures - modifications on the purine ring



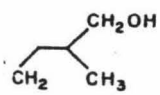
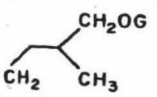
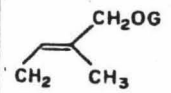
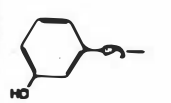
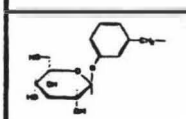
R1	R3	R4	Common name	Abbreviation
	glucosyl	H ribosyl ribotide glucosyl alanyl	dihydrozeatin dihydrozeatin riboside dihydrozeatin riboside-5'-monophosphate dihydrozeatin-9-glucoside dihydrozeatin-9-alanine dihydrozeatin-7-glucoside	DZ DZR DZRNT DZ9G DZ9A DZ7G
		H ribosyl	dihydrozeatin-O-glucoside dihydrozeatin riboside-O-glucoside	DZOG DZROG
		H ribosyl	t-zeatin-O-glucoside t-zeatin riboside-O-glucoside	ZOG ZROG
		H ribosyl ribotide glucosyl glucopyranosyl-1,3-ribosyl	meta-topolin meta-topolin riboside meta-topolin riboside-5'-monophosphate meta-topolin 9-glucoside meta-topolin 9-(glucopyranosyl-1,3-ribosyl)	mT mTR mTR5'P mT9G mT9RG
		H ribosyl	meta-topolin-O-glucoside meta-topolin riboside o-glucoside	mTOG mTROG

Figure 1.4 Cytokinin structures - modification on the side chain

been proposed: indirect (from tRNA) and direct (*de novo*) cytokinin biosynthetic pathways.

Studies on the indirect biosynthetic route of cytokinins via isopentenylated tRNA (Klemen and Klambt, 1974) were initiated following the discovery of the cytokinin isopentenyladenosine (iPA) in two $tRNA^{ser}$ species from yeast in 1966 (Zachau et al., 1966; Bieman et al., 1966). Much data has been obtained over the last three decades relating to the location of cytokinin moieties in tRNA molecules, their occurrence in different tRNA species of different organisms and the pathways involved in their biosynthesis. Cytokinin as a constituent of a tRNA molecule is always located adjacent to the 3′-end of the anticodon of the tRNA codon starting with U (Sprinzl et al., 1991; Murai, 1994). Cytokinins have been found in tRNAs from virtually all organisms examined (Skoog and Armstrong, 1970; Greene, 1980). They are regarded as "bound" forms of cytokinins, released only by alkaline hydrolysis (Letham and Palni, 1983).

Investigations into the incorporation of potential cytokinin precursors into tRNA indicated that the biosynthesis of tRNA cytokinins takes place during post-transcriptional tRNA processing (Hall, 1973). The first step is isopentenylation of adenine (always at position 37, A37) to form a cytokinin-active nucleoside (tRNAiPA). The reaction is catalysed by Δ^2-isopentenyl pyrophosphate(Δ^2- iPP):tRNA isopentenyl transferase (EC 2.5.1.8) (Holtz and Klambt; 1975; 1978), using Δ^2-iPP and preformed unmodified tRNA as substrates (Murai, 1994; Taller, 1994). Δ^2-iPP is provided by the mevalonic acid pathway which is also the starting compound for biosynthesis of several other plant hormones including GAs, ABA and brassinosteroids (Prinsen et al., 1997). The isopentenylated tRNA may be further modified to produce other forms of tRNA cytokinins (Murai, 1994; Taller, 1994; Prinsen et al. 1997).

Some investigators have suggested that the "free" cytokinins extracted using aqueous alcoholic solvents could be formed by the release of the "bound" tRNA cytokinins (Hall, 1970; Skoog and Armstrong, 1970; Chen et al., 1976; Murai, 1994; Morris, 1995). However, with the exception of iPA, an obvious objection to this proposal has been that *cis*-ZR is the predominant cytokinin in the tRNA of plants and plant-associated bacteria

(Morris, 1995) while *trans*-ZR is the major isomer of "free" ZR in higher plants (Letham and Palni, 1983). Also, the rate of tRNA turn-over has been reported to be too slow to account for the pool of free cytokinins (Trewavas, 1970; Hall, 1973). For instance, the rate of incorporation of externally applied [^{14}C]-adenine into free cytokinins is too high to be explained by 'tRNA turnover' (Stuchbury et al., 1979). However, Bassil et al. (1993) have recently partially purified a *cis-trans*-isomerase for zeatin from *P. vulgaris* endosperm, indicating that tRNA cannot be ruled out as a possible source of free cytokinins.

The direct (*de novo*) cytokinin biosynthetic pathway has been proposed to be via the condensation of Δ^2-iPP and 5′-AMP to form isopentenyladenosine-5′-monophosphate (iPNT) (Chen, 1982). Subsequent modifications to the iPNT molecule through dephosphorylation, deribosylation and/or side chain hydroxylation lead to the formation of iPA, iP, ZNT, ZR and Z (Jameson, 1994; Morris, 1995; Chen, 1997). The key emzyme that catalyses the condensation step is Δ^2-iPP:5′AMP-Δ^2-isopentenyltransferase (or isopentenyltransferase, IPT). A number of genes (*ipt, tzs, ptz and fas1*) encoding isopentenyltransferases have been isolated and cloned from several different plant pathogens (see McKenzie, 1996). Transformation of plants with isopentenyltransferase genes such as *ipt* has confirmed the function of these isolated genes in manufacturing cytokinins in plants (Smigocki and Owens, 1988; Medford et al., 1989; Smart et al., 1991; Smigock, 1991; McKenzie et al., 1994; 1998; Faiss et al. 1997). Unfortunately, attempts to isolate the homologous isopentenyltransferase genes from higher plants have, so far, been unsuccessful. However, IPT enzyme activity has been demonstrated in plants (Chen and Melitz, 1979; Nishinari and Syono, 1980; Chen, 1982; Reinecke et al., 1991; 1992; Blackwell and Horgan, 1994), although the enzyme itself has not been purified to homogeneity due to its instability. Therefore, it is possible that plants have the same pathway of cytokinin synthesis as that in several plant pathogens.

Recently, 6-benzylaminopurine (BA) and its riboside (BAR) have been recognised as naturally-occuring cytokinins (Ernst et al., 1983; Nandi et al., 1989a,b; Vahala et al. 1993; van Staden and Crouch, 1996; Strnad, 1997). Unfortunately, nothing is known about the *de novo* biosynthesis of the BA class cytokinins. However, the big difference

in the side chain structure of BA from the other groups of cytokinins should indicate that it has a different biosynthetic pathway from the isopentenyl five-carboned side chain cytokinins, i.e. different enzymatic systems for the formation of the benzyl side chain and the condensation of the side chain with adenine.

1.2.3 Cytokinin metabolism

Once the cytokinin nucleotide iPNT is synthesised, this compound can be readily converted to many other forms of cytokinins through various enzyme-catalysed modifications of the purine ring and the side chain of the molecule (reviewed by Letham and Palni, 1983; Jameson, 1994; McGaw and Burch, 1995; Chen, 1997).

1.2.3.1. Interconversions of cytokinin free bases, ribosides and nucleotides: The interconversion of cytokinin bases, nucleosides and nucleotides plays a central part in cytokinin metabolism. Endogenous cytokinin analyses have shown that these compounds are widely present in plant tissues (Letham and Palni, 1983; Nooden and Letham, 1993; Jameson, 1994; McGaw and Burch 1995; Hammerton, 1996), including the tissues of gymnosperms (Van Staden, 1978; Doumas and Zaerr, 1988; Doumas et al. 1989; Bollmark et al., 1995; Moritz and Sundberg, 1996). Feeding experiments with radioactively-labeled cytokinin bases have shown that the conversion from iPNT to ZNT is very rapid (Stuchbury et al., 1979; Palni et al., 1983; Palni and Horgan, 1983). This conclusion is supported by the facts that, in higher plants, the hydroxylated forms of cytokinins (the zeatin group) are the major forms accumulated in most plant tissues and that *ipt*-transformed plant tissues accumulate high levels of hydroxylated cytokinins and not the non-hydroxylated (the isopentenyladenine group) forms even though these are the direct product of isopentenyltransferase activity (Smigocki and Owens, 1988; McKenzie et al., 1994; 1998; Redig et al., 1996). The enzymes, microsomal mixed function oxidases, that catalyse the hydroxylation of the side chain of iP forms to the Z forms have been isolated from cauliflowers (Chen and Leisner, 1984)

The modifications on the purine rings of cytokinin free bases, ribosides and nucleotides can be catalysed by the same enzyme systems as those for adenine/adenosine/adenosine monophosphate interconversions (Chen and Ekert, 1977; Chen and Kristopeit, 1981a,

1981b; Jameson, 1994; Chen, 1997). Both iPNT and ZNT can be converted to their corresponding ribosides through dephosphorylation catalysed by 5′-nucleotidase (Chen and Kristopeit 1981a, 1981b; Burch and Stuchbury, 1986, 1987).

Once cytokinin ribosides are formed they can, subsequently, be converted to cytokinin bases in plant tissues (Letham and Palni, 1983; Jameson, 1994). Adenosine nucleosidase, an enzyme catalysing the irreversible hydrolysis of adenosine to adenine, may be responsible for the deribosylation of cytokinin ribosides (i.e. nucleosides). Nucleosidase activity from partially purified enzyme has been demonstrated in wheat germ (Chen and Kristopeit, 1981b) and tomato roots and leaves (Burch and Stuchbery, 1986).

Exogenously-fed cytokinin free bases can also be converted to ribosides and/or nucleotides in plant tissues (reviewed by Jameson, 1994; McGaw and Burch, 1995; Chen, 1997). The reaction where cytokinin free bases are ribosylated to form ribosides is catalysed by adenosine phosphorylase (Chen and Petschow, 1978) and the phosphorylation of ribosides is catalysed by adenosine kinase (Chen and Eckert, 1977). In a number of plants, an alternative pathway has been found where adenine phosphoribosyltransferase catalyses the direct phosphorylation of cytokinin bases to nucleotides (Doree and Guern, 1973; Chen et al., 1982; Burch and Stuchbury, 1987; Moffatt et al., 1991). Recently, Schnorr et al. (1996) have cloned a gene cDNA (termed the *ATapt2* gene) from *Arabidopsis thaliana* which encodes an adenine phosphoribosyltransferase. This enzyme has a high specificity for cytokinins and may play a role in converting cytokinin bases to their corresponding nucleotides.

1.2.3.2 Reduction of the double bond on the side chain: Dihydrozeatin-*O*-glucoside, dihydrozeatin riboside-*O*-glucoside and dihydrozeatin 3-, 7- and 9-glucoside are often found in plant tissues and are frequently identified as metabolites of applied zeatin and zeatin riboside (Letham and Palni, 1983; Jameson, 1994; McGaw and Burch, 1995). So far, experimental evidence indicates that the reduction of the double bond on the side chain takes place at the levels of zeatin, zeatin riboside or zeatin nucleotide (Mok et al., 1988; Singh et al., 1988; Martin et al., 1989). For example, Martin et al. (1989) isolated

a reductase from *Phaseolus coccineus* embryos which was shown to be highly specific for *trans*-zeatin only.

1.2.3.3 Conjugation: Conjugation of cytokinins can take place on the purine ring or on the hydroxylated side chain. Cytokinins can be glucosylated at the 3, 7 and 9 positions of the purine ring (Fox et al., 1971; 1973; Parker and Letham, 1973; Letham et al., 1975; Letham and Palni, 1983; McGaw et al., 1984). Glucosyltransferases which catalyse the formation of 7- and 9- glucosides of benzyladenine have been isolated from radish (Entsch and Letham, 1979; Entsch et al., 1979). Cytokinins with a hydroxylated side chain are often conjugated to form the *O*-glucoside and other *O*-conjugates (reviewed by Jameson, 1994). An enzyme, zeatin *O*-glucosyltransferase, has been isolated from immature seeds of *Phaseolus lunatus*, which recognises *trans*-zeatin, but not dihydrozeatin, *cis*-zeatin or zeatin riboside (Dixon et al., 1989). An enzyme that catalyses the formation of *O*-xylosides has been isolated and the gene cDNA has recently been cloned from *Phaseolus vulgaris* (Turner et al., 1987; Martin et al., 1997). The enzyme converts zeatin to *O*-xylosylzeatin.

Other novel forms of cytokinins have been identified by different investigators through endogenous cytokinin analysis or exogenous cytokinin feeding experiments. Taylor et al. (1984) isolated a ribosylzeatin glycoside from buds of *Pinus radiata* in which the hexosyl moiety is attached to the ribosyl moiety rather than to the side chain hydroxyl (i.e. ZR-G). A similar hexosyl conjugate of ZR was also detected in buds of Douglas fir (*Pseudotsuga menziesii*) (Morris et al., 1990). The analyses using immunoaffinity and mass spectral techniques indicated that the compound was also different from ZROG with the hexosyl moiety being attached to the ribosyl group of the molecule (Morris et al., 1990). Due to the ease of hydrolysis by β-glucosidase, the hexosyl group in both cases is believed to be glucose.

During investigations into the metabolism of ^{14}C-BA by shoot cultures of *Gerbera jamesonii*, a novel metabolite similar to the structures of the novel glucosides in *P. radiata* and *P. menziesii* was identified (Horgan,1985). β-glucosidase treatment of the metabolite released BA riboside. Blakesley et al. (1990) has identified the metabolite as

6-benzylamino-9-(glucosylribosyl)purine through analyses of mass spectra of the metabolite. More recently, studies of BA metabolism in *Petunia* leaf explants during shoot organogenesis revealed the presence of an abundant novel BA conjugate (Auer et al., 1992a and b). Using mass spectrometry, chemical methods of carbohydrate analysis, UV absorption spectra and Fourier transformed infrared spectra, Auer and Cohen (1993) have identified the novel conjugate as 6-benzylamino-9-[*O*-glucopyranosyl-(1→3)-ribofuranosyl]-purine. All these results indicate that there may be a common enzyme that catalyses the attachment of a glucosyl to the ribosyl group of cytokinins in a wide range of plant species.

Also of interest, Kobayashi et al. (1997) has isolated a novel cytokinin from coconut milk (one of the earliest sources of cytokinin activity) and, by using NMR techniques, mass spectrometry and HPLC sugar analysis, the novel form has been identified as a polysaccharide conjugate of ZR, 14-O-{3-O-[β-D-galactopyranosyl-(1→2)-α-D-galactopyranosyl-(1→3)-α-L-arabinofuranosyl]-4-O-(α-L-arabinofuranosyl)-β-D-galactopyranosyl}-trans-zeatin riboside (G_3A_2-ZR), indicating that a much more complex pathway of cytokinin conjugation is present in plants.

1.2.3.4 Hydrolysis: The hydrolysis of cytokinin ribotides and ribosides to their corresponding ribosides and free bases has been discussed in Section 1.2.3.1. Although N-glucoside conjugates are extremely stable to hydrolysis treatment (Parker and Letham, 1973; Gawer et al., 1977; Parker et al., 1978), the *O*-glucosides are readily hydrolysed by enzymes such as β-glucosidase (McGaw and Burch, 1995).

1.2.3.5 Oxidation: The N^6-side chains of exogenously applied iP, iPA, Z and ZR can be readily cleaved by cytokinin oxidase in plant tissues and this results in irreversible destruction of cytokinin activity (Jameson, 1994). *In vitro*, 7- and 9-glucosyl cytokinins have also been found to be susceptible to cytokinin oxidase (Jameson, 1994). Generally, the Δ^2 double bond on the side chain of cytokinins is required for cytokinin oxidases to cleave the side chain. Reduction of the double bond, replacement of the side chain with benzyl or conjugation of the side chain with an *O*-glucosyl moiety makes cytokinins

resistant to cytokinin oxidases. Cytokinin oxidase has been reviewed recently by Armstrong (1994), Hare and van Staden (1994) and Jones and Schreiber (1997).

1.2.4 Functions, activity and modes of action of cytokinins

Cytokinins were named for their ability to promote cell division and growth of plant tissues cultured *in vitro*. However, cytokinins also have powerful effects on a wide variety of biochemical, physiological and developmental processes. These include promotion of nutrient mobilisation, promotion of leaf expansion, delay of senescence, promotion of chloroplast development, release from seed dormancy, release from bud apical dominance, induction of shoot formation (Skoog and Miller, 1957; Letham and Palni, 1983; Horgan, 1984; Mok, 1994), the involvement in plant responses to environmental stress and pathogens (Letham, 1994; Hare and van Staden, 1997) and even enhancement of nodulation (Yahalom et al., 1990; Taller and Sturtevant, 1991).

The activity of individual cytokinins in the modulation of these various processes in higher plants varies according to their different molecular configurations. Accordingly, Letham and Palni (1983) have proposed that all the different cytokinins could be categorised into: 1. active forms, i.e. the molecular species that bind to a receptor to evoke a growth or physiological response; 2. translocation forms; 3. storage forms that would release free cytokinin when required; 4. detoxification forms which are produced when exogenous cytokinin levels are so high as to be toxic; 5. deactivation products formed to lower endogenous cytokinin levels.

For the detection of cytokinin activity, various bioassays such as tobacco, soybean, carrot and bean callus bioassays (Miller, 1963; Murashige and Skoog, 1962; Shaw et al., 1971) have been indispensable in determining cytokinin structure-activity relationships. It has been shown that the cytokinin free bases (Z, DZ and iP) and ribosides (ZR, DZR and iPA) are very active in different bioassays (reviewed by Letham and Palni, 1983). Many studies have shown that cytokinin ribosides are predominant forms in cytokinin translocation via xylem from roots to shoots in higher plants (Hautala et al., 1986; Jameson et al., 1987; Grayling and Hanke, 1992; Singh et al., 1992; Letham, 1994). Feeding experiments with radioactively-labeled exogenous cytokinins have shown that

the cytokinin nucleotides are often the predominant metabolites formed immediately following uptake (Knypl et al., 1985; Singh et al., 1988; Letham and Zhang, 1989; Moffatt et al., 1991). The nucleotides are subsequently rapidly metabolised to the other cytokinin forms. Therefore, cytokinin nucleotides are believed to be formed to facilitate uptake across the cellular membranes (Singh et al., 1988).

Cytokinin 7- and 9-glucosides are very stable in plant tissues and may serve as deactivation or detoxification forms (inactive forms) which reduce active cytokinin levels (Parker and Letham, 1973; Gawer et al., 1977; Letham et al., 1975; Letham and Palni, 1983; Letham and Gollnow, 1985; Jameson, 1994; McGaw and Burch, 1995). While cytokinin activity was significantly reduced by 7- and 9-glucosylation in nearly all bioassays, 3-glucosylation had little effect on activity (Letham et al., 1983; Parker et al., 1975; van Staden and Drewes, 1992). This may be due to the fact that the 3-glucoside has been shown to be susceptible to hydrolysis by β-glucosidase (Letham et al., 1975; Parker et al., 1975; van Staden and Drewes, 1992). The *O*-glucosides are not substrates for cytokinin oxidase and are highly active in bioassays (McGaw and Horgan, 1983; Letham et al., 1983; McGaw et al., 1985). A decline in *O*-glucosides has also been observed to be correlated with plant growth and development in some species (Smith and van Staden, 1978; van Staden and Dimalla, 1978; Jameson et al., 1982; Brzobohaty et al., 1993, 1994). Therefore, *O*-glucosides are believed to be the storage forms of active cytokinins. The conversion from *O*-glucosides to active cytokinins is catalysed by β-glucosidase (Wang et al., 1977; Falk and Rask, 1995).

A role for the novel cytokinin conjugates is yet to be determined. However, the facts that ZR-G was identified from bud materials (Taylor et al., 1984; Blakesley et al., 1990; Morris et al., 1990) and that ZR-G formation was associated with shoot organogenesis in *Petunia* (Auer and Cohen, 1993) indicate that ZR-G may play a part in morphogenesis in plants.

Despite a large number of reports concerning the diverse physiological roles of cytokinins, little is known about the mechanism(s) of cytokinin action. Cloning cytokinin-responsive genes, analysing their expression patterns and identifying the

functions of the gene products should provide a useful means for investigating the mechanism(s) of cytokinin action at the molecular level. There has been some experimental evidence showing that cytokinins can either increase or decrease gene expression activity in higher plants (see reviews by Schmülling et al., 1997; Hare and van Staden, 1997). Most of the studied genes were identified by either differential screening of cDNA libraries or by Northern blot analysis using already-isolated genes as probes to test their reactions towards cytokinins.

Regulation of gene expression by cytokinins may occur at different levels during gene expression. Dominov et al. (1992) demonstrated that an increase in transcription rate largely accounted for the accumulation of the cytokinin-induced pLS216 gene (of unknown function) mRNA in tobacco suspension culture. By using nuclear run-off transcription assays, Lu et al. (1990; 1992) showed that the cytokinin enhancement of the accumulation of nitrate reductase transcript in etiolated barley leaves was regulated at the transcriptional level. A similar conclusion was also reached by Andersen et al. (1996) from investigation of the regulation of hydroxypyruvate reductase gene expression by cytokinin in etiolated pumpkin cotyledons. Furthermore, Lu et al. (1990) and Andersen et al. (1996) found that the cytokinin-dependent mRNA accumulation of nitrate reductase and hydroxypyruvate reductase displayed a linear dose-response in the range of 10 nM to 100 μM BA in these etiolated tissues. When *Arabidopsis* was grown on cytokinin-containing medium, genes of chalcone synthase (*CHS*) and dihydroflavonol reductase (*DFR*), coding for enzymes of the anthocyanin biosynthetic pathway, displayed 10-fold and 34-fold higher transcriptional rates respectively in nuclear run-off assays (Deikman and Hammer, 1995), clearly demonstrating that the regulation of these two genes by cytokinin takes place at the transcriptional level.

Post-transcriptional regulation has also been demonstrated in an investigation on the transcription and stability of cytokinin-induced mRNAs encoding the small subunit of ribulose-1,5-biphosphate carboxylase/oxygenase (*rbcS*) and chlorophyll a/b binding protein (*cab*) in *Lemna gibba*. Cytokinin treatment slowed the loss of *rbcS* and *cab* mRNAs specifically (Flores and Tobin, 1986, 1988). By comparing data obtained from Northern blot and nuclear run-off assays, Silver et al. (1996) showed that although the

mRNA of the early nodulin gene *SrEnod2* of *Sesbania rostrata* accumulated over the time of cytokinin treatment no change in transcriptional rate was detected, strongly indicating post-transcriptional regulation of this gene expression by cytokinin.

Not only do cytokinins enhance gene expression, they can repress the expression of some genes as well. Teramoto et al. (1994, 1995) have shown that BA, Z and iP caused a 90% reduction of the expression of CR9 and CR20 (of unknown function) in the etiolated cotyledons of *Cucumis sativus* L. as quickly as 4 h after treatment. Estruch et al. (1993) showed that the expression of floral homeotic genes (*DEFA, PLENA and GLO* homologs) in *ipt* transgenic tobacco displayed a 95% reduction due to a 600-fold increase in endogenous cytokinins.

The upstream regulation of gene expression by cytokinins remains unknown. Cytokinin receptors, fluctuations in the concentration of intracellular Ca^{2+} and change in protein phosphorylation may be involved in cytokinin signalling systems (reviewed by Hare and van Staden, 1997; Schmülling et al., 1997).

In conclusion, phase change of higher plants, i.e. from a juvenile form to a mature one, leads to forms which have distinctive morphological and physiological attributes. It has been demonstrated that the plant hormone, cytokinin, may play a role in controlling expression of phase-related characteristics in some species. In *Pinus radiata*, cytokinin applied to mature buds in culture induces the production of juvenile characteristics such as primary needles. However, a relationship between the endogenous cytokinins, their alterations in the buds and phase change of the trees is unknown. Further, little work has been performed investigating the regulatory effects of cytokinins on gene expression during phase change. Therefore, achievement of the aims listed in the overview should provide vital information towards understanding phase change in *P. radiata*.

Chapter 2 Extraction, purification, isolation and identification of endogenous cytokinins in the buds of *Pinus radiata*

2.1 Introduction

Analysis of cytokinins in plant tissues has historically been laborious and time consuming due to the extremely low levels and diverse molecular structures of the cytokinins. In general, cytokinin analysis involves the preparation of cytokinin samples and the detection of individual cytokinins. Sample preparation accounts for a large proportion of the time and effort expended on performing each analysis (Hedden, 1993) prior to bioassay (Meilan et al., 1993), RIA (MacDonald and Morris, 1985) and/or GC-MS detection (Letham et al., 1991; Soejima, 1992, 1995; Horgan, 1995). The initial anticipation that cytokinins could be quantified by radioimmunoassay of crude plant extracts (Weiler, 1979, 1980; Weiler and Spanier, 1981) was, unfortunately, not justified. Detailed analysis requires the sample to be well purified and the individual cytokinins to be separated, so that not only is interference in the assay avoided but also problems associated with differential cross-reactivity of individual cytokinins with the antibodies in the immunoassay (Hedden, 1993).

The procedures for sample preparation which have been employed in the analysis of the cytokinins include extraction, purification and separation of cytokinins. Firstly, extraction of the cytokinins should be carried out carefully to prevent enzymatic and chemical changes and to increase extraction efficiency (Tokota et al., 1980). Homogenising plant tissue in methanol has been a traditional extraction method and is still widely used (Parker and Letham, 1973; Scott et al., 1980; Morris et al., 1990; Dietrich et al., 1995). However, cytokinin nucleotides may be hydrolysed in methanol extracts, because phosphatases can function in such solutions (Bieleski, 1964; Horgan and Scott, 1987). To eliminate the problem associated with phosphatases, some authors routinely use Bieleski's solution (methanol : chloroform : acetic acid : water; 12:5:1:2) to extract cytokinins from plant tissues at -20°C (Bieleski, 1964; Meilan et al., 1993; von Schwartzenberg et al., 1994). A modified version is also used (Jameson et al., 1987; Singh et al., 1988; Day et al., 1995; Wang et al., 1995).

Insoluble polyvinylpolypyrrolidone (PVPP) may be used for sample purification and solid-phase extraction (Cappiello and King, 1990). PVPP has also been used in the purification of nucleotides and nucleosides in plant samples (Guinn, 1973). It has been shown that cytokinins can readily be eluted from a PVPP column at low pH (3.5) while phenolics are strongly retained (Biddington and Thomas, 1976). Methanol is a very efficient solvent with which to elute cytokinins from PVPP columns (Mousdale and Knee, 1979).

To detect a full spectrum of cytokinins, it is necessary to separate nucleotide cytokinins from the other forms. This is relatively easy to perform as the additional phosphate moiety on the nucleotide cytokinins aquires negative charges over a range of pH. Separation is usually achieved by passage of sample through a cellulose phosphate column (Parker and Letham, 1974; Scott et al., 1980; Summons et al., 1983; Scott and Horgan, 1984; Badenoch-Jones et al., 1984; Meilan et al., 1993). However, because a large bed volume is required, a large volume of eluate is unavoidable for both nucleotide fraction (acid wash) and the fraction containing the cytokinin free bases, ribosides and glucosides (basic wash). Therefore, a considerable volume of the aqueous eluate has to, subsequently, be reduced to a few millilitres by rotary evaporation, which is time-consuming and difficult to manipulate when the sample volume becomes small. More importantly, due to acid (pH 3.0) and basic (pH 9-10) washes having to be employed as eluents, the application of the solid-phase extraction technique, using C_{18}, is not practical because nucleotides and some of the ionised cytokinins would not be well retained by C_{18} (Guinn and Brummett, 1990). Processing of multiple samples at one time, therefore, is difficult to perform.

DE52 anion exchange has been used in sample purification as a precolumn to an immunoaffinity column (MacDonald and Morris 1985; Doumas, 1989; Morris et al., 1990; Morris et al., 1991; Dietrich et al., 1995) to remove nucleotides and other impurities. DEAE cellulose has also been utilised in the purification of the cytokinin nucleotide fraction derived from cellulose phosphate column (Summons et al., 1983; Badenoch-Jones et al., 1984). The features of DEAE cellulose allow, at neutral pH, the free bases, ribosides and glucosides to pass through and at the same time nucleotides are

retained. In addition, at neutral pH a column of C_{18} connected directly to the DE52 column will trap the cytokinins passing through DE52. Since their first use in cytokinin extraction (Morris et al., 1976), open columns of C_{18} have been widely used and the linkage of DE52 and C_{18} columns has been reported previously (Jameson and Morris 1989).

Bulk separation of cytokinin glucosides from bases and ribosides needs to be achieved if *O*-glucosides are of interest as well. Partitioning against 1-butanol was believed to be an excellent step with the basic cytokinins being extracted into 1-butanol and nucleotides remaining in the aqueous phase. However, *O*-glucosides do not partition well into 1-butanol (Horgan and Scott 1987). Thin layer chromatography (TLC) has been shown by D. S. Letham's group to be very useful in the separation of the glucosides from the bases and ribosides (e.g. Hocart et al., 1990) but, probably because of manipulation difficulties, this technique has not been widely adopted. Recently, a powerful protocol has been successfully developed to separate the glucosides from the free bases and ribosides (Lewis et al., 1996) using amine column HPLC (normal phase HPLC)

Although a number of overseas groups still assay complex mixtures of cytokinins (Schmülling et al., 1989; Prinsen, et al., 1994; Thomas, et al., 1995), separation into the individual forms by reverse phase HPLC is believed by several groups to be an essential step in achieving accurate results (e.g. MacDonald and Morris, 1981; Badenoch-Jones et al., 1984, 1987; MacDonald and Morris, 1985; Horgan and Scott, 1987; Jameson et al., 1987; Morris et al., 1991; Horgan, 1995). Reverse phase HPLC can efficiently separate not only individual cytokinins from one another but also from impurities which may impact upon the detection of cytokinins by the RIA (Banowetz, 1994; Eason et al., 1996).

While reverse phase HPLC can lead to the separation of the individual forms of the different cytokinins, the identification and quantification of cytokinins can not be accurately determined merely by the ordinary UV detector of the HPLC, as even extensively purified cytokinin samples will still contain co-eluting UV absorbing

substances. Some researchers have used diode-array UV spectral detector linked to the HPLC to quantify cytokinins after immunoaffinity purification (Schreiber, 1990; Nicander et al., 1993; Dieleman et al., 1997). However, great care must be taken when this this technique is used as any trace co-eluted UV absorbing substances will change the shape of the spectrum of the cytokinins. Bioassay, immunoassay or mass spectrometry is usually required to detect cytokinins.

Bioassay was the first technique used in the quantitative analysis of cytokinins in plants. Despite the limitations of slowness, variability of responses, interference by other factors and low sensitivity (Reeve and Crozier, 1980; Horgan and Scott, 1987), various bioassay methods such as tobacco callus (Murashige and Skoog, 1962), soybean callus (Miller, 1963), and carrot callus (Shaw et al., 1971) have played an important part in detecting novel cytokinins and quantifying them. Although quantification of cytokinins is now commonly carried out by immunoassay (Banowetz, 1994), bioassays are still an indispensable means for determining structure-activity relationships of novel cytokinins.

Immunoassays offer specificity, high sensitivity and convenience, and therefore are a popular method for cytokinin analysis. Although immunoassays have been developed in different forms such as radioimmunoassays (RIAs) (Weiler, 1980; Badenoch-Jones et al., 1984; MacDonald and Morris, 1985), enzyme-linked immunosorbent assays (ELISAs) (Cahill et al., 1986; Trione and Sayavedro-Soto, 1988; Pelese et al., 1989; Jameson and Morris, 1989) and scintillation proximity immunoassay (Wang et al., 1995), radioimmunoassays are the most commonly used technique. Since RIA was introduced into cytokinin analysis (Weiler 1980), the technique has been advanced greatly in speed, sensitivity and sample handling capability (MacDonald and Morris 1985; Badenoch-Jones et al., 1987; Jameson and Morris 1989; Morris et al., 1991). However, sequential additions of the buffer, labelled analogue of cytokinins and antibody necessary for the assays still take up a large portion of the time and effort of the core RIA operation.

While the most significant development has been the steady increase in the use of immunoassays, gas chromatography-coupled mass spectrometry (GC-MS) and liquid chromatography-coupled mass spectrometry (LC-MS) have become more and more

important tools for both qualitative and quantitative analyses of cytokinins (Morris, 1977; Scott and Horgan, 1984; Yang et al., 1993; Hedden, 1993; Moritz, 1996). Although GC-MS has been shown to be a reliable and sensitive method for the analysis of plant hormones (Hedden, 1993; Moritz, 1996), the necessary derivatisation of the cytokinins is problematic since, depending on the conditions, multiple derivatives can occur (Palni et al., 1983).

The application of LC-MS does not require those compounds with a low volatility to be derivatised. Unfortunately, LC-MS using thermospray or fast-atom bombardment interfaces does not give a detection limit low enough for routine cytokinin analysis (Hedden, 1993; Imbault et al., 1993). Yang et al. (1993) demonstrated for the first time that the application of LC-MS using atmospheric pressure chemical ionisation (LC-APCI/MS) allowed the analysis of cytokinins at a biologically relevant concentrations starting from less than 1 g plant material. More recently, Prinsen et al. (1995) carried out quantitative analysis of cytokinins using electrospray tandem mass spectrometry and achieved a detection limit of 1 pmol zeatin riboside. Furthermore, tandem mass spectrometry (MS/MS) is a valuable technique for structural characterisation.

In this phase of the work, my aim was to obtain an efficient multiple sample processing capability and to shorten the whole analysis time in order to deal with a large number of samples. All the procedures for analysis of cytokinins in the buds of *Pinus radiata* consisted of the following steps: extraction, removal of gel-forming substances using a PVPP column, purification and separation of cytokinin nucleotides from the free bases, ribosides and glucosides by employing PVPP-DE52-C_{18} columns linked in series; bulk separation of cytokinin glucosides from the bases and ribosides by using a normal phase (amine column) and HPLC; separation of individual cytokinins using reverse phase C_{18} HPLC; identification and quantification of the cytokinins by RIAs; and, finally, characterisation of cytokinin structures by ES MS/MS.

2.2 Materials and Methods

All the procedures used for the analysis of cytokinins are outlined as a flow chart in Fig. 2.1.

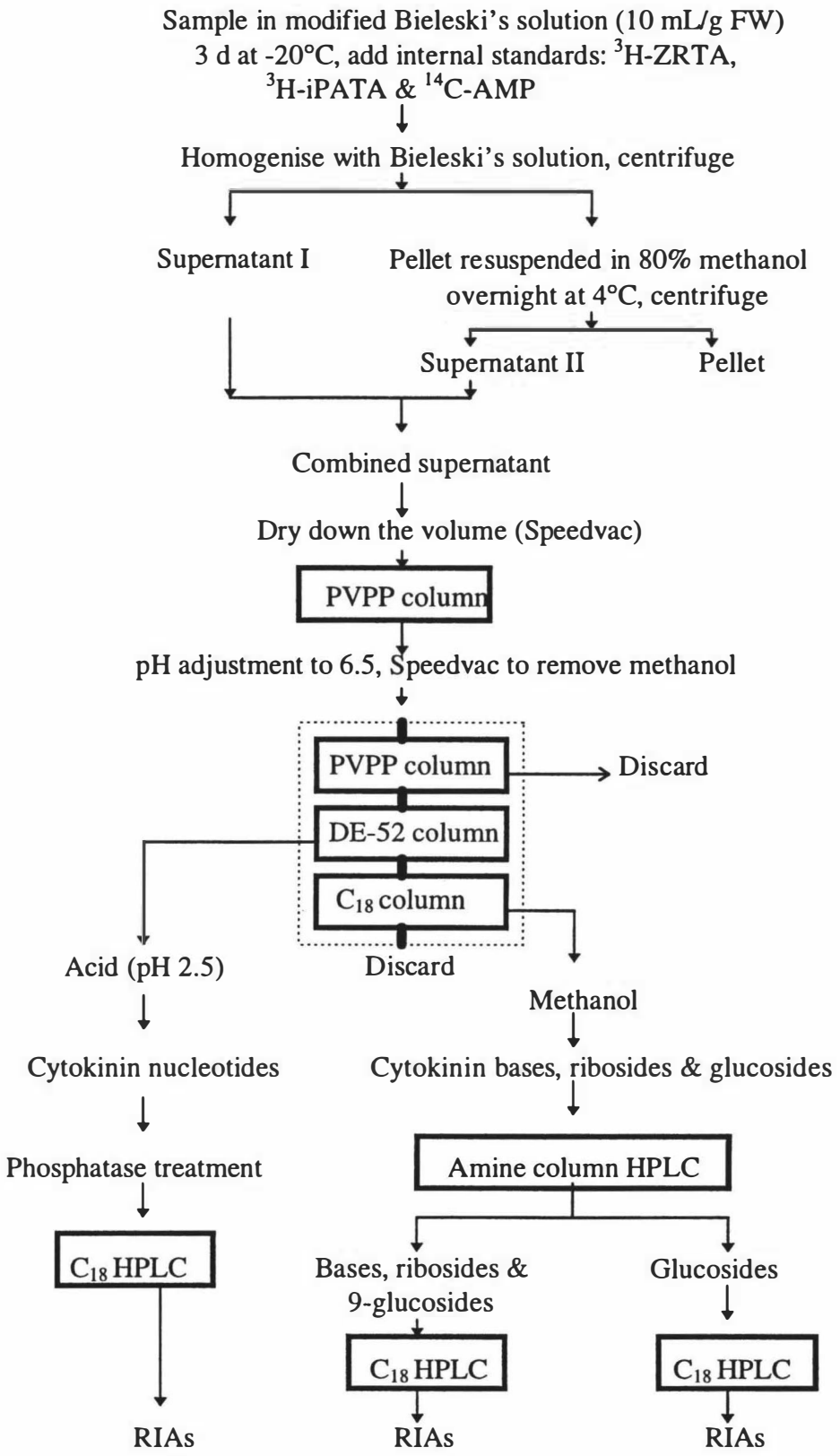


Figure 2.1. Procedures used for extraction, purification, separation and quantification of different individual cytokinins in *Pinus radiata* buds. ▭ indicates column used; ⋯ indicates columns are linked.

2.2.1 Plant materials

Six to 10 g of field-grown mature buds, juvenile buds or tissue cultured bud fragments of *Pinus radiata* were used in the development of cytokinin analysis protocols and the identification of endogenous cytokinins present in the buds of *P. radiata*. Mature buds were collected on September 27, 1994 (5-year-old), May 9, 1995 (8-year-old) and April 1, 1997 (mixture of 9-year-old and 5-year-old) from the first- and second-order branches in the upper crown of the trees. At each collection of the mature buds, one terminal bud and its surrounding whorl buds were excised from one branch, the scales pealed off and the buds dropped immediately into modified Bieleski's solution (methanol : H_2O : HAc; 70 : 30 : 3 v/v/v, 10 mL g^{-1} FW) which had been pre-chilled to -20°C. For juvenile material, the leader buds of one-year-old seedlings were collected on May 9, 1995. After the primary needles were peeled off, the seedling buds were pooled in pre-chilled Bieleski's solution. Both the mature trees and the seedlings were grown in the nursery of The New Zealand Forest Research Institute (NZFRI), Rotorua, New Zealand. Tissue cultured buds of *P. radiata* were prepared at the micropropagation unit of NZFRI according to methods described previously (Horgan 1987) and harvested into pre-chilled Bieleski's solution (-20°C). To destroy activity of enzymes such as phosphatases and glucosidases, all the harvested buds were stored in Bieleski's solution at -20°C for at least three days. Duplicate samples were used in all analyses.

2.2.2 Extraction of cytokinins

2.2.2.1 Equipment and chemicals: All experimental procedures were carried out in disposable polypropylene plasticware such as 50 mL conical tubes, column barrels, Eppendorf tubes and pipette tips to minimise losses of cytokinins. Any glassware used, such as Pasteur pipettes, was silanised to help reduce adsorption of cytokinins to the glass surface. When necessary, extract volumes were reduced using a Savant SVC200 Speedvac condenser with care being taken not to warm samples or reduce to complete dryness (unless stated otherwise). A Heraeus Sepatech biofuge A centrifuge and an IEC Micromax centrifuge were used for centrifuging large and small volume samples respectively.

2.2.2.2 Extraction of endogenous cytokinins: After remaining in the Bieleski's solution under -20°C for at least three days, the buds were homogenised in the original Bieleski's solution in which the bud materials were kept. To reduce the losses of cytokinins, both the mortar and pestle were rinsed twice with 80% methanol and the rinses were combined with the homogenate. ^{3}H-ZR-trialcohol (^{3}H-ZRTA), ^{3}H-iPA-trialcohol (^{3}H-iPATA) and ^{14}C-AMP internal standards (30,000 to 50,000 cpm each) had been added to sample bottles prior to sample homogenisation to enable losses of cytokinins during extraction and purification procedures to be estimated. Subsequently, the homogenates were centrifuged at 7000 *g* for 30 min at 4°C and the resulting supernatant was decanted into a 50 mL Falcon tube and stored at 4°C. The pellet was resuspended in 80% methanol (5 mL g^{-1} FW) and extracted overnight at 4°C before the sample was centrifuged again. The second supernatant was removed and combined with the first supernatant. The combined supernatants were then reduced *in vacuo* (Savant Speedvac) to around 2 mL and stored at -20°C until required.

2.2.3 Purification and separation of cytokinins

2.2.3.1 Polyvinylpolypyrrolidone column chromatography: Dry polyvinylpolypyrrolidone (PVPP) powder was slurried with acidified methanol (methanol : 0.5N HAc; 80:20 v/v). The slurry was then allowed to settle for 1 min before fine particles were carefully decanted. The resulting slurry was packed under gravity into a 30 mL syringe barrel (Terumo®) which had two discs of No.4 filter paper placed in the bottom to hold the PVPP in place. The final bed volume of the column was adjusted to 15 - 20 mL and the packed column was then conditioned with 2 bed volumes of acidified methanol prior to the application of a solution of standards consisting of ^{3}H-ZR-trialcohol, ^{3}H-iPA-trialcohol and ^{14}C-AMP (30,000 to 50,000 cpm each) or a true sample containing the three radio-labelled internal standards (Section 2.2.2.2). The standards or the true samples were then eluted by adding three bed volumes of the acidified methanol. To monitor the elution profile of cytokinins through the column, 1 mL fractions were collected and the radioactivity in a small proportion (usually 50 µL) of each fraction measured after mixing with 1 mL scintillation cocktail. Otherwise, the eluate was evaporated *in vacuo* to near dryness (but complete dryness was avoided) and re-dissolved with 5 mL water. The solution was subsequently

adjusted to pH 6.5 with 0.1 N NaOH and finally evaporated *in vacuo* to obtain a volume of 2 mL in preparation for application to the PVPP-DE52-C_{18} column complex.

2.2.3.2 PVPP-DE52-C_{18} column complex chromatography: The sample purified through the PVPP column (Section 2.2.3.1) was subsequently allowed to pass through connected PVPP, DE52 and C_{18} columns. This column complex was used for both further purification and the separation of phosphorylated cytokinins from all the other cytokinin forms. The columns were individually packed and then connected in series as shown in Fig. 2.1.

2.2.3.2a Small PVPP column packing and preconditioning: The PVPP column used here was made by slurrying PVPP powder in 10 mM ammonium acetate buffer (pH 6.5) and packing the slurry into a 30 mL syringe barrel to a final bed volume of 5 to 10 mL.

2.2.3.2b DE52 column packing and preconditioning: Anion exchange cellulose DE52 (Whatman) was prepared according to the manufacturer's instructions and finally equilibrated in 10 mM ammonium acetate buffer (pH 6.5). The DE52 was packed under gravity into a 30 mL syringe barrel to a final bed volume of 20 mL. The column was conditioned with an additional three bed volumes of 10 mM ammonium acetate buffer (pH 6.5) before direct use or being linked to the PVPP and C_{18} columns.

When the properties of DE52 were being examined, the DE52 columns were used independently and radio-labelled cytokinin standards were applied directly to the columns. To test the feasibility of using DE52 for actual samples, a pre-column of PVPP (Section 2.2.3.2a) was linked to DE52 and actual sample mixed with radio-labelled internal standards was applied to the linked PVPP column. The columns were subsequently eluted with 10 mM ammonium acetate buffer and the eluate fractions were monitored for both ^{3}H and ^{14}C using liquid scintillation counting (Wallac). After being eluted with 10 mM ammonium sulphate, the DE52 column was eluted again with acetic acid (pH 2.5) to recover the cytokinin nucleotides retained by the column. The fractions (1 mL) of the acid wash were collected and ^{3}H and ^{14}C activities in every fraction were measured using liquid scintillation counting (Wallac).

2.2.3.2c C_{18} column packing and pre-conditioning: The third column in the series was a small octadecylsilica (C_{18}, Bondesil Analytichem International) column which was used to collect cytokinins being eluted from the DE52 column in the column complex. C_{18} powder was packed into a 10 mL syringe barrel (up to 4 mL mark) which had a pre-fitted filter in the bottom (Alltech). The column was preconditioned with 20 BVs of methanol, followed by 20 BVs of 10 mM ammonium acetate (pH 6.5).

2.2.3.2d Linkage of PVPP, DE52 and C_{18} columns: After each column was packed and conditioned, the PVPP, DE52 and C_{18} columns were connected in series with needles and bungs as shown in Fig. 2.1. Subsequently, 20 mL of 10 mM buffer (pH 6.5) was applied to condition the entire column complex.

2.2.3.2e Sample application, elution and cytokinin recovery: Samples (2 mL) were applied to the top column of the series through an attached 50 mL syringe barrel which afterwards served as a solvent reservoir. Subsequently, the complex was eluted with 60 mL (3 BV DE52 column) of 10 mM ammonium acetate buffer (pH 6.5) and then the columns disconnected. The PVPP column was discarded, the DE52 column was subsequently eluted with acetic acid (45 mL, pH 2.5) to recover phosphorylated cytokinins, and the C_{18} column was flushed with 15 mL 80% methanol to elute free bases, ribosides and glucosides. The free base, riboside plus glucoside fraction was then dried and taken up in 50 µl of 50% acetonitrile prior to amine column HPLC (Section 2.2.3.3a). The fraction of phosphorylated cytokinins (acid wash) was evaporated to complete dryness *in vacuo* (Savant, Speedvac), re-dissolved in phosphatase reaction solution and subjected to phosphatase treatment (Section 2.2.4.1).

2.2.3.3 High performance liquid chromatography: For both normal and reverse phase high performance liquid chromatography (HPLC), a Waters 600E Multisolvent delivery system, including a 600-MS system controller, a Waters U6K injector port and 490E programmable multiwavelength UV detector set at 269 nm, was used to separate individual cytokinins. HPLC grade solvents were used, diluted with Milli Q water and filtered through 0.45 µm Nylon membrane filter (Millipore). To prevent problems with

air bubbles, all the solvents were sparged for at least 10 min at 100 mL min^{-1} helium prior to use and followed with a 30 mL min^{-1} helium sparging during the entire HPLC run. Data generated by the detector were collected and processed on an IBM compatible computer using Delta software (V.4.06 Digital Solutions Ltd. Australia).

2.2.3.3a Normal phase HPLC: An amine column (Alphasil $5NH_2$, 250 mm × 4.6 mm, HPLC Technology) was used to achieve bulk separation of cytokinin glucosides (excluding iP9G) from all cytokinin free bases and ribosides (but including iP9G) according to a gradient protocol developed by Lewis et al. (1996). The cytokinin free base and riboside bulk fraction was collected over the period of 1 - 13 min and the cytokinin glucoside bulk fraction from 13 - 28 min. Both fractions were then evaporated to near dryness and taken up in 50 µl of 25% methanol respectively in preparation for C_{18} HPLC. The separation of glucosides from the other cytokinins was verified by running cytokinin standards on HPLC at the beginning and end of every 4 - 8 sample HPLC runs. The cytokinin standard solution used for normal phase HPLC included Z, DZ, *cis*Z, ZR, DZR, *cis*ZR, iP, iPA, iP9G, ZOG, DZOG, ZROG, DZROG, Z9G and DZ9G (around 10 -50 ng of the individual cytokinins/50µL injection). The solution contained the same concentration of acetonitrile (50% v/v) as used in dissolving samples. After the cytokinin standard run at the beginning, the injection port was washed at least three times with 150 µL 100% HPLC methanol and two blank solvent runs followed to clean the column prior to sample application.

2.2.3.3b Reverse phase HPLC: The HPLC mobile phase gradient used in this experiment was an adaptation of those described in MacDonald et al.(1985), Jameson and Morris (1989) and Lewis et al. (1996). The separation of individual cytokinins in each bulk fraction (cytokinin free bases/ribosides, cytokinin glucosides and dephosphorelated cytokinin nucleotides) was achieved on an octadecyl silica C_{18} column (Beckman Ultrasphere 5 µm, 250 mm × 4.6 mm) with a triethylamine/acetic acid buffer (pH 3.5, referred to as HPLC buffer)/methanol/acetonitrile gradient. Briefly, the C_{18} HPLC column was equilibrated in HPLC buffer (40 mM triethylammonium acetate (TEA, pH 3.5) containing 20% methanol. The samples were eluted with mobile phase gradients: 20% methanol over 1 min, 20-22.4% over 4 min, 22.4-31.2% methanol

over 4 min, 31.2-32% over 5 min, 32-32.8% over 5 min, 32.8% methanol to 20% acetonitrile over 1 min, 20-23.2% acetonitrile over 5 min, 23.2-24% acetonitrile over 5 min, 24.0% acetonitrile to 80% methanol over 10 min. The flow rate was 1 mL min^{-1}. Fractions (30 seconds) were collected in 1.5 mL Eppendorf tubes. A cytokinin standard, which contained about 20 ng each of Z9G, DZ9G, Z, DZ, *cis*Z, ZR, DZR, kinetin, iP9G, iP and iPA, was run prior to the first sample application and after every fourth sample run to enable retention times of compounds exhibiting cross-reactivity in the RIA to be compared with retention times of authentic standards. After the standard run, the injection port was cleaned at least three times with 150 µL HPLC methanol. Two injections of buffer always followed the cytokinin standard run and the fractions collected following the second buffer injection were subjected to RIA for contamination check.

2.2.3.4 Purification and separation of cytokinin glucosides by immunoaffinity spin-columns: The procedures for purification and separation of novel cytokinin glucosides and traditional *O*-glucosides are outlined in Fig. 2.2.

2.2.3.4a Preparation of anti-cytokinin antibodies: One gram of each of the lyophilised hybridoma culture fluids of antibody tZR3 which has a broad cross-reactivity for Z, ZR, DZ, DZR, Z9G and DZ9G, and iPA3 which has strong specificity for iP and iPA were reconstituted in 5 mL half-strength PBS buffer (pH 7.2). The tZR3 and iPA3 antibodies were gifts to Professor P. E. Jameson from Dr G. M. Banowetz. Subsequently, the antibody solutions were adjusted to contain 50% saturated concentration of ammonium sulphate and remained on ice for 1 h with frequent gentle shaking. The solutions were then subjected to centrifugation at 3000 g for 15 min at 4°C. The resulting pellets containing anti-tZR3 or anti-iPA3 antibody were re-suspended in 2 mL half-strength PBS buffer and dialysed against 2 L half-strength PBS buffer with constant gentle stirring at 4°C for 48 h; three buffer changes were made during the dialysis. The dialysed solutions were then passed through a 5 mL DE23 column (which had been conditioned in half-strength PBS for 15 h), with half-strength PBS as eluting solution, 1.5 mL fractions were collected. To determine the fractions which had antibody activity, 50 µL aliquots were taken from every fraction and mixed with 300 µL RIA

buffer (Section 2.2.5.1) containing 2500 cpm ^{3}H-tZRTA for tZR3 or 2500 cpm ^{3}H-iPATA for iPA3. Based on the RIA results, all the antibody-containing fractions of tZR3 or iPA3 were combined and concentrated using 15 mL Manosep™ concentrators with a protein cut-off point of 10K. Finally, 1 mL each of purified and concentrated tZR3 and iPA3 were obtained and ready for use.

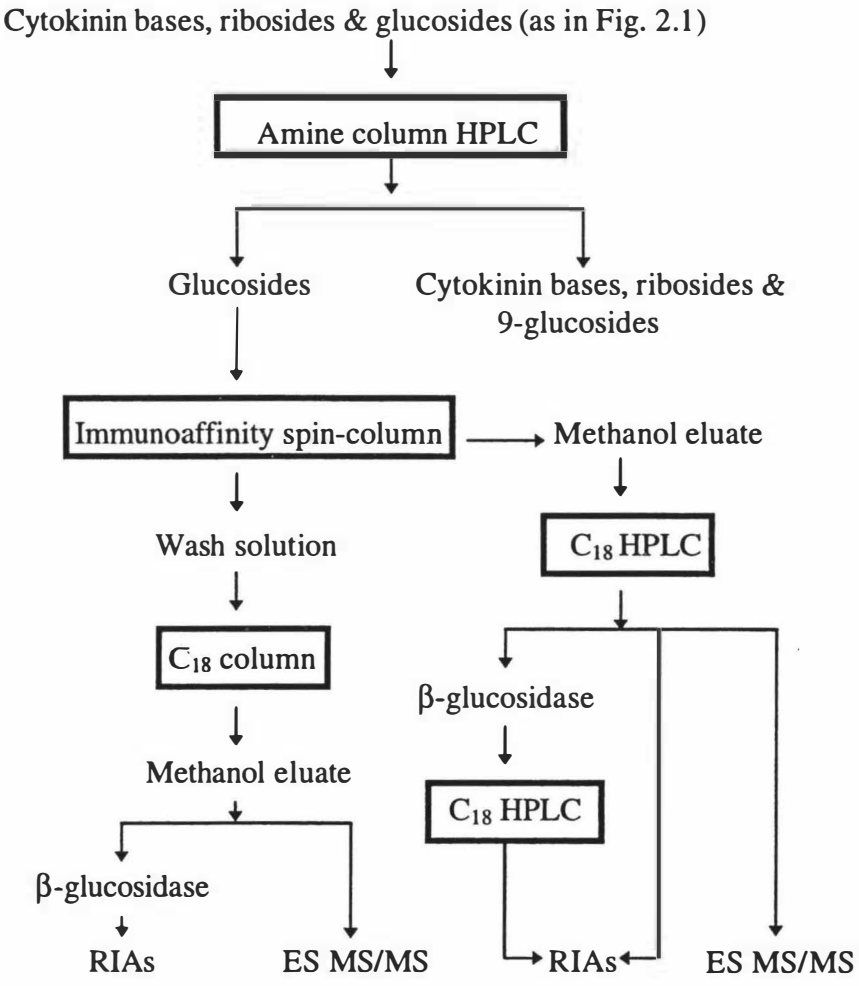


Figure 2.2. The procedures for purification and separation of novel cytokinin glucosides from traditional *O*-glucosides. ▭ indicates column was used.

2.2.3.4b Purification and separation of cytokinin glucosides: To purify novel cytokinin glucosides and separate them from traditional *O*-glucosides, the glucosides were collected between 12 and 28 min from the amine column HPLC (Fig. 2.1), dried and re-dissolved in 1 mL 50 mM PBS containing 0.5 mL purified tZR3, 0.5 mL iPA3

antibodies and 0.14 M NaCl. Subsequently, a portion of the solution was transferred into 0.5 mL micro-concentrator which had a protein cut-off point of 10K (Nanosep™, PALL FILTRON) and spun at 12,000 rpm in a micro-centrifuge until near dryness. This process was repeated until all the solution had passed through the concentrator. In this way, two cytokinin pools were produced: traditional *O*-glucosides, which were not retained by the antibodies, passed through the membrane of the concentrator. However, all the other glucosides cross-reactive with the antibodies, including novel glucoside cytokinins, were retained by the antibodies and remained in the concentrator because the antibodies were not able to pass through the membrane of the micro-concentrator (Fig. 2.2).

To identify *O*-glucosides and avoid contamination by antibody cross-reactive cytokinin glucosides, the solution which had passed through the antibody-containing concentrator was combined and the volume of it was reduced *in vacuo* using the Savant Speedvac. Subsequently, the same process of purification of the solution using the immunoaffinity micro-concentrator was repeated two more times. The resulting solution which had gone through the antibody-containing micro-concentrator three times was passed through a C_{18} column (3 mL BV). The methanol eluates of the C_{18} were concentrated *in vacuo*. This bulk fraction was then checked by RIA (Section 2.2.5.1) to confirm the absence of cross-reactive cytokinins and stored at 4°C until required for identification of the traditional *O*-glucosides.

The antibodies remaining in the micro-concentrator were washed twice with 0.5 mL 50 mM PBS containing 0.14 M NaCl. Subsequently, the cytokinin glucosides retained by tZR3 and iPA3 antibodies were recovered by spinning 0.7 mL methanol (0.5 mL the first time and 0.2 mL the second time) through the micro-concentrators at 12000 rpm. The methanol eluates were evaporated in a Savant Speedvac to reduce the volume and stored at 4°C until required for novel cytokinin glucoside analysis. The antibodies were recycled by being immediately re-suspended in water and washed with 0.5 mL water three times after methanol elution of the cytokinins and finally dissolved in 1 mL 50 mM PBS containing 0.14 M NaCl.

2.2.4 Enzyme treatments

2.2.4.1 Alkaline phosphatase treatment: To measure individual cytokinin nucleotides, the phosphate groups on these molecules were cleaved by alkaline phosphatase before the separation of the corresponding dephosphorylated forms through C_{18} HPLC. Cytokinin nucleotide fractions eluted from the DEAE columns (Fig. 2.1) were evaporated to complete dryness *in vacuo* (Savant Speedvac) and then dissolved in 5 mL phosphatase reaction solution containing 0.1 M ethanolamine (pH 9.5), 0.1 mL 40 mM $MgCl_2$ and 20 µL alkaline phosphatase (Sigma EC 3.1.3.1). Subsequently, the reaction solution was subjected to incubation at 37°C overnight (*ca.* 12 h) (Hocart et al., 1990). After incubation, the reaction solution was loaded onto a preconditioned C_{18} column with 3 mL BV and washed with 9 mL Milli Q water. The dephosphorylated cytokinins were eluted from the column with 15 mL of 80% methanol. The eluate was evaporated to near dryness and re-dissolved in 50 µL 25% methanol prior to C_{18} HPLC.

2.2.4.2 β-glucosidase treatment: To remove the glucosyl group from cytokinin glucosides with a β-glucoside bond linkage, fractions containing cytokinin glucosides were evaporated to dryness and re-dissolved in 0.5 mL to 5 mL (according to sample size) β-glucosidase reaction solution containing 0.3 mg/L β-glucosidase (from sweet almonds, Boehringer Mannheim) in 50 mM sodium acetate buffer (pH 5.4) (Jameson et al., 1982). After 22 h incubation at 25°C, the reaction solution was either dried down, taken up in 50 µL 25% methanol (when sample volume was small) and directly applied to C_{18} HPLC or passed through a C_{18} open column to extract de-glucosylated cytokinins (when sample volume was large) prior to application to C_{18} HPLC.

2.2.5 Detection and identification of cytokinins

2.2.5.1 Radioimmunoassay (RIA): Two monoclonal antibodies were used for the RIA: clone 16 which cross-reacted with hydroxylated cytokinins such as Z, DZ, ZR, DZR, and Z9G, and clone 12 which is cross-reactive with non-hydroxylated cytokinins such as iP, iPA and iP9G (Trione et al., 1985; Lewis, 1994) (Appendix A).

A streamlined protocol was developed based on more traditional RIA procedures (MacDonald and Morris 1985, Jameson and Morris 1989). The dried fractions from C_{18}

HPLC in 1.5 mL Eppendorf tubes were dissolved in 450 µl antibody-antigen reaction solution which included RIA buffer [(50 mM sodium phosphate buffer with 0.14 M NaCl, pH 7.2, 0.1% w/v gelatine (Difco), 0.01% ovalbumin (Sigma Grade V)], ^{3}H-ZR-trialcohol or ^{3}H-iPA-trialcohol with radioactive strength being adjusted to 5000 cpm per 450 µl reaction solution; and clone 12 or clone 16 providing approximately 2500 cpms binding (B0) per 450 µL reaction solution. Samples were incubated in the reaction solution for 30 min at room temperature, followed by addition of 600 µl 90% saturated ammonium sulphate (pH 7.0) and mixed well. After 15 minutes, the tubes were centrifuged at 10,000 rpm for 2 min and the supematants aspirated. Antigen was released by addition of 50 µl methanol. Finally, the released radioactivity was quantified using liquid scintillation counting (Wallac) after addition of 1 mL scintillation cocktail (Optiphase Hi-Safe 2, Wallac). Fractions collected during C_{18} HPLC from 0 to 25.5 min were assayed with clone 16 using ^{3}H-ZRTA as competitor and fractions eluting from 26 to 40 min were assayed with clone 12 using ^{3}H-iPATA as competitor.

Standard curves were always included in each batch of RIAs to enable an accurate calculation of the amount of cytokinin in the samples of the same batch. A series of dilutions in triplicate of ZR (when clone 16 was used) or iPA (when clone 12 was used) between 0 and 100 pmol were prepared by diluting a 10^{-3} M stock solution with 50% HPLC methanol into 1.5 mL Eppendorf tubes (triplicate). Subsequently, the tubes were dried using the Speedvac, and the RIA reaction solution added as described above for samples.

2.2.5.2 Electrospray mass spectrometry: The confirmation of cytokinin identities was carried out on a PE SCIEX API 300 LC/MS/MS mass spectrometer (Perkin-Elmer Sciex Instruments) which is a triple quadrupole instrument equipped with an atmospheric pressure ionisation (API) source. Sample solutions were introduced into a fused silica capillary tube (id 1 -2 µm) using a syringe pump (Harvard model 2400-001) at a rate of 5 µL per minute. Electrospray was performed by applying a potential between 4.5 and 5 KV to a stainless syringe needle into which the capillary was fed. The capillary tube was kept flush with the tip of the syringe needle. A coaxial air spray was applied along

the sprayer to assist liquid nebulisation, and this was usually adjusted to 0.7 L/min. The conditions for ionisation were optimised to an ion spray voltage at 5KV, and orifice voltage at 30 volts and a focussing RNG voltage at 100 volts. While these condition were used for straight mass analysis of all cytokinins, occassionally the conditions were optimised for individual molecules. All samples were dissolved in 50% (v/v) methanol/water. The instrumental scale of Q1 and Q3 for the mass to charge ratio (m/z) was calibrated with a polypropylene glycol standard (PPG standard). The mass scans were usually carried out in Q1 between the region of 50 and 700 m/z with a step length of 0.1 amu and a dwell time of 1 ms. To obtain structural information, the mass spectrum of components of a particular cytokinin were also measured by the third quadrupole after fragmentation of the molecular ion using collision induced dispersion (CID). Molecular and component masses were determined from the measured m/z values for the protonated molecules and components.

To obtain standard mass spectra of different forms of cytokinins, standard cytokinins ($[^2H_5]$-Z, $[^2H_3]$-DZ, $[^2H_5]$-ZR, $[^2H_3]$-DZR (Apex Chemicals, England), ZOG, ZROG, DZROG, iP, iPA and iP9G (Sigma)) dissolved in 50% methanol/milli Q (v/v) at 10^{-5} - 10^{-6} M concentration were introduced into the instrument through the electrospray interface by direct injection with a microsyringe at 5 μL/min. The mass spectra of both parent ions (quasi-molecular ions) and product ions produced from the parent ions by CID were determined.

The cytokinin samples used for mass spectrometry were derived either from the mature buds of field-grown trees [collected on May 9, 1995 (8-year-old trees) and April 1, 1997 (mixture of 9-year-old and 5-year-old trees)] or from tissue cultured material. Cytokinin extraction, purification and isolation were carried out as described in Section 2.2.3 (outlined in Fig. 2.1 and 2.2). For the novel cytokinin glucosides designated by x, the fractions containing ZRx, DZRx and iPAx collected from C_{18} HPLC were completely dried and re-dissolved in 50% methanol/Milli Q water (v/v). Individual fractions were analysed by mass spectrometry as described above. The identification of the quasi-molecular ions $[M+H^+]$ of the cytokinins was carried out using the first quadrupole mass detector. Peaks were then selected for further analysis by MS/MS. To do this,

conditions were optimised for the selected m/z (optimising ion spray, orifice and ring voltages). These ions are then directed into the collision cell where they collide with neutral gas molecules (the flow of gas molecules into the collision cell was also optimised for the analyte). The collision energy was varied in order to bring about fragments of the parent molecules and the third quadrupole recorded all the fragment ions. To identify traditional cytokinin *O*-glucosides, the bulk fractions purified using tZR3 and iPA3 antibodies as described in Section 2.2.3.4b and Fig. 2.2 were dissolved in 50% methanol/water (v/v) and then introduced into the mass spectrometer the same way as described for the standards and other samples.

2.3 Results

2.3.1 PVPP column analysis

To test the performance of PVPP columns in purifying cytokinin extracts, a mixture of standard ^{3}H-ZRTA, ^{3}H-iPATA and ^{14}C-AMP (serving as internal markers of non-phosphorylated cytokinins and cytokinin nucleotides respectively) or cytokinin extract samples spiked with ^{3}H-ZRTA, ^{3}H-iPATA and ^{14}C-AMP were applied to the columns (as described in Section 2.2.3.1). In both cases, it was found that the ^{3}H-labelled cytokinins and the ^{14}C-labelled nucleotide were very easily eluted off the columns when acidic methanol was used as the eluent (Fig. 2.3A and B). For a 20 mL bed volume PVPP column, cytokinins were detected in the eluate fractions after only 10 mL (half BV) eluent had passed through the columns. All the non-phosphorylated cytokinins and nucleotide were completely eluted off the columns with 20 mL eluting solvent (1 BV). Moreover, the nucleotide co-eluted with other forms of cytokinins (Fig. 2.3A and B). While cytokinins and nucleotide quickly passed through the PVPP columns, it was observed that a layer of pink-brownish precipitate was arrested on the top of the columns and substances associated with a yellow colouration were retained by the upper parts of the columns.

2.3.2 Separation of nucleotides from the other forms of cytokinin using DE52

As described in Sections 2.2.3.2b - d, DE52 columns were tested to separate nucleotides from all the other non-phosphorylated forms of cytokinins. Distinctive separation of ^{14}C-AMP from ^{3}H-ZRTA and ^{3}H-iPATA in the standard mixture was achieved by DE52

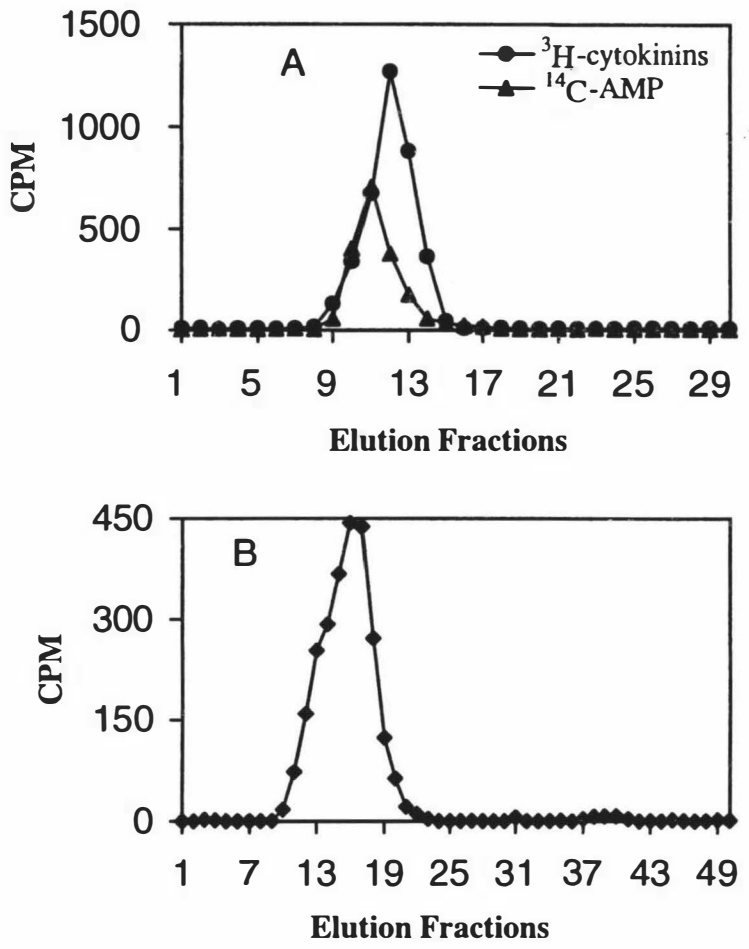


Figure 2.3. Elution profiles of cytokinins through PVPP columns. A: Elution profile of a mixture of ^{3}H-ZRTA, ^{3}H-iPATA and ^{14}C-AMP on PVPP column; B: actual sample (field tree buds) with internal standards ^{3}H-ZRTA, ^{3}H-iPATA and ^{14}C-AMP on PVPP column. Column: PVPP, 20 ml bed volume packed in a 30 ml polypropylene syringe barrel. Mobile phase: 80% methanol in 0.5 N acetic acid. Flow rate: unrestricted. 1 mL fractions were collected. Detection: 50 µL eluate of every fraction was mixed with 1 ml scintillation cocktail and radioactivity determined using a Wallac Liquid Scintillation Counter. Combined ^{3}H and ^{14}C activity was used in B. Different amounts of radioactivity were added in A and B and no correction was made to the values shown in the figures.

columns (Fig. 2.4A). When 10 mM ammonium acetate buffer (pH 6.5) was used to elute the columns, ^{3}H-ZRTA and ^{3}H-iPATA cytokinins began to appear in the eluate fractions after only half a BV of the buffer had gone through the columns and 2 BV of the buffer was sufficient to elute all the ^{3}H-ZRTA and ^{3}H-iPATA cytokinins from the columns. Meanwhile, it was observed that ^{14}C-AMP was strongly retained by the DE52 during elution, with no significant ^{14}C-AMP being found in the buffer fractions. To elute ^{14}C-AMP from the DE52 columns, 1.5 BV of acetic acid (pH 2.5) was required before any ^{14}C-AMP appeared in eluate fractions and a further 1.5 BV of the acid (pH 2.5) was essential for the complete recovery of ^{14}C-AMP (Fig. 2.4A).

To examine the elution profile of plant samples on DE52 columns, the extracts were spiked with ^{3}H-ZRTA, ^{3}H-iPATA and ^{14}C-AMP and were subsequently run through a DE52 column. The DE52 column had a pre-column of PVPP to remove gel-like substances. The same separation profiles were obtained with the spiked samples (Fig 2.4B) as observed in the standards despite an increase by about 12 mL in the requirement of 10 mM ammonium acetate buffer (pH 6.5) due to the addition of the small PVPP column.

2.3.3 Recoveries of cytokinins after passage through the PVPP-DE52-C_{18} column series

The performance of the entire model PVPP-DE52-C_{18} column complex was also assessed by examining recoveries of internal markers (^{3}H-ZRTA, ^{3}H-iPATA and ^{14}C-AMP) from the individual columns of the PVPP-DE52-C_{18} linked series. The results are displayed in Table 2.1. DE52, which trapped the nucleotide, gave more than a 90% recovery for nucleotide, determined as ^{14}C radioactivity and this was shown to be repeatable. The average recovery for the non-phosphorylated cytokinins from the C_{18} columns was more than 75% and also gave repeatable results. The PVPP pre-columns were eluted with 80% methanol after being disconnected from the series, but no radioactivity was detected in the methanol eluate.

2.3.4 HPLC separation of cytokinins

2.3.4.1 Bulk separation of cytokinins: An efficient normal phase HPLC protocol

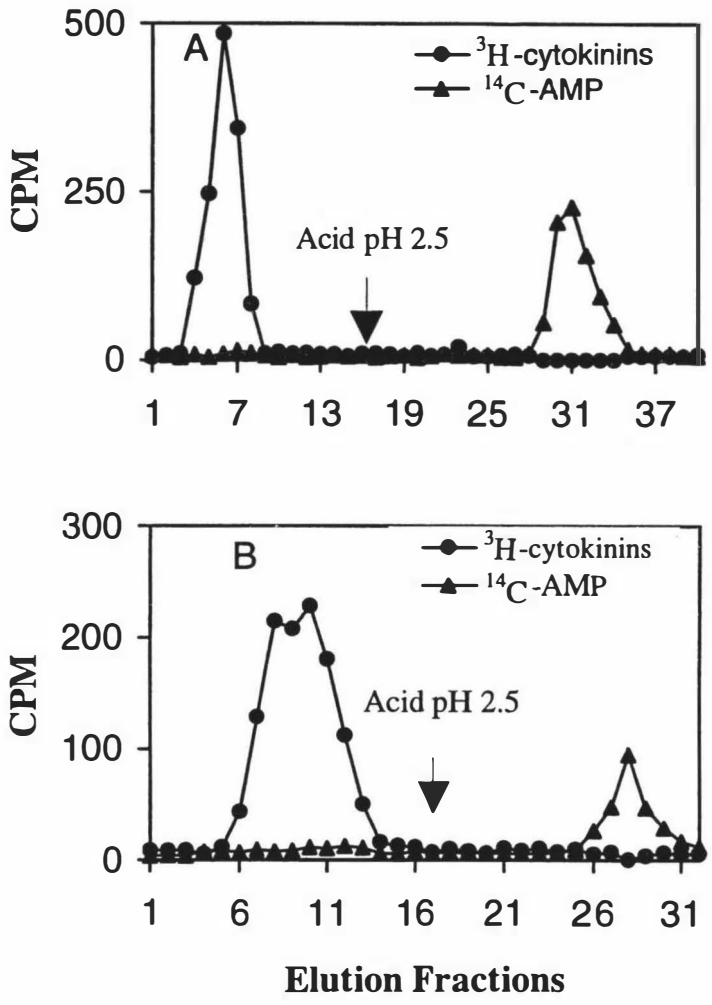


Figure 2.4 Separation profiles of cytokinins through DE52 columns. A: a standard mixture of ^{3}H-ZRTA, ^{3}H-iPATA and ^{14}C-AMP; B: an actual sample (field tree buds) containing internal standards ^{3}H-ZRTA, ^{3}H-iPATA and ^{14}C-AMP through a DE52 column which had a PVPP pre-column (10 mL BV). DE52 column was 20 mL bed volume; 10 mM ammonium acetate buffer (pH 6.5) was used to elute ^{3}H-ZRTA and ^{3}H-iPATA; 1 N acetic acid (pH 2.5) was used to elute ^{14}C-AMP. The flow rate was unrestricted and 4 mL fractions were collected. 50 μL eluate from every fraction was mixed with 1 mL scintillation cocktail and radioactivity was determined using Wallac Liquid Scintillation Counter. Values in the figures were not corrected.

Table 2.1. The performance of the entire model PVPP-DE52-C_{18} column complex. Tissue cultured micro-shoots were collected and extracted as described in Section 2.2.2.2 but radio-labelled internal standards were added after the homogenisation of samples. Other conditions were as described in Section 2.2.3. Phosphorylated cytokinins were traced as ^{14}C-AMP and non-phosphorylated cytokinins were traced as ^{3}H-ZRTA and ^{3}H-iPATA.

	Complex number	Volume resulted	Recovery (%)	Error(%)
DE52 (releasing	Rep. 1	45 mL	93.8	0.4
Phosphorylated cytokinins)	Rep. 2	45 mL	90.8	
C_{18} (releasing Non-	Rep. 1	15 mL	76.7	3.3
phosphorylated cytokinins)	Rep. 2	15 mL	77.0	

developed by Lewis et al.(1996) was used to separate cytokinin free bases and ribosides from cytokinin glucosides. According to the separation profile of cytokinin standards obtained using this protocol (Fig. 2.5), a clear separation of the free bases and ribosides (minute 0 - 9.0) from the glucosides (minutes 13.0 - 28.0) could be achieved with the exception of iP9G (Rt = 10.65). Because iP9G was cross-reactive with clone 12 antibodies, and eluted earlier than iP or iPA on a reverse phase C_{18} column, it was pooled with the free bases and ribosides.

2.3.4.2 Separation of individual cytokinins: Reverse phase HPLC was employed to separate individual cytokinins (Section 2.2.3.3b). A distinctive separation of individual standard cytokinins which were cross-reactive with clone 16 or clone 12 was achieved (Fig. 2.6). As marked in Fig. 2.6, the retention times of the internal standards ^{3}H-ZRTA, ^{3}H-iPATA and ^{14}C-adenosine (product of ^{14}C-AMP following treatment with phosphatase) were clearly different from other cytokinins. The only exception was that DZ9G and ^{3}H-ZRTA were not clearly separated.

2.3.5 Radioimmunoassay

A streamlined radioimmunoassay protocol (Section 2.2.5.1) was used to detect and quantify the individual cytokinins extracted from the *P. radiata* materials (as described in Section 2.2.1) following separation of individual cytokinins by reverse phase HPLC. Because three steps in the traditional RIA protocol were reduced in the streamlined

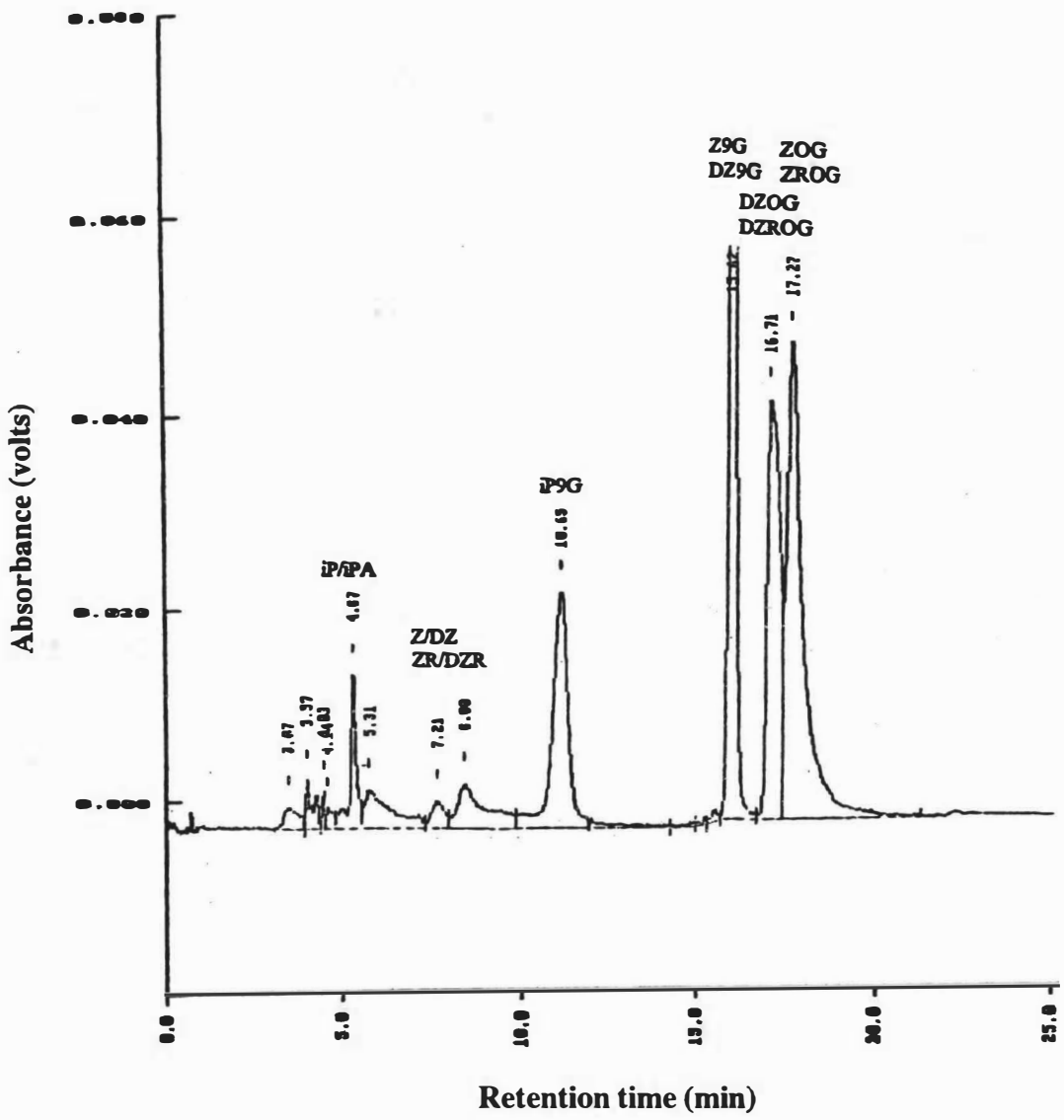


Figure 2.5 Normal phase HPLC separation of cytokinin standards. A clear cut was made to separate glucosides (excluding iP9G) from free bases and ribosides at 13 min. Column: Alphasil 5NH_2, 5 μm, 250 × 4.6 mm. Detection: absorbance at 269 nm. Other conditions were according to the protocol described previously (Lewis et al., 1996).

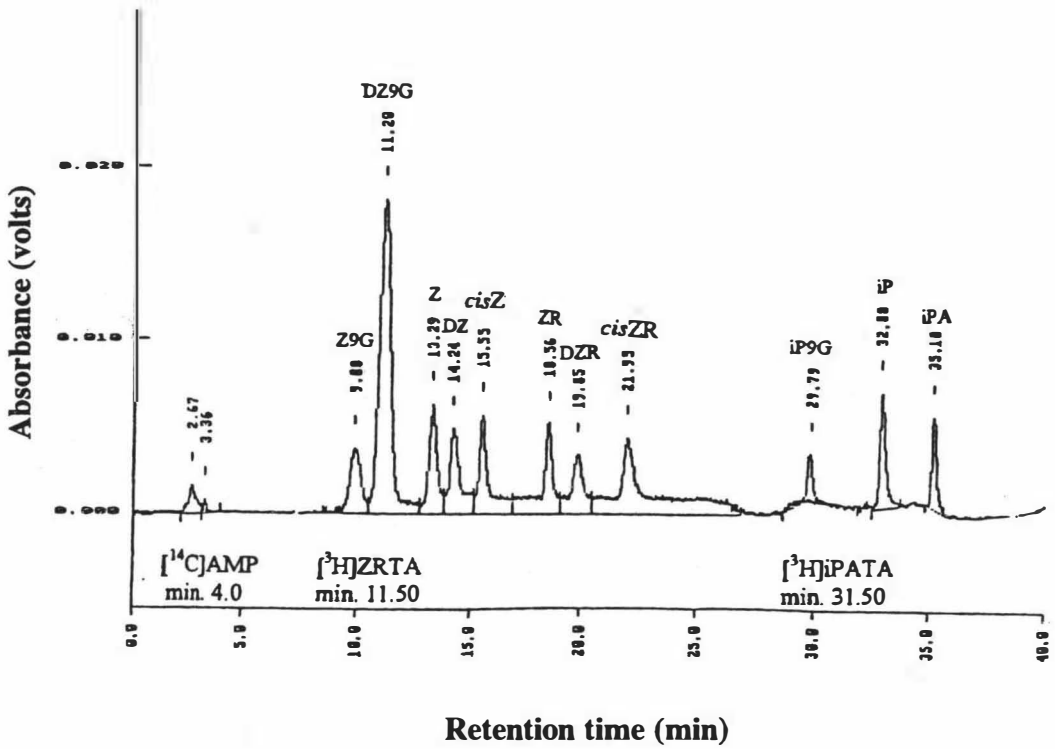


Figure 2.6 Reverse phase HPLC separation of cytokinin standards. Column: 250 × 4.6 mm octadecyl silica C_{18} Altex. Mobile phase: 40 mM HPLC buffer (pH 3.5)/methanol/acetonitrile (Section 2.2.3.3b). Flow rate: 1 ml min^{-1}. Detection: absorbance at 269 nm.

protocol to a one step operation (RIA buffer, radioactive labelled competitor and antibodies were pre-mixed), the time and labour required for the streamlined RIA protocol was reduced significantly compared to the traditional procedures. Consequently, 700 to 1000 RIA tubes were able to be handled per assay compared with 250 to 300 tubes for the traditional procedure. Moreover, due to the RIA reaction solution being aliquotted once as a larger volume (i.e. one 450 μL aliquots compared to three individual, smaller aliquots in the traditional protocol), more accurate aliquots and fewer aliquotting errors were achieved. As a result, the baseline of the RIAs had much less background noise compared with the baseline obtained from the traditional RIA protocol.

To quantify the cytokinins using RIA, a particular standard curve for clone 16 against ZR or for clone 12 against iPA always accompanied each batch of RIAs. Fig. 2.7A and B demonstrate representative standard curves for clone 16 and clone 12 respectively. Cytokinin concentrations were calculated according to the linear regions of the standard curves. Therefore, calculation equations as shown in Fig 2.7C and D were obtained from the transformation of the linear regions of these standard curves and used in quantifying cytokinins. The basic form of the calculation equations was $y = ax + b$, where y represents B/B0 ratio; x is Log(pmol of ZR or iPA); a is the slope of the straight line the equation represents and b is the interception of the line on the Y axis (Fig 2.7C and D).

2.3.6 Mass spectra of standard cytokinins

Mass spectrometry of standard cytokinins was carried out as described in Section 2.2.5.2. Table 2.2 shows the diagnostic positive ions of cytokinins generated by tandem (MS/MS) mass spectrometry. All standard cytokinins produced quasi-molecular ions $[M + H]^+$ as parent peaks. More structural information for individual cytokinins was obtained following analysis of the daughter ions produced by fragmentation of the parent peaks.

The iP had a quasi-molecular ion $[M+H]^+$ at m/z 204 mass units and iPA at m/z 336 mass units. After fragmentation, two product ions at m/z 148 and 136 were formed from the iP parent ion and three product ions at m/z 204, 148 and 136 from the iPA

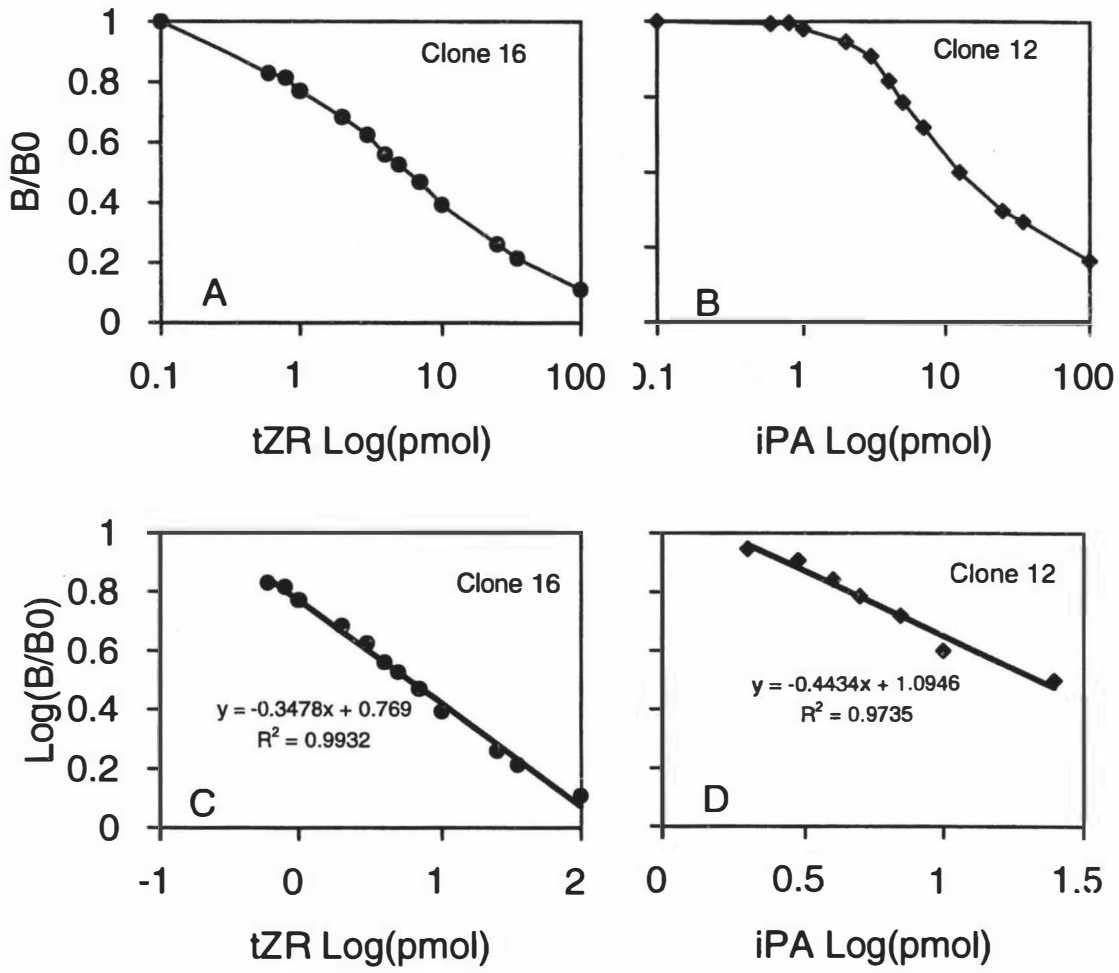


Figure 2.7 Examples of radioimmunoassay standard curves for antibodies employed in RIAs in this thesis. A: a standard curve for clone 16 against ZR; B: a standard curve for clone 12 against iPA; C: a calculation equation derived from the linear part of the clone 16 standard curve in A; D: a calculation equation for clone 12 transformed from the linear region in the sandard curve in B. B = binding of ^{3}H-ZRTA or ^{3}H-iPATA in the presence of ZR or iPA; B0 = binding of ^{3}H-ZRTA or ^{3}H-iPATA in the absence of any labeled ZR or iPA. X axis in A and B also has a log scale.

Table 2.2 Diagnostic positive ions in MS/MS spectra of standard cytokinins.

Cytokinin forms	Parent $[M + H]^+$ ion	Daughter ions m/z
Isopentenyladenine	204	148, 136
Isopentenyladenosine	336	204, 148, 136
Isopentenyladenine-9-glucoside	366	204, 148, 136
Zeatin-*O*-glucoside	382	220, 202, 148, 136
Zeatin riboside-*O*-glucoside	514	382, 352, 220, 202, 148, 136
$^2H_3$-dihydrozeatin	225	
$^2H_3$-dihydrozeatin riboside	357	225
Dihydrozeatin riboside-*O*-glucoside	516	384, 354, 222

parent ion. The product ions at m/z 148 and 136 indicate partial (m/z 148) or complete (m/z 136) loss of the side chain from iP and iPA and the product ion at m/z 204 from iPA was due to the cleavage of the ribosyl group off the molecule. A typical MS/MS spectrum of iPA is displayed in Fig. 2.8.

The Z-like cytokinins also lost their side chain either partially (m/z) 148, or completely (m/z 136) in a way that was similar to iP-like cytokinins. The composition of the zeatin-containing cytokinins was determined using tandem mass spectrometry to identify characteristic daughter ions. For example, zeatin riboside-*O*-glucoside produced a quasi-molecular ion at m/z 514 and daughter ions at m/z 382, 352, 220, 202, 148 and 136. Among the product ions, the ion at m/z 382 indicates the formation of $[ZOG + H]^+$ due to the loss of the ribosyl group from ZROG. The ion at m/z 352 represents the formation of $[ZR + H]^+$ due to the loss of the glucosyl group from the ZROG. The ion at m/z 220 indicates $[Z + H]^+$. The ion at m/z 202 indicates that a cyclisation of the side chain has occurred with the loss of H_2O from the zeatin. The ions at m/z 148 and 136 indicate the partial and complete losses of the five-carboned side chain from the zeatin (Fig. 2.9). Although it was clear on the MS/MS spectrum that a ribose unit was cleaved from molecules of the ribosides, an ion peak representing the ribose moiety was not observed. In contrast to the zeatin- and iP-containing cytokinins, the MS/MS spectrum of dihydrozeatin-*O*- glucoside (Fig. 2.10) shows strong ion peaks

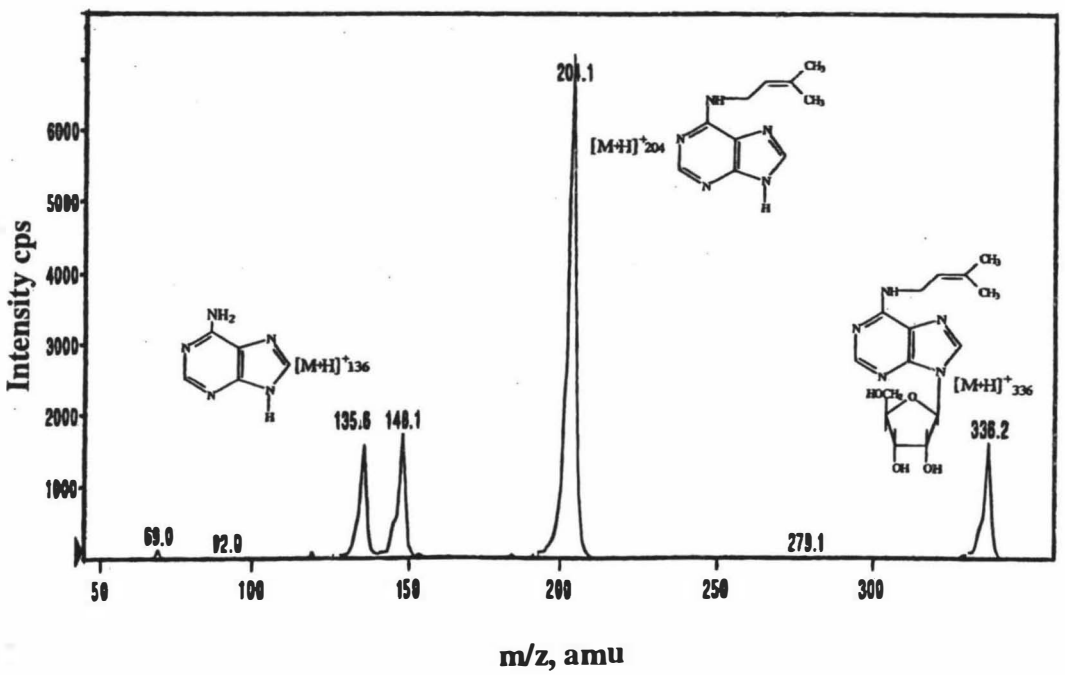


Figure 2.8 MS/MS spectrum of isopentenyladenosine.

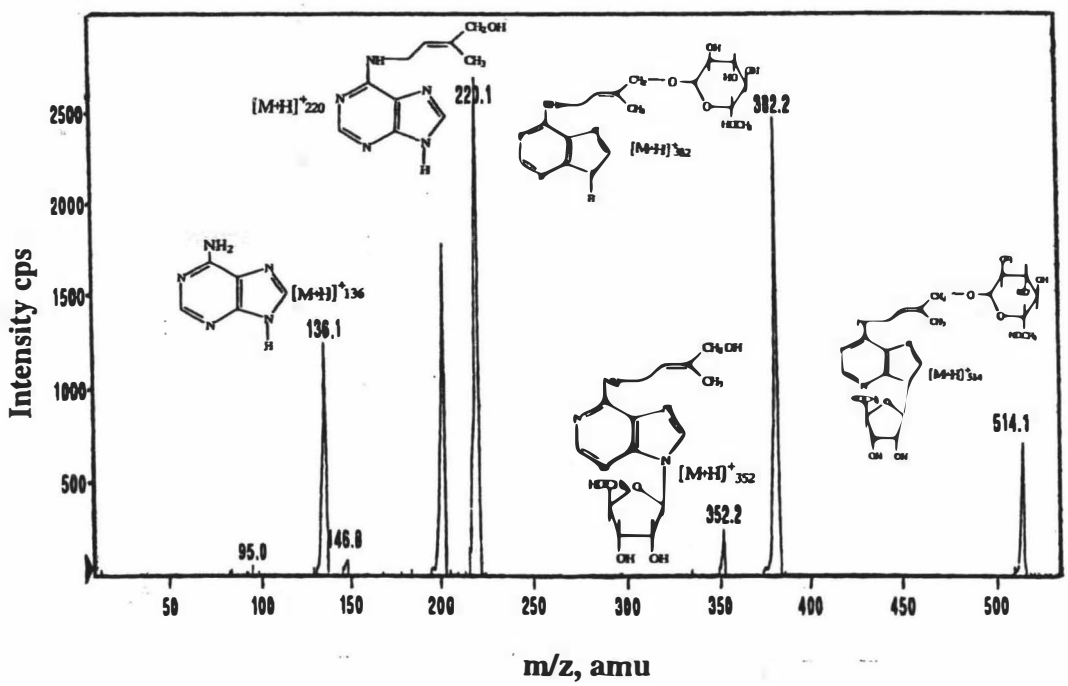


Figure 2.9 MS/MS spectrum of zeatin riboside-*O*-glucoside.

at m/z 384 $[DZOG + H]^+$ and 222 $[DZ + H]^+$. However, no ions with m/z values of 148 and 136 were observed using the same conditions as those used in Fig. 2.9 for ZROG fragmentation, indicating the stability of the five-carbon side chain in contrast to the relatively easy fragmentation of the glucosyl and ribosyl groups from DZROG. The MS/MS spectra of both ZROG and DZROG showed that the ion peaks representing the ZOG (m/z 382, Fig. 2.9) and DZOG (m/z 384 which was not observed, Fig. 2.10) are much stronger than the peaks representing ZR (m/z 352, Fig. 2.9) and DZR (m/z 354, Fig. 2.10) respectively. This indicates that the *O*-glucoside bonds on the side chains are more stable than the N-ribosyl bonds on the purine rings.

2.3.7 Endogenous cytokinins in the buds of *P. radiata*

2.3.7.1 Cytokinins detected by RIAs: Bulk fractions of cytokinin free bases and ribosides, dephosphorylated cytokinins, and glucosides, isolated from the bud material (as described in Section 2.2.1) using the procedures outlined in Fig. 2.1, were each fractionated on C_{18} HPLC and collected in 0.5 min fractions (Section 2.2.3.3b). Fig. 2.11 shows representative RIA histograms of individual 0.5 min fractions of the HPLC eluate with clone 16 or clone 12 (Section 2.2.5.1). By comparison of the retention times between sample RIA peaks and standard cytokinin peaks, it was determined that ZR, DZR, iP and iPA were the free base and riboside cytokinins present in the buds of *P. radiata* (Fig. 2.11A). The detection limit in the RIA was ca 1 pmol.

Cytokinin nucleotides were dephosphorylated (Section 2.2.4.1) prior to reverse phase HPLC step so that their identities could be determined by comparison with the retention times of standard cytokinins. As marked in Fig 2.11B, three of the RIA peaks were identical to the retention times of ZR, DZR and iPA and accordingly I concluded that ZR nucleotide, DZR nucleotide and iPA nucleotide are present in the bud tissues. Interestingly, the retention times of the other three peaks in Fig. 2.11B did not correspond to any known form of cytokinin nucleotide but matched those of the cytokinin glucoside peaks shown in Fig. 2.11C. Therefore, these peaks were regarded as novel cytokinin nucleotide peaks, and designated as ZRx nucleotide, DZRx nucleotide and iPAx nucleotide as it was subsequently found that these cytokinins contained ZR, DZR and iPA components respectively (Section 2.3.7.2 and 2.3.7.3).

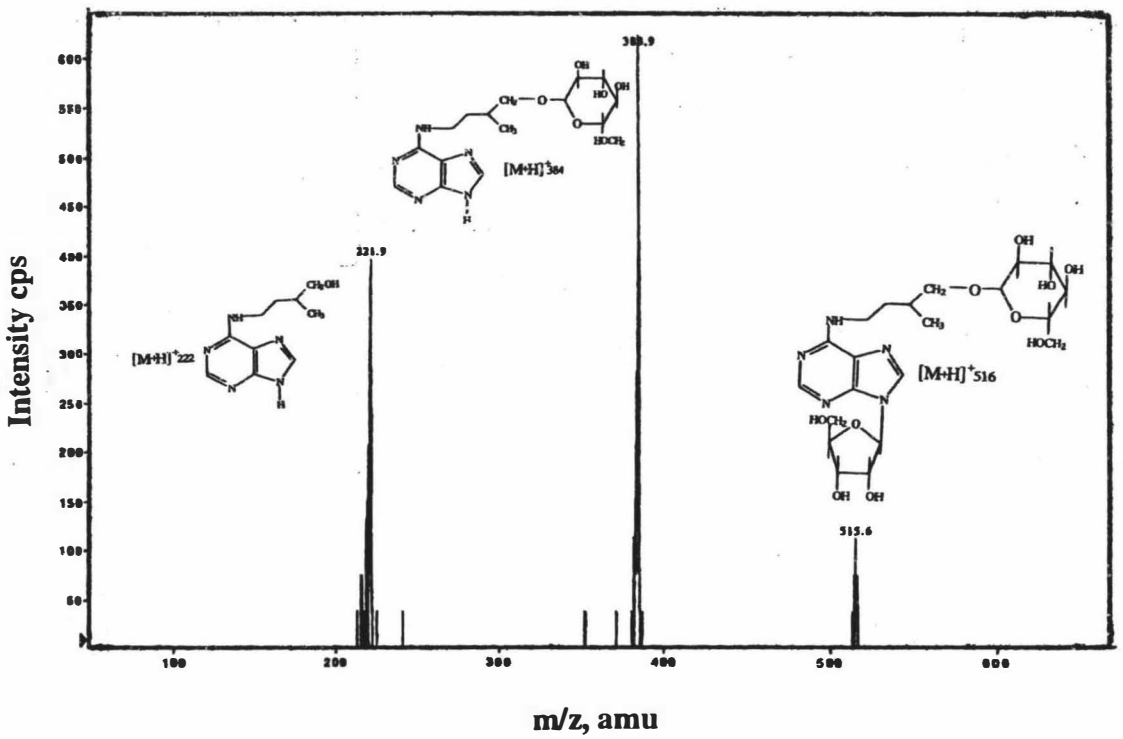


Figure 2.10 MS/MS spectrum of dihydrozeatin riboside-*O*-glucoside.

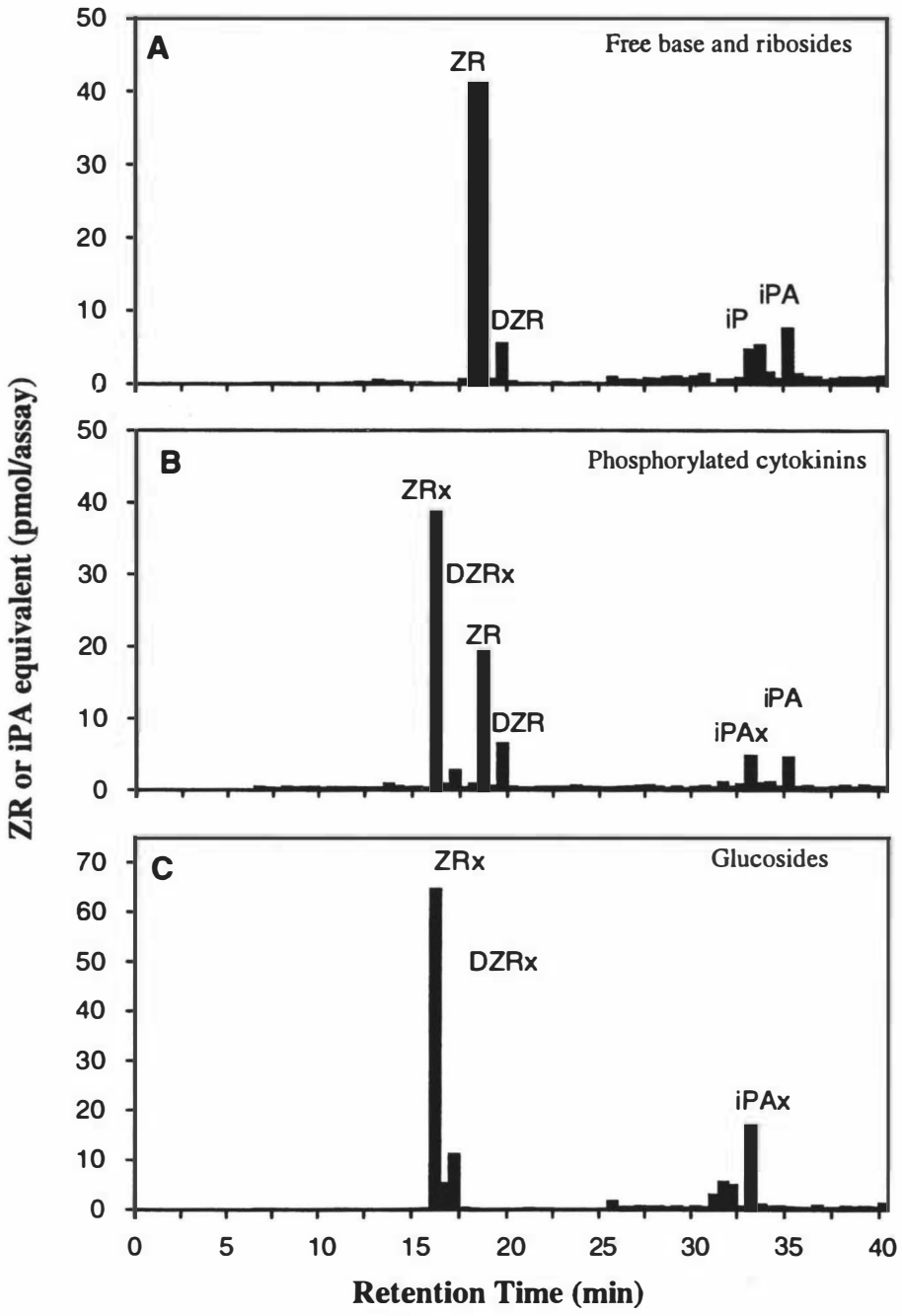


Figure 2.11 Cytokinins detected in 8-year-old mature buds of *Pinus radiata* using RIAs. HPLC fractions 0-25.5 min were assayed with clone 16 and 26-40 min assayed with clone 12. A: free bases and ribosides; B: cytokinins released from phosphorylated cytokinins after treating with alkaline phosphatase; C: cytokinin glucosides. Data shown in the figure were not corrected for losses or cross reactivity.

Figure 2.11C shows the profiles of glucosides detected with RIAs after C_{18} HPLC. These glucosides were neither traditional *O*-glucosides nor N-glucosides, because traditional *O*-glucosides were not able to cross-react with the anti-cytokinin antibodies used and the retention times of any cross-reactive N-glucosides were very different from those of the cytokinin glucoside peaks shown in Fig 2.11C under the HPLC conditions used in this thesis (Section 2.2.3.3b and Fig. 2.6). As mentioned earlier, these peaks had identical retention times to those of the dephosphorylated novel cytokinin nucleotide peaks shown in Fig. 2.11B. Therefore they are designated as ZRx, DZRx and iPAx (Fig. 2.11C).

2.3.7.2 Evidence of novel cytokinin glucosides by HPLC analyses and enzyme treatments: Since the RIA results displayed in Fig. 2.11 revealed the presence of three major novel cytokinin glucosides (which had been pooled in the glucoside fraction from the amine column HPLC, Section 2.2.3.3a) and their respective phosphorylated forms, the novel glucosides were further purified and isolated using an immunoaffinity method (Section 2.2.3.4b and Fig. 2.2). This step was necessary to eliminate any possible contamination by traditional *O*-glucosides. The solid line in Fig. 2.12 shows the separation profile of the immunoaffinity-purified novel cytokinin glucosides on C_{18} HPLC. Two major UV-absorbing peaks with retention times at 16.1 and 17.0 min cross-reacted with clone 16 (Fig. 2.13A, dark bars) and two peaks with retention times at 31 and 33 min cross-reacted with clone 12 (Fig. 2.13B, light bars).

After the sample fractions containing ZRx, DZRx and iPAx were treated with β-glucosidase (Section 2.2.4.2), both HPLC spectra (Fig. 2.12, broken line) and RIA analyses (Fig. 2.13A and B, light bars) demonstrated that ZR, DZR and iPA were released from ZRx, DZRx and iPAx respectively. These data indicate that the three novel cytokinin glucosides are conjugated with a hexosyl group (most likely a glucosyl) somewhere on the ribosyl group of their molecules.

2.3.7.3 Confirmation of novel cytokinin glucosides by mass spectrometry: For unequivocal identification of the novel cytokinin glucosides, representative samples of the individual fractions containing ZRx (16 min), DZRx (17 min) or iPAx (33 min)

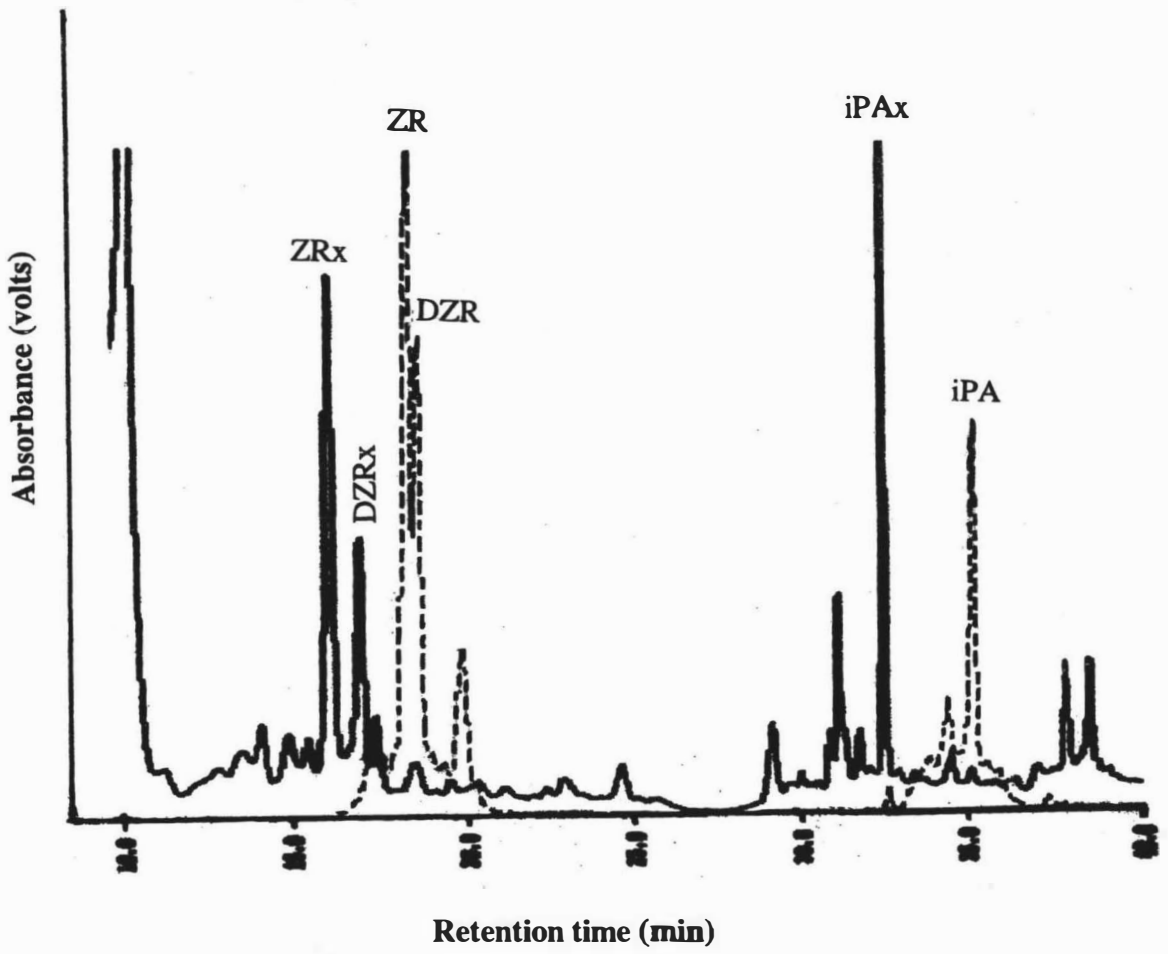


Figure 2.12 HPLC spectra of the immunoaffinity-purified novel cytokinin glucosides before (solid line) and after β-glucosidase treatment (broken line)

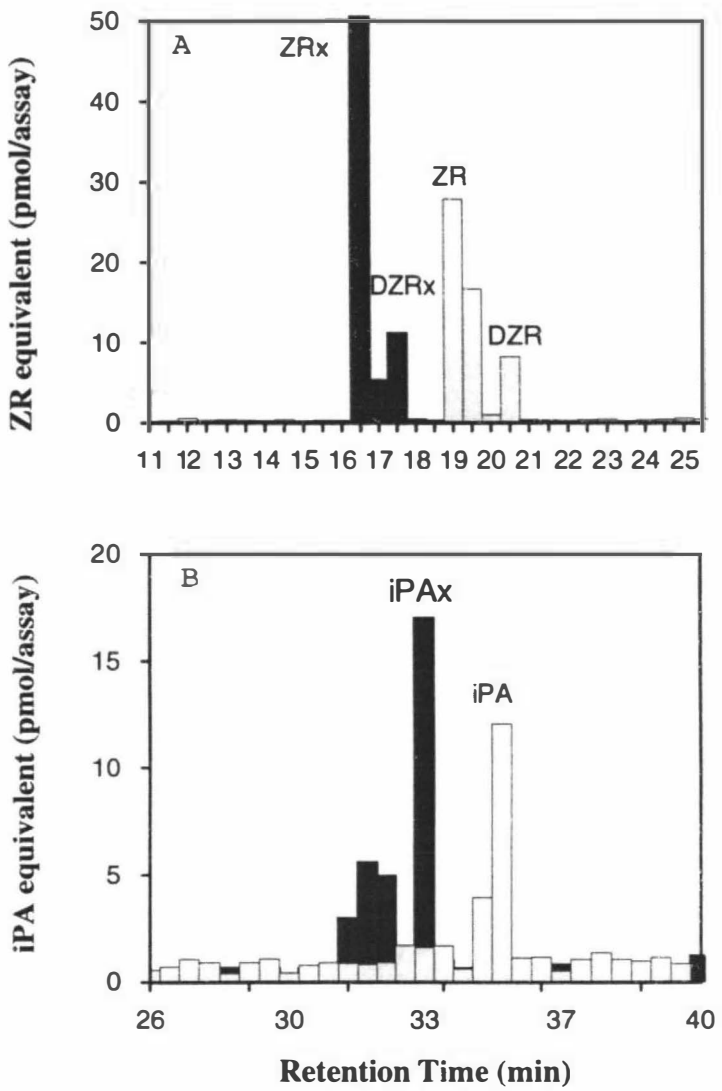


Figure 2.13 RIA profiles of individual 0.5 min fractions of the HPLC eluate of the imunoaffinity-purified novel cytokinin glucosides before (dark bars) and after (light bars) β-glucosidase treatment. A, assayed with antibody against ZR-like cytokinins (clone 16) and B, assayed with antibody against iP-like cytokinins (clone 12).

were subject to ES MS/MS analysis (Section 2.2.5.2) respectively following C_{18} reverse phase HPLC separation. The compound corresponding to retention time at 31 min was not further pursued because RIA activity was very weak after β-glucosidase treatment Fig. 2.13B).

ZRx produced a quasi-molecular ion peak at m/z 514 (Fig. 2.14) which was identical to that of standard ZROG(Fig. 2.9), indicating that ZRx and ZROG had the same molecular mass and they both have a molecular weight of 513. After fragmentation, ZRx produced product ions at m/z 352, 220, 202, 148, 136 (Fig. 2.14), indicating that ZRx and ZROG share the common structural fragments of zeatin riboside (m/z 352), zeatin (m/z 220) and purine (m/z 136). The absence of a product ion at m/z 382 from ZRx (Fig. 2.14) provided definitive evidence that the structural arrangements of the hexose moiety between ZRx and ZROG were very different. Although it was clear on the MS/MS spectrum that a hexose unit was present on the molecule, hexose was not positively charged under the conditions used. The lack of the ion at m/z 382 clearly shows the glucosyl in the ZRx must be linked to the ribosyl group rather than to the side chain.

Fig. 2.15 shows the MS/MS spectrum of DZRx. Similar to the standard DZROG displayed in Fig. 2.10, DZRx compound was more stable than ZRx, producing fewer ion fragments under the same ionisation conditions as those used in analysing ZRx. Only two product ion peaks at m/z 354 and 222 were observed after the fragmentation of the quasi-molecular ion (m/z 516) of DZRx (Fig 2.15). The ion at m/z 354 indicates the presence of ribosyldihydrozeatin in the DZRx molecule while the ion at m/z 222 confirmed that dihydrozeatin was part of the DZRx molecule. By comparison with the spectrum of standard DZROG (Fig. 2.10), it is very clear that the glucosyl group is linked to the ribosyl of DZRx molecule rather than to the side chain because no peak at m/z 384 was observed in the mass spectrum of DZRx. The ion at m/z 384 is characteristic of DZOG (Table 2.2).

The mass spectrum of the HPLC fraction containing iPAx gave a quasi-molecular ion at m/z 498 (Fig. 2.16). After fragmentation of this parent ion, a MS/MS spectrum of

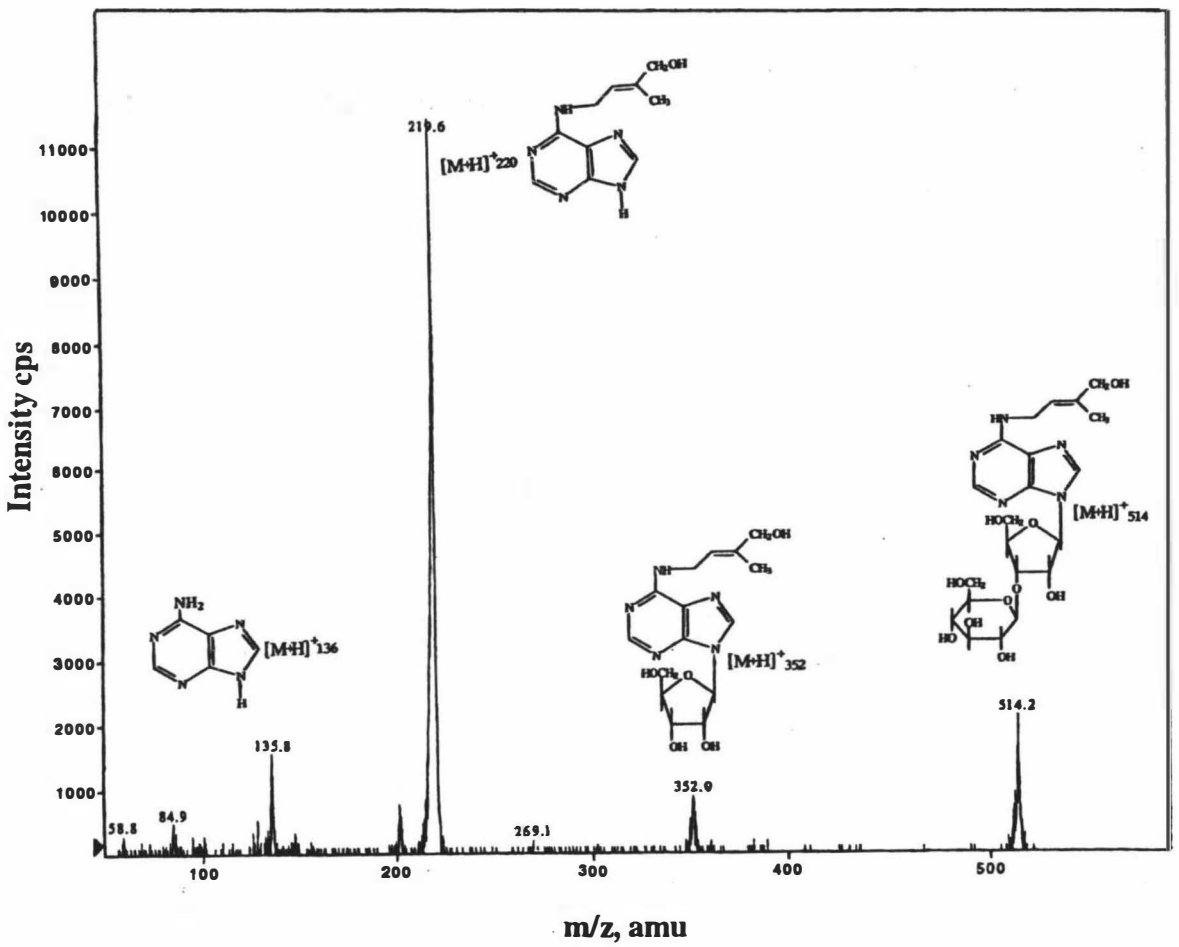


Figure 2.14 MS/MS spectrum of novel cytokinin ZRx.

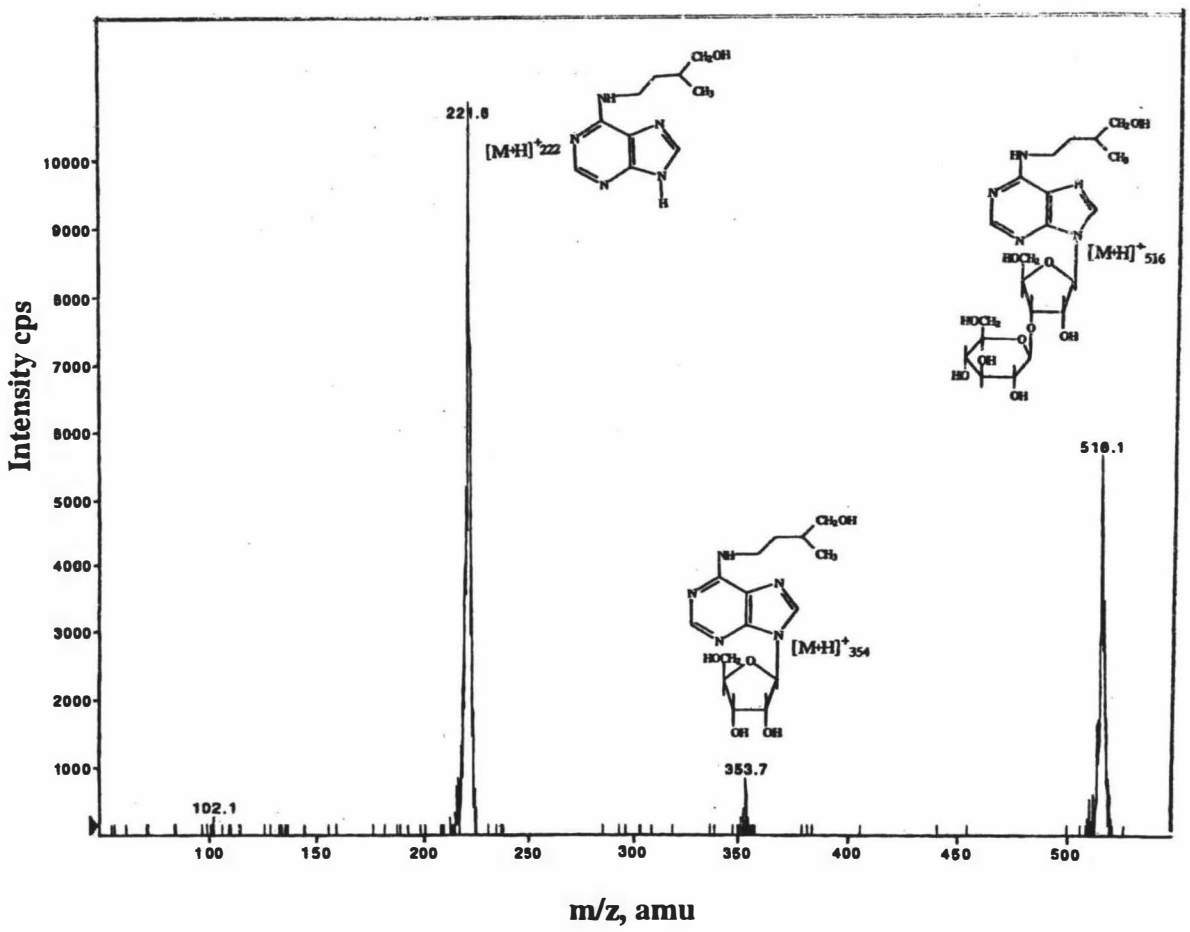


Figure 2.15 MS/MS spectrum of novel cytokinin DZRx.

product ions identical to that of iPA fragments (Fig. 2.16 and Fig. 2.8) was produced, including the ions at m/z 336, 204, 148 and 136. Comparison between the mass spectra in Fig. 2.16 and Fig. 2.8 revealed that iPA (m/z 336), iP (m/z 204) and adenine (m/z 136) were the components of the iPAx molecule. Because the mass difference between iPAx and the biggest fragment iPA was 162 units, which is equivalent to the mass of one hexosyl unit, there must be a hexosyl group attached to the ribosyl of the iPAx molecule.

Figure 2.17 shows proposed structures of the novel cytokinins of ZR-G, DZR-G, iPA-G and their phosphorylated forms.

2.3.7.4 No evidence of traditional O-glucosides: After the removal of cross-reactive glucoside cytokinins by the novel immunoaffinity method (Section 2.2.3.4b), the fractions which would contain the traditional *O*-glucosides were treated with β-glucosidase (Section 2.2.4.2) and checked with RIAs (Section 2.2.5.1). There was no sign from RIA results indicating the existence of traditional *O*-glucosides. To confirm these results, mass spectrometry was used. Fig. 2.18 shows the mass spectra of the fraction which would contain *O*-glucosides if any were present. There were no significant peaks which would correspond to ZOG (m/z 382), DZOG (m/z 384), ZROG (m/z 514) or DZROG (m/z 516) (Fig. 2.18A and B). Therefore, no evidence has been found that traditional *O*-glucosides were present in the buds of *P. radiata*. The detection limit of the MS was approximately 1 pmol.

2.4 Discussion

2.4.1 Methodology of cytokinin analysis

A wide spectrum of cytokinins has been found in the mature buds from *P. radiata* trees. It is striking that so many novel cytokinins and their corresponding phosphorylated forms have been discovered. The results obtained in this thesis indicate that the strategy and the techniques employed in this work were effective.

The initial extraction methodology is a key step in cytokinin analysis as chemical and/or enzymatic modification to cytokinin molecules may occur during the extraction process

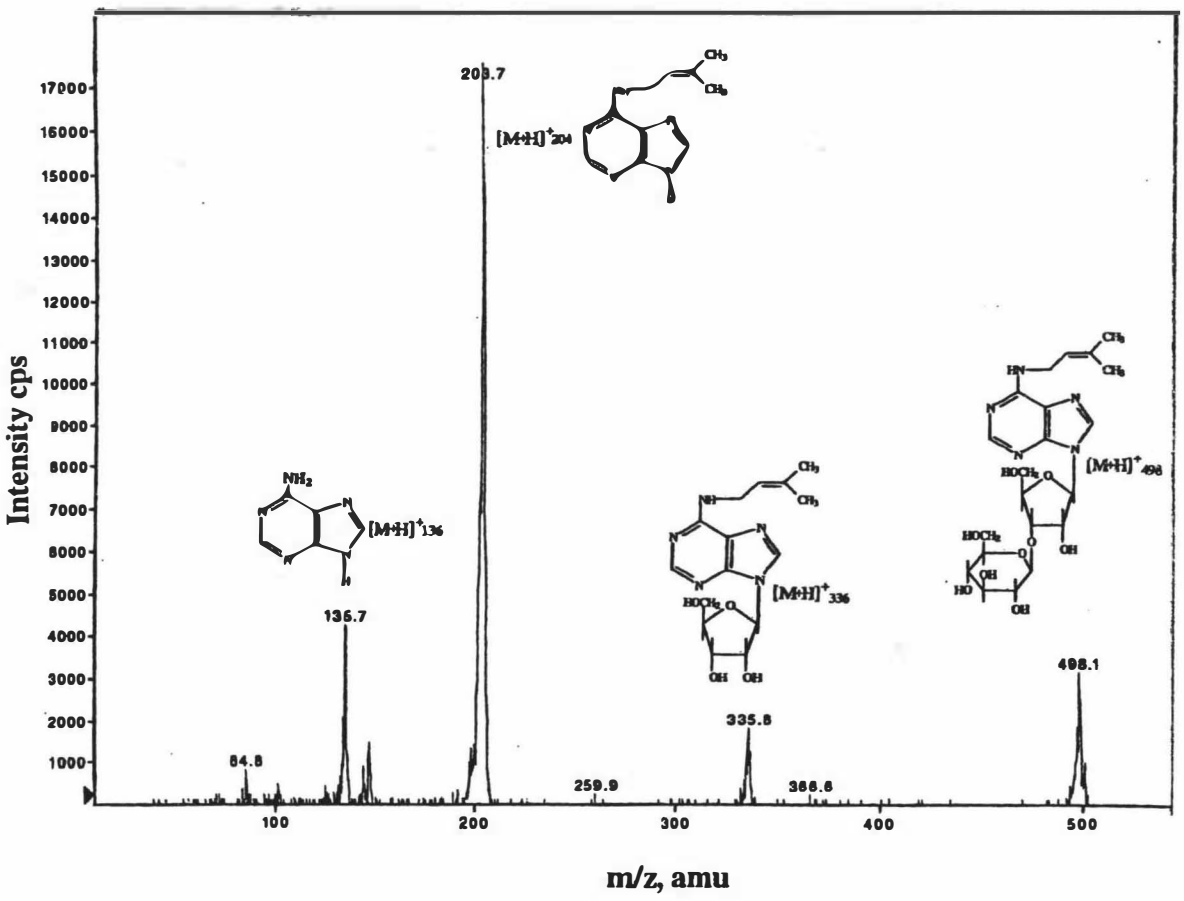


Figure 2.16 MS/MS spectrum of novel cytokinin iPAx.

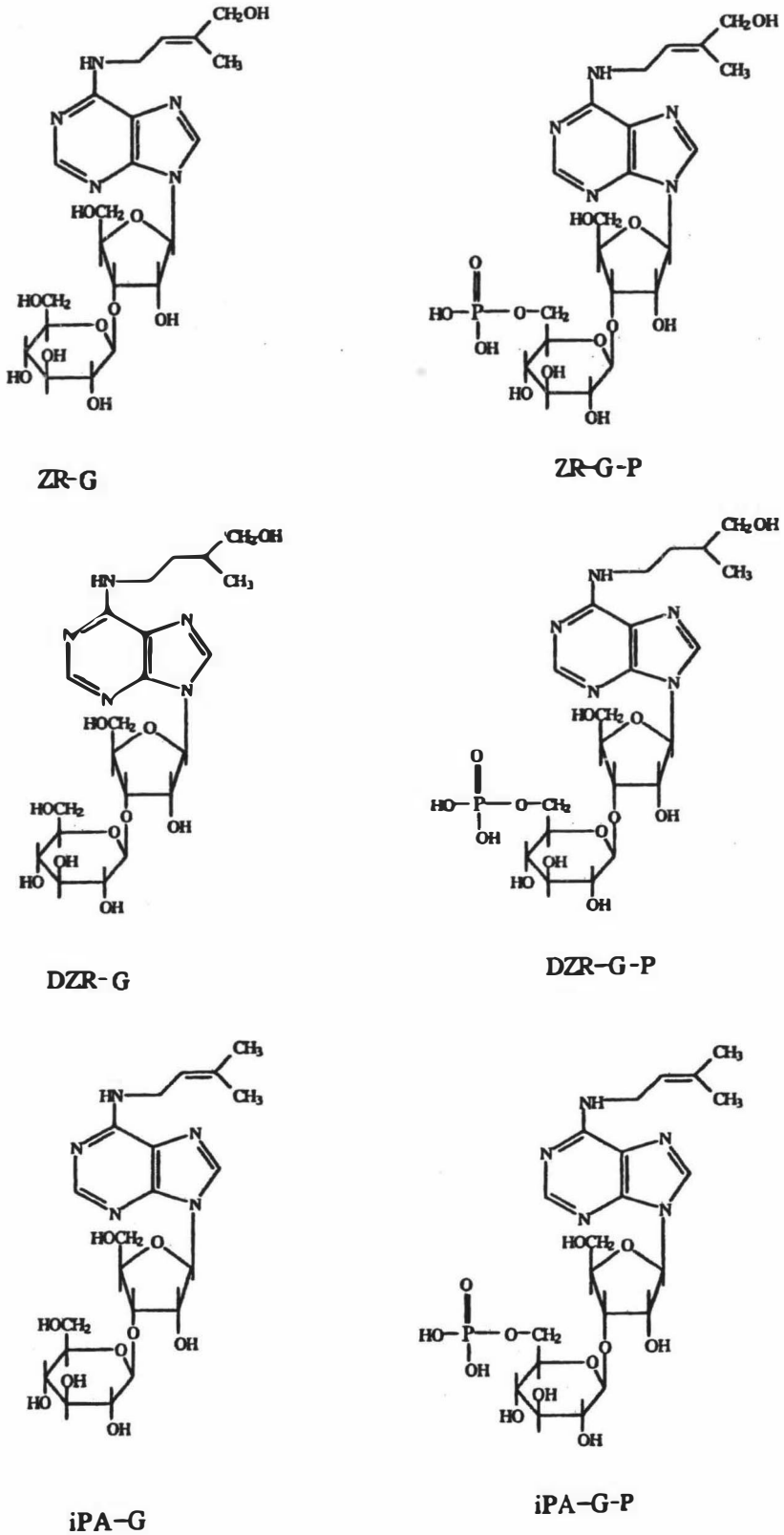


Figure 2.17 Proposed structures of identified novel cytokinins derived from buds of *Pinus radiata*.

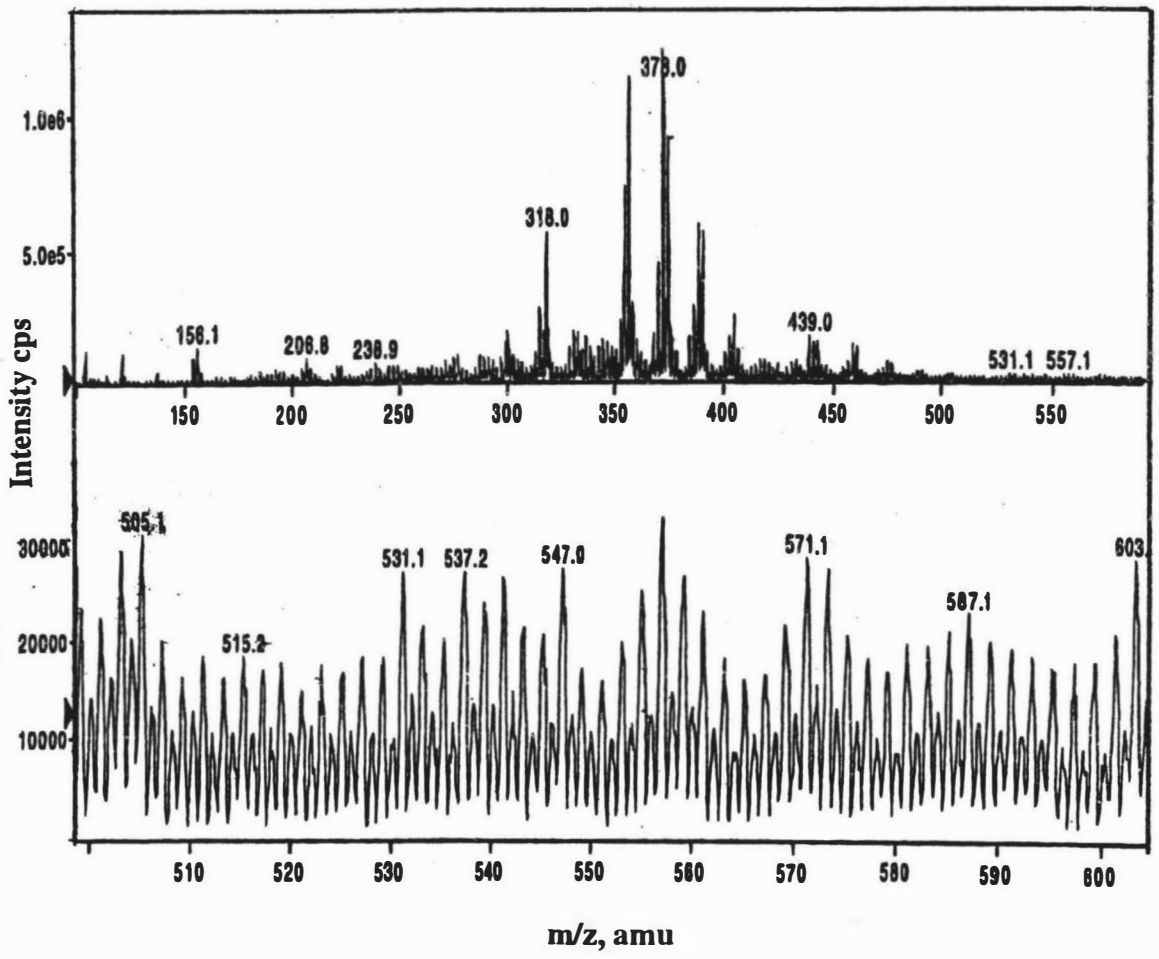


Figure 2.18 Mass spectra of the fraction that would contain traditional *O*-glucosides if any were present.

(Tokota et al., 1980). It had been found that non-specific phosphatase activity persisted even in aqueous alcohol solvents, causing conversion of cytokinin nucleotides to cytokinin nucleosides (Laloue and Pethe, 1982, Letham, 1978, Horgan and Scott 1987). Bieleski (1964) discovered that acidic organic solvents could efficiently inactivate phosphatases if plant tissues were kept at low temperature. The original Bieleski's solution (methanol : chloroform : acetic acid : water; 12:5:1:2) and a modified Bieleski's solution (Section 2.2.1) were both successfully adapted later for cytokinin extraction (Jameson et al., 1987, Singh et al., 1988, Meilan et al., 1993, von Schwartzenberg et al., 1994, Day et al., 1995, Wang et al., 1995). Although Bieleski's solvent is able to inactivate phosphatase, 80 % methanol is regarded as a more efficient solvent for cytokinin extraction (Horgan and Scott 1987). Therefore, the double-solvent extracting strategy used in this work (Section 2.2.2.2 and Fig. 2.1) should have the advantages of both inactivating phosphatases and, subsequently, effectively extracting cytokinins.

In preliminary experiments, a highly coloured gel was formed when the sample extract was reduced to about 0.5 ml volume. This problem still remained even after the sample had been passed through the PVPP-DE52-C_{18} complex. By testing solubility, it was found that gel was more readily dissolved in water than in methanol. Therefore, PVPP columns with acidified methanol as the eluting solvent were employed to test the possibility that acidified methanol could be used to flush cytokinins off the column quickly enough to leave the gel-forming impurities behind on the column. Indeed, all forms of cytokinins were readily eluted from PVPP columns (Fig. 2.3A and B) using this method. The fact that the gelling problem disappeared after this step was in agreement with the fact that visible solid substances and pigments were retained by the PVPP columns under the eluting conditions used. Biddington and Thomas (1976) showed that low pH can accelerate cytokinin elution from PVP columns and coincidentally enhance binding of phenolics to PVP.

The use of the PVPP-DE52-C_{18} column complex allowed further purification of sample extracts and separation of phosphorylated cytokinins from the other forms of cytokinins to take place in one passage. The PVPP column in the complex retained significant

amounts of green and yellow pigments when sample dissolved in buffer or water was percolated through at pH 6.5. These pigments may be different in binding characteristics from those which bound to the PVPP under acidic methanol eluting conditions. It is important to use a small PVPP column volume (no more than 10 mL in this thesis) in the column complex because, compared with acidic methanol as an eluting solvent, a much larger volume of eluting solution is required before any cytokinin can be eluted off PVPP column when aqueous buffer with a neutral pH is used as the eluting solution (Baddington and Thomas, 1976). In addition, a large PVPP column reduces the elution rate quite significantly.

It has been shown in this thesis, as an alternative to a cellulose phosphate column, that anion exchangers such as DE52 can be successfully used in separating cytokinin nucleotides from the other forms of cytokinins (Fig. 2.4). The results (Section 2.3.2) indicate that cytokinin nucleotides can be retained very strongly by DE52 at pH 6.5. Using the complex developed in this experiment, simultaneous processing of multiple samples (8 -10 samples) was achieved. In addition, compared to the several hundred millilitres of eluate normally collected from cellulose phosphate columns, the small volumes of the phosphorylated fraction (45 mL) derived from the DE52 column and the non-phosphorylated cytokinin fraction (15 mL of 80% methanol) collected from the small C_{18} columns, meant that the samples could be dried quickly using a Savant Speedvac (12 samples at one time). Therefore, the use of a rotary evaporator could be eliminated. As shown in Table 2.1, good recoveries and reproducibility were obtained in model systems using the column complex. Compared with 60 -80 % recoveries reported using immunoaffinity columns (MacDonald and Morris 1985; Doumas et al., 1989), our average recovery was reasonably good (77% - 100% for non-phosphorylated cytokinins after the C_{18} column of the complex; > 90% for phosphorylated forms). Very clean samples were obtained at this stage.

Cellulose phosphate chromatography has been widely adapted for separating cytokinin nucleotides from the free bases, ribosides and glucosides. When cellulose phosphate chromatography is employed, acidic solutions with pH 3.0-3.1 are used to elute cytokinin nucleotides from cellulose phosphate columns, while the non-phosphorylated

cytokinins are bound to the column under these conditions (Parker and Letham, 1973). As a result, the cytokinin nucleotides are separated from the rest of the cytokinin forms. However, a certain proportion of positively charged nucleotides may be present under these acidic conditions (pH 3.0 - 3.1), because the phosphate group on cytokinin nucleotides may have no negative charges at pH 3.0 - 3.1 while the amino groups at position 6 of cytokinins (pK_a 4.2, Horgan and Scott 1987) will be positively charged. Therefore the cytokinin nucleotide molecule can carry an overall positive charge. For instance, the phosphate group of the AMP molecule, which is widely used as a internal marker of nucleotides, has two pK_a values for the phosphate groups on the molecule. The dissociation of H^+ from the first phosphate group of AMP (pK_a 3.8, Dawson et al., 1986) does not readily occur at pH 3.0 - 3.1 while the amino group at position 6 of AMP can easily accept a H^+ becoming positive, so that the whole molecule is positively charged. Therefore, great care needs to be taken when cellulose phosphate is used for the purpose of nucleotide separation, especially with the use of ^{14}C-AMP as an internal standard.

In contrast to cellulose phosphate, when anion exchangers such as DE52 are used, the sample is required to be at neutral pH. Under neutral pH, phosphorylated cytokinins carry a double negative charge while the other cytokinin forms do not carry any charge. Thus phosphorylated cytokinins will bind to the columns, while the other forms of cytokinin readily pass through the columns. It is, therefore, much easier to get clear separation between phosphorylated cytokinins and the other forms using an appropriate anion exchanger column than a cation exchanger column such as cellulose phosphate column.

Furthermore, a low (for the nucleotides) or high pH (for the free bases, ribosides and glucosides) is needed to elute cytokinins from cellulose phosphate columns. This makes it impossible to link a C_{18} column underneath a cellulose phosphate column to collect cytokinins due to the ionisation caused by these conditions. However, the linkage of a C_{18} column to a DE52 column has been successfully used (Jameson and Morris, 1989). A DE52 column linked in the PVPP-DE52-C_{18} complex has provided a very useful approach for small-sized sample processing. As with ion exchange chromatography,

overloading can be a problem (it was not observed in this thesis) when the ratio between sample size and column bed volume of DE52 becomes too high. To avoid an overloading problem, when a large sample size is required in cytokinin analysis, an alternative to using DE52 could be DE53 (twice the exchange capacity of DE52) or other large capacity anion exchanger such as DEAE-Sephadex A52 (three and half times the exchange capacity of DE52).

Since the radioimmunoassay technique was first applied to the analysis of cytokinins (Weiler, 1980) it has been a valuable technique for both qualitative and quantitative detection of cytokinins, especially in combination with HPLC techniques (McDonald and Morris, 1985). However, the multiple steps of the addition of separately prepared RIA reaction ingredients (such as the buffer, the antibody and the tritiated competitor cytokinin) made the analysis very labour-intensive and time-consuming. More importantly, due to the errors caused by aliquotting antibody and tritiated competitor in small volume (50 μL) and the highly sensitivity nature of the RIA technique, the baseline of RIA histograms is often found to be very noisy. The advantages of using the streamlined RIA protocol over traditional procedures include more accurate and repeatable aliquotting in larger volume, shorter analysis time and reduced labour requirement.

Immunoaffinity chromatography has been widely used in purifying cytokinins (Davis et al., 1986; MacDonald and Morris, 1985; Doumas et al., 1989; Nicander et al., 1993; Hammerton et al 1996). Traditionally, to make an immunoaffinity column, anti-cytokinin antibodies must be immobilised to a support such as cyanogen bromide-activated cellulose or Sepharose (McDonald and Morris, 1985, Nicander et al., 1993). In this thesis, a much simpler, faster and more convenient immunoaffinity purification method was developed (Section 2.2.3.4b). This technique takes advantages of the fact that the membrane of a Nanosep™ spin column does not allow antibodies to pass through. The antibodies are simply retained in the spin column in a favourable buffer solution rather than being chemically linked to support material. Therefore, the antibody activity can remain at maximum level and the antibodies can be easily

recycled. The cytokinins retained by the correspoding antibodies can be easily dissociated using methanol.

Electrospray is a very useful ionisation technique that allows molecules of low volatile such as cytokinins to be introduced into a mass spectrometer without methylation or acetylation (Moritz, 1996). Prinsen et al. (1995) demonstrated a successful application of ES MS/MS to cytokinin analysis. In this work, the technique has also been successfully used to identify the novel cytokinins in the buds of *P. radiata*. The detection limit as determined using standard cytokinins was similar to that reported by Prinsen et al. (1995) of about 1 pmol.

2.4.2 Endogenous cytokinins in the buds of *P. radiata*

It has been observed that *trans*-zeatin riboside (ZR) is present in the buds of mature *Pinus radiata* used in this thesis (Section 2.2.1). This is consistent with the previous report that ZR was identified in the field buds of this species as determined by HPLC and GC-MS techniques (Taylor et al., 1984). ZR has also been reported to be present in other coniferous species. In *Pseudotsuga menziesii*, ZR was detected in vegetative and reproductive buds (Morris et al., 1990) and in roots (Doumas et al., 1989). ZR was shown to be the most abundant cytokinin in buds of *Picea abies* (Bollmark et al., 1995; Chen et al., 1996) and in buds of *Picea sitchensis* (Lorenzi et al., 1975).

Dihydrozeatin riboside (DZR) was detected in the buds of *P. radiata* in this work. DZR has not been reported previously in *P. radiata*, nor in most other conifers investigated (Lorenzi et al., 1975; Morris et al., 1990; Bollmark et al., 1995; Chen et al., 1996) except in the roots of *Pseudotsuga menziesii* (Doumas et al., 1989). DZR is resistant to cytokinin oxidase due to the lack of a Δ^2 double bond in its N^6 side chain (Jameson, 1994). Therefore, DZR may be important in maintaining a certain level of cytokinin activity or be involved in controlling cytokinin turnover in buds of *P. radiata*.

Attempts to identify isopentenyladenosine (iPA) in *P. radiata* have not been made by previous researchers (Taylor et al., 1984). In this thesis, iPA has been detected in buds, which is in line with the results obtained from other conifers such as *Pseudotsuga*

menziesii (Doumas et al., 1989; Morris, et al., 1990) and *Picea abies* (Bollmark et al., 1995 and Chen et al., 1996).

Isopentenyladenine (iP) was the only free base cytokinin detected in the buds used in this work. The identification of iP in *P. radiata* was not pursued by previous researchers (Taylor et al., 1984). In *Pseudotsuga menziesii,* although iP was not found in the roots (Doumas et al., 1989), it was observed to be present in vegetative and reproductive buds (Morris et al., 1990).

Three novel cytokinin hexose conjugates (ZRx, DZRx and iPAx) have been isolated from the buds of *P. radiata* in this work (Fig. 2.11C). Because ZR, DZR and iPA were released when the three novel cytokinins were treated with β-glucosidase (Fig. 2.12 and 2.13), the hexosyl moieties are most likely to be glucose. The strong cross reactivities of ZRx and DZRx with clone 16, and iPAx with clone 12 (Fig. 2.11C and 2.13), indicate that the glucosyl moieties are not linked with the side chains. This was particularly obvious in iPAx, because it does not have a hydroxyl on the side chain for a glucosyl moiety to attach with.

The evidence derived from the MS/MS spectra also clearly demonstrates that the glucosyl moieties were linked to the ribosyl groups on the novel cytokinin forms. Although the novel ZRx and DZRx had molecular weights identical to the traditional ZROG and DZROG respectively, their MS/MS spectra were significantly different. For instance, ZROG and DZROG produced strong ion peaks corresponding to ZOG (at m/z 382; Fig. 2.9) and DZOG (at m/z 384; Fig. 2.10) but very weak peaks corresponding to ZR (m/z 352; Fig. 2.9) and DZR (m/z 354; Fig. 2.10) respectively, indicating ZOG and DZOG structures are much more stable than ZR and DZR under the MS/MS conditions used. In contrast, the novel ZRx and DZRx did not produce any peaks corresponding to ZOG or DZOG but only peaks corresponding to ZR (m/z 352; Fig. 2.14) or DZR (m/z 354; Fig. 2.15). These facts directly confirmed that the glucosyl moiety on these novel cytokinins is linked to the ribosyl group of the molecule. Likewise, the MS/MS spectrum of iPAx also confirmed that the glucosyl is attached to the ribosyl group of the molecule (Fig. 2.16). Although it was clear that a hexose was cleaved from ZROG,

DZROG, ZRx, DZRx and iPAx (Figs. 2.9, 2.10, 2.14, 2.15 and 2.16), the hexose was not charged under the conditions used. In fact, no sugar ion was observed under the conditions used in this work.

According to the results derived from the immunoaffinity isolation (Fig. 2.2), RIA (Fig. 2.11), enzyme treatments, HPLC techniques (Fig. 2.12 and 2.13), and ES MS/MS (Fig. 2.14 - 2.16), I propose the structures of the three novel cytokinin glucosides as ZR-G, DZR-G and iPA-G as shown in Fig. 2.17. Although the exact linkage position between the glucosyl and the ribosyl group in the three novel glucosides is yet to be determined, it is likely to be a glucosyl-(1→3)-ribosyl linkage as Auer and Cohen (1993) reported such a linkage in the novel glucoside of benzylaminopurine riboside in other plants. ZR-G is most likely to be the same compound as that found by previous researchers in *P. radiata* (Taylor et al., 1984) and in *Pseudotsuga menziesii* (Doumas et al., 1986; Morris et al., 1990). However, DZR-G and iPA-G have not been reported before in any species.

It is most interesting that, although abundant novel cytokinin glucosides have been detected in the buds of *P. radiata*, no traditional *O*-glucosides were detected, indicating *P. radiata* may have a different enzymatic system for the conjugation of cytokinins to glucose. More data are needed to determine whether this metabolism phenomenon is widely present in other plant species. Previously, the novel glucoside of benzylaminopurine riboside was reported in *Gerbera jamesonii* (Horgan, 1985; Blakesley et al., 1990) and petunia (Auer and Cohen, 1993) and the similar novel glucoside of ZR was reported in radiata pine (*Pinus radiata*) (Taylor et al., 1984) and Douglas fir (*Pseudotsuga menziesii*) (Morris et al., 1990), indicating that this particular enzymatic system may be widespread.

Cytokinin nucleotides are believed to be very important in cytokinin metabolism (Letham and Palni, 1983; Jameson, 1994). Unfortunately, those working on cytokinins in conifers (Taylor et al., 1984; Doumas et al., 1986; 1989; Morris et al., 1990; Bollmark et al., 1995; Chen et al., 1996; Moritz and Sundberg, 1996) have also ignored the nucleotide cytokinins. In this work, zeatin nucleotide, dihydrozeatin nucleotide and

isopentenyladenine nucleotide have been detected (Fig. 2.11B). Interestingly, treatment of phosphorylated cytokinins released the three novel cytokinin glucosides (Fig. 2.11B), indicating that the phosphorylated forms of these novel cytokinins may also play important roles in the growth and development of *P. radiata*. The proposed linkage of the phosphate groups on these molecules is shown in Fig. 2.17; the exact position of the phosphate on the sugar groups is yet to be determined. This work clearly indicates that not only cytokinin nucleotides but other forms of phosphorylated cytokinins are also important and must be included in any cytokinin analysis.

Chapter 3 Cytokinins and phase change in *Pinus radiata*

3.1 Introduction

Data presented in Chapter 2 revealed that there is a wide range of endogenous cytokinins in buds of *Pinus radiata*. The fact that application of the cytokinin, 6-benzylaminopurine (BA), converts mature buds of *P. radiata* cultured *in vitro* from production of mature phase traits to those of juvenile phase traits (Horgan, 1987) strongly indicates that cytokinins may be a key factor in the regulation of phase change in this species. Therefore, a quantitative analysis of endogenous cytokinins in buds from trees of different maturation status as well as a study of the metabolism of exogenous cytokinin during the "rejuvenation" period of mature buds *in vitro* was carried out to provide information on the involvment of cytokinins in phase change in *P. radiata.*

Four years old *P. radiata* trees can produce both buds with juvenile characteristics (i.e. bearing primary needles) and buds which do not produce primary needles but produce the bud scales characteristic of mature trees. For convenience in this thesis, the apparently juvenile buds from four-year-old trees are referred to as "juvenile" buds (J4) and the apparently mature buds as "mature" buds (M4). The term "rejuvenation" is used to describe the morphological fact that exogenous cytokinin induces mature buds to produce juvenile characteristics (i.e. primary needles) under experimental conditions *in vitro.*

3.2 Materials and methods

3.2.1 Cytokinin analyses of field-grown buds

All buds collected from trees in the field were collected on the same date (May 9, 1995) from seedling, four-year-old and eight-year-old *Pinus radiata* trees growing at the New Zealand Forest Research Institute (NZFRI) nursery, Rotorua, New Zealand. Two replicate bud samples were always taken to represent each age group of trees. In the case of juvenile buds from seedlings, the terminal buds were collected from about 100 seedlings. To provide samples of mature buds from eight-year-old trees, 24 individual

trees in one block were selected and the terminal buds were harvested from the secondary branches in the upper crowns of the trees. Due to the fact that four-year-old trees produce both juvenile and mature buds, 25 four-year-old trees were selected, and both "juvenile" and "mature" buds were collected from secondary branches in the upper crowns of these trees.

In all cases, buds for cytokinin analyses were excised from the trees and put into pre-chilled (-20°C) modified Bieleski's solvent (Jameson et al., 1987) contained in 250 mL polypropylene bottles immediately after primary needles (in the case of juvenile buds) or bud scales (in the case of mature buds) were peeled off. The harvested buds in the Bieleski's solvent (approximately 5 mL Bieleski's solvent g^{-1} FW) were stored at -20°C until required, but a minimum of three days. The exact weight for each sample was calculated according to the difference in the weight of the tubes before and after the sample was put in. Around 6 g of buds were collected for every sample except for the "juvenile" buds from four-year-old trees. Because this material was limited, each of the replicate samples in this case was 1.7g. The buds excised from the trees were randomly allocated across the two replicates.

3.2.2 Tissue culture material for cytokinin analyses

The tissue culture system for *P. radiata* developed at the NZFRI (Horgan, 1987) was used to investigate cytokinin-induced "rejuvenation" in this work. Buds collected from the same 24 individual eight-year-old mature trees as described in Section 3.2.1 were surface-sterilised in 500 mL 50% bleach (v/v) for 15 min with constant stirring on a magnetic stirrer. The buds were subsequently rinsed three times in a large volume of sterile water (at least 300 mL each time). The bud scales were then peeled off and the buds cross-sectioned into 5 mm fragments. The bud fragments were subsequently placed onto modified Le Poivre (LP) media (Horgan, 1987, Appendix B) with either 5.0 mg/L 6-benzylaminopurine (BA) (medium LP5) or without addition of exogenous cytokinin (medium LPCH). The plant material was cultured for the first 30 d in petri dishes in a growth room at the NZFRI with a 24°C/20°C day/night temperature regime, a 16 h photoperiod and light ~~tensity~~ intensity of *ca.* 60 $\mu Em^{-2} s^{-1}$.

After 30 d in culture, around 10 g of two replicate samples representing BA-treated (LP5) and non-cytokinin treated (LPCH) materials were harvested into pre-chilled (-20°C) Bieleski's solvent and kept at -20°C for at least 3 d prior to cytokinin extraction. The remaining bud fragments which had been maintained on LP5 and LPCH media for the 30 d were further subcultured onto fresh LP5 and LPCH media respectively. After a further 20 d in culture in a Sanyo growth chamber at Massey University, approximately 3.0 g samples with two replicates were harvested into pre-chilled (-20°C) Bieleski's solvent and kept at -20°C for at least 3 d.

3.2.3. Bud material for morphological studies

Buds from seedlings, four-year-old and eight-year-old trees (as described in Section 3.2.1) were collected for morphological comparisons. To analyse the morphology of the buds produced by the needle fascicle meristems of mature eight-year-old trees, the terminal buds on some of the secondary branches in the upper crown of the trees were removed and the emerging buds from the bases (fascicle meristems) of the secondary needle clusters were collected approximately six months later.

To determine the time course of morphological changes at the cellular level during tissue culture, mature buds derived from the eight-year-old trees were cultured as described in Section 3.2.2. The bud fragments cultured on BA-containing and cytokinin-free media were sampled at days 0 (field buds), 3, 9, 15 and 25 and fixed in formalin-alcohol-glacial acetic acid (FAA, 5:90:5 v/v/v). The bud fragments which had been cultured on BA-containing or cytokinin-free media for 30 days were further subcultured for a further 20 days on either BA-containing or cytokinin-free media respectively. These subcultured buds were then harvested and fixed in FAA.

3.2.4. Light microscopy

After fixation, all the buds were washed with 70% ethanol for 5 min. and then placed in fresh 70% ethanol for 30 min. Dehydration was carried out using Johansen's ethanol/ tertiary butyl alcohol (TBA) method (Johansen, 1940) by transferring the buds through an 85% (TBA/95% ethanol/water: 7:10:3 v/v/v) , 95% (TBA/95% ethanol: 11:9 v/v) and 100% (TBA/100% ethanol: 15:5 v/v) ethanol/TBA series for 2 h in each. The buds

were then transferred to vials containing 100% TBA and placed in an oven (56°C) for 36 h, with changes of TBA approximately every 12 h. The buds were subsequently transferred from TAB to TAB/paraffin oil (50:50 v/v) for 1 h, the buds then transferred together with some TBA/paraffin liquid onto solid wax in prepared vials. After 2 h, the buds were transferred to fresh melted wax and allowed to remain in pure wax over a period of 24 h at 60°C with three fresh wax changes. Finally, the buds were embedded in wax in the desired orientation and sectioned at 8 μM using a Leica rotary microtome. The wax ribbons were mounted on glass microscope slides using 10% P.V.A. White Resin Glue (National Starch & Chemical NZ Ltd). Wax was removed from sample sections on glass slides by immersing the slides in xylol for 10 min. The glass slides were double stained with safranin and fast green. The double stained sections were examined under a Zeiss bright field microscope and photographs were taken using Tungsten Fujichrome film.

3.2.5. Cytokinin analyses

For quantification of cytokinins in the field and tissue cultured materials, the extraction, purification, separation and detection of individual endogenous cytokinins were carried out according to the procedures described in Chapter 2.

Because of the use of BA during tissue culture, the concentrations of BA and its derivatives in the cultured materials were also analysed. Consequently, the retention times on C_{18} HPLC column of BA and its known derivatives (BA, BA riboside, BA3G, BA7G, BA9G and BA riboside nucleotide) were determined using the C_{18} HPLC protocol described in Section 2.3.3.2. Cross-reactivities of BA and its derivatives with clone 12 and clone 16 were also determined with the standard RIA protocols described for cytokinin standard curves in Section 2.5.1.

For cytokinin quantification, the differential cross-reactivities of Clone 12 or Clone 16 with individual cytokinins, including BA and its derivatives, were integrated into the calculations for concentrations of the individual forms. However, no correction for cross reactivity could be made for the novel forms of the cytokinins described in Chapter 2 due to a lack of standards.

3.3 Results

3.3.1 Changes in bud morphology during phase change in field-grown trees

During growth and development of *P. radiata*, bud and leaf morphology changes dramatically from the juvenile phase to the mature phase. As displayed in Fig. 3.1A, seedling buds produce primary needles which are photosynthetically functional single leaves. Needle fascicle primordia are found in the axil of some of the primary needles. When trees develop to full maturity (eight-year-old trees), the buds of the mature trees become tightly contained in scales without any photosynthetically functional primary needles being produced; the buds generate only needle fascicles which are made up of a bundle of three secondary needles surrounding a normally quiescent meristem (Fig 3.1B). *P. radiata* trees at the age of four years can apparently give rise to both "juvenile" (Fig. 3.1C, the buds appearing morphologically similar to the seedling buds) and "mature" (Fig. 3.1D, the buds appearing morphologically similar to the mature buds of the eight-year-old trees) buds. While the apparently "juvenile" buds initiated large numbers of primary needles, these needles were usually much shorter (<1.5 cm) than the primary needles generated by seedlings (around 3.0 cm).

The features of mature buds were very stable in the fully matured trees (eight years old). When the terminal buds of the secondary branches of the fully matured 8-year-old trees were removed, the fascicle meristems in the bases of secondary needle clusters on the portions of the branches below the terminal buds were released from apical dominance and grew out. These elongating axillary buds (Fig. 3.1E) were morphologically the same as the mature terminal buds of the eight-year-old trees from which they came (Fig. 3.1B and E).

More detailed examination using light microscopy revealed that mature buds from the field could actually initiate primordia equivalent to primary needle primordia. However, instead of developing into primary needles, these primordia developed into scales. The epidermal cells of these scale-bound primordia were stained by safranine (Fig. 3.3M). After about five scales had differentiated, the axillary meristems of the mature buds started to differentiate secondary needle primordia (Fig.3.3A and B).

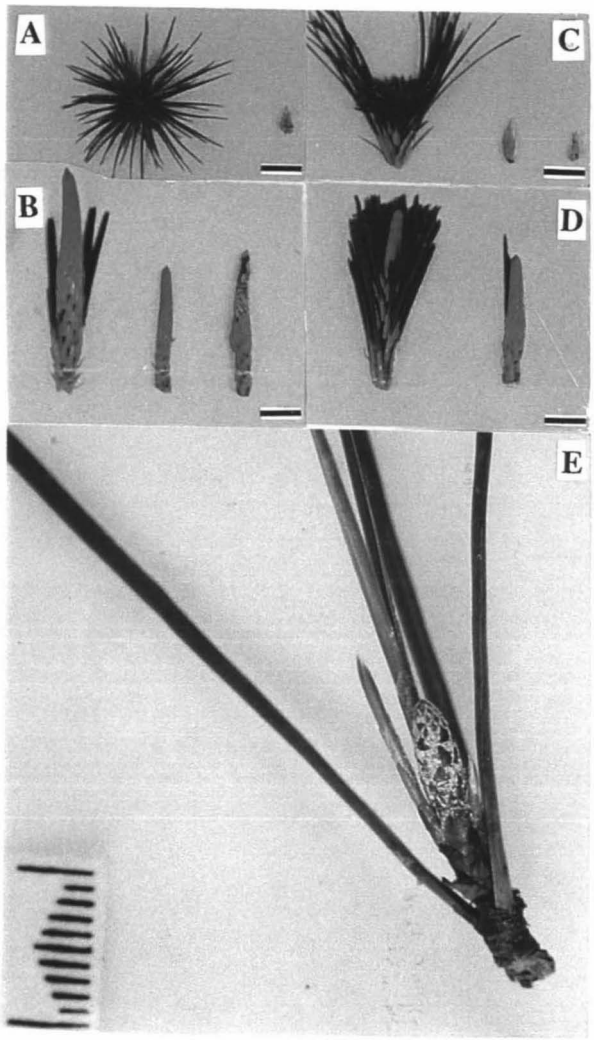


Figure 3.1 Morphology of buds from juvenile and mature phases in *P. radiata*. A: juvenile buds from one-year-old seedlings (S); B: mature buds from eight-year-old trees (M8); C: "juvenile" buds from four-year-old trees (J4); D: "mature" buds from four-year-old trees (M4); E: mature bud emerging from the base of a needle fascicle following removal of the terminal buds from a branch of an eight-year-old mature tree. The bars represent 1 cm.

3.3.2. Changes in bud morphology during cytokinin-induced "rejuvenation" *in vitro*

Although the features of mature buds were very stable in the field, it had been observed that *in vitro*, in the presence of BA, mature buds "rejuvenated" and produced juvenile bud morphology (Horgan, 1987). A detailed time course study was begun to examine the morphological and anatomical changes occurring in mature buds in tissue culture on cytokinin-containing medium. Mature buds on cytokinin-free medium were used for the purposes of comparison.

During the first 30 days of culture, treatment with BA caused the mature bud fragments to initiate primary needles from the axillary meristems in the bases of needle fascicles. However, the initiated primary needles were not able to elongate if BA was continuously present in the culture medium (Fig. 3.2A). Exogenous BA not only caused primary needle initiation from the mature buds but it also inhibited secondary needle development *in vitro*. BA completely prevented secondary needle primordia developing into secondary needles and caused elongating secondary needles to abort. When the mature bud fragments which had been cultured on cytokinin-containing medium for 30 days were transferred onto cytokinin-free medium, the initiated primary needles started to elongate to become fully developed primary needles (Fig.3.2B).

However, without BA, the mature buds *in vitro* continued to display mature features such as the secondary needle primordia developing into secondary needles without primary needle production (Fig. 3.2C). In addition, it was observed that, after the secondary needles were fully developed, the fascicle meristems sitting in the bases of the secondary needle clusters started to generate axillary buds. These buds were also contained in scales without developing into primary needles. This observation was in agreement with what had been observed in mature trees (Fig. 3.1E). However, if the mature buds were transferred from cytokinin-free medium onto cytokinin-containing medium after the secondary needles were fully developed, primary needles were then initiated by the meristems in the bases of those fully developed secondary needle clusters (Fig. 3.2E).

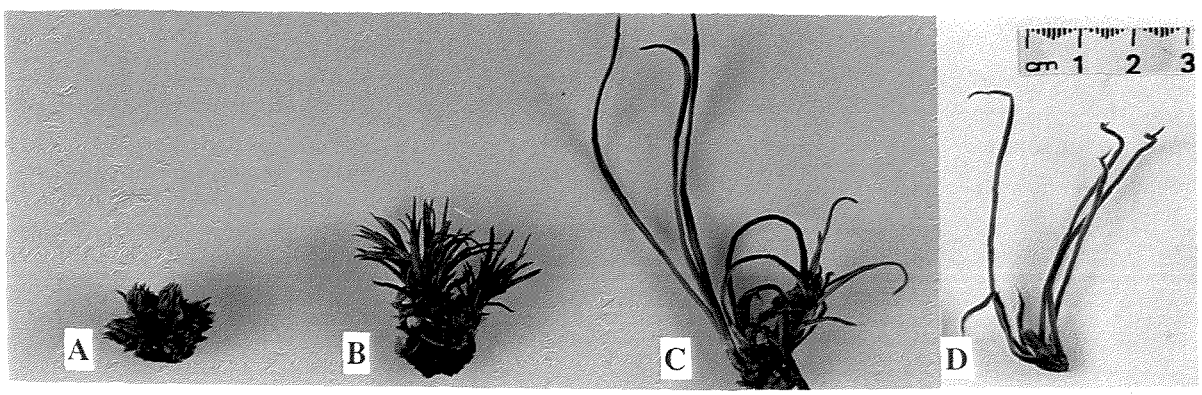


Figure 3.2 Changes in morphology of eight-year-old mature buds under different cytokinin treatment regimes. A: bud cultured on medium containing 5.0 mg/L BA for 30 days followed by subculture onto identical fresh medium for a further 20 days; B: bud cultured on medium containing 5.0 mg/L BA for 30 days followed by subculture onto cytokinin-free medium for a further 20 days; C: bud cultured on cytokinin-free medium for 30 days followed by subculture onto fresh cytokinin-free medium for a further 20 days; D: bud cultured on cytokinin-free medium for 30 days followed by subculture onto medium containing 5.0 mg/L BA for a further 20 days.

Even more detailed information is revealed using anatomical examination. In tissue culture on cytokinin-free medium, secondary needle primordia continue to complete their preset developmental program. The elongation of secondary needle primordia is shown in Fig. 3.3B, D, F and H. Although the secondary needle primordia developed rapidly to form fully elongated secondary needles, the fascicle meristems in the bases of the secondary needle primordia were observed to remain quiescent over the initial 25 days in culture in the absence of BA (Fig. 3.3B - H). However, when the mature bud fragments were subcultured onto the same medium (i.e. without BA) for a further 20 days (i.e. total 50 days on cytokinin-free medium), some of the previously quiescent fascicle meristems began to develop. As shown in Fig. 3.3J, the meristems initiated primary needle primordia, but these needle primordia did not elongate. Instead, they formed curve-shaped leaves, covering the meristems. The most striking feature observed in these primary needle primordia was that their epidermal cells were heavily stained by safranine which is similar to that which had been observed in the scale primordia in the mature field buds (Fig. 3.3M).

The developmental events for the mature buds cultured in the presence of BA were found to be very different from those described above for mature buds cultured in the absence of BA. From Day 3, the anatomical features of the mature bud fragments cultured on BA began to show differences compared to those without BA (Fig. 3.3 A and B). In the presence of BA, it was observed that secondary needle primordia stopped elongation at Day 3 (Fig. 3.3A). When the buds cultured on BA reached Day 9, the secondary needle primordia could no longer be observed in the mature bud sections. Instead, numerous primary needles were being initiated from enlarged meristems (Fig. 3.3C). The bud fragments were continuing to initiate primary needles at days 15 and 25 (Fig. 3.3E and G), but the elongation of the primary needles was largely retarded. Although numerous primary needles were produced, a typical apical meristem structure was rarely observed in the microtomed sections of the buds cultured with BA after 25 days (Fig. 3.3L). However,when the mature buds which had been cultured on cytokinin-containing medium for 30 days were subcultured onto cytokinin-free medium for another 20 days, the retarded primary needles vigorously underwent elongation and an apical meristem dome was produced similar to the apical meristem structure of a seedling (Fig. 3.3I; Riding, 1972). When the mature buds which had been cultured on

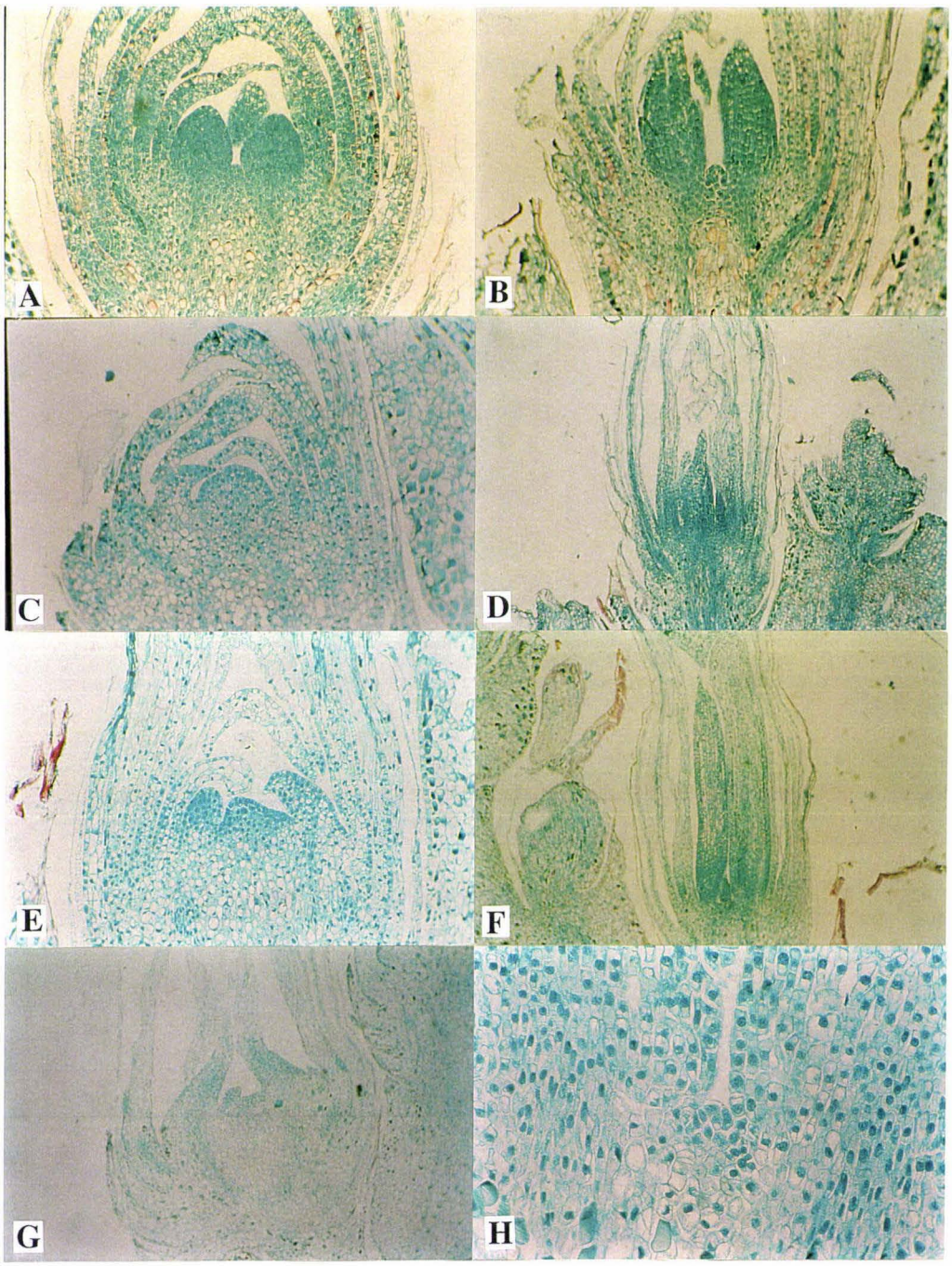


Figure 3.3 See next page for caption.

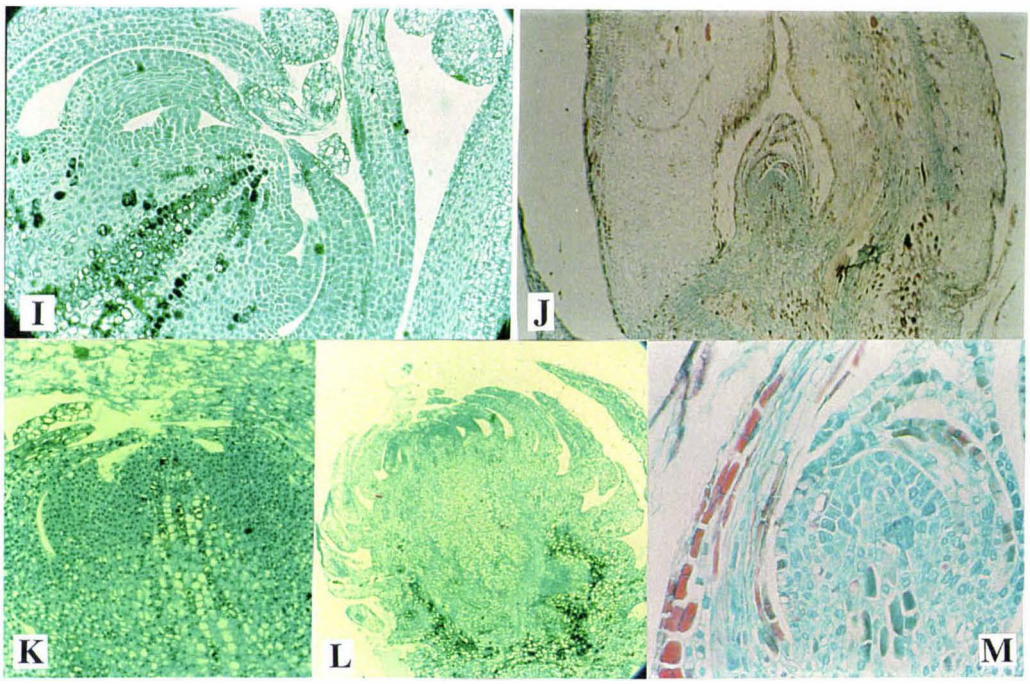


Figure 3.3 Anatomical changes in fascicle meristems and needle primordia of mature buds during culture with 5.0 mg/L BA or without exogenous cytokinin. A: bud cultured with BA for 3 d, x191; B: bud cultured without cytokinin for 3 d, x191; C: bud cultured with BA 9 d, x191; D: bud cultured without cytokinin for 9 d, x76.4; E: bud cultured with BA for 15 d, x191; F: bud cultured without cytokinin for 15 d, x76.4; G: bud cultured with BA for 25 d, x76.4; H: bud cultured without cytokinin for 25 d, x477.5; I: bud cultured with BA for 30 d followed by subculture without cytokinin for a further 20 d, x122.4; J: bud cultured without cytokinin for 30 d followed by subculture onto fresh cytokinin-free medium for a further 20 d, x305.6; K: bud cultured without cytokinin for 30 d followed by subculture onto medium containing BA for a further 20 d, x305.6; L: bud cultured with BA for 30 d followed by subculture onto fresh medium containing BA for a further 20 d, x76.4; M: fascicle meristem and needle primordia in a field-grown bud from an eight-year-old mature tree, x477.5.

cytokinin-free medium for 30 days were transferred onto BA-containing medium for another 20 days, the fascicle meristems in the bases of secondary needle clusters became significantly enlarged and started to initiate primary needles (Fig. 3.2D and Fig. 3.3K). No accumulation of the safranine-stainable substances was observed in the epidermal cells of the primary needle primordia initiated or elongated from the buds which had been exposed to exogenous cytokinin (Fig. 3.3I, K and L).

3.3.3 Quantitative analyses of cytokinins in field-grown mature and juvenile buds

The identification of the endogenous cytokinins from the buds of *Pinus radiata* is reported in Chapter 2. In this chapter, the changes in concentration of the different forms of endogenous cytokinins are reported.

3.3.3.1. Cytokinin free bases and ribosides: The highest total concentration of cytokinin free base and ribosides was measured in the seedling buds and the lowest was found in the eight-year-old mature buds (Fig. 3.4A). The levels of the total cytokinin free base and ribosides in the four-year-old "juvenile" and "mature" buds were between the two extremes (Fig 3.4A), with the four-year-old "juvenile" buds having a higher total concentration of cytokinin free base and ribosides than the four-year-old "mature" buds (Fig. 3.4A). The concentration of individual cytokinin free base and ribosides in the juvenile and mature buds is shown in Fig. 3.4B and C. ZR and DZR were at a greater concentration in the seedling buds than in the eight-year-old mature buds. The ZR concentration in both the "juvenile" and "mature" buds from the four-year-old trees was also higher than in the mature buds from eight-year-old trees (Fig. 3.4B and C). However, the level of ZR in the "juvenile" buds from four-year-old trees was lower than in the "mature" buds of the same age (Fig. 3.4C). The sampling from the four-year-old trees corresponded to the time when the "juvenile" buds were initiating large numbers of primary needles.

The only free base cytokinin detected in the field buds was iP. Fig. 3.4D sumarises iP concentration in juvenile and mature buds, showing both seedling and the "juvenile" buds had higher concentrations of iP than did the mature buds from either four-year-old or eight-year-old trees. The concentration of iPA was also found to be greater in the

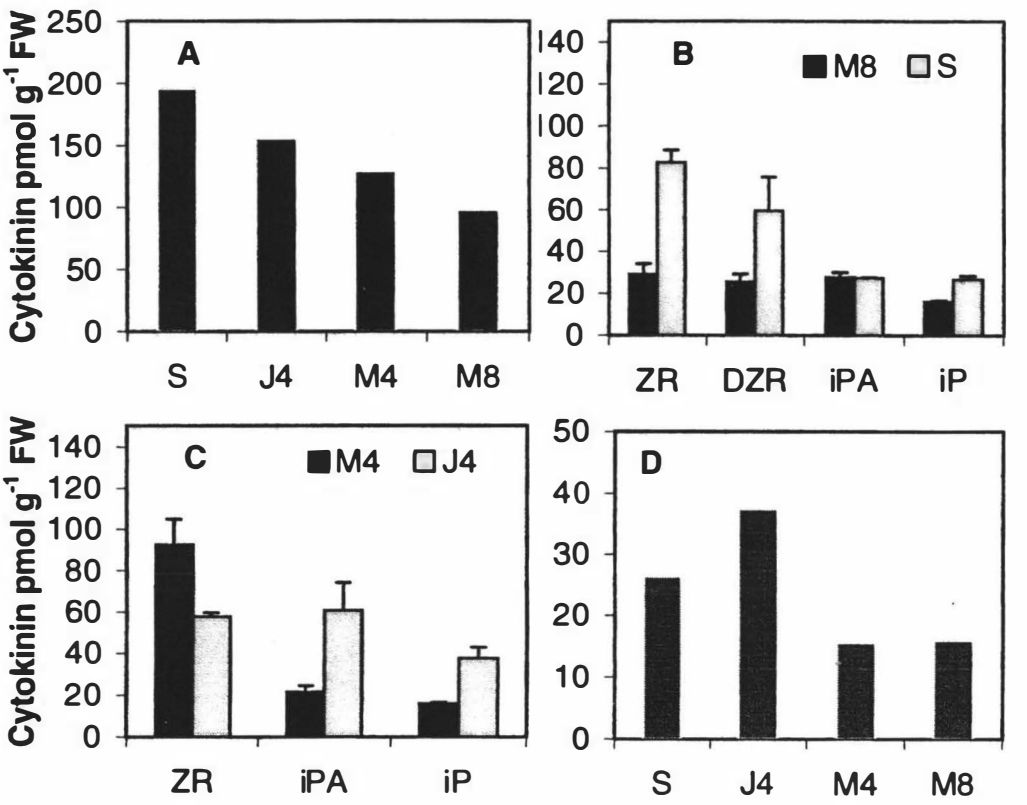


Figure 3.4 Concentration of endogenous cytokinin free base and ribosides in the buds from *P. radiata* trees. A: the total concentration of cytokinin free base and ribosides in the buds of different maturation phases; B: the concentrations of individual cytokinin free base and ribosides in the buds from seedlings and eight-year-old trees; C: the concentrations of free base and ribosides in "juvenile" and "mature" buds from the same plot of four-year-old trees; D: the concentration of cytokinin free base in the buds of different maturation phases. S, one-year-old seedling buds; J4, "juvenile" buds from four-year-old trees; M4, "mature" buds from four-year-old trees; M8, mature buds from eight-year-old trees.

"juvenile" buds (J4) than in the "mature" buds (M4) from four-year-old trees. But, there was no significant difference between the seedling buds and the mature buds from four-year-old and eight-year-old trees.

3.3.3.2. Cytokinin nucleotides: The procedure for analysing the nucleotides is described in Chapter 2, but no corrections for antibody cross reactivities could be made for the novel nucleotides ZR-G-P, DZR-G-P and iPA-G-P due to a lack of standards for these forms.

The total concentration of cytokinin nucleotides was much higher in the mature buds from the eight-year-old trees or the "mature" buds from the four-year-old trees than in the juvenile buds from seedlings or "juvenile" buds from four-year-old trees (Fig. 3.5A). Zeatin nucleotide and the novel phosphorylated form of zeatin riboside glucoside were the most abundant forms of phosphorylated cytokinins in the mature buds from both eight-year-old and four-year-old trees and their levels were very high in these mature buds (Fig. 3.5B and C). However, it was found that the concentrations of these phosphorylated forms were either very low or below detection limits in both seedling buds and "juvenile" buds from four-year-old trees (Fig. 3.5B and C).

Even the less abundant phosphorylated forms of cytokinins such as DZR-P, DZR-G-P and iPA-P also exhibited a higher concentration in the mature or "mature" (M4) buds than in the seedling or the "juvenile" (J4) buds (Fig. 3.5B and C). In fact, the phosphorylated form of iPA glucoside (iPA-G-P) was the only phosphorylated cytokinin detected that had a slightly higher concentration in the "juvenile" buds than in the "mature" buds (Fig. 3.5C).

3.3.3.3 Glucosylated cytokinins: As described in Chapter 2, all cytokinins which had a glucosyl attachment on the molecule differed from the traditional *O*-glucosides. The quantitative measurements of the novel cytokinin glucosides were carried out directly by RIAs without them being converted to their corresponding ribosides after separation on C_{18} HPLC. Due to a lack of standards for these novel cytokinin glucosides, no corrections for cross reactivities of these novel cytokinins with clone 12 and clone 16

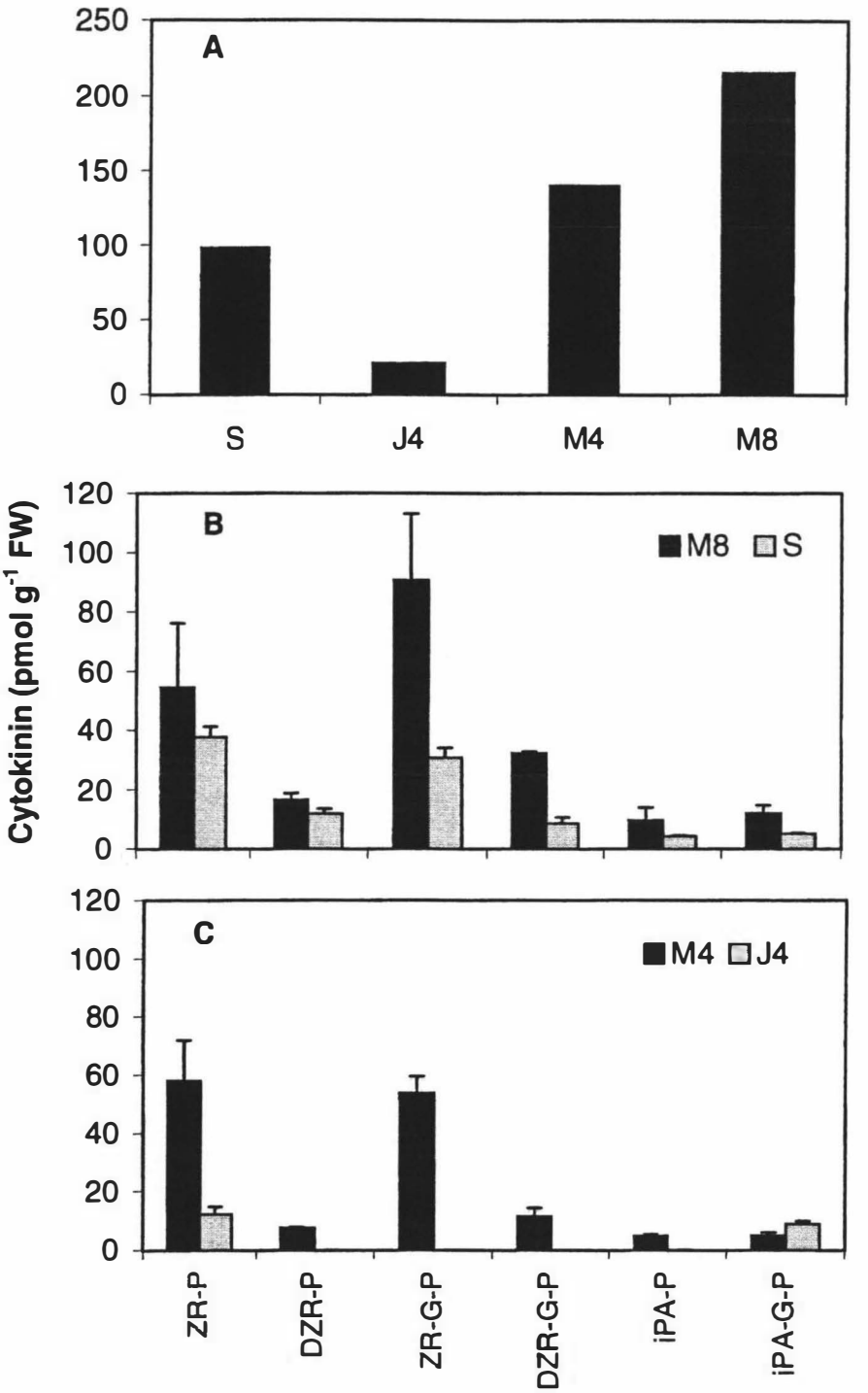


Figure 3.5 The concentration of endogenous phosphorylated cytokinins in the buds from *P. radiata* trees. The values represent the corresponding forms released after alkaline phosphatase treatment of the phosphorylated cytokinins. A: total concentration of phosphorylated cytokinins in the buds of different maturation phases; B: the concentration of individual phosphorylated cytokinins in the buds of seedling and mature trees; C: the concentration of individual phosphorylated cytokinins in the "mature" and "juvenile" buds from the same plot of four-year-old trees. S, seedling buds; J4, "juvenile" buds from four-year-old trees; M4, "mature" buds from the four-year-old trees; M8, mature buds from the eight-year-old trees.

could be made. Therefore, the results listed in the Table 3.1 are not absolute values. However, this does not impact on any conclusions drawn based on the comparisons of these glucosylated cytokinins.

The results in Table 3.1 show that the total levels of novel glucosylated cytokinins were higher in the eight-year-old mature and four-year-old "mature" buds than in the seedling and the four-year-old "juvenile" buds. For individual glucosylated cytokinins, ZR-G and DZR-G displayed the same patterns as those of the total glucosylated cytokinins. In contrast, the concentration of iPA-G was higher in the juvenile buds than in the mature buds, particularly in the case of the "juvenile" buds from four-year-old trees (Table 3.1).

Table 3.1 The concentrations of glucosylated cytokinins in the different types of buds. Where DE error are shown, the values represent the means of two samples.

Bud type	ZR-G	DZR-G	iPA-G	Total
Seedlings (S)	353.9 ± 13.8	16.2 ± 5.1	45.3 ± 8.0	415.4
Juvenile (J4)	291.2 ± 5.5	14.7	54.6 ± 10.9	360.5
Mature (M4)	438.0 ± 23.9	45.2 ± 2.9	30.6	513.8
Mature (M8)	468.3 ± 2.0	37.4 ± 4.3	41.5± 5.2	547.2

3.3.4 Metabolism of BA during cytokinin-induced "rejuvenation" *in vitro*

As described in Section 3.3.2, exogenously applied BA induces the rejuvenation of mature *P. radiata* buds in culture. To help understand how BA is involved in inducing this phase reversion, the metabolism of BA in the buds in culture was analysed.

3.3.4.1. Identification of BA metabolites: Buds cultured in the presence of BA were obtained as described in Section 3.2.2. The extraction, purification and HPLC separation of BA and its metabolites followed the same procedures for endogenous cytokinin analysis as detailed in Chapter 2. During preliminary separation trials on C_{18} HPLC using standard BA and BA derivatives, it was found that the retention times of BA and BA riboside overlapped with iP and iPA respectively. Further, the retention times of BA3G and BA7G almost overlapped with those of zeatin and zeatin riboside respectively. BA9G was well separated from the other BA derivatives and other known

endogenous cytokinins. Retention times for standards of BA and its derivatives on C_{18} HPLC are listed in the Table 3.2. Because the chromatographic properties of BA, BA riboside and BA9G were very similar to iP, iPA and iP-9-G, the internal standard, ^{3}H-iPATA, was used to monitor losses of BA, BA riboside and BA9G during the analysis process.

Due to the overlaps of the retention times of BA and BA derivatives with some of the endogenous cytokinins, cross reactivities of clone 12 and clone 16 with BA and BA derivatives were determined using RIAs as described in Section 2.5.2. The results of cross reactivities of clone 12 and clone 16 with BA and BA derivatives are contained in Table 3.2. No cross reactivity was detected between clone 16 and BA and its derivatives such as BA3G or BA7G (Fig. 3.6 and Table 3.2). Therefore, BA and its derivatives did not influence the measurements of zeatin-like cytokinins with clone 16 antibody. However, strong cross reactivities of clone 12 with BA, BA riboside and BA9G were detected (Fig. 3.6 and Table 3.2). Therefore, clone 12 was employed to detect BA, BA riboside and BA9G as well as iP-like cytokinins. Finally, ES MS/MS as described in Section 2.5.2 was used to confirm the identities of BA and its metabolites extracted from tissue cultured materials.

The bulk fraction which would contain BA, BA riboside plus BA9G collected following HPLC using the amine column (Section 2.3.4.1) was further fractionated through C_{18} HPLC. Six major peaks were revealed by RIAs with clone 12 from the bud fragments exposed to BA for 30 d (Fig. 3.7A). Three of these peaks were tentatively identified as BA9G, BA and BAR by comparison with the retention times of authentic standards. The remaining three peaks were unidentified metabolites, designated as C_2, C_3 and C_4, with retention times of 32.5, 36.5 and 40.0 min. When the bud fragments which had been exposed to BA for 30 d were then subcultured onto the same medium for another 20 d, the patterns of BA metabolites were basically the same except that the peak representing BA9G dropped to an extremely low level and another peak, designed as C_1, at a retention time of 28.5 - 29.0 min. was produced (Fig. 3.7B). When the mature bud fragments were cultured on cytokinin-free medium for 20 d following their 30 d exposure to BA, the pattern of BA metabolites remained similar to that produced by the bud fragments cultured on BA-containing medium for 30 d,

Table 3.2 HPLC retention times and cross reactivity of the two monoclonal antibodies, clone 16 and clone 12, with 6-benzylaminopurine and its derivatives.

Cytokinin	Retention Time (min) on C_{18} HPLC	Cross Reactivity (%) Clone 16	Clone 12
Z	13.29	34	1.7
BA3G	14.41	0	-
BA7G	18.48	0	-
ZR	18.56	100	-
BANT	19.42	0	-
BA9G	26.14	0.6	122.0
BA	33.47	0.4	45.6
BAR	35.24	0.5	78.1
iPA	35.18	-	100
iP	32.88	0	93
iP9G	29.79	-	131

except that the unidentified compound C_4 dropped to a very low level.

The fractions believed to be BA, BAR and BA9G were confirmed using ES MS/MS. In Fig 3.8 A, the molecular ion of 226 m/z corresponds to the molecular mass (225) of BA + H^+. Although iP co-eluted with BA on C_{18} HPLC, no peak at 204 m/z [iP+H^+] was observed in these fractions, indicating the iP level was extremely low if present, at least in comparison with that of BA in these cultured buds. In Fig. 3.8B, the ion peak at 358 m/z corresponds to the molecular mass of BAR + H^+ and the peak at 226 corresponds to the mass of BA plus H^+ which is most likely a daughter ion of the BAR under the MS ionisation conditions used in this work (Fig. 3.8B). Although BAR had the same retention time as iPA, no ion peak corresponding to the iPA molecular mass [336, iPA+H^+] was observed. Fig 3.8 C presents the MS/MS spectrum of the fraction containing BA9G. The ion peak of 388 m/z corresponds to the mass of BA9G + H^+ and the daughter ion at 226 m/z confirms that BA again is a component of the compound with a mass of 388 m/z.

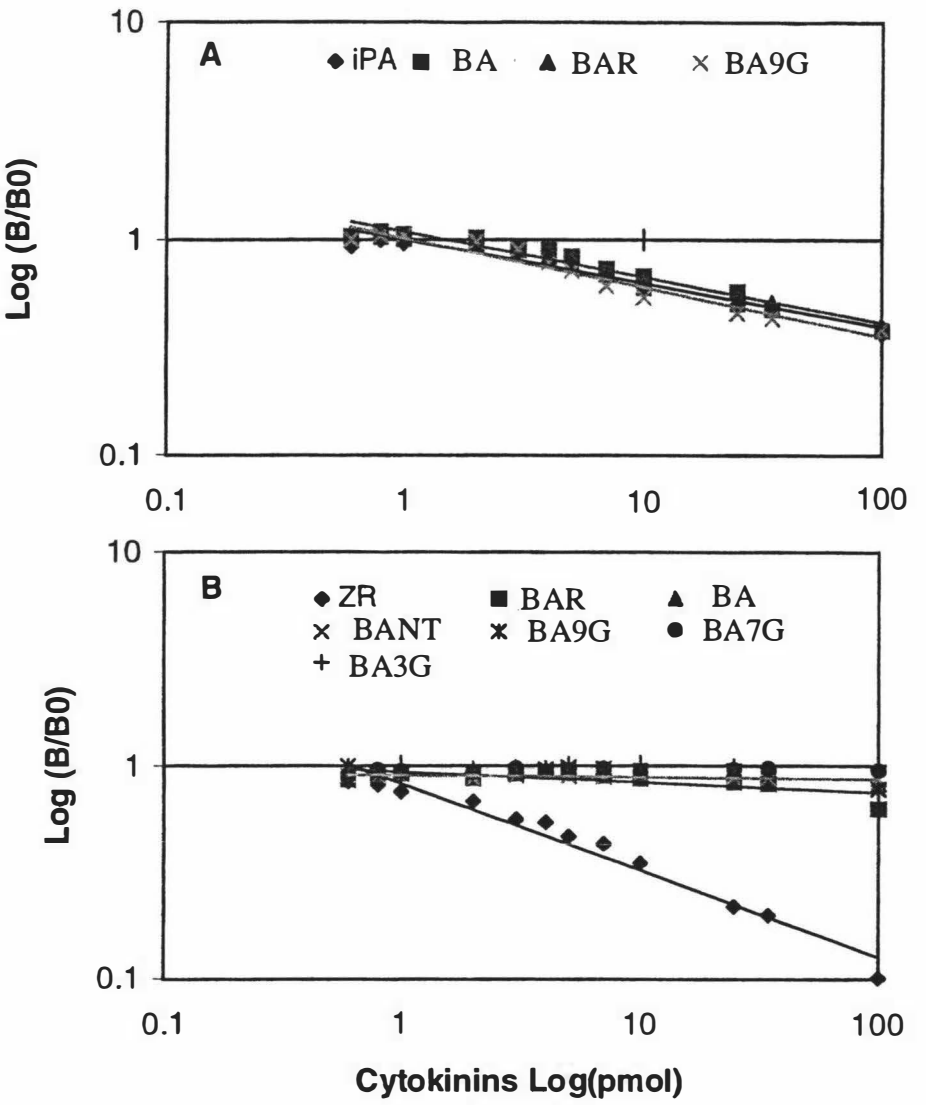


Figure 3.6 Logit transformation curves of the cross reactivity of BA and its derivatives to clone 12 and clone 16. A: cross reactivity against clone 12 antibody; B: cross reactivity against clone 16.

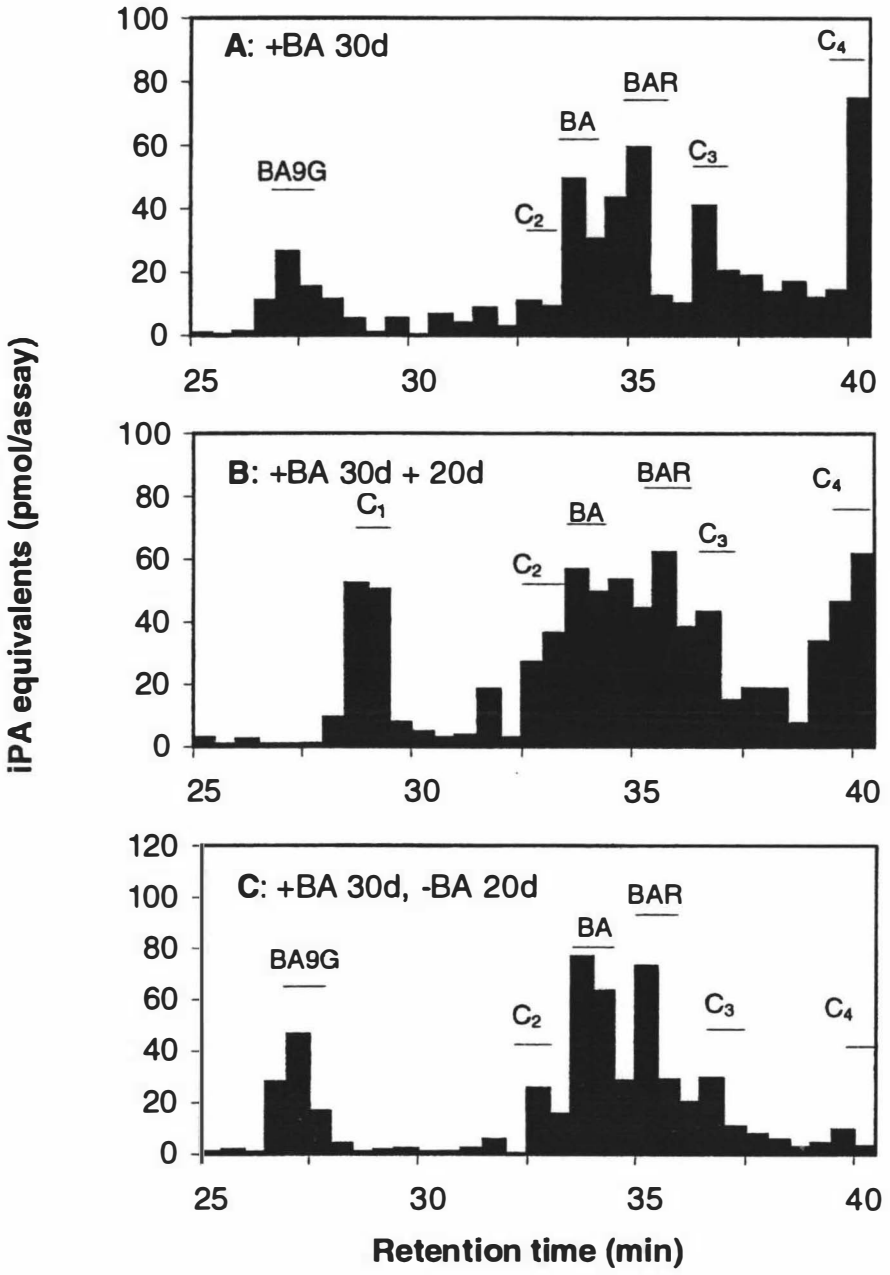


Figure 3.7 The qualitative profiles of BA metabolites (free base, ribosides and 9-glucosides) extracted from the bud fragments cultured under different BA regimes. A: buds from eight-year-old mature trees cultured on BA-containing medium for 30 days; B: buds from eight-year-old mature trees cultured on BA-containing medium for 30 days and subcultured onto fresh BA-containing medium for a further 20 days; C: buds from eight-year-old mature trees cultured on BA-containing medium for 30 days and subcultured onto fresh cytokinin-free medium for a further 20 days.

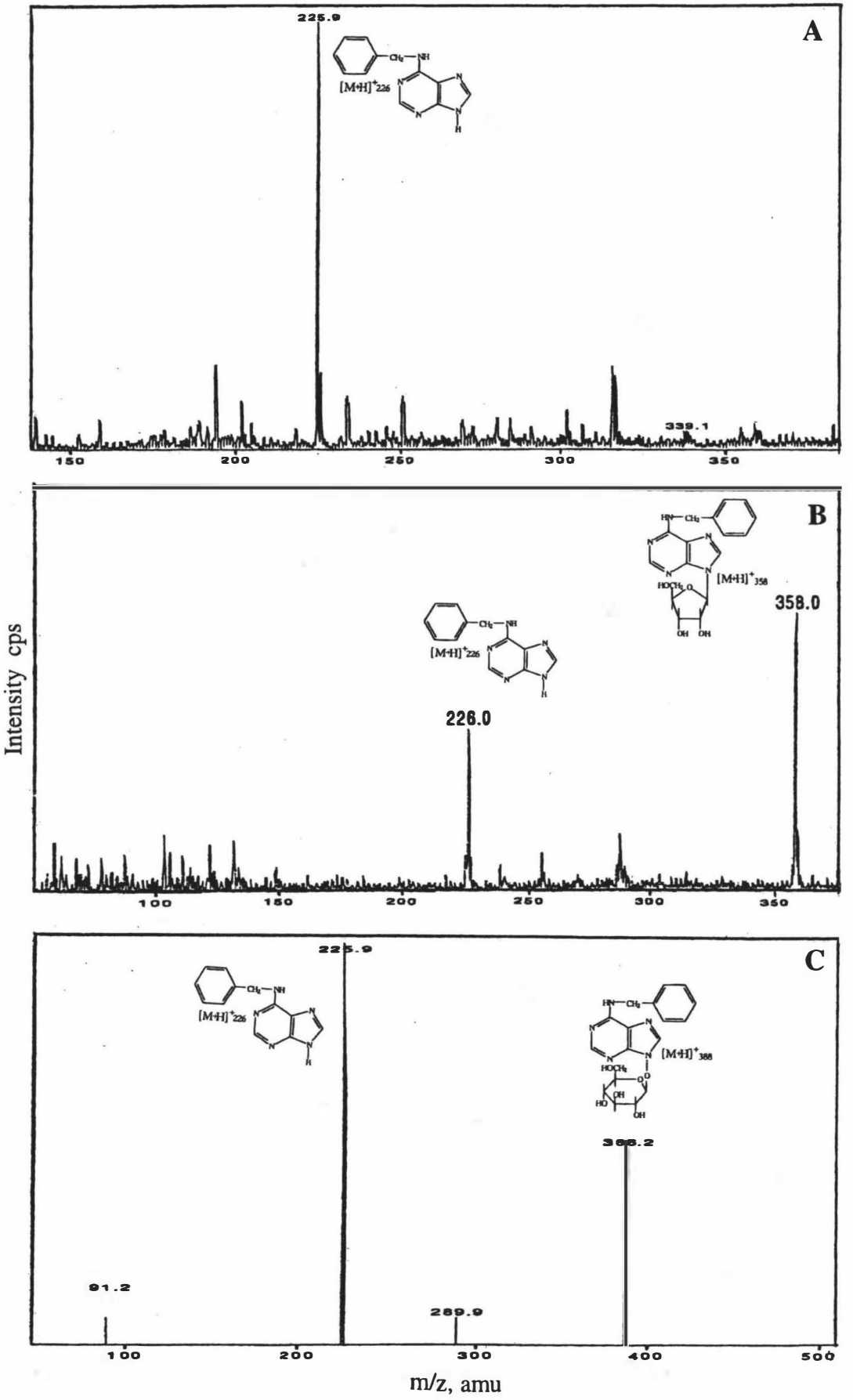


Figure 3.8 Electrospray MS spectrum of 6-benzylaminopurine (A), 6-benzylaminopurine riboside (B) and electrospray MS/MS spectrum of 6-benzylaminopurine glucoside (C).

The bulk fraction of BA glucosides collected using the amine column (Section 2.3.4.1) was further subjected to reverse phase C_{18} HPLC to fractionate individual BA glucosides. Fig. 3.9A displays the C_{18} HPLC-RIA profile of BA glucosides derived from the "rejuvenated" mature buds cultured on BA-containing medium for 30 days while Fig. 3.9B shows the glucoside profiles in the "rejuvenated" mature buds which had been cultured on the medium containing BA for 30 days and subcultured onto fresh cytokinin-free medium for a further 20 days. The buds which had been constantly cultured on BA-containing medium for 30 + 20 days produced a BA glucoside profile similar to that displayed in Fig. 3.9A (data not shown). In all cases, the BA glucoside profiles detected by RIAs using clone 12 antibody showed that four major compounds (C_1, C_5, BAR-G and C_6) were present in the "rejuvenated" buds. The peak marked as C_1 may be the same compound as that designated C_1 in Fig. 3.7B as they had the same retention times on C_{18} HPLC. The unidentified metabolites C_5 and C_6 were observed at a retention time of 31.5 and 35.0 min respectively. One of the most abundant metabolites present in the bulk fraction of glucosides was a novel BA conjugate tentatively designated as BAR-G. The MS/MS spectrum of the fraction containing this novel glucosylated BA riboside is presented in Fig. 3.10. The ion peak at 520 m/z $[M+H^+]$ indicates that the molecular mass of the glucosylated BA riboside is 519. The daughter ions at 226 m/z and 358 m/z correspond to BA and the BA riboside respectively.

This novel glucosylated BA riboside is the same compound as isolated from *Gerbera jamesonii* callus by Blakesley et al. (1990) and *Petunia* tissue cultured explants by Auer and Cohen (1993). Although the novel glucosylated iPA had the same retention time on C_{18} HPLC, the MS spectrum of the fraction displayed only an extremely low peak at 498 m/z which would correspond to the novel iPA glucoside.

Figure 3.11 summarises the structures of identified BA metabolites extracted from buds cultured on BA-containing medium.

Bulk fractions of the phosphorylated BA metabolites were obtained following the procedure described in Section 2.6.2. Subsequently, the bulk fractions containing

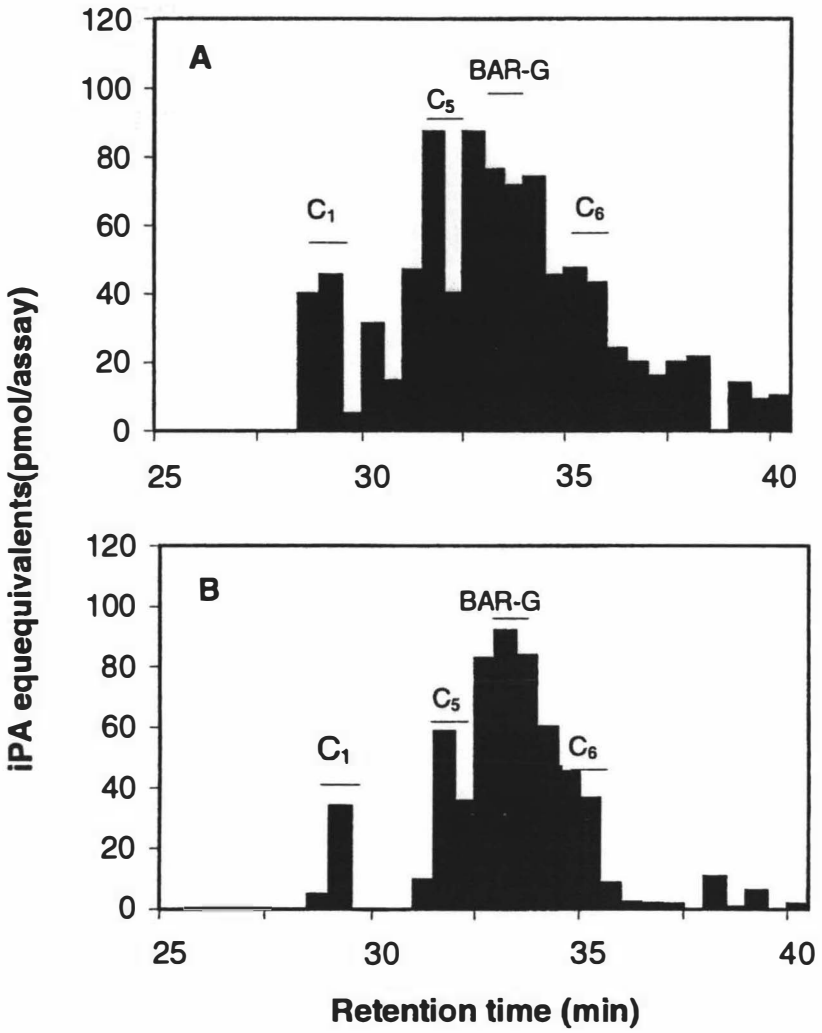


Figure 3.9 The quantitative HPLC-RIAs profiles of BA glucosides extracted from bud fragments cultured on BA-containing medium. A: BA glucoside profile derived from the buds cultured on BA-containing medium for 30 days; B: BA glucoside profile derived from the buds cultured on BA-containing medium for 30 days and subcultured onto fresh cytokinin-free medium for a further 20 days.

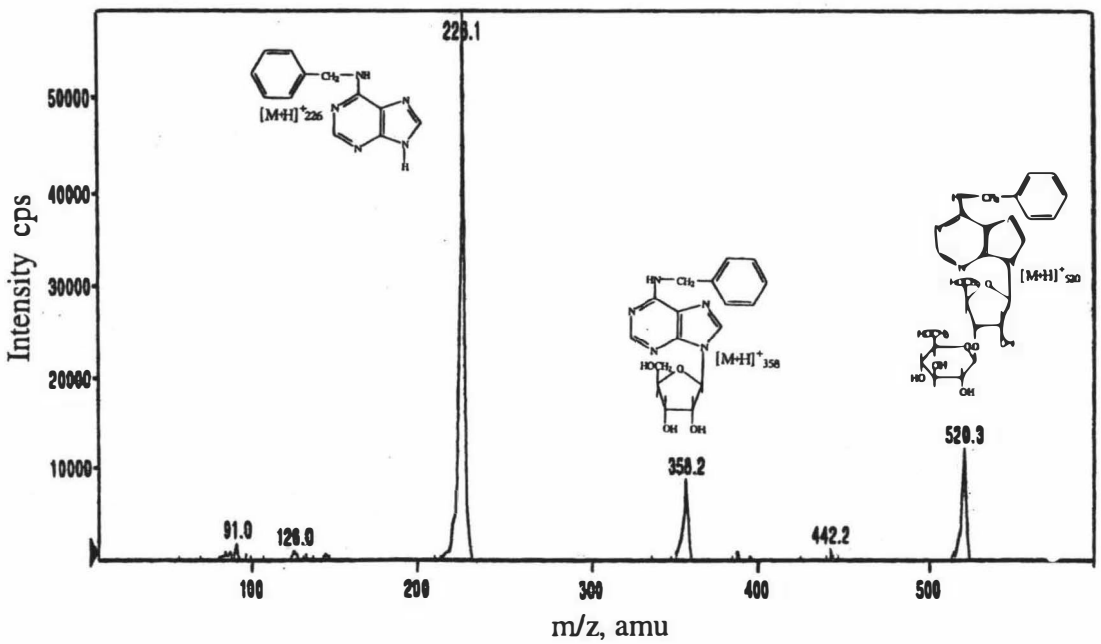


Figure 3.10 Electrospray MS/MS spectrum of 6-benzylaminopurine-9-ribosyl-glucoside BAR-G

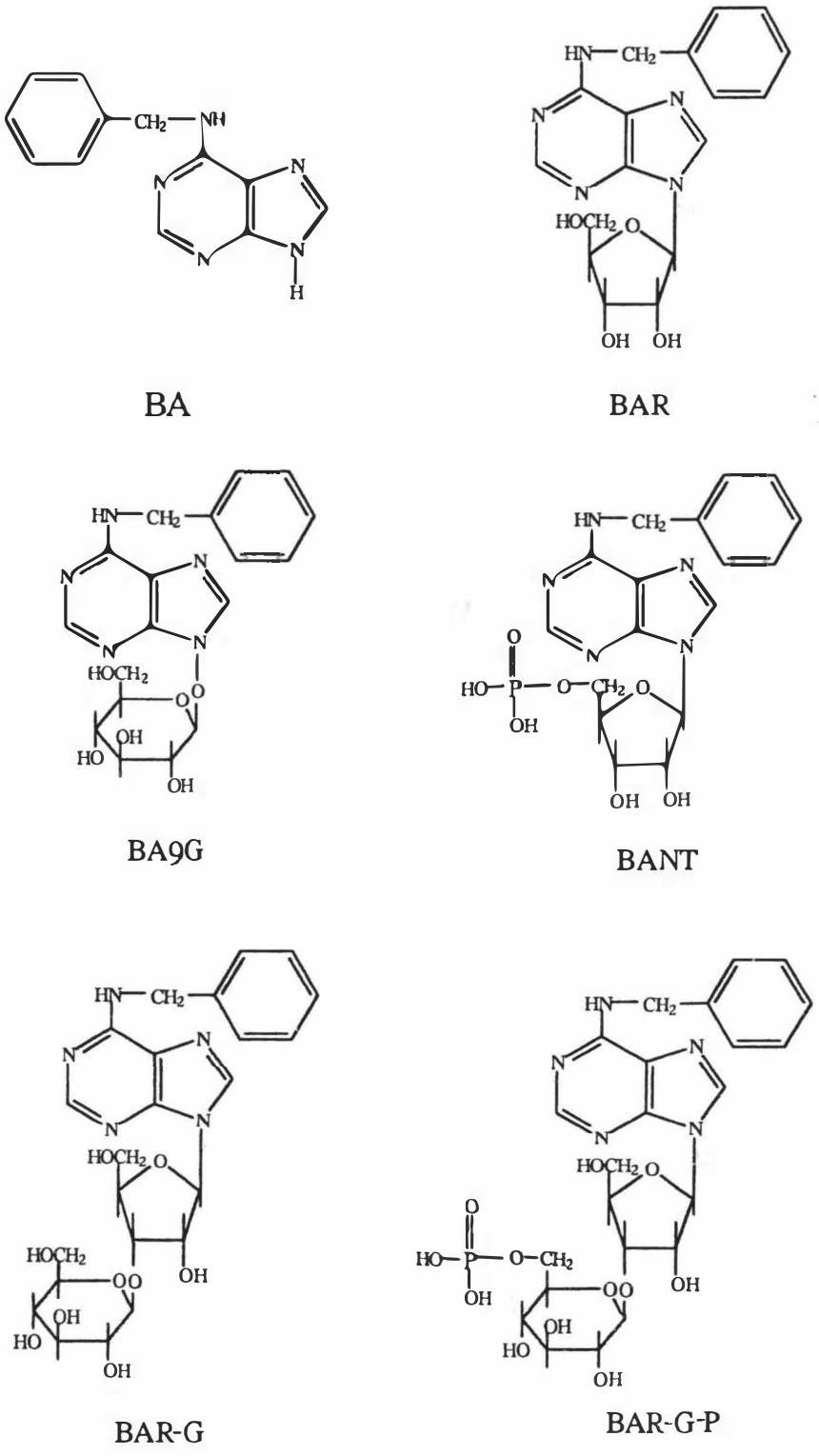


Figure 3.11 Proposed structures of identified BA metabolites derived from buds cultured on medium containing 5.0 mg/L exogenous BA during the "rejuvenation" of mature buds from eight-year-old mature trees of *Pinus radiata*.

phosphorylated BA metabolites were treated with alkaline phosphatase (Section 2.4.1) prior to C_{18} HPLC separation of the released dephosphorylated products. The RIA profiles of the C_{18} HPLC fractions revealed that phosphatase treatment of the bulk nucleotide fraction led to the production of two major compounds in cultured buds exposed to all BA treatment regimes which corresponded to the retention times of BA riboside and the BAR-G on the C_{18} HPLC column (Fig. 3.12A, B and C).

3.3.4.2 Alterations in the concentrations of BA metabolites during "rejuvenation": When BA and its riboside, glucosides, phosphorylated forms and six unidentified metabolites are plotted as percentages of the total metabolite pool in the cultured buds under different BA treatment regimes, patterns of metabolism are revealed (Fig. 3.13A). Although BA was metabolised into many different forms, a relatively high (13.2 %) proportion of BA remained unmetabolised by the bud fragments after 30 d culture on the medium containing 5.0 mg/L BA, even increasing slightly (to 14.2 %) after prolonged culture (30+20 d) on BA. Interestingly, the relative abundance of BA did not decrease but increased to 17.5 % of the whole metabolite pool in the buds cultured for a further 20 days following transfer to cytokinin-free medium after 30 d on BA. The BAR was also abundant in the metabolite pool (12.4%) when the bud fragments were cultured for 30 d on the medium containing 5.0 mg/L BA. However, the abundance of BAR decreased slightly (10.1 %) when the bud fragments were subcultured on the medium containing 5.0 mg/L BA for a further 20 d following 30 d culture on the same medium. The abundance of BAR also decreased (down to 7.3 %) in the bud fragments after subculturing onto cytokinin-free medium for 20 d (following 30 d culture on the medium containing 5.0 mg/L BA). The abundance of BA9G in the metabolite pool was found to be similar in the bud fragments cultured on the medium containing 5.0 mg/L BA for 30 d (5.4 %) or when subcultured onto cytokinin-free medium for 20 d (following the 30 d culture on the medium containing BA) (7.3 %). Surprisingly, prolonged exposure of the bud fragments to BA (30+20 d) resulted in a marked decrease in the abundance of BA9G in the metabolite pool (Fig. 3.13A). The novel conjugate BAR-G also decreased from 22.9 % of the metabolite pool at Day 30 on BA to 15.3 % at Day 30+20 on BA. However, the abundance of BAR-G increased (31.8 %) following

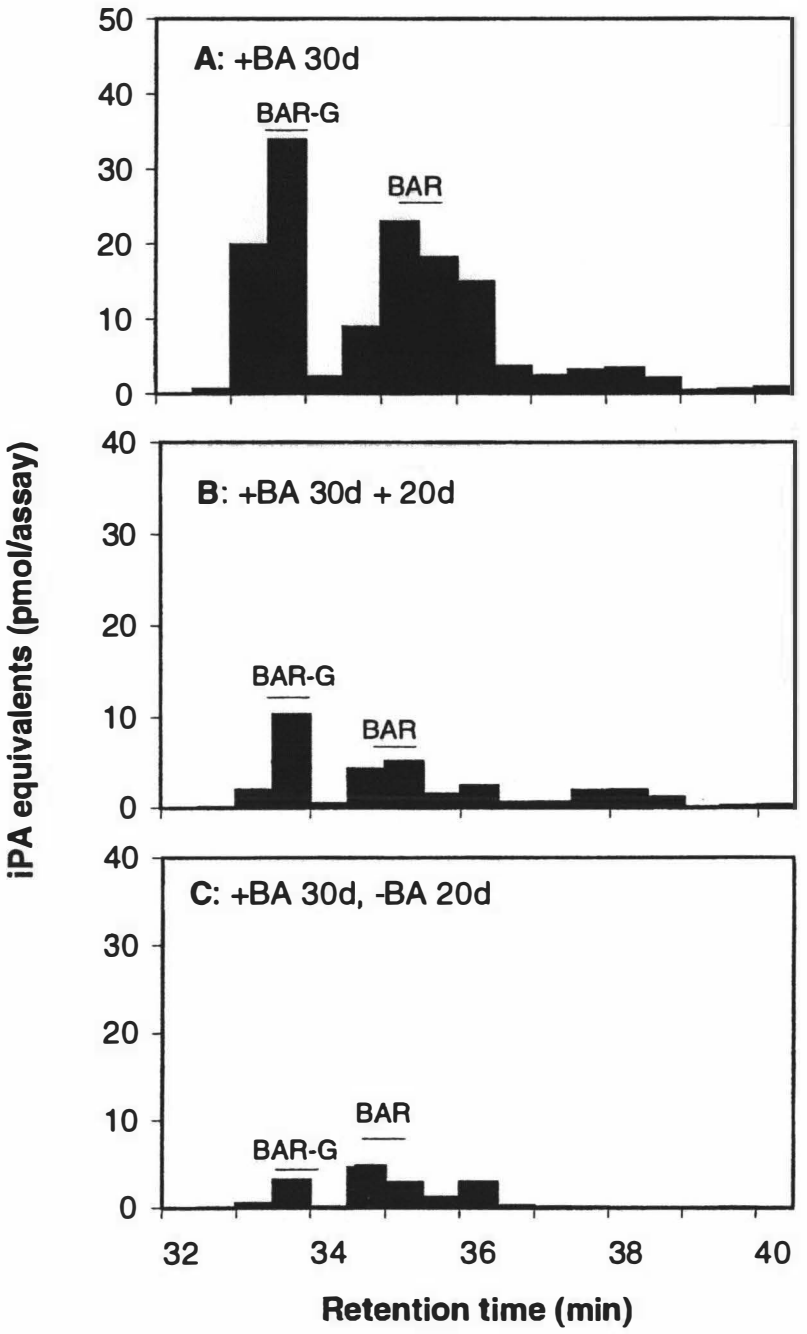


Figure 3.12 The qualitative HPLC-RIAs profiles of phosphorylated BA metabolites extracted from the bud fragments cultured under different BA regimes. The values represent the corresponding forms released after alkaline phosphatase treatment of the phosphorylated BA metabolites. A: buds from eight-year-old mature trees cultured on BA-containing medium for 30 days; B: buds from eight-year-old mature trees cultured on BA-containing medium for 30 days and subcultured onto fresh BA-containing medium for a further 20 days; C: buds from eight-year-old mature trees cultured on BA-containing medium for 30 days and subcultured onto fresh cytokinin-free medium for a further 20 days.

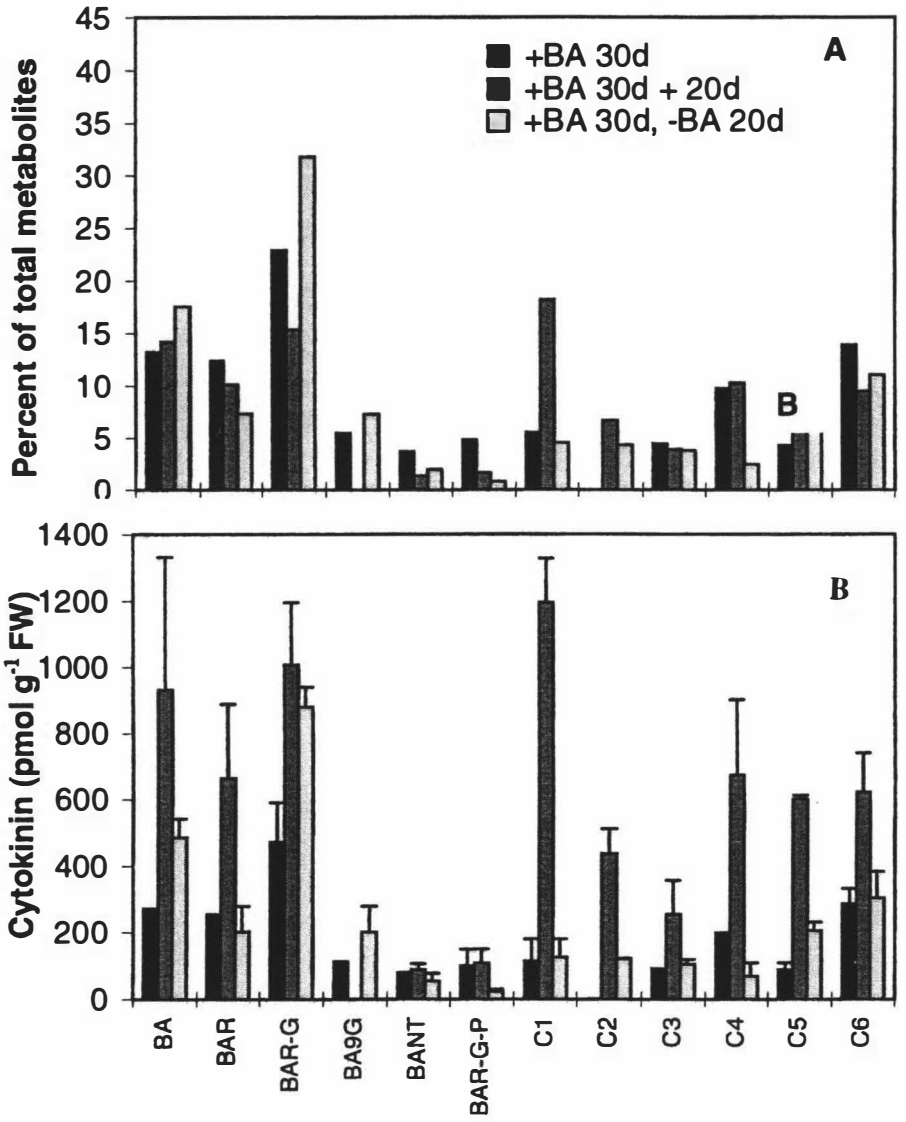


Figure 3.13 Comparisons of individual metabolites of BA extracted from the bud fragments under different exogenous BA regimes in culture. A: abundance patterns of individual BA metabolites in the total metabolite pools extracted from the bud fragments under different BA regimes in culture; B: the concentrations of individual BA metabolites in the bud fragments under different BA regimes in culture.

the withdrawal of BA from the culture medium Fig. 3.13A).

The abundance of BA nucleotides was very low in comparison with the other metabolites of BA, and was below 5.0 % of the total metabolite pool (Fig. 3.13A). Among the unidentified compounds, C_1 increased during prolonged culture on BA-containing medium from 5.5 % at Day 30 on the medium containing 5.0 mg/L BA to 18.2 % at Day 30+20 on the same BA-containing medium. The abundance of C_4 was strongly associated with the presence of an exogenous supply of BA; withdrawal of BA resulted in a marked drop in the abundance of C_4 relative to the total metabolite pool (Fig. 3.13A).

The comparison of the concentrations of BA metabolites under different BA treatment regimes is displayed in Fig. 3.13B. It is important to note that over time a number of bud fragments were dying, but that these fragments were not readily recognised at day 30. These fragments had the effect of diluting the concentration of BA metabolites leading to an apparently lower concentration of BA metabolites in the buds sampled at day 30 than in the buds which were subcultured onto BA-containing medium for 20 d following a 30 d culture on the same medium. With the exception of BA9G, removal of exogenous BA from the culture medium caused the concentrations of BA metabolites within the buds to drop. However, the responses of individual BA metabolites were not all the same. The concentrations of BA and BAR decreased more (47.9.0% and 69.4% respectively) compared to BAR-G which showed only a slight decrease (12.6%) in 20 days after the withdrawal of BA from the medium. Among the unidentified compounds, C_1 and C_4 showed the most marked decrease (89.6% for C_1 and 90.0% for C_4) in concentration in the buds 20 d after removal of BA from the culture media (Fig. 3.13B).

3.3.5 Effect of BA treatment on endogenous cytokinin content

Because of the co-elution of iP, iPA and [9R-G]iPA with BA, BAR and BAR-G respectively, it was not possible to distinguish iP, iPA and [9R-G]iPA from BA, BAR and BAR-G using the clone 12 antibody because it was able to cross-react strongly with both iP-like and BA-like cytokinins (Table 3.2). However, while the MS spectra of the fractions corresponding to the retention times of BA/iP, BAR/iPA and BAR-G/[9R-

G]iPA showed very high peaks corresponding to BA (Fig. 3.8A), BAR (Fig. 3.8B) and BAR-G (Fig 3.8C), these spectra did not reveal any significant peaks corresponding to iP (204 m/z) (Fig. 3.8A), iPA (336 m/z) (Fig. 3.8B) or [9R-G]iPA (498 m/z) (data not shown), indicating that the content of iP-like cytokinins was insignificant compared to BA-like cytokinins in the bud fragments cultured on BA-containing medium.

There was a significant decrease in the concentration of ZR and DZR in the bud fragments exposed to exogenous BA (Fig. 3.14A). After 30 days of culture on BA-containing medium, the content of ZR and DZR in the bud materials dropped 6.2- and 4.9-fold respectively. When the bud fragments were continually subcultured on BA-containing or transferred onto cytokinin-free medium for another 20 days, both ZR and DZR remained at a low level.

Figure 3.14 B and C displays the changes in the concentrations of the novel glucosides ZR-G and DZR-G. ZR-G decreased 5.5-fold in the first 30 days of culture on BA-containing medium. When the bud fragments were exposed to BA for 30 + 20 days or transferred to cytokinin-free medium for another 20 days following a 30 d culture on BA, ZR-G concentration remained low. The novel glucoside DZR-G showed a similar (but less significant) trend to ZR-G (Fig. 3.14 B and C).

Although significant concentrations of endogenous phosphorylated cytokinins were detected in the mature buds of eight-year-old trees (Fig. 3.5B) and, in particular, the concentration of the zeatin nucleotides and phosphorylated ZR-G were very high, no ZR-like phosphorylated cytokinins were detected in the "rejuvenated" buds after 30 days of culture on BA-containing medium (the lower limit of detection in this experimental system was approximately 0.5 pmol). Zeatin nucleotide remained below the detection limit in the subcultured buds with or without exogenous BA application (data not shown).

3.3.6 Changes in endogenous cytokinins in the buds on cytokinin-free medium

When the mature buds from eight-year-old trees were cultured on medium without exogenous cytokinin, the mature buds continued their mature phase, producing only secondary needles. The changes in endogenous cytokinin free base and ribosides during

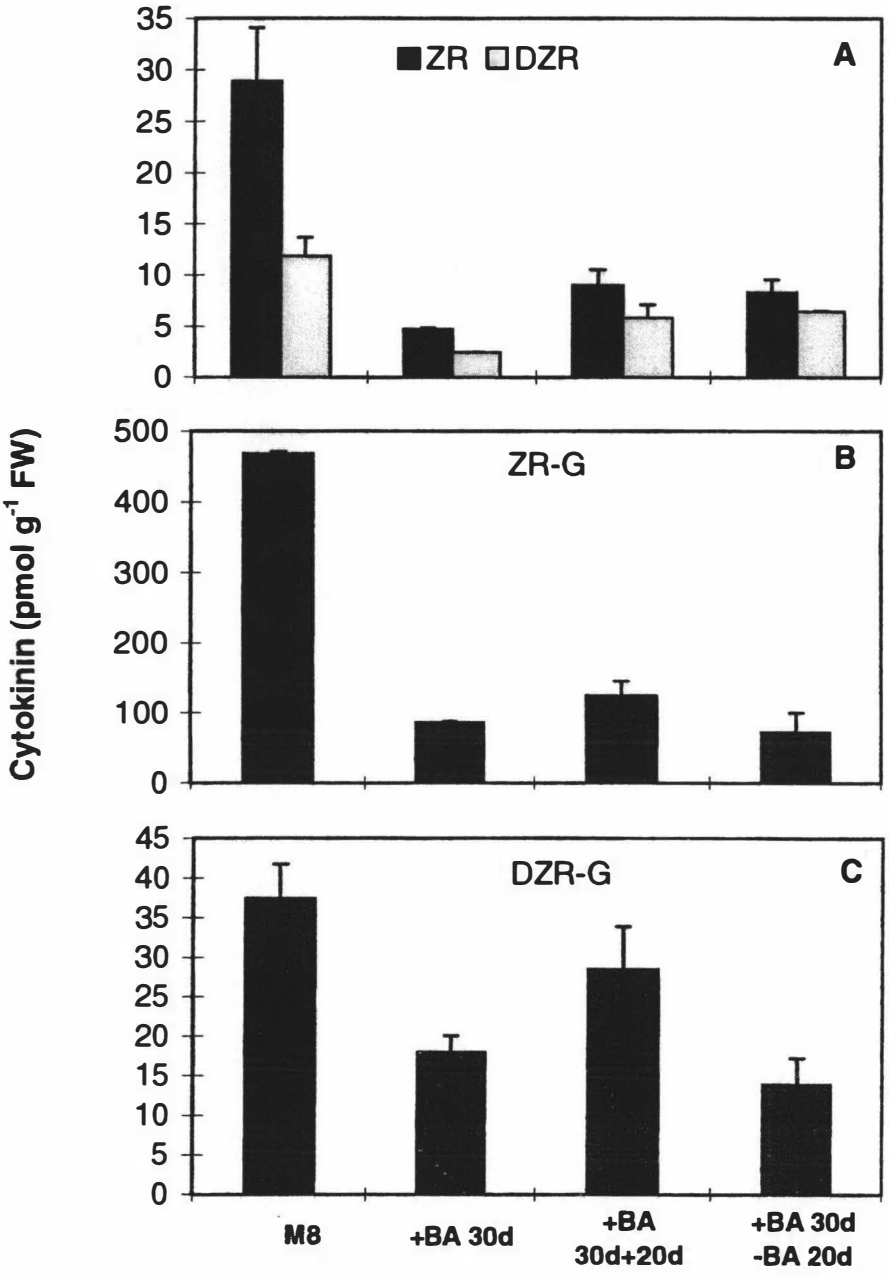


Figure 3.14 Endogenous cytokinins extracted from eight-year-old mature buds (M8) and the M8 bud fragments cultured under different BA regimes. A: the concentrations of zeatin riboside (ZR) and dihydrozeatin riboside (DZR); B: the concentration of zeatin riboside glucoside (ZR-G); C: the concentration of dihydrozeatin riboside glucoside (DZR-G). The values in B and C are not corrected for cross reactivity against clone 16.

this culture period are shown in Fig. 3.15. Unlike the situation in the buds cultured on BA-containing medium, there was no decrease in the concentration of ZR and DZR in the mature buds in the first 30 days of culture on cytokinin-free medium. In fact, the concentration of both ZR and DZR increased slightly (Fig. 3.15A). Morphologically, this first 30 days in culture corresponded to the time period when the mature buds were vigorously developing secondary needles. However, the content of ZR and DZR in the bud fragments decreased significantly 20 days after the buds were further subcultured onto cytokinin-free medium (Fig. 3.15A), a time when the secondary needle development was largely completed. In contrast to ZR and DZR, the concentration of iPA in the buds gradually increased over the 30 + 20 days of culture on cytokinin-free medium while the concentration of the free base cytokinin, iP, remained constantly low (Fig. 3.15B).

The concentration of both ZR-G and DZR-G in the cultured mature buds at Days 30 and 30 + 20 in the absence of exogenous BA decreased dramatically to a very low level (Fig. 3.16A and B), a similar trend as in the buds cultured on BA-containing medium (Fig. 3.14B and C). However, the concentration of the novel iPA-G over the same time period increased steadily (Fig. 3.16C).

3.4. Discussion

3.4.1. Heteroblasty and phase change in *P. radiata*

In higher plants phase-specific traits may be qualitative or quantitative in nature and may change at different rates (Hackett, 1985; Poethig, 1990; Greenwood and Hutchison, 1993; Greenwood, 1995; Lawson and Poethig, 1995; Telfer et al., 1997). In *P. radiata*, the transition from the juvenile to the mature phase may cause changes in diameter growth rate, tree form, foliage morphology and onset of reproductive organs and alter the efficiency of rooting of cuttings (Horgan et al., 1997). In *P. radiata*, changes in bud morphology are one of the most striking features observed during maturation (Fig. 3.1), with buds producing primary needles during their juvenile phase and being sealed in scales in their mature phase.

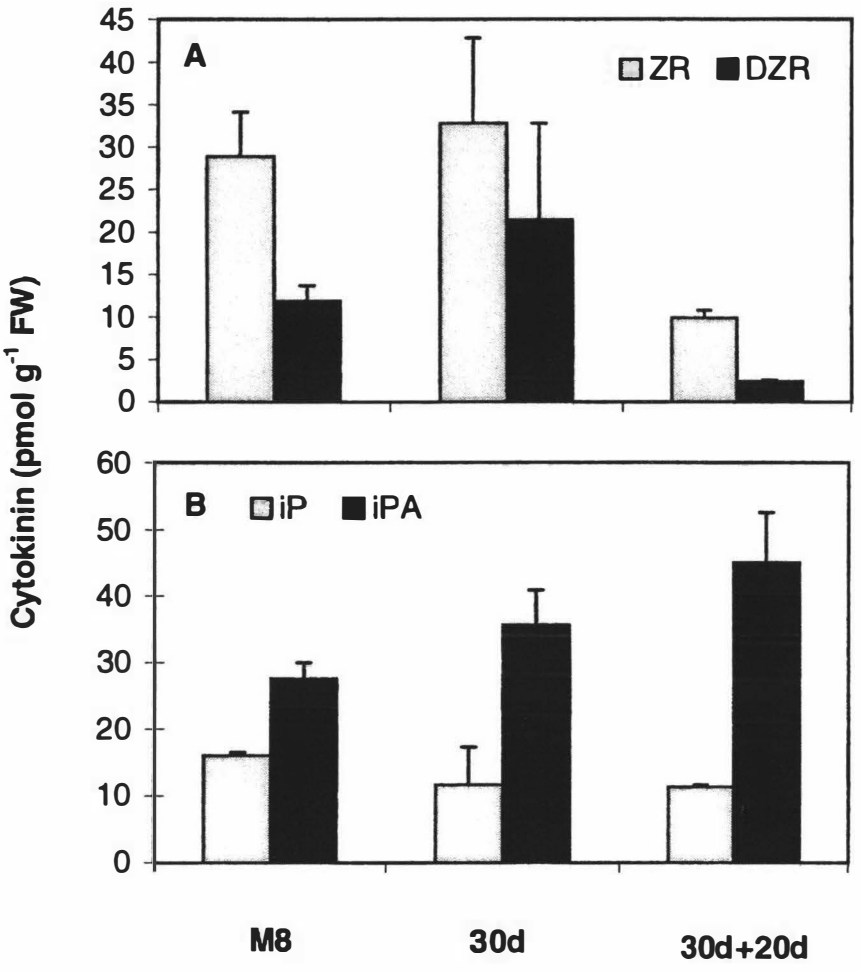


Figure 3.15 Changes in the concentration of endogenous cytokinin free base and ribosides in the buds from eight-year-old mature trees (M8) and the M8 bud fragments cultured on cytokinin-free medium for different time periods. A: endogenous zeatin riboside (ZR) and dihydrozeatin riboside (DZR); B: endogenous isopentenyladenine (iP) and isopentenyladenosine (iPA).

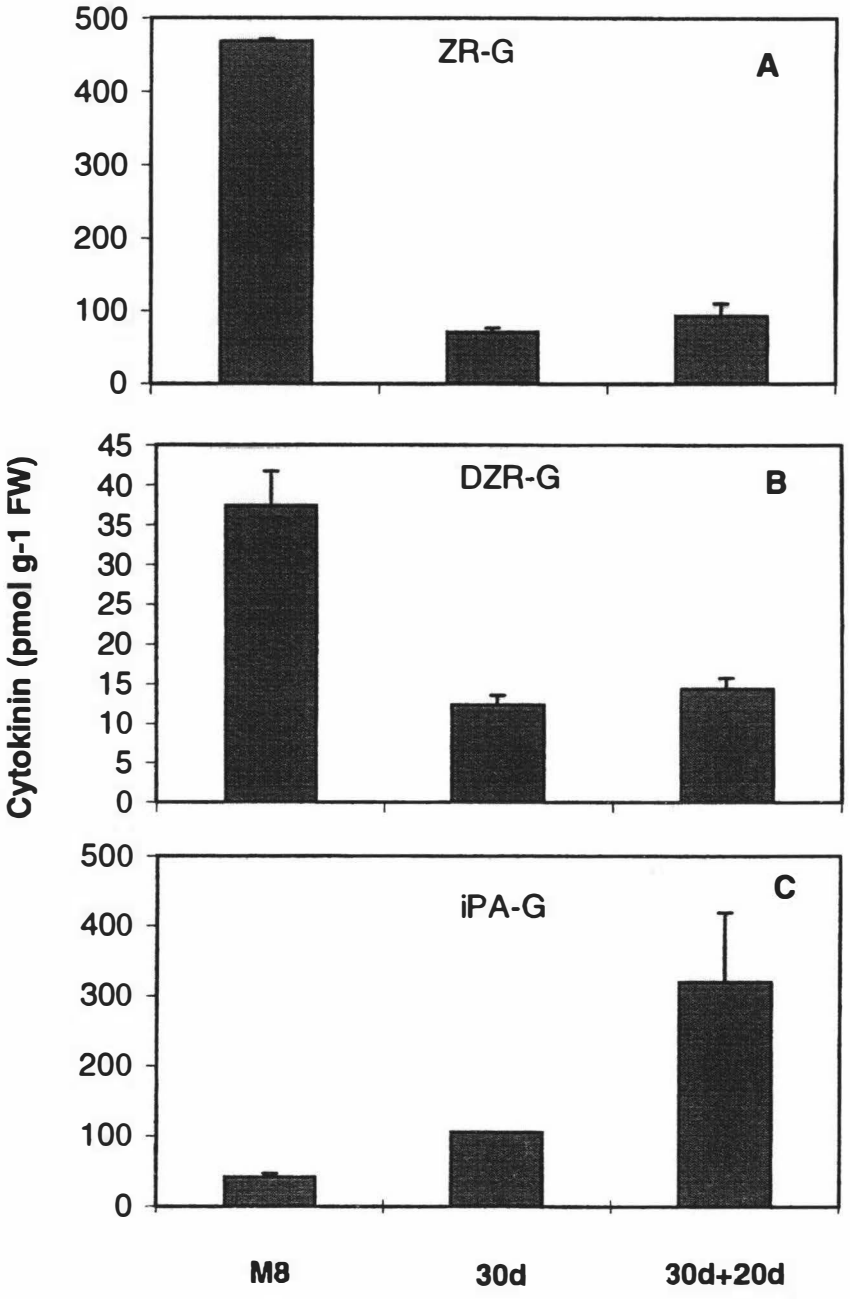


Figure 3.16 Changes in the concentrations of cytokinin glucosides in eight-year-old mature buds (M8) and the M8 buds which had been cultured either on cytokinin-free medium for 30 days (30 d) or on cytokinin-free medium for 30 d followed by subculture onto fresh cytokinin-free medium for a further 20 d (30d+20d). The values shown in the figures are not corrected for cross reactivity against antibodies. A: changes in the concentration of zeatin ribosylglucoside (ZR-G); B: changes in the concentration of dihydrozeatin ribosylglucoside (DZR-G); C: change in the concentration of isopentenyladenosine glucoside (iPA-G).

P. radiata displays a series of changes in leaf morphology during its development. It forms about eight cotyledons (varying from 5 to 12) during embryogenesis (Scott, 1960). Primary needle formation starts on the apical meristems as early as during seed germination (Riding 1972) and continues during the juvenile phase (Fig. 3.1A and C). Primary needles are main photosynthetic organs during early seedling development. When trees have reached maturity (for example, 8 years old), apical meristems initiate primordia which develop into scales (cataphylls) rather than primary leaves (Bollmann and Sweet, 1976, 1979; Fig. 3.1B, D and E). It is important to note that the conversion from production of primary needles to scales may be due to a gradual modification to the growth and development of the primary needle primordia. For instance, although the primordia on apical meristems from both one-year-old seedlings and four-year-old trees have the determination to form primary needles, the vigorously produced primary needles from seedlings (Fig. 3.1 A) are at least twice as long as those produced by the four-year-old trees (Fig. 3.1A and C). Physiologically, the difference appears even earlier: the content of chlorophyll in the primary needles from two-year-old trees was less than 50 % of that in the seedlings (see Table 4.3). The conversion of primary needles to scales during the transition from juvenile to mature seems to be a common feature in pine species. For example, in Jack pine (*Pinus banksiana* lamb), the conversion from initiation of primary needles to initiation of scales occurs within three years (Browne, 1995).

The origin of secondary needles is different from that of primary needles. Secondary needles derive from the fascicle meristems (axillary meristems) produced in the axils of primary needles or cataphylls (scale leaves) while the primary needles or scale leaves are generated by apical meristems. The secondary needles are the sole photosynthetic organs in *P. radiata* when trees reach full maturity. The fact that the shoots induced from the fascicle meristems develop features identical to those produced by the apical meristems (Fig. 3.1 B and E) indicates that the fate of the foliar primordia initiated from the fascicle meristems in *P. radiata*, just like those from apical meristems, is also determined by the maturation state of the tree.

Poethig (1990) proposed that juvenile and mature phases are specified by independently regulated, overlapping programs that modify the expression of a common set of processes required for shoot growth. Supporting evidence for this hypothesis comes from studies in maize (Poethig, 1990), *Arabidopsis* (Telfer et al., 1997; Van Lijsebettens and Clarke, 1998), and English ivy (Hackett and Murray, 1993). In *P. radiata*, primary needles, such as those of four-year-old trees, with morphology between fully photosynthetically functional primary needles and scales can be viewed as transitional leaves with mixed identities, implying the presence of overlapping programs between juvenile and mature phases. Presumably, there must be regulatory signal(s) involved in controlling the phase change process.

Plant hormones are widely involved in plant growth and development and are known to influence some of the characteristics associated with phase change (Zimmerman et al., 1985; Day et al., 1995; Carswell et al., 1996). It is possible that they may act as signals in controlling phase change in higher plants.

3.4.2. Endogenous cytokinin metabolism and phase change

Qualitative aspects of endogenous cytokinins in *P. radiata* have been discussed in Chapter 2. Following the procedures detailed in Chapter 2, a full spectrum of endogenous cytokinins including the novel cytokinins has been quantitatively analysed. The patterns of cytokinin metabolism are very complex but significant differences are seen between the buds from juvenile and mature trees.

Seedling buds contained the highest combined concentration of cytokinin free base and ribosides while buds from eight-year-old trees displayed the lowest level (Fig. 3.4A). A similar trend has been found in other species (Hendry et al., 1982; Galoch 1985; Day et al., 1995; Perrin et al., 1997). For instance, Galoch (1985) found marked variations in the content of plant hormones in buds and apical sections of birch shoots. The juvenile vegetative trees showed the highest level of cytokinins, and fully mature 15-year-old plants exhibited the lowest level.

The fact that trees of *P. radiata* at four years old are able to produce both juvenile and mature buds (Fig. 3.1C and D) means that these trees are at a transitional stage between the juvenile and a fully mature phase. In agreement with this fact, the combined concentration of cytokinin free base and ribosides in the buds of four-year-old trees is also lower than in seedling buds but higher than in the buds from eight-year-old mature trees (Fig. 3.4A). Therefore, the general trend for free base and ribosides in the buds from *P. radiata* (seedling buds>juvenile (J4) buds>mature (M4) buds>mature (M8) buds (Fig. 3.4A)) is indicative of an involvement of cytokinin metabolism in the phase change. A remarkably similar trend for the combined cytokinin free base and ribosides has also been detected between juvenile, transitional and mature tissues in an evergreen woody angiosperm species native to New Zealand (Day et al., 1995).

Cytokinin free bases and ribosides are believed to contribute to the pool of 'active' cytokinins (Letham and Palni, 1983). However, their exact 'roles' have not been fully elucidated. Although high concentrations of ZR were found in buds from one-year-old seedlings and four-year-old transitional trees (Fig. 3.4B and C), a high concentration of ZR alone is not likely in itself to be sufficient to control the formation of primary needles. For example, the mature buds from four-year-old trees also contained higher ZR than did the juvenile (J4) buds (Fig. 3.4C). However, as shown in Fig. 3.4D, the high level of iP (the only free base cytokinin detected in the field buds) in the seedling and juvenile (J4) buds appeared more closely associated with the production of juvenile characteristic-primary needles, and this is in agreement with the fact that both mature (M8) and apparently mature (M4) buds have a relatively lower iP concentration (Fig. 3.4D). The data from tissue-cultured mature buds also support this contention, because the iP concentration in the tissue-cultured mature buds was consistantly low (Fig. 3.15B): a low level of iP is highly correlated with mature bud morphology .

A wide range of phosphorylated cytokinins was detected because of the use of the modified Bieleski's solvent which can effectively inactivate phosphatases (Bieleski, 1964; Jameson et al., 1987). The differences in phosphorylated cytokinins between the buds of mature and juvenile trees are striking (Fig. 3.5). The total level of nucleotides in the seedling or "juvenile" (J4) buds are significantly lower than in either of the

mature buds analysed (i.e. M8 and M4) (Fig. 3.5), especially for the two major species, zeatin riboside nucleotide and the novel phosphorylated zeatin riboside glucoside (Fig. 3.5B and C). The results strongly demonstrate the importance of measuring the phosphorylated cytokinins in cytokinin analysis. Previous studies have shown that cytokinin nucleotide breakdown can occur during extraction due to the use of inadequate extraction solutions such as aqueous methanol or ethanol (Bieleski, 1964; Horgan and Scott, 1987). Also, cytokinin nucleotide degradation was observed in preliminary work for this thesis. If the adjustment of sample pH was carried out by using concentrated ammonia in a small volume of sample solution, degradation of the phosphate group from cytokinin nucleotides resulted in higher levels of the corresponding cytokinin nucleosides being detected.

Although it has been suggested that nucleotide cytokinins may be mainly associated with cytokinin uptake and transport across membranes (Singh et al., 1988; Letham and Palni, 1983; Jameson, 1994; McGaw and Burch, 1995), the low levels of phosphorylated cytokinins in the seedling and "juvenile" (J4) buds may also mean more phosphorylated cytokinins are utilised in juvenile than in mature buds. This is the first set of data showing such diverse forms of phosphorylated cytokinins, several of which have not been reported before. Although the relationship of these six phosphorylated cytokinins to overall cytokinin biosynthesis or metabolism remains to be determined, it is widely accepted that iPA nucleotide is the first step of cytokinin synthesis (Jameson, 1994; McGaw and Burch, 1995).

Despite the different concentrations in different bud types, similar patterns of the individual phosphorylated cytokinins measured in the buds from seedlings, four-year-old mature buds (M4) and eight-year-old mature buds (M8) (Fig. 3.5B and C) may indicate that the metabolic pathways for the phosphorylated cytokinins may be similar but the general metabolism rates very different between the mature and juvenile buds from the field. A fast turnover rate of phosphorylated cytokinins in seedling or "juvenile" (J4) buds may be associated with active production of primary needles displayed by these juvenile buds. Moreover, phosphorylated cytokinins were not detected in the mature buds in culture without additional cytokinin in the medium,

indicating that cultured mature buds also have a rapid turnover of phosphorylated cytokinins to meet their fast growth in culture. Therefore, the phosphorylated cytokinins on their own seem more likely to be associated with growth rather than phase.

Another distinct feature of the results reported in this thesis is that an extremely high level of novel cytokinin glucosides (ZR-G, DZR-G and iPA-G) was detected in the buds of *P. radiata* (Table 3.1). The functions of these novel cytokinin glucosides remain to be determined. Although *O*-glucosides are widely present in higher plants and algi (de Nys et al., 1990) and are believed to be very important as storage forms and in regulating the levels of active cytokinins (Jameson, 1994; Brzobohaty et al., 1994; Auer, 1997), no *O*-glucosides were detected in *P. radiata,* reflecting the fact that cytokinin metabolism is different in this coniferous species.

The higher concentrations of ZR-G and DZR-G in mature (M8) or "mature" (M4) buds and the lower concentrations in seedling and the "juvenile" (J4) buds from the field showed a trend similar to the nucleotides (Table 3.1 and Fig. 3.5). However, the novel glucoside iPA-G displayed the opposite pattern (Table 3.1). Although the reduced level of ZR-G and DZR-G and increased level of iPA-G appear to be associated with juvenile buds in the field, the mature buds cultured on medium without additional cytokinin (the condition allowing the mature buds to continue the development of mature phase) also exhibited a decreasing concentration of ZR-G and DZR-G and an increasing concentration of iPA-G. Therefore, these data indicate that the reduction in ZR-G and DZR-G and the increase in iPA-G may be more associated with growth processes of the buds, irrespective of the phase of development.

Furthermore, although the mature buds on cytokinin-free medium have low concentrations of phosphorylated cytokinins, ZR-G and DZR-G and high concentration of iPA-G, the concentration of free base cytokinin iP is also low, and it is hypothesised, has not reached the critical concentration or a critical ratio between the different forms (probably a threshold) for the production of juvenile traits. Consequently, these cultured buds continued their mature phase.

3.4.3 Cytokinin-induced "rejuvenation" *in vitro* in *P. radiata*

In *P. radiata*, micropropagated shoots from mature trees develop a juvenile morphology (producing primary needles) in tissue culture in response to cytokinin in the medium (Horgan, 1987; Fig. 3.2A, B and D). Without exogenous cytokinin, the mature buds in culture continue producing secondary needles but no primary needles (Fig. 3.2C). This phenomenon strongly indicates that cytokinins may be involved in regulating phase change in *P. radiata*. More detailed anatomical examination of the cultured buds shown in Fig 3.2 revealed that the phase reversion of the mature buds caused by exogenous cytokinin in tissue culture was through cessation of secondary needle formation, activation of fascicle meristems and stimulation of primary needle initiation (Fig. 3.3A, C, E, G, I, K and L). However, exogenous cytokinin is not needed continuously to maintain the reversion from the mature to the juvenile state. Not only does continuous exposure to exogenous cytokinin halt the elongation of these newly initiated primary needles, it results in an atypical apical meristem (Fig. 3.3L). However, following withdrawal of cytokinin from the medium after 30 days, the atypical apical meristems then attain a morphology (Fig. 3.3I) which is very similar to that of the meristems from seedlings (Riding, 1972), indicating that reversal of phase change has occurred at the meristem level in terms of primary needle production.

One way to explain the effect of cytokinin on the apparent "rejuvenation" of *P. radiata* is that cytokinin can reset the fate of the meristems and/or foliar primordia in mature buds. For instance, although the fascicle meristems in mature buds on cytokinin-free culture medium could also be activated to initiate foliar primordia (after secondary needles had fully developed) (Fig. 3.3J), these meristems were much smaller and the epidermis of the scale-bound primordia initiated by these meristems was heavily stained by safranine (Fig. 3.3J) in comparison with those cytokinin-activated meristems and the epidermis of foliar primordia (Fig. 3.3I, K and L) which did not accumulate safranine-stable compounds. The heavy accumulation of safranine-stainable substances in the epidermal cells may be an early sign that these foliar primordia will develop as scales covering the mature buds. This contention is in agreement with the fact that the field-grown mature buds also accumulate safranine-stainable substances in the epidermis of their scale-bound primordia (Fig. 3.3M). Therefore, the action of cytokinin on the shift

from the production of scales to primary needles may be associated with attributes located in the epidermal cells of these foliar primordia.

Indeed, studies carried out in other species have shown that leaf epidermal cell traits can be used as phase change markers and are well correlated with other phase-specific features. For example, the density and/or distribution of leaf epidermal cell trichomes is different between juvenile and mature leaves in *Arabidopsis* (Chien and Sussex, 1996; Telfer et al., 1997; Van Lijsebettens and Clarke, 1998), maize (Lawson and Poethig, 1995; Poethig, 1997) and some woody species (Schaffalitzky de Muckadell, 1954; Wareing and Frydman, 1976; Brand and Lineberger, 1992). In addition to morphological aspects, epicuticular wax accumulation is also one of the most obvious epidermal traits that show significant differences between juvenile and mature leaves in maize (Moose and Sisco, 1994, 1996; Evans and Poethig, 1995).

Although exogenous cytokinins are able to cause rejuvenation of mature buds, it is important to note that these shoots mature faster than *true* juvenile trees after the shoots have been planted in the nursery bed (Horgan, 1987; Horgan et al., 1997). This indicates that cytokinin may affect some switches of phase change, such as the one determining primary needle production, but not all of them. In this physiological context, rejuvenated characteristics are therefore able to mature more quickly when the influence of cytokinin is removed.

3.4.4. Metabolism of BA during cytokinin-induced "rejuvenation" *in vitro*

I have confirmed the previous observation (Horgan, 1987) that BA is able to induce phase reversion of mature buds of *P. radiata in vitro* (Fig. 3.2 and 3.3). The question then arises as to how BA causes the mature buds to revert to juvenile development. Because approximately four weeks of *in vitro* culture on BA-containing medium had been used previously to induce mature bud fragments to produce shoots with primary needles at NZFRI (Horgan, 1987), experimental time frames were therefore selected to coincide with observed developmental stages. For instance, BA metabolites were extracted and characterised from buds after 30 days of exposure to BA-containing

medium to correspond to primary needle initiation; 30 days exposure to BA plus 20 additional days on cytokinin-free medium to correspond to primary needle elongation; and 30 days of exposure to BA plus an additional 20 days of subculture on fresh BA-containing medium to reflect the inhibition of primary needle elongation. It has been found in this work that BA is metabolised to an extensive range of products in the bud fragments of *P. radiata* during the culture period. Six of these products have been identified as BA, BAR, BA9G, BAR-G, BANT and BAR-G-P (Fig. 3.11). Structures for the remaining six peaks identified in Fig. 3.7 and Fig. 3.9 have not been determined. They could be the products of further modification to the molecules of the identified BA derivatives shown in Fig. 3.11.

It has been reported that BA can be rapidly taken up by different tissue types of various plant species *in vitro* (Minocha and Nissen 1982; Biondi and Canciani 1984; Auer et al., 1992b; Werbrouck et al., 1995) including explants of pine (Vogelmann et al., 1984). After uptake, plant tissues convert BA into a great diversity of metabolites (reviewed by van Staden and Crouch, 1996). The results obtained in this work (Fig. 3.13) reflect an even greater diversity of metabolites in *P. radiata* than those found in other plant systems (Laloue et al., 1981; Laloue and Pethe, 1982; Letham et al., 1982; Letham and Gollnow,1985; Van der Krieken et al., 1988, 1990; Auer et al., 1992b; Van Staden and Drewes, 1992; Werbrouck et al., 1995).

The free base BA is believed to be the most active form in many systems (Van Staden and Crouch, 1996) and has been identified as a naturally-occurring cytokinin (Nandi et al., 1989a and 1989b). In *P. radiata*, the high concentration of BA maintained in the cultured mature buds (Fig. 3.13) during phase reversion, even after the withdrawal of BA from the culture medium, is highly correlated with the production of primary needles (a juvenile feature). BAR has been confirmed to have relatively low biological activity compared to the free base in a variety of bioassay systems (van Staden and Crouch, 1996). Further, in growing tobacco cell cultures, Laloue and Pethe (1982) found that only the free base exhibited activity. Therefore, the high level of BAR in the metabolite pool (Fig. 3.13) may serve as a source of BA through interconversion, although BAR itself may also be an active form *per se* (Peters and Beck, 1992). The ability to maintain high levels of BA and BAR for a prolonged period in the mature

buds during culture is a distinct feature of *P. radiata* that differs significantly from the levels found in other cultured plant tissues (Van der Krieken et al., 1988, 1990; Auer et al., 1992b; Werbrouck et al., 1995). For example, in *Petunia*, the combined level of BA and BAR dropped dramatically after their levels peaked within the first day of culture and were not detectable after six days of culture even in the presence of BA in the culture medium (Auer et al., 1992b). However, the level of BA and BAR in the cultured mature buds of *P. radiata* remained consistently high during the 30-day culture on BA-containing medium; even 20 days after the withdrawal of BA from the culture medium the level was still relatively high (Fig. 3.13).

BA9G has been regarded as an inactivation or detoxification form of BA (Van Staden and Crouch, 1996) and has been isolated as a naturally-occurring cytokinin form (Nandi et al., 1989a). The production of BA9G has been demonstrated in many experimental systems (Wilson et al., 1974; Zhang et al., 1987; Van Staden et al., 1990; Auer et al., 1992a, 1992b; Blakesley and Constantine 1992; Feito et al., 1994, 1995; Werbrouck et al., 1995). A common trend in these systems is that BA9G accumulates to a very high level while BA and/or BAR decrease to a very low level after long term culture on BA-containing media (Auer et al., 1992a, 1992b; Feito et al., 1994, 1995; Werbrouck et al., 1995). However, the data obtained in this work showed that the ability of cultured *P. radiata* buds to convert BA to BA9G was very limited because BA9G was only a minor metabolite compared to BA, BAR and the BAR-G (Fig. 3.13). This is in agreement with the fact that no endogenous 9-glucosides were detected in the field buds of *P. radiata*, indicating that BA metabolism in *P. radiata* has a regulatory mechanism that is different from the other higher plant species studied (Wilson et al., 1974; Zhang et al., 1987; Van Staden et al., 1990; Auer et al., 1992a, 1992b; Blakesley and Constantine 1992; Feito et al., 1994, 1995; Werbrouck et al., 1995).

The MS/MS spectrum clearly demonstrates that one of the novel BAR glucosides found in the cultured buds of *P. radiata* is BAR-G (Fig. 3.10). This novel form of cytokinin is the same compound as identified from *Gerbera* shoot culture by Horgan (1985) and Blakesley et al. (1990) and from *Petunia* culture by Auer et al. (Auer etal., 1992a, 1992b; Auer and Cohen, 1993). The biological function of this novel form is unknown.

However, the fact that it accumulated to a high level in the actively growing cultured buds (Fig. 3.13) indicates that the metabolic behaviour of this cytokinin may be similar to iPA-G (Fig. 3.16C) which accumulated in the bud fragments cultured totally on cytokinin-free medium. Auer and Cohen (1993) suggested that BAR-G accumulation was associated with bud organogenesis in *Petunia*, which may also be the case in *P. radiata*.

The BA nucleotide has been widely detected in other plant systems (Van Staden and Crouch, 1996) and was identified in tissue-cultured buds of *P. radiata*. However, the phosphorylated form of BAR-G identified in this study has not been reported before. The relatively low level of phosphorylated cytokinins detected in this study may mean that there is a very rapid turnover rate for phosphorylated BA metabolites. BA nucleotide and phosphorylated BAR-G may serve as intermediates for interconversion between the base, BAR, BAR-G and the other forms of cytokinins in the cultured mature buds. It has been shown in other plant experimental systems that BA nucleotide may play a central role in the regulation of the levels of the various metabolic forms of cytokinins as they are readily interconverted to the riboside and to the base (Laloue et al., 1981; Laloue and Pethe, 1982; Van Staden and Crouch 1996).

The unidentified C_1 and C_4 may, particularly, be responsible for the inhibition of primary needle elongation, because a marked reduction in the levels of C_1 and C_4 compounds was highly associated with the elongation of primary needles (Fig. 3.13).

In summary, the accumulation of high levels of active cytokinins such as the free base BA in the cultured mature buds may be the cause of the reversion from scale production (mature feature) to primary needle production (juvenile feature). This contention is in agreement with the fact that higher levels of the endogenous active free base, iP, were detected in the juvenile buds than in the mature buds of field-grown trees. The metabolite patterns observed during the 'rejuvenation" of mature buds in culture may lead to the maintenance of critical levels of active cytokinins.

The question now is whether BA activity is through its influence on the concentration of endogenous cytokinins. Unfortunately, there are limited studies on this issue. Feito et al. (1994) claimed that prolonged exposure to BA induces an increase in the level of endogenous cytokinins in *Actinidia deliciosa* tissues and similar results have been reported by other researchers in other systems (Kuiper et al., 1989; Kataeva et al., 1991; Vankova et al., 1991). However, the data (Fig. 3.14) obtained from this study shows a decline in endogenous cytokinin concentration in the buds treated with exogenous BA, supporting the idea that exogenously applied BA acts 'per se' and not through endogenous cytokinins (Feito et al., 1995; Centeno et al., 1996). More recently, it has been observed that another synthetic cytokinin-like compound, CPPU ((N-2-chloro-4-pyridyl)-phenylurea), also caused a decrease in endogenous cytokinin concentration while it promoted fruit development in kiwifruit (*Actinidia deliciosa*) (Lewis et al., 1996).

The decline in the level of endogenous cytokinins caused by exogenous cytokinins such as BA or CPPU may be due to enhanced cytokinin oxidase activity by these exogenous cytokinins (Chatfield and Armstrong 1986; Kamínek and Armstrong 1990). It is also possible that a high concentration of exogenous cytokinin absorbed into plant tissues can down-regulate endogenous cytokinin biosynthesis through feedback mechanisms.

Chapter 4 Identification of cytokinin-responsive genes using mRNA differential display and characterisation of gene expression

4.1 Introduction

As reported by Horgan (1987) and seen in the previous chapter of this thesis, the development of mature buds of *Pinus radiata* can be reversed by exogenous cytokinin ***in vitro***. The cytokinin 6-benzylaminopurine induces mature buds in culture to display juvenile morphology. Anatomical analyses revealed that this phasic reversal of the mature buds *in vitro* induced by exogenous cytokinin was due to meristem enlargement, primary needle initiation and secondary needle elimination at the shoot apex (Chapter 3). Moreover, the endogenous cytokinin analyses (Chapter 3) also indicated that there was a correlation between the endogenous cytokinin metabolism and the development of primary needles which are specific to the juvenile phase.

Studies on phasic development for other woody species (Hackett et al., 1987, Marc and Hackett, 1991, Marc and Hackett, 1992) and herbaceous plants (Poethig, 1990) have indicated that the apical and subapical meristem regions of the shoot apex are the sites of origin of phase-related characteristics (Poethig, 1990). Cytokinins appear to modulate the genes that are involved in imparting a fate of primary needle development on the mature shoot meristems of *P. radiata*. Identification of cytokinin-responsive gene(s) that are involved, directly or indirectly, in such determination of phase-related characteristics will help towards an understanding of the mechanism of phase change.

4.2 Materials and Methods

4.2.1 Plant materials

Individual mature trees of 9 and 4 years old held at the NZFRI nursery were marked and used as the sources of bud materials. Consequently, for each experiment, the plant materials used for different treatments came from the same individual trees to guarantee the comparison of gene expression in response to cytokinin within the same genotype. All the buds were collected from the secondary and tertiary branches of the upper crown of individual trees. The buds collected from the field trees were either used directly for

RNA and DNA isolation or cultured *in vitro* subject to experimental treatments. For direct use, the field buds were immediately put in liquid nitrogen and stored at -80°C after collection.

4.2.1.1 Tissue culture: For tissue culture, the harvested field-grown buds from 9- and 4-year-old trees were surface-sterilised in 500 mL of 50% bleach (Franklin Limited, NZ) for 15 min with constant stirring on a magnetic stirrer. The buds were then rinsed three times in a large volume of sterile water (at least 300 mL each time), after which the bud scales were peeled off and the bud cross-sectioned into 5 mm fragments. These were placed onto both cytokinin-containing (LP5) and non-cytokinin LP (LPCH) nutrient media which were solidified with 0.8% Difco agar (Appendix B). Cytokinin supplements in the culture media and the environmental conditions of the cultures differed according to the purpose of each experiment.

4.2.1.2 Tissue cultured materials for RNA and DNA isolation:
(i) For mRNA differential display, the field buds and the bud fragments which had been cultured on either BA-containing or cytokinin-free medium for periods of 72 h and 25 d were used for total RNA extraction. The cultures were maintained in a specialised tissue culture room at 25°C under a 16/8 h fluorescent light/dark cycle.

(ii) For the time course Northern analyses, bud fragments were cultured on either BA-containing or cytokinin-free medium in a growth chamber at 25°C, either in compete darkness or a 16/8 h fluorescent light/dark cycle. The bud fragments were harvested in liquid nitrogen at the time points of 0 h (field bud fragments), 12 h, 24 h, 48 h, 72 h, 7 d and 25 d and then stored at -80°C until required for RNA extraction. Also, at the same time of collecting materials at day 25, some buds cultured on BA-containing medium were transferred onto cytokinin-free medium and they were then harvested after 20 further days of culture.

(iii) For dose response Northern analyses, the bud fragments from a four year-old tree were cultured on cytokinin-free medium for 48 h in a growth chamber at 25°C in complete darkness and then transferred onto LP nutrient medium supplied with a series

of concentrations of isopentenyl adenosine (iPA) (0, 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M) or 6-benzylaminopurine (BA) (0, 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M). After 24 h culture under the same conditions, the fragments were harvested in liquid nitrogen and then stored at -80°C until required for RNA extraction.

4.2.2 RNA extraction

To prevent RNA degradation during extraction, all the glassware was well washed using detergent and wrapped with tinfoil and baked in a dry air oven at 180°C for at least 4 h to destroy RNase activities. Disposable plastic tubes (50 mL conical tubes and Eppendorf vials) and tips were autoclaved at 121°C for 20 min. Previously unopened stocks of chemicals were used to make solutions. Clean disposable gloves were worn during the procedure. The Milli Q water used was always collected directly from the Milli Q system and autoclaved at 121°C for 20 min but not DEPC-treated.

RNA extraction was achieved by using a modified protocol according to Chang et al. (1993). One half to two grams of plant material which was stored at -80°C or immediately frozen in liquid nitrogen after harvest was weighed out in a liquid nitrogen pre-chilled mortar and ground to a fine powder using mortar and pestle in the presence of liquid nitrogen. Each fine tissue powder was then transferred with a pre-chilled spatula into a 50 mL sterile screw-capped disposable polypropylene tube which was being chilled in liquid nitrogen until all the samples had been ground.

Immediately prior to the extraction buffer being added to the ground sample, 0.3 mL of β-mercaptoethanol (BDH) was added to 15 mL of extraction buffer which had been pre-warmed to 65°C. The buffer mixture was then poured into the tubes containing the ground samples. Mixing was completed by inverting the tubes quickly several times, and then vortexing for 1 min. Subsequently, 15 mL of chloroform:isoamylalcohol (24:1 v/v) was added to and mixed with the buffer and the resultant mixture was vortexed for 1 min. The mixture was subsequently incubated in a water bath at 55°C for 15 min with occasional shaking. The tubes were removed from the water bath and kept on ice until the next step took place (usually less than 30 min). Subsequently, the samples were centrifuged at 5500 *g* for 15 min at 4°C. The supernatants were transferred into another

set of sterile disposable polypropylene tubes, taking great care to avoid transfering solid debris from the interface. The supernatant was partitioned a second time against chloroform by adding an equal volume of chloroform:isoamylalchohol (24:1 v/v). After vortexing (about 10 s), the mixture was centrifuged for 15 min at 7000 *g* at 4°C.

The supernatants were carefully moved into a new set of fresh sterile disposable 50 mL polypropylene tubes with accurate measurement of the supernatant volume. One fourth volume of 10 M LiCl (BDH, stored at -20°C) was added to each supernatant. After mixing, supernatant and LiCl was left overnight (around 12 to 16 hours) at 4°C to precipitate RNA. The tubes were centrifuged for 30 min at 8000 *g* at 4° to precipitate RNA. The supernatants were removed and the RNA pellets were washed with 3 mL of chilled 70 % ethanol. The tubes were centrifuged for 10 min at 9000 *g* at 4°C and then the supernatants were carefully removed to prevent loss of the RNA pellets.

The pellets were then dried briefly by inverting the tubes on clean paper towels and the pellets then dissolved in 500 µL of SSTE prior to transfer to 1.5 mL Eppendorf tubes. An equal volume of chloroform:isoamylalcohol (24:1 v/v) was added to each tube and vortexed for 30 s. The tubes were then centrifuged for 15 min at 10000 rpm at room temperature in a microcentrifuge. The aqueous phases were then transferred to a new set of Eppendorf tubes and mixed with 2.5 volumes of pre-chilled 100% ethanol (Analar), followed by a 2 h precipitation at -20°C or 20 min at -80°C.

The mixtures were centrifuged for 20 min at 12000 rpm in a microcentrifuge and the supernatants were removed. The RNA pellets were air-dried or dried using a Speedvac, dissolved in 200 -500 µl of autoclaved Milli Q water. After the concentration was determined using a spectrophotometer, the RNA solution was aliquotted to 50 µL fractions and sored at -80°C until required..

Extraction buffer:
2 % CTAB (cetyltrimethylammonium bromide)
2 % SDS (sodium decyl)
1 % soluble PVP
0.1 M Tris-HCI (pH 8.0)
0.025 M EDTA (pH 8.0)
2.0 M NaCI
The buffer mixture was autoclaved at 121° for 20 min and stored at room temperature until required.

SSTE buffer: 1.0 M NaCl
0.5% SDS
0.01 M Tris-HCl (pH 8.0)
0.001 M EDTA (pH 8.0)
The buffer mixture was autoclaved at 121° for 20 min and stored at room temperature until required.

4.2.3 Genomic DNA extraction

The DNA extraction protocol was developed during the study based on the modified RNA extraction procedure. One half to 1 g field-grown buds or tissue-cultured material was ground under liquid nitrogen. The fine tissue powder was transferred to 15 mL extraction buffer pre-warmed to 65°C and was added with 0.3 mL of β-mercaptoethanol, as detailed for the RNA extraction (Section 4.2.2). After slow but thorough mixing, an equal volume of chloroform:isoamyl ethanol (24:1; v/v) was added to the extraction mix and further mixed by slow inversion. The extraction mixture was then incubated for 15 min at 55°C. The tubes were inverted several times during the incubation. After centrifugation at 9000 *g* for 15 min, the aqueous phases were transferred to another set of fresh centrifuge tubes or disposable polypropylene tubes. Subsequently, 0.5 volume of 100% ethanol was added and mixed with the aqueous phases to precipitate DNA. The silky DNA precipitate was harvested either by centrifugation at 9000 *g* for 5 min or scooped out using pipette tips. The DNA pellets were then washed with 75% ethanol and followed with 100% ethanol. Finally, the DNA pellets were dried by inverting the tube on a paper towel. The DNA was subsequently dissolved in 500 µl of sterile Milli Q water. Possible contamination by RNA was eliminated by RNase treatment at a concentration of 10 µg/mL DNAse-free RNase at 37°C for 50 - 60 min. The RNase protein was later removed by chloroform partitioning. The quality and quantity of the prepared DNA were examined as described below (Section 4.2.4).

4.2.4 Determination of DNA and RNA concentration and quality by spectrophotometry

DNA and RNA preparations were diluted to 1 mL final volume with sterile Milli Q water. The baseline of the spectrophotometer was set using identical sterile water to that used in diluting the samples and absorbance of the diluted preparations in quartz

curvetts with a 1 cm light path was determined from 200 nm to 320 nm. The ratio of absorbance at 260 nm and 280 nm and the absorbance spectrum from 200 nm to 320 nm were used to determine the purity of DNA and RNA preparations. Pure RNA has a ratio of 2.0 at OD_{260}/OD_{280} and pure DNA has a OD_{260}/OD_{280} ratio of 1.8. The concentration of RNA and DNA in the preparations was calculated on the basis that an OD of 1 with a 1 cm light path at 260 nm equals approximately 40 µg/mL of RNA or 50 µg/mL of double stranded DNA.

4.2.5 Differential display of mRNA

A modified protocol of the mRNA differential display technique was based on the method developed by Liang et al. (Liang et al., 1992, 1993; Oh et al., 1995). The precautions detailed for RNA extraction (Section 4.2.2) were taken throughout the first strand cDNA syntheses.

4.2.5.1 The first strand cDNA synthesis: Total RNA derived from field-grown buds, tissue-cultured bud fragments with cytokinin treatment and tissue-cultured bud fragments without cytokinin treatment were utilised as templates for synthesis of the first strand cDNA. The 5′-T_{12}VN (Operon Technologies; where V=A, C and G and N=A, C, G and T) was used as primer for reverse transcription (RT) reaction. For each RT reaction, 5 µg of total RNA and 50.1 pmol of 5-T_{12}VN 3′ primer were mixed in a PCR tube at a volume of 10 µL in sterile water and the RNA/primer mixture was denatured at 70°C for 10 min. Subsequently, the tube was removed from the 70°C water bath and put immediately on ice for at least 5 min. The contents were collected in the bottom of the PCR tube by a brief centrifugal pulse and 4.0 µL 5 × RT buffer, 2.0 µL 0.1 M DTT, 1.0 µL 10 mM dNTP mix and 2.0 µL RNase inhibitor added and mixed gently by pipetting up and down. The reaction mixture was incubated for three minutes at 42°C. After the incubation, 1.0 µL Superscrit II RNase H^- reverse transcritase (GIBCOBRL) was added, mixed well by pipetting up and down and incubated for 50 min at 42°C water bath to synthesise first strand cDNA. To inactivate the reverse transcription enzyme, the temperature of the incubation was increased to 70°C and kept at the same temperature for 15 min. The synthesised first strand cDNA solution was used immediately or kept at -20°C until required for amplifying cDNAs.

4.2.5.2 Differential display PCR: The first strand cDNAs generated by RT reaction were amplified by polymerase chain reaction (PCR) using an anchor primer 5′ TTTTTTTTTTTTVC 3′ or 5′ TTTTTTTTTTTTVG 3′ (Operon) and a random primer (5′ CGGCARGTNACNTT 3′, Life Technology) which is able to bind to different positions of the cDNAs. As a result of the random primer, the amplified cDNA populations of different sequences could be of various length. To carry out PCR amplification, two Master PCR mixes were prepared in order to reduce pipetting errors and save time. To each of the PCR tubes (the number of PCR tubes was the same as required by the sample numbers) 48.0 µl of Master I PCR mix was added, followed by the additions of 2.0 µL of first strand cDNA solution from each of the different sample RT reactions. These tubes were preheated to 94°C for 5 sec in a PCR thermocycler (Perkin-Elmer Cetus DNA Thermal Cycler) and 50 µL of Master II PCR mix was then added to each tube with quick pipetting up and down to mix the PCR reaction solution. The PCR reaction was performed for 40 cycles of 30 sec at 94°C, 42°C for 1 min 30 sec, 72°C for 1 min 30 sec and finally incubated at 72°C for 10 min to complete extension of the cDNAs. The PCR reaction solutions were kept at 4°C on hold in the PCR thermocycler before removal to a -20°C freezer.

Master I PCR mix:

dNTP (10 mM)	0.5 µL
Water	37.5 µL
Anchor primer (30 µM)	8.33 µL
Random primer (30 µm)	1.67 µL

Master II PCR mix:

PCR buffer with MgCI	10.0 µL
Expand polymerase	1.0 µL
water	39.0 µL

4.2.5.3 cDNA display electrophoresis: The different size classes of PCR-amplified cDNA populations were separated by running 20 µL of each of the PCR reaction solutions side by side on a 1.5% agarose gel which contained 0.6 µg/mL ethidium bromide. The gels were run in 1× TAE buffer at 50-80 V for 2.5-4 hours and the cDNA bands were visualised with UV light stimulation and photographed with Polaroid 667

film. Samples were loaded after being mixed with SUDS loading buffer at a 5:1 ratio (v/v).

10×TAE buffer for 1 L:	48.5 g Tri(hydroxylmethyl)aminomethane (Tris) 11.3 mL glacial acetic acid 100 mL 0.5 M EDTA (pH 8.0)
DNA loading buffer(SUDS):	1% (w/v) sodium dodecyl sulphate (SDS) 50% (w/v) glycerol 0.025% (w/v) bromophenol blue 100 mM EDTA (pH 8.0)

4.2.6 Cloning of selected cDNA sequences

The Invitrogen® TA cloning® system was used to clone the cDNAs. The TA cloning system provides a quick, one-step cloning strategy for the direct insertion of a PCR product into a plasmid vector. The cloning system takes advantage of the non-template dependant activity of thermostable polymerases used in PCR that add a single deoxyadenosine to the 3′ ends of duplex molecules. These 3′ A-overhangs are used to insert the PCR product into a vector which contains single 3′ T-overhangs at its insertion site. The vector supplied with the kit is a linear molecule with 3′ T-overhangs at its insertion site.

4.2.6.1 cDNA preparation: Selected bands of interests from the differential display gels were excised into 1.5 mL centrifuge tubes, 100 µl of sterile water added and followed by boiling for 5 min to extract the cDNA. After centrifugation, the supernatants containing the cDNAs were stored at -20°C until further usage.

4.2.6.2 Ligation: The concentration of PCR products in the supernatants were estimated by running 5 µL of the supernatants on an agarose gel with a mass ladder (Low DNA mass™ ladder, Life Technologies). The amount of the PCR product used was determined by the formula provided in the manufacturer's instruction. The ligation reaction solution was 15 µL containing 1× ligation buffer (vial TA2), 50 ng of pCR™ II vector (vial TA3), 10 ng of PCR product and 1.5 µL of T4 DNA ligase (vial 5). The ligation reaction was incubated at 12°C overnight.

4.2.6.3 Plasmid transformation of E. coli: A vial containing 50 µL of TA cloning™ OneShot™ competent cells thawed on ice was mixed with 2.0 µL of 0.5 M β-mercaptoethanol very gently and followed by 2.0 µL of ligation reaction added to the tube. After gentle tapping to mix the contents, the tube was kept on ice for 30 min and subsequently treated for exactly 30 sec at 42°C. After the heat shock, the tube was immediately replaced on ice for 2 min and then 450 µL of pre-warmed SOC medium was added to the tube. The contents were subsequently shaken at 37°C for exactly 1 h at 225 rpm in a rotary shaking incubator. After the 1 h incubation, 25 µL, 100 µL and 300 µL of transformation solution from each transformation vial were spread on separate and labelled LB agar plates containing 50 µg/mL kanamycin and incubated overnight at 37°C. Colonies on the LB plates were numbered and stored at 4°C for plasmid isolation, restriction analysis and sequencing.

SOC medium (Sambrook et al, 1989):	20.0 g trypton (Difco) 5.0 g yeast extract (Difco) 0.5 g NaCl 2.5 mM Kcl 10 mM MgCl2 20 mM glucose pH 7.0, autoclave.
LB medium:	10.0 g trypton (Difco) 5.0 g yeast extract (Difco) 10.0 g NaCl adjust pH to 7.0, autoclave.

4.2.7. Plasmid extraction and purification

The methods used follow closely those of Sambrook et al. (1989). To amplify plasmids with the cDNA inserts, the individual colonies were inoculated into 10 mL LB broth medium containing 100 µg/mL ampicillin and incubated overnight at 37°C at 225 rpm in a rotory shaking incubator. Next morning the cultures were centrifuged at 3000 g for 5 min to harvest the bacteria. After the supernatants were removed, the tubes were inverted on paper towels for 10 min to allow the medium to drain completely from the pellets. Subsequently, 200 µL of buffer (solution I) was added to resuspend the pellets by pipetting up and down. The resuspension was then transferred to 1.5 mL Eppendorf tubes and vortexed. After 5 min on ice, 400 µL of lysis solution (solution II) was added into the Eppendorf tubes and the tubes were rapidly inverted (not shaken or vortexed)

five times and kept on ice for 5 min. Following the ice incubation, 300 µL of solution III was added to the tubes, the mixtures were vortexed vigorously and subsequently centrifuged for 5 min at 12000 rpm. The supernatants were transferred into another set of 1.5 mL microcentrifuge tubes and mixed by vortexing with 0.5 mL of chloroform:isoamyl ethanol (24:1; v/v). After centrifugation, 750 µL of the supernatants were transferred into another set of 1.5 mL tubes and mixed with the same volume of 100% isopropanol. The mixtures were then put in a -20°C freezer for at least 20 min. Centrifugation for 15 min at 13000 rpm was then carried out to precipitate the plasmid. After the supernatants were removed by aspirating, the plasmid pellets were washed with 70% ethanol and dried using a Speedvac (Savant SVC200) and finally redissolved in 50 µL of sterile water and stored at -20°C.

Solution I:	50 mM glucose 25 mM Tris (pH 8.0) 10 mM EDTA (pH 8.0) Autoclaved at 121°C for 20 min and stored at -20°C.
Solution II:	0.2 M NaOH 1% SDS Fresh preparation.
Solution III:	3 M potassium acetate 2 M acetic acid Autoclaved at 121C for 20 min and stored at -20°C.

4.2.8. Confirmation of cDNA inserts

About 0.5 to 1 µg of each plasmid preparation was digested with *Eco*RI (Boehringer Mannnheim) at 37°C for 3-4 h in a 10 µL of total volume of digestion solution which contained 1× buffer H, 5 units of *Eco*RI and 10 µg RNase. Subsequently, the digestion solution was mixed with 2 µL of loading buffer (SUDS) and fractionated on 1.0% agarose gel to confirm the existence of cDNA inserts. A 50 bp ladder DNA molecular weight marker (XIII, Boehringer Mannheim) was used to size released cDNA inserts. Plasmid preparation treated the same way as described above but without adding *Eco*RI restriction enzyme was included as uncut plasmid controls.

4.2.9. Grouping of the cloned cDNA sequences

The restriction-enzyme fingerprinting approach (Shoham et al., 1996) was employed to determine if the cloned single band was actually composed of one species or multiple

species of cDNAs. If the cloned band has multispecies of cDNA, then they can be grouped according to restriction-enzyme fingerprinting patterns. The restriction-enzyme fingerprinting was performed by digesting 1 μg of RNA-free plasmid DNA (confirmed in Section 4.2.8), extracted from each of the cDNA-containing colonies derived from a single selected band, with both *Eco*RI and a tetra-nucleotide recognition site enzyme, *Sau*3AI. Five units of *Eco*RI and five units of *Sau*3AI were used in 10.0 μL of reaction solution with 1× buffer A (Boehringer Mannheim). The digested DNA samples were then mixed with 2.0 μL of SUDS loading buffer and fractionated side by side on a 3.0% agarose gel containing 0.67 μg/mL ethidium bromide. The band patterns were revealed under UV light and photographed using Polaroid 667 film.

4.2.10. Sequencing of the cloned cDNA

Sequencing was carried out by employing both automated sequencer and manual sequencing protocols.

4.2.10.1 Plasmid DNA preparation: For sequencing, the plasmid DNA prepared as detailed in Section 4.2.3.8 was further purified using an additional chloroform partition. Twenty microlitres of the plasmid DNA was mixed with 2.0 μL of RNase stock solution (10 mg/mL) in a 1.5 mL Eppendorf tube and incubated in a 37°C water bath for 30 min. After the incubation, 378 μL of sterile water and 200 μL of solution III described in Section 4.2.3.8 were added to the tube. After being briefly vortexed, 500 μL of chloroform:isoamyl alcohol (24:1; v/v) was added to the tube and then the mixture was vortexed for 30 sec prior to a 10 min centrifuge at 12000 rpm at 4°C. Subsequently, 500 μL of the supernatant was carefully transferred to a new Eppendorf tube, well mixed with equal volume of 100% isopropanol and placed in a -20°C freezer for 20 min. After the cold precipitation, the mixture was centrifuged for 10 min at 13000 rpm at 4°C and the resulting plasmid DNA pellet was washed with 1 mL cold 70% ethanol. Finally the plasmid DNA pellet was dried using a Speedvac and dissolved in 20 μL of sterile water. The quality and concentration of the plasmid DNA was verified by both the methods detailed in Section 4.2.4 as well as running 1.0 μL of the 20 μL plasmid DNA on a 1.2% agarose gel along with 8.0 μL of DNA mass ladder (Life Technologies). The original

plasmid DNA preparations were diluted to the optimum concentration either for automated sequencing or manual sequencing.

4.2.10.2 Automated sequencing: Sequences of both strands of the cDNAs cloned in the pCRII vectors were obtained by the Centre for Gene Research at the University of Otago using the Applied Biosystems Automated Sequencer. The intact plasmid DNA at a concentration of 200 ng/μL was provided. M_{13} forward and reverse primers (New England Biolab) were used in the sequencing.

4.2.10.3 Manual sequencing: Sequenase Version 2.0 (USB) was used for manual DNA sequencing. Three microlitres of 2 M NaOH and 1 μl of 0.5 M EDTA (pH 8.0) were added to 26 μl template solution containing about 3 μg of the plasmid DNA with cDNA insert to be sequenced. This mixture was incubated for 30 min at 37°C. Subsequently, 3 μL of 3 M NaOH and 90 μL of 95% ethanol were added to the mixture and plasmid DNA was precipitated by centrifugation for 10 min at 10000 rpm in a microcentrifuge. The DNA pellet was dried using a Speedvac, redisolved in 7 μL of sterile water, 1 μL of M13 forward or reverse primer (New England Biolab) and 2 μL of the 5× sequenase sequencing buffer. After mixing well, the solution was incubated at 65°C for 5 min and then allowed to cool down to 35°C. Following the annealing incubation, 5.5 μL of labelling reaction solution containing 1 μL of 0.1 M DTT, 2 μL of diluted 5× labelling mix , 2 μL of Sequenase buffer (Pharmacia Biotech.) and 0.5 μL of ^{35}S-dATP was added to the template/primer annealing tube and incubated at room temperature for 5 min. Subsequently 3.5 μL of this template/primer/labelling mix was transferred to each of the four termination tubes which contained 2.5 μL of the appropriate ddNTP and incubated for 5 min at 37°C. Finally, 4 μL of stop solution was added to each tube and the reaction tubes were kept at -20°C until required for sequencing gel.

^{35}S-labelled sequence fragments of different sizes generated from the sequencing reaction were separated on 6.0% polyacrylamide gel. The gel was poured with 75 mL of acrylamide mix containing 60 μL of TEMED and 400 μL of 10% ammonium persulphate. After denaturing in boiling water for 5 min, 5 μL of the sequencing reactions were loaded onto sequencing gel which had been pre-run for 1 h in 1× TBE

running buffer. When the dark blue dye (the xylene cyanol FF) had run completely off the bottom of the gel (long run), 5 µL of the same set of sequencing reactions were loaded for the short run. After the bromophenol blue dye front had just run off the gel, the gel was transferred onto a sheet of 3 mm blotting paper, vacuum-dried at 80°C, wrapped with GladWrap plastic membrane and exposed to Fuji medical X-ray film overnight at room temperature. The nucleotide sequences were manually read by putting the X-ray film against a white background.

Acrylamide mix:	Urea 36.0 g Stock acrylamide solution containing 38.0% (w/v) acrylamide and 2.0% (w/v) bis-acrylamide 11.25 ml Amberlite MB-1 3-4 g After filtering through 45 µm Millipore filter, 7.5 ml 10× TBE was added and water to 75 ml.
10× TBE running buffer:	Tris, 162.0 g; Boric acid, 27.5 g; Na_2EDTA, 9.5 g. Adjusted to 1 L with Milli Q water.

4.2.11 Northern analyses

4.2.11.1 Fractionation of total RNA by electrophoresis: Total RNA was separated on 1.2% denaturing agarose gel containing 4.5% formaldehyde. The gel apparatus used was soaked overnight with detergent and thoroughly rinsed with RO water. Subsequently, the comb and the gel tray (15 × 15 cm or 20 × 25 cm in size) were also soaked with 0.1 N HCl for at least 3 h before being rinsed with sterile water and then 95% ethanol. If the volumes of RNA samples were bigger than the gel well allowed, they were snap-frozen in liquid nitrogen and dried down to the volume desired using the SpeedVac. For loading, the samples and standard RNA size ladder (0.24 - 9.5 kb RNA ladder, Life Technology) were denatured at 65°C for 12 min after being mixed with loading buffer and, immediately after the heat treatment, cooled on ice at least 2 min before loading. Gels containing 4.5% formaldehyde were run in 1× RNA running buffer at 80 V for 4 - 5 h in a fume hood and photographed using Polaroid 667 film under UV light.

4.2.11.2 Gel blotting: Fractionated RNAs on gels were transferred onto Nylon membranes (Hybond+, Amersham) according to the downward alkaline transfer method (Chomczynski, 1992) for 3-4 h. After neutralising with neutralising phosphate buffer

for 15 min, the membranes were placed in a UV light box (UV Stratalinker® 2400) with the RNA side facing towards UV light and treated with UV light for 2 min to cross-link RNA to the membranes. The membranes were each placed between two pieces of 3 mm blotting paper, sealed in plastic bags and stored at 4°C until required.

4.2.11.3 Preparation of [α-^{32}P]dCTP-labelled probe: Ready-To-Go DNA labelling kit (Pharmacia Biotech Inc, USA) was used to label the probe. Insert of cDNAs used for preparing probes were produced by digesting the corresponding cDNA-containing plasmids and the cDNA insert was subsequently isolated on 1.0% agarose gels. The purified band of cDNA insert on the gel was excised and put in 1.5 mL tube with 50 µL of water. After freezing and thawing, the tube was centrifuged at 13000 rpm for 10 min. The supernatant was stored at -20°C and directly used as template in the probe-labelling. Around 40 ng of the template cDNA in a volume of 25 µl was denatured for 3 min in boiling water and then immediately placed on ice to cool. The 25 µL of denatured template cDNA solution and 5 µL of [α-^{32}P]dCTP (3000 Ci/mmol, ICN Pharmaceuticals Inc, California) were added to 20 µL of reconstituted reaction mix which had been kept on ice for 10 to 30 min and then incubated at 37°C for 30 min. The labelled cDNA probes were purified using ProbeQuantTM G-50 microcolumns by following instructions provided by the manufacturer (Pharmacia Biotech).

4.2.11.4 Prehybridation and hybridisation: The membranes were sealed in plastic bags with 30 to 60 mL of hybridisation solution without obvious air bubbles and pre-hybridised for 30 min in a water bath at 65°C with gentle agitation. Subsequently, the pre-hybridisation plastic bags were cut and a small opening made on one corner and the purified ^{32}P-labelled DNA probe was added into the pre-hybridisation plastic bags by pipette. After careful removal of all air bubbles, the opening was sealed with plastic sealer and the membranes were subjected to overnight hybridisation at the same conditions employed for the pre-hybridisation. A 639 bp fragment of *Prcr5* cDNA, a 930 bp fragment of *Prcab* cDNA and a 300 bp fragment of 28S-Pr*RNA* cDNA were used as hybridisation probes. Levels of 28S-RNA were used as loading control between lanes.

After overnight hybridisation, the membranes were washed for 30 min at 65°C twice with washing solution I and twice with washing solution II (high stringency wash). The membranes were then blotted on 3-mm blotting paper briefly to remove excess washing solution, sealed in plastic bags before being dried and exposed to Fuji X-ray medical films.

Loading dye:	0.01 g bromophenol blue 0.01 g xylene cyanol in 100 ml formamide store at -20°C
RNA loading buffer:	1250 µL loading dye 425 µL formaldehyde 250 µL 10× MSE 10 µL 10 mg /mL ethidium bromide
10× MSE:	41.8 g MOPS 6.8 g NaOAC 20 ml 0.5 M EDTA dissolve in water and adjust pH to 7.0 with NaOH, add water to 1 L. Autoclave, store at room temperature.
RNA gel running buffer:	100 mL 10× MSE 17.85 mL formaldehyde make up to 1L with sterile water
Transfer solution (pH 11.4 - 11.45):	3 M NaCI 8 mM NaOH
Neutralising buffer (pH 7.2):	0.2 M sodium phosphate buffer
Hybridisation solution:	0.25 M phosphate buffer (pH 7.2) 1 mM EDTA 7% SDS
Washing solution I:	40 mM phosphate buffer (pH 7.2) 1 mM EDTA (pH 8.0) 5% SDS
Washing solution II:	40 mM phosphate buffer (pH 7.2) 1 mM EDTA (pH 8.0) 1% SDS

4.2.11.5 Stripping hybridised ^{32}P-labelled probes off Northern blot membranes:

Northern blot membranes were boiled in stripping solution for 3× 10 min and the membranes were then exposed to Fuji X-ray medical film to ensure that stripping of the probe from the membranes had been successful.

stripping solution:	0.1 × SSPE 0.1% SDS

4.2.12 Southern analyses

4.2.12.1 Genomic DNA digestion by restriction enzymes and electrophoresis: Thirty micrograms of RNA-free genomic DNA in 100 µL of reaction solution was treated with 1 µL of restriction enzyme *Eco*RI, *Hind* III and *Xba* I at 37°C overnight to obtain complete digestion of the DNA. Next morning, the digested genomic DNA was precipitated by adding two volumes of 100% ethanol. The pellet was then washed with 75% and 100% ethanol. The air-dried DNA was then dissolved in 20 µL of sterile water. Subsequently, 5 µL of SUDS was added to the DNA samples and the solution mixed. The DNA samples were fractionated on 1% agarose gel with a size of 15 × 15 cm gel overnight at 25 V in 1× TAE running buffer overnight. Ethidium bromide was added to the gel at 0.67 µg/mL. DNA marker was run at the same time to indicate the sizes of DNA fragments. After being photographed with Polaroid 667 film, the Southern gel was transferred onto Hybond+ nylon membranes (Amersham) and the blotting was carried out using the downward alkaline transfer method (Chomczynski, 1992) for 3-4 hours. Subsequently, the membranes were removed, neutralised and cross-linked with UV light. Probe formation, pre-hybridisation and hybridisation were carried out using the same conditions as described in Section 4.2.11 for Northern analysis.

4.2.12.2 Stripping hybridised ^{32}P-labelled probes off Southern blot membranes: Stripping of the probes from Southern membranes was carried out by shaking the membranes in 0.4 M NaOH for 15 min at 30°C. Subsequently, the membranes were neutralised in 0.1 M Tris-HCI buffer (pH 7.2). Success of the stripping was confirmed by autoradiography.

4.2.13 Sequence data analysis:

The sequence data obtained in this thesis were analysed using the relevant programmes of GCG software package (Genetic Computer Group, Inc., Wisconsin). GenBank database was used to search for homologous nucleic acid sequences of cloned cDNA sequences and the predicted polypeptide amino acid sequences from the cloned cDNA

coding region were compared with the protein sequences held in the SwissProt, PIR protein databases.

4.3 Results

4.3.1 Quality and yields of total RNA preparation

In the preliminary RNA extraction trials using the original protocol from Chang et al. (1993), the yield obtained from our plant materials was mostly below 100 $\mu g\ g^{-1}$ FW and the ratio of OD_{260}/OD_{280} was below 1.5. Therefore, the original protocol was modified as detailed in Section 4.2.2, mainly by eliminating spermidine, reducing soluble PVP concentration from 2.0% to 1.0%, adding another detergent (SDS 2.0%) and treating the extraction mixture of plant material and the extraction buffer plus chloroform at a temperature of 55°C for 15 min. As a result of the modification, the yield obtained, as shown in Table 4.1, was greatly increased, in most cases being above 200 $\mu g\ g^{-1}$ FW.

Table 4.1 The quality and the quantity of total RNA obtained using modified RNA extraction procedures.

Materials	Yields (μg/g FW)	OD_{260}/OD_{280}
Field mature buds	113-380	1.65-1.75
Tissue cultured buds + BA	290-500	1.70-1.90
Tissue cultured buds - CK	390-500	1.70-1.90
Seedling buds	100 - 185	1.76 -1.78
Plantlet buds	158-224	1.75-1.98

The RNA pellets obtained at the final stage of the extraction were white and easily dissolved in water. To guarantee the quality for downstream steps, three methods were used to check RNA preparations (Section 4.2.4). As listed in Table 4.1, the ratio at OD_{260}/OD_{280} was above 1.70, indicating that there was no significant contamination by proteins and polysaccharides. An example of the absorbance spectra obtained from RNA samples is shown in Fig. 4.1, showing the typical shape of pure nucleic acid. The 280-nm side of the figure indicates no protein and polysaccharide contamination and the valley at 230 nm shows no salt contamination.

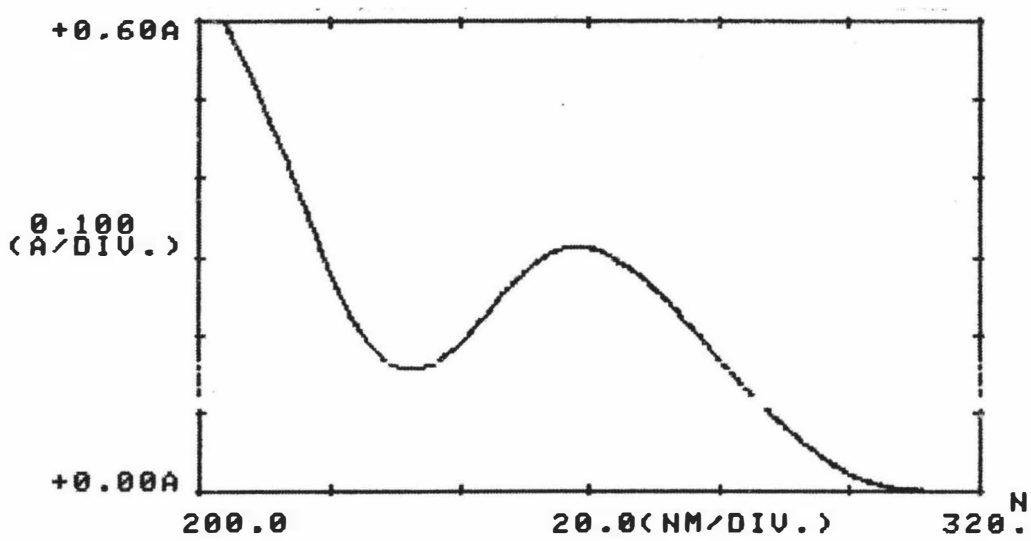


Figure 4.1 UV-light absorbance spectrum of purified total RNA from the buds of *P. radiata*.

The intactness of the RNA molecules of each sample was inspected by use of 1.2% denaturing agarose gel eletrophoresis (Section 4.2.11.1). The fractionated total RNA visualised with ethidium bromide shows no significant degradation (Fig. 4.2). It was also found that the profiles of total RNA obtained from the primary and the secondary needles of all ages differed from those isolated from the bud materials. Apart from the two major bands at 28 S and 18 S, the total RNA from needles had three extra distinctive bands (Figure 4.2 C)

4.3.2 mRNA differential display

The conditions as detailed in Section 4.2.5 for the first strand cDNA synthesis and the amplification of the cDNAs produced clear patterns of cDNAs from mRNA of 9-year-old mature buds and the mature buds that had received different cytokinin treatment for different periods of time (Fig. 4.3 and 4.4). As a result of using longer degenerate random primers and ethidium bromide staining, many fewer bands were visualised (Fig. 4.3 and 4.4) compared to the original technique (Liang and Pardee, 1992, Liang et al., 1993; Oh et al., 1995). With the same degenerate random primer (5′ CGGCARGTNACNTT 3′), differences at the 3′ end base of the anchored primers (5′ T_{12}VG 3′or 5′ T_{12}VC 3′) resulted in different profiles of the amplified cDNA populations (Fig. 4.3 and 4.4).

In the case of T_{12}VG, the band at ~650 bp as indicated by an arrow in Fig. 4.3 can not be seen in the lane of the field mature buds while its brightness was much stronger from the mature buds which had been cultured with cytokinin for 3 d and 25 d (Lanes 4 and 6) than that from the mature buds cultured without cytokinin (Lanes 5 and 7). This band was later designated as *Pinus radiata cytokinin-responsive* gene (*Prcr5*) band.

The Figure 4.4 shows the patterns of amplified cDNA generated by using 5′ CGGCARGTNACNTT 3′ and 5′ T_{12}VC3′. The top band showed similar intensities in both cytokinin-treated and non-cytokinin-treated sample lanes (Lanes 3, 4, 5 and 6) but not in the field mature bud lane (Lane 2). This band was later designated as *Pinus radiata chlorophyll a/b binding protein gene* (*Prcab*) band.

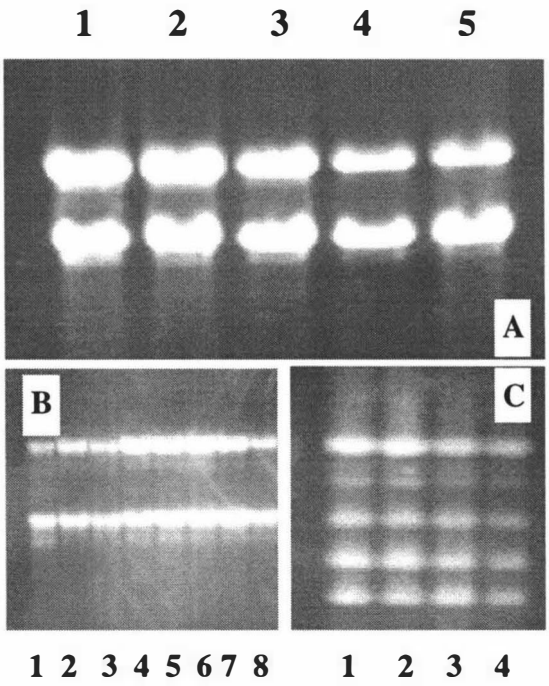


Figure 4.2 Electrophoresis of total RNA on a 1.2% denaturing agarose gel. The two major bands of 28S and 18S rRNA indicate no significant degradation. A: total RNA preparations for the first strand cDNA synthesis. Lane 1, field mature buds. Lane 2, mature bud fragments cultured on exogenous cytokinin-containing media for 3 d. Lane 3, mature bud fragments cultured on cytokinin-free media for 3 d. Lane 4, mature bud fragments cultured on cytokinin-containing media for 25 d. Lane 5, mature bud fragments cultured on cytokinin-free media for 25 d. B: total RNA prepared for Northern analysis. Lane 1 - 5, the same types of plant material as used in A but differrent extraction batch. Lane 6, mature bud fragments cultured on cytokinin-free media for 20 d after 25 d culture on cytokinin-containing media. Lane 7, seedling buds. Lane 8, plantlet on cytokinin-free culture medium. C: total RNA obtained from needles. Lane 1, primary needles from seedlings. Lane 2, primary needles from 2.5-year-old trees. Lane 3, secondary needles from seedlings. Lane 4, secondary needles from 2.5-year-old trees.

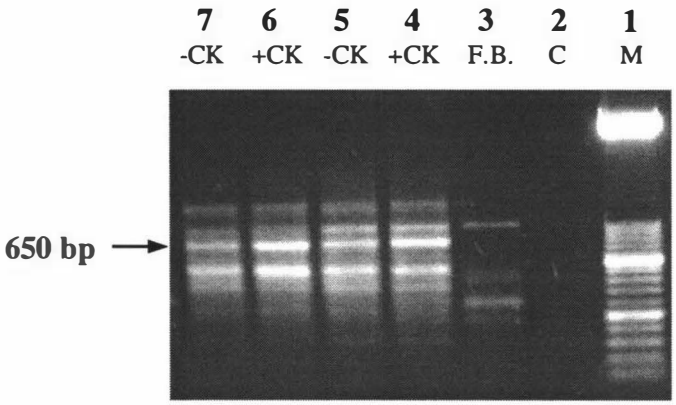


Figure 4.3 The differential display profiles of cDNAs amplified using $T_{12}VG$ as anchored primer, 5' CGGCARGTNACNTT 3' as random primer and the first strand cDNA as template. Gel concentration: 1.5% agarose containing 0.6 μg/ml ethidium bromide. Plant material sources: Lane 1, double stranded DNA size marker (M). Lane 2, PCR reaction without any first strand cDNA template (C). Lane 3, field mature buds (F.B.). Lane 4, mature bud fragments cultured on cytokinin-containing media for 3 d (+CK). Lane 5, mature bud fragments cultured on cytokinin-free media for 3 d (-CK). Lane 6, mature bud fragments cultured on cytokinin-containing media for 25 d (+CK). Lane 7, mature bud fragments cultured on cytokinin-free media for 25 d (-CK). The band of ca. 650 bp as indicated by an arrow is a cytokinin responsive band.

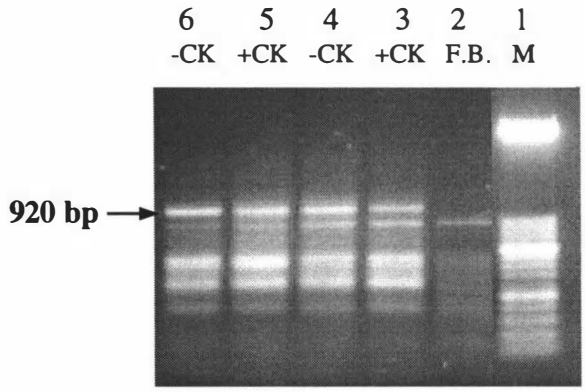


Figure 4.4 The differential display patterns of cDNAs multiplied using $T_{12}VC$ as anchored primer, 5' CGGCARGTNACNTT 3' as random primer and the first strand cDNA as template. Agarose gel was 1.5 % containing 0.6 μg/ml ethidium bromide. Lane 1, double strand DNA size marker. Lane 2, field mature buds (F.B.). Lane 3, mature bud fragments cultured on cytokinin-containing media for 3 d. Lane 4, mature bud fragments cultured on cytokinin-free media for 3 d. Lane 5, mature bud fragments cultured on cytokinin-containg media for 25 d. Lane 6, mature bud fragments cultured on cytokinin-free media for 25 d. The band at the size indicated by an arrow was not expressed in the mature field buds.

4.3.3 Cloning of *Prcr5* and *Prcab* bands

4.3.3.1. cDNA extraction from display agarose gel: The bands of *Prcr5* and *Prcab* cDNA were sliced out and the cDNA was extracted from the agarose gel slice as detailed in Section 4.2.6.1. The cDNA concentration was then determined by running the cDNA solutions along with a standard DNA mass ladder (DNA Mass™ Ladder, Life Technologies). After staining with ethidium bromide, the fluorescence intensities of the bands were compared (e.g. Fig. 4.5). A range between 20 and 200 ng cDNA was estimated and the cDNA solution was then diluted according to subsequent uses.

4.3.3.2. Cloning of the cDNA: The cDNA sequences derived from the *Prcr5* and *Prcab* bands were directly ligated into the pCRII vector as described in Section 4.2.6.2. Subsequently, the recombinant constructs of the *Prcr5* and *Prcab* cDNA were used to transform *E. coli* as described in Section 4.2.6.3. Putative recombinants were selected by culturing transformed *E. coli* on kanamycin-containing LB plates as detailed in Section 4.2.6.3. Thirty-four positive colonies for *Prcr5* and twenty-one colonies for *Prcab* were produced.

To confirm that the positive colonies possessed cDNA inserts, the numbered colonies of both *Prcr5* and *Prcab* were cultured individually in LB broth overnight as detailed in Section 4.2.7. Subsequently, the plasmid was extracted and purified as described in Section 4.2.7. The purified plasmid DNA was then digested with *Eco*RI as detailed in Section 4.2.8. After electrophoresis of the digestion solution, those clones with the inserts of *Prcr5* or *Prcab* cDNAs were identified as displayed in Fig. 4.6 and 4.7. Those colonies with an insert of ~640 or ~920 were regarded as true positive clones with *Prcr5* or *Prcab* cDNA inserts respectively. All the other colonies with no inserts or different insert sizes were either self ligation of the vector or false ligation with non-specific background contamination cDNAs. Fourteen clones of *Prcr5* (Fig. 4.6) and six clones of *Prcab* gene cDNA (Fig. 4.7) were obtained.

4.3.4 cDNA sequence grouping of single size band

Based on Figures 4.6 and 4.7, those colonies having inserts with different expected sizes were eliminated as false colonies. However, the clones containing the same size of

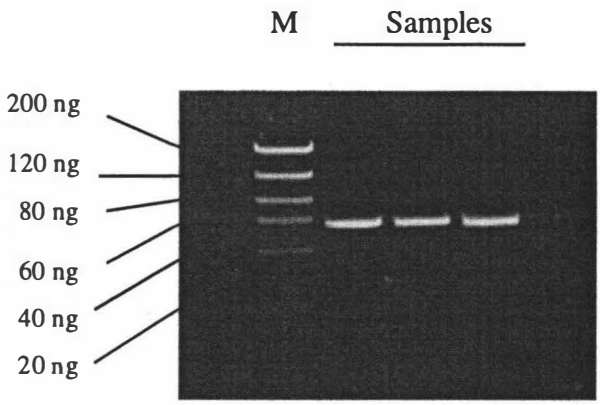


Figure 4.5 Estimation of sample plasmid DNA mass by comparing fluorescence of sample and mass ladder standards. The mass standard and the sample were run on 1.0% agarose gel and subsequently stained with ethidium bromide for 30 min. After 10 min destaining in running buffer, the photo was taken by Polaroid film 667.

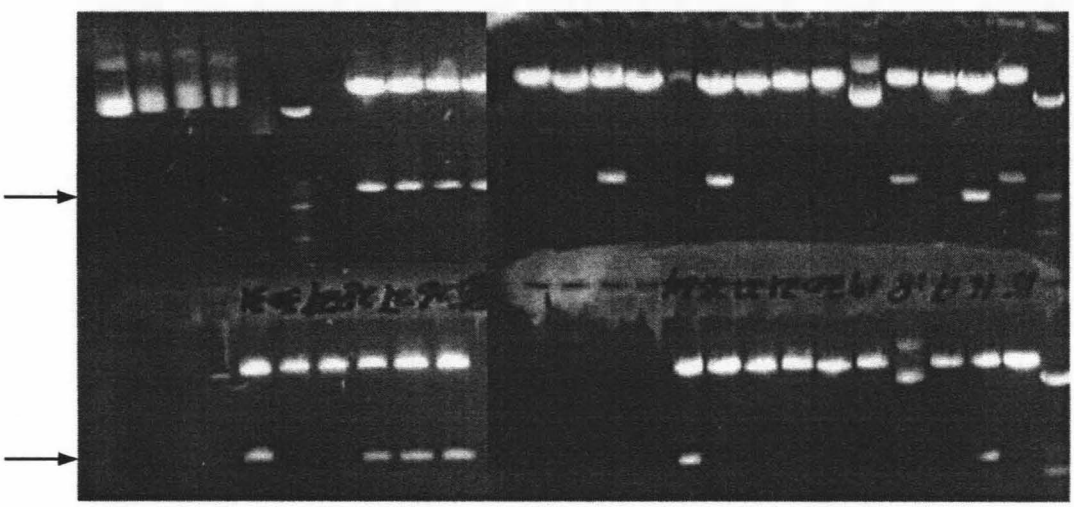


Figure 4.6 Confirmation of the presence of *Prcr* cDNA inserts in TA-cloning pCRII vectors derived from transformed *E. coli* colonies. Plasmid DNA prepared from every colony was digested with restriction enzyme *Eco*RI and run on 1.2% agarose gel containing 0.6 μg/ml ethidium bromide. Fourteen clones which contain target cDNA (around 650 bp indicated by an arrow) were identified.

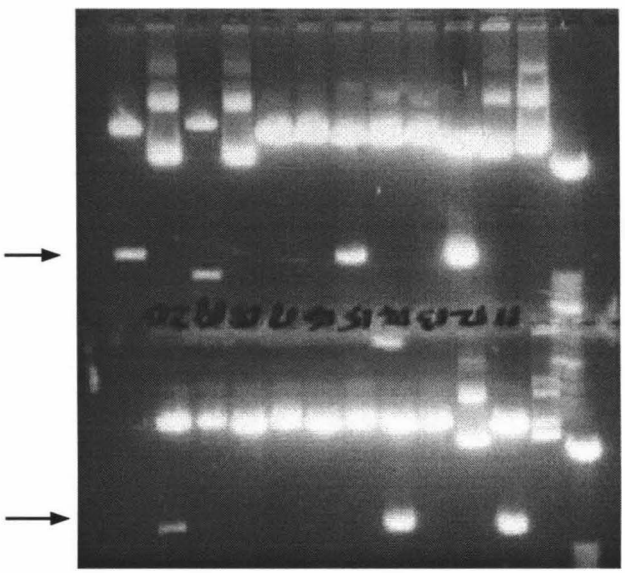


Figure 4.7 Confirmation of the presence of *Prcab* cDNA inserts in TA-cloning pCRII vectors derived from transformed *E. coli* colonies. Plasmid DNA prepared from every colony was digested with restriction enzyme *Eco*RI and run on 1.2% agarose gel containing 0.6 μg/ml ethidium bromide. Six clones which contain target cDNA (around 920 bp indicated by an arrow) were identified.

inserts do not necessarily have the same sequence. To assess if the inserts with the same size were actually composed of one or multiple cDNA sequences, a double restriction enzyme digestion was carried out as described by Shoham et al. (1996) and detailed in Section 4.2.9. Figure. 4.8A shows that the 14 cloned *Prcr5* cDNA gave the same enzyme digestion profiles, indicating that these clones belonged to the same cDNA species. This was in agreement with all the sequencing data obtained later. In contrast to Figure 4.8A, the fingerprinting profiles of another cloned band shown in Fig. 4.8B indicated several different groups of cDNA species were present in one single band.

4.3.5 Characterisation of *Prcr5* cDNA and *Prcab* cDNA

Prcr5 and *Prcab* inserts cloned in pCRII vectors were sequenced in both automated and manual ways as detailed in Section 4.2.10. The cDNA strands were sequenced in both directions.

4.3.5.1. Nucleotide sequence of Prcr5 cDNA: Nine of the 14 cloned *Prcr5* cDNAs were sequenced and they had 100% homology at the nucleotide level, which is in agreement with the result obtained from double restriction enzyme fingerprinting (Fig. 4.8A). The sequences of the *Prcr5* cDNAs consisted of 639 bp nucleotides which contained a 363 bp open reading frame (ORF) and a 276 bp entire 3′ untranslated region (UTR) (Fig. 4.9). In the 3′ UTR, a polyadenylation signal AATAAA is located 20 bp upstream of the polyA tail attachment (Fig. 4.9). According to analyses by Northern blotting, the entire *Prcr5* mRNA should be about 750 bp in size (Fig. 4.19). Therefore, the cloned *Prcr5* cDNA does not include the 5′ untranslated region and the region coding for N-terminal amino acids, which all together is about 110 bp nucleotides.

4.3.5.2: Amino acid sequence of the deduced polypeptide from the cDNA: The deduced PrCR5 peptide sequence shown in Fig. 4.9 was obtained with the PUBLISH programme of the GCG. The peptide consists of 121 amino acids. The calculation with ISOELECTRIC programme of the GCG software package indicated that the deduced PrCR5 is a basic peptide having an isoelectric point of 8.08. The Fig. 4.10 produced with the PEPPLOT programme of the GCG showed that the peptide is likely to be hydrophilic.

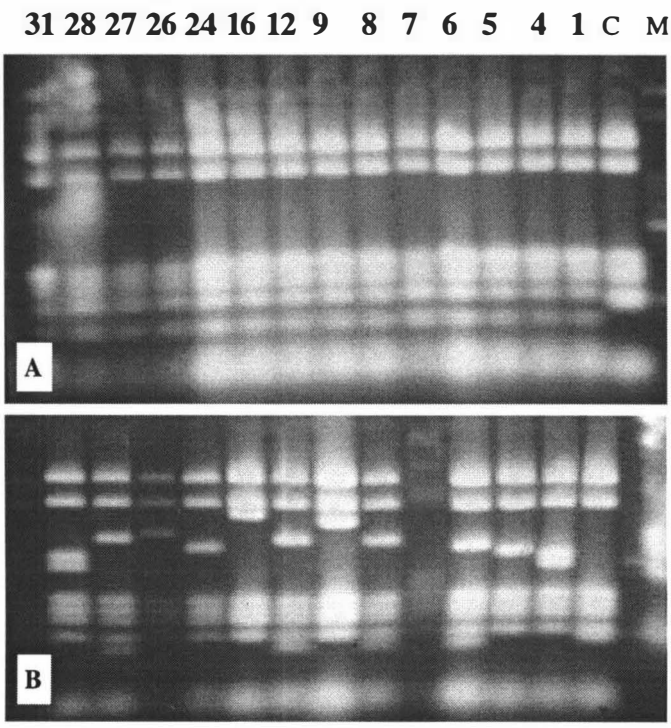


Figure 4.8 cDNA sequence grouping by restriction enzyme digestion fingerprinting. Plasmid preparation of each positive clone was digested with *Eco*RI and *Sau*3AI and then run on 3% agarose gel containing 0.6 μg/ml ethidium bromide. A: fingerprint display of fourteen true positive clones. The clones were numbered as shown on the top of each lane. Lane C indicates a control of pCRII plasmid DNA. Lane M is the DNA size marker. The patterns of the fourteen clones indicate that they may all have the same sequence. B: Restriction enzyme fingerprinting of clones whose plasmids derived from a different single band. The profiles in different lanes indicate that they have different sequences.

```
     Random primer
1    CGGCAGGTTACGTTAATTCAAGGAGATGGAGGCGTGGGCAGCATCAGGCAGATCAACTTC  60
     ArgGlnValThrLeuIleGlnGlyAspGlyGlyValGlySerIleArgGlnIleAsnPhe

61   ACCCCCGCTCACAAGGATTTCAGCTTCATCAAGGAGCGAGTGGATGAAATTGACGAGGAA  120
     ThrProAlaHisLysAspPheSerPheIleLysGluArgValAspGluIleAspGluGlu

121  AAAATGGTGTTGAAGTATACAAATATCGAAGGAGGGGTGCTGGGAAAGAAATTGAGTGCT  180
     LysMetValLeuLysTyrThrAsnIleGluGlyGlyValLeuGlyLysLysLeuSerAla

181  GCGAAGTTTGAGCTCAAATTCGTTCCCAGGAAAGAAGGGGGATGTGTCACCAGCTGGATC  240
     AlaLysPheGluLeuLysPheValProArgLysGluGlyGlyCysValThrSerTrpIle


241  TGTAATTACGAGACCCTTCCTGGTGCTCAGCTTGAAGAAAGCAAAGCAAAAGAGATAAAG  300
     CysAsnTyrGluThrLeuProGlyAlaGlnLeuGluGluSerLysAlaLysGluIleLys


301  GAAAATAGCATTGCCATGTTGAAGAAGATCGAGCAGTATCTGCTCTCCAATCCTTCTTTA  360
     GluAsnSerIleAlaMetLeuLysLysIleGluGlnTyrLeuLeuSerAsnProSerLeu

361  TACTGATGATCAATATATCTCTATGTGCATCTGTCTTCGCATGCATAAATAGTTTAGTGC  420
     TyrEndEnd

421  CGAGTATTATATGAATGAAACGGCTAAGAGTGTGCAAAATAAAGAAAGGTGATAGTATCC  480

481  CTTCGGCCGATCCTCCATCGTTCGCGTTATTTCTGGGCTGTAGCATGCCCTCTATGTGTT  540

541  TGCAATCGTAGAGTATGTGTACCTTTTTCGTCACATGAATGTGTGTAGCTCTTTCCATAT  600

601  AAATAAATTATGTGTACCTTTTTCGCCAAAAAAAAAAAA  639
                              Anchored primer
```

Figure 4.9 Sequence of cloned *Prcr5* cDNA and deduced amino acid sequence of the protein PrCR5.

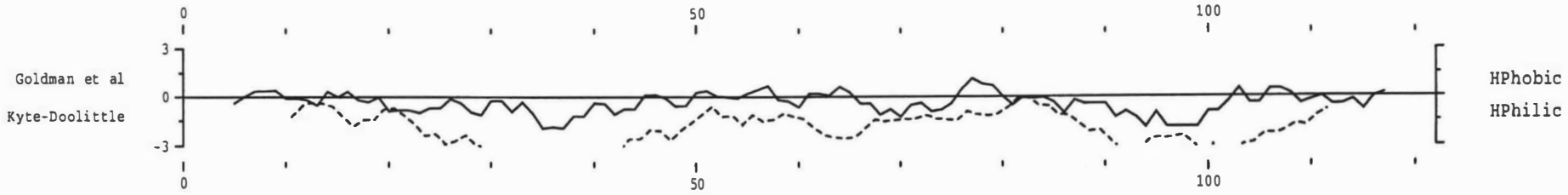


Figure 4.10 Hydropathy plot of the deduced amino acid sequence of PrCR5.

4.3.5.3 Relationship of Prcr5 to other gene sequences: At the nucleotide level, the search using the BLAST programme of GCG indicated that *Prcr5* had homologies with several sequences, which code for a pathogenesis-related protein (PR-10). Within a 263 bp coding region of *Prcr5* cDNA, there was 53 % identity with a potato pSTH-2 mRNA (GB_PL: M25155). In addition, in a 47 bp coding region, *Prcr5* had 81% identity with an *Asparagus officinalis* early flowering protein mRNA. No similarity was found between the 3′ untranslated regions of *Prcr5* cDNA and any other sequences in the GenBank database.

Although it was difficult to find homologous sequences at the nucleotide level, many more sequences homologous to *Prcr5* gene were found at the amino acid level from Genebank, SwissProt and PIR protein databases. The similarity and identity were calculated with the BESTFIT programme of GCG (Table 4.2). The most homologous protein sequence to the deduced PrCR5 amino acid sequence was an early flowering protein in *Asparagus* (Yeo et al. 1996; GenBank U69983), having a 51.7% similarity and 44.8 % identity. As shown in Table 4.2, the protein sequences that PrCR5 has homology with are mainly from three groups of proteins: pathogenesis-related protein-

Table 4.2 Similarities and identities between the deduced polypeptide sequence of *Prcr5* and the other protein sequences

Sequences	Similarity (%)	Identity (%)	PI
PrCR5	100	100	8.08
AoEFP (early flower protein)	51.7	44.8	4.76
SbPR-10	50.9	40.4	5.34
AoPR1(PR-10)	49.6	42.6	6.26
Cor a 1 (pollen allergen)	49.2	33.3	6.13
Bet v1 (RNase)	48.7	37.4	6.36
STH-2 (PR-10)	48.2	36.4	5.64
Mal d 1 (pollen allergen)	47.9	33.6	6.52
Parsley2 (PR-10)	46.2	34.5	4.31
Ginseng2 RNase	46.1	33.0	4.36

* Protein sequence accession numbers: AoEFP, GB: U69983; SbPR-10, GB: U60764; AoPR1, SW: Q05736; Cor a1, SP: Q39454; Bet v1, SP: Q39427; STH-2, SW: P17642; Mal d1, SP: Q43549; Parsley2, SW: P27538; Ginseng2, Moiseyev et al. (1997).

10 (PR-10), ginseng RNase and pollen allergen proteins. However, the isoelectric point of PrCR5 is much higher (PI 8.08) than those of the related proteins listed in Table 4.2.

Pair-based comparison of PrCR5 with other groups of protein sequences was performed with the PILEUP programme of GCG. The PrCR5 amino acid sequence was aligned using progressive and pairwise method with an *Asparagus* early flowering protein (AoEFP, U69983), an *Asparagus* pathogenesis-related protein (AoPR1, Q05736), a birch pollen allergen, Betv1 (Q39427), Betv 1C (P43176), a parsley intracellular protein 2, a ginseng RNase2, a *Corylus avellana* major allergen (Cor a1, Q39454), a *Malus domestica* major allergen (Mal d1, Q43549) and a *Solanum tuberosum* pathogenesis-related protein 2 (STH-2, P17642) (Fig. 4.11). As highlighted in the Fig. 4.11, all the sequences share some common patterns (or conserved patterns) of amino acids, indicating they might have similar functions in these plant species.

The dendrogram (Fig. 4.12) shows the clustering relationships of the PrCR5 protein with other related proteins. As indicated in the figure 4.12, the most related sequences to PrCR5 protein are AoEFP and AoPR1. However, there are no big difference in the relatedness distances between PrCR5 and any other sequences in comparison with AoEFP and AoPR1.

4.3.5.4. Comparison of 3′ untranslated region of Prcr5 with other sequences: No sequences in the GenBank database were found to be homologous with the nucleotide sequence of the 3′ untranslated region of *Prcr5* mRNA.

4.3.6 Characterisation of *Prcab* gene cDNA

4.3.6.1 Characterisation of the sequence: Three clones of *Prcab* cDNA were sequenced and had 100% homology to one another. The cDNA sequence was composed of 916 bp nucleotides (Fig. 4.13) with a 642 bp open reading frame and a 274 bp 3′ untranslated region. The polyadenylation signal AATAAA is situated 22 bp upstream of the polyadenylate tail. According to the Northern analyses (Fig 4.23), the complete mRNA would be 1.10 Kbp. By deduction, the cloned cDNA is a partial cDNA containing an entire 3′ untranslated region. The deduced peptide sequence of PrCAB from the partial *Prcab* cDNA was obtained with the PUBLISH programme and consists of 214 amino acids (Fig. 4.13).

```
            1                                                   50
  PetcrPR2  MGAVTTDVEV ASSVPAQTIY KGFLLDMDNI IPKVLPQAIK SIEIISGDGG
GS RNase 2  ~GVQKTETGA ISPVPAEKLF KGSFLDMDTV VPKAFPEGIK SVQVLEGNGG
    Betv1c  ~GVFNYESET TSVIPAARLF KAFILEGDTL IPKVAPQAIS SVENIEGNGG
     Betv1  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~IS SVENIEGNGG
     Cor a1  MGVFNYETET TSVIPPARLF KRFVLDSDNL IPKVAPKAIK SIEIIEGNGG
     Mal d1  MGVFNYETEF TSVIPPARLF NAFVLDADNL IPKIAPQAVK SAEILEGDGG
      STH-2  MAVFTFEDQT TSPVAPATLY KALVKDADTI VPK.AVDSFK SVEIVEGNGG
      AoEFP  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~EIVS SASVVAVDGG
      AoPR1  MSSGSWSHEV AVNVAAGRMF KAAMLDWHNL GPKIVPDFIA GGSVVSGDGS
     PrCR5  ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~R QVTLIQGDGG

            51                                                  100
  PetcrPR2  AGTIKKVTLG EVS.QFTVVK QRIDEIDAEA LKYSYSIIEG DLLL..GIIE
GS RNase 2  VGTIKNVTLG DAT.PFNTMK TRIDAIDEHA FTYTYTIIGG DILL..DIIE
    Betv1c  PGTIKKITFP EGS.PFKYVK ERVDEVDHAN FKYSYSMIEG GALG..DTLE
     Betv1  PGTIKKITFP EGS.PFKYVK ERVDEVDHAN FKYSYSMIEG GALG..DTLE
     Cor a1  PGTIKKICFD EGS.PFNYIK QKVEEIDQAN FSYRYSVIEG DALS..DKLE
     Mal d1  VGTIKKINFG EGS.TYSYVK HRIDGVDKEN FVYKYSVIEG DAIS..ETIE
      STH-2  .GSIKKMNFV EGS.PIKYLK HKIHVVDDKN LVTKYSMIEG DVLG..DKLE
      AoEFP  VGSIRQINFT S.AMPFPYLK ERLDFVDEAN FECKSSLIEG GDLGT..KLE
      AoPR1  VGTIREIKIN NPAIPFSYVK ERLDFVDHDK FEVKQTLVEG GGLGK..MFE
     PrCR5  VGSIRQINFT PAHKDFSFIK ERVDEIDEEK MVLKYTNIEG GVLGK..KLS

            101                                                 150
  PetcrPR2  SITSKFTVVP T.DGGCIVKN TTIYTPIGDA VIPENVKEA TEQSGMVFKA
GS RNase 2  SIENHFKIVP T.DGGSTITQ TTIYNTIGDA VIPENIKDA TDKSIQLFKA
    Betv1c  KICNEIKIVA TPDGGSILKI SNKYHTKGDQ EMKAEHMKAI KEKGEALLRA
     Betv1  KICNEIKLVA TPDGGSILKI SNKYHTKGDH EMKAEHMKAI KEKAEALLRA
     Cor a1  KINYEIKIVA SPHGGSILKS ISKYHTIGDH ELKDEQIKAG KEKASGLFKA
     Mal d1  KISYETKLVA S.GSGSVIKS TSHYHTKSDV EIKEEHVKAG KEKASHLFKL
      STH-2  SISYDLKFEA HGNGGCVCKS ITEYHTKGDY VLKDEEHNEG QKQGMELFKI
      AoEFP  SASSHFKLVP SSNGGCVVKL EGIFKALPGV ETTDEVAKG. KEMMTNAIKA
      AoPR1  CATTHFKFEP SSNGGCLVKV TASYKILPGV ..ADESAKA. KEGITNHMKA
     PrCR5  AAKFELKFVP RKEGGCVTSW ICNYETLPGA QLEESKAKEI KENSIAMLKK

            151       163
  PetcrPR2  IEAYLLANPG AY~
GS RNase 2  VEAYLLAN~~ ~~~
    Betv1c  VESYLLAHSD AYN
     Betv1  VESYLLAHSD AYN
     Cor a1  VEGYLLAHSD AYN
     Mal d1  IENYLLAHSD AYN
      STH-2  VEAYLLANPS VYA
      AoEFP  AEAYLVANPT AYA
      AoPR1  TEAYLLANPT AYV
     PrCR5  IEQYLLSNPS LY~
```

Figure 4.11 Common patterns of conserved amino acids between the deduced PrCR5 protein sequence and other PR-10 protein sequences. The multiple alignment was achieved using PILEUP of the GCG package with a gap weight of 12 and a gap length weight of 4. The conserved amino acids are highlighted as **bold**. Petcr PR2: *Petroselinum crispum* pathogenesis-related protein 2, accession no. P27538. GS RNase 2: Ginseng RNase 2, Moiseyev et al., (1997). Betv1c: *Betula verrucosa* major pollen allergen, accession no. P43176. Betv1: *Betula verrucosa* major pollen allergen, accession no. Q39427. Cor a1: *Corylus avellana* major allergen, accession no. Q39454. Mal d1: *Malus domestica* major allergen, accession no. Q43549. STH-2: *Solanum tuberosum* pathogenesis-related protein, accession no. P17642. AoEFP: *Asparagus officinalis* early flowering protein, accession no. U69983. AoPR1: *Asparagus officinalis* pathogenesis-related protein, accession no. Q05736.

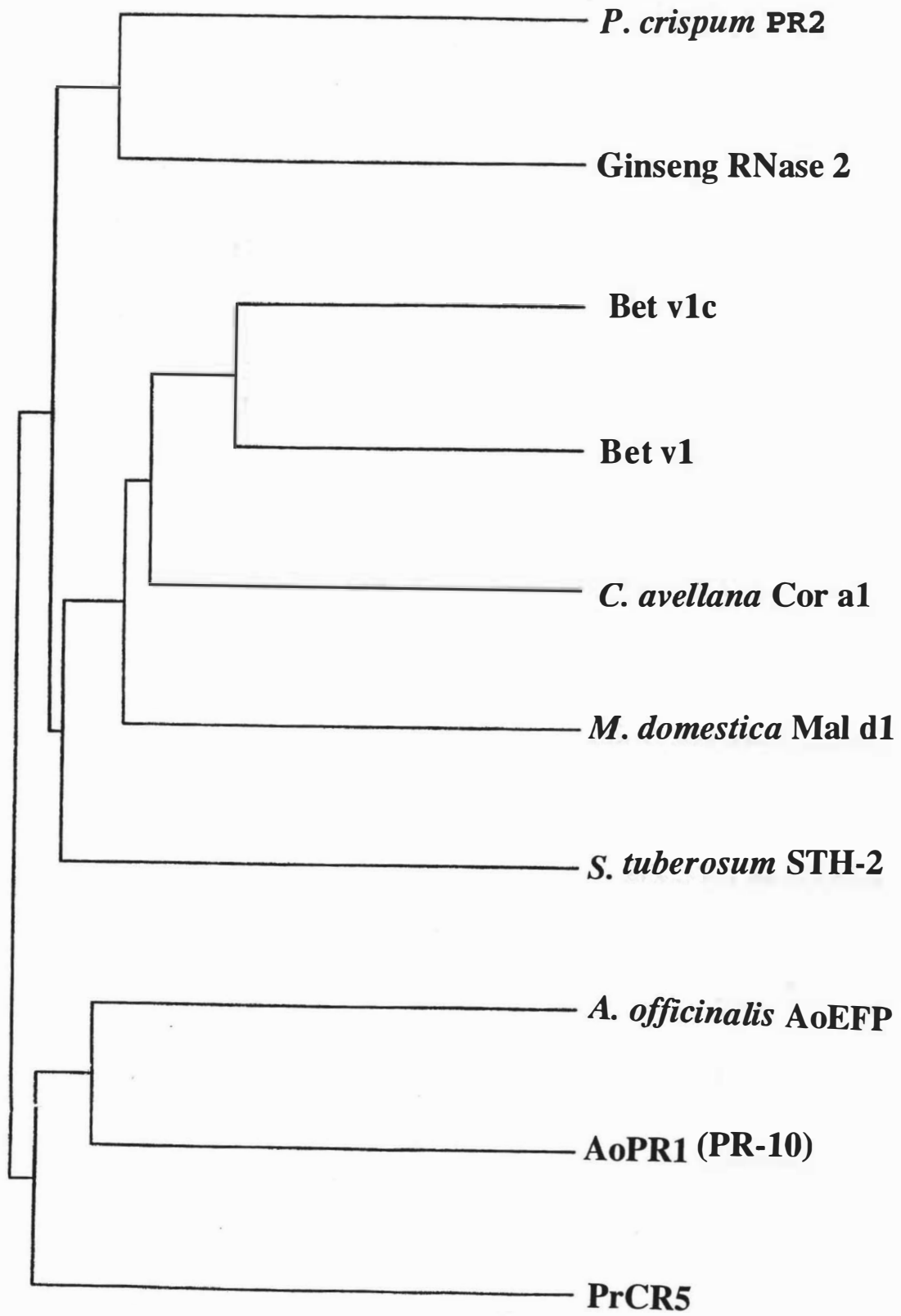


Figure 4.12 Dendogram displaying the relatedness of the deduced PrCR5 protein sequence to other related proteins. The distance along the horizontal axis is proportional to the difference between sequences. The vertical axis has no meaning. See figure 4.11 for accession numbers.

```
     Random primer
1    CGGCAAGTTACGTTCACCATGGCAACAGCTTCGGCCATCCAAAGCTCAAGCTTGGCAGGC  60
     ArgGlnValThrPheThrMetAlaThrAlaSerAlaIleGlnSerSerSerLeuAlaGly

61   CAGACCCTCCTAAGGCCGCAACAGAATGAGCTCGTCAAGAAAGTGGGCACGTCGCAGGCT 120
     GlnThrLeuLeuArgProGlnGlnAsnGluLeuValLysLysValGlyThrSerGlnAla

121  CGAATCACCATGCGAAGAACCGTAAGGAGCGCCCCCGAGAGCATTTGGTATGGACCTGAC 180
     ArgIleThrMetArgArgThrValArgSerAlaProGluSerIleTrpTyrGlyProAsp

181  CGCCCCAAGTACCTAGGCCCCTTCTCGGAACAGACGCCGTCATATCTCACCGGAGAATTT 240
     ArgProLysTyrLeuGlyProPheSerGluGlnThrProSerTyrLeuThrGlyGluPhe

241  CCCGGCGACTACGGGTGGGACACTGCCGGCCTCTCGGCGGATCCAGAGACCTTCGCAAAA 300
     ProGlyAspTyrGlyTrpAspThrAlaGlyLeuSerAlaAspProGluThrPheAlaLys

301  AACAGAGAGCTGGAGGTGATCCACTGCAGATGGGCCATGTTGGGAGCGCTCGGCTGCGTT 360
     AsnArgGluLeuGluValIleHisCysArgTrpAlaMetLeuGlyAlaLeuGlyCysVal

361  TTCCCGGAGCTGTTGGCCAAAAATGGGGTTGAAATTTGGGGAAGCTGTGTGGTTCAAGGC 420
     PheProGluLeuLeuAlaLysAsnGlyValGluIleTrpGlySerCysValValGlnGly

421  CGGGGCGCAGATATTCTCAGAGGGAGGCTTGACTATTTGGGGAACCCCAACCTGATCCAC 480
     ArgGlyAlaAspIleLeuArgGlyArgLeuAspTyrLeuGlyAsnProAsnLeuIleHis

481  GCGCAGAGCATTCTAGCCATCTGGGCCTGCCAGGTTGTTCTCATGGGATTGATTGAAGGA 540
     AlaGlnSerIleLeuAlaIleTrpAlaCysGlnValValLeuMetGlyLeuIleGluGly

541  GAGTGGGAGGAGGACCACTTGGAGAGGGGTTGGACCCTCTGTACCCAGGGGGTGCCTTTG 600
     GluTrpGluGluAspHisLeuGluArgGlyTrpThrLeuCysThrGlnGlyValProLeu

601  ACCCACTGGGGCTGGCCGACGACCCAGAGGCCTTCGCGGAGCTGAAGGTGAAGGAGATTA 660
     ThrHisTrpGlyTrpProThrThrGlnArgProSerArgSerEnd

661  AGAACGGTCGGCTGGCCATGTTCTCCATGTTCGGTTTCTTCGTTCAGGCAATCGTGACCG 720

721  GAAAGGGCCCCATTGAAAATNTTTACGACCACTTGGCGGACCCCGCTGCCAACAATGCCT 780

781  GGGCCTACGCCACCAATTTCGTTCCTGGCAAGTGAAGGGAAGTGACGGAAAATAAAAGAG 840

841  GCCTGTGATCTGTGCATTAATCATTTGACAGCCTTAGTGTTAATAAAATATTTTCTTTCA 900

901  CCTGGAAAAAAAAAAAAAA  919
        Anchored primer
```

Figure 4.13 Nucleotide sequence of cloned *Prcab* cDNA and the deduced amino acid sequence of the protein PrCAB.

4.3.6.2 Relationship to other genes: At the nucleotide level, the *Prcab* has more than 90% identical nucleotide bases with the complete *cab* mRNA of Japanese black pine (*Pinus thunbergii*). At the amino acid level, the identity remains as high as 87%. The difference between *Prcab* and the other *cab* sequences mainly occurred at the 3′ side of the cDNA sequence and the C terminal of the peptide sequence (Fig. 4.14).

4.3.7 Genomic analysis of the cloned *Prcr5* and *Prcab* genes

4.3.7.1. DNA preparations: Around 475 μg g^{-1} FW of genomic DNA was isolated from field grown mature buds using the method described in Section 4.2.3. The quality of the isolated DNA was determined by both spectrophotometry as detailed in Section 4.2.4 and by restriction enzyme digestion (Fig. 4.15). An at least 1.7 average ratio at OD_{260}/OD_{280} and typical shape of UV light absorbance spectrum similar to Fig. 4.1 indicated the genomic DNA obtained was of good quality. It was further confirmed that the isolated DNA was of high quality as the DNA was readily digested by all three different restriction enzymes (Fig. 4.15).

4.3.7.2. Southern analyses: Southern analyses were performed to study the arrangement of the genes whose partial cDNAs had been cloned. The genomic DNA isolated from the buds of one 9-year-old and one 4-year-old (clone G27 tree) was digested with *Eco*RI, *Hind* III and *Xba* I (Section 4.2.12). After electrophoresis, the gel was blotted onto Nylon membrane (Section 4.2.12). Subsequently, the membranes were probed with either ^{32}P-labelled *Prcr5* cDNA or ^{32}P-labelled *Prcab* cDNA.

The Southern hybridisation as described in Section 4.2.11.4 with ^{32}P-labelled *Prcr5* indicated that the *Prcr5* and *Prcab* are each represented by a multigene family *in P. radiata* (Fig. 4.16 and 4.17). Multiple hybridising bands were observed in every restriction enzyme digestion lane. These data suggest that the sequences hybridising to *Prcr5* and *Prcab* probes are present in multiple copies in the genome. The bands hybridising to *Prcab* showed the same patterns between the two trees tested while the patterns revealed with the *Prcr5* probe varied in the *Eco*RI digestion lanes between the two trees examined. Although there is a possibility that the multiple bands could be due to multiple introns within the gene, it is unlikely to be the situation in the *Prcr5* gene because of its fairly small size (about 750 bp in full) and the high washing stringency

```
          1                                                    50
Q40956    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~QQNELVK KVGASQARIT
Q40934    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~QARIT
Q00984    ~~~~~~~~~~ ~~~MATASAI QSSSLAGQTL LRPQQNELVK KVGTAQARIT
P10049    ~~~~~~~~~~ ~~~MATASAI QSSSLAGQTL LRPQQNELVK KVGTAQARIT
P15193    MATTMASCGI GSRCAFAGAQ LSSVKPQNNQ LLGVG.GAHG EARLTMRKAT
PrCAB1    ~~~~~~~~RQV TFTMATASAI QSSSLAGQTL LRPQQNELVK KVGTSQARIT

          51                                                  100
Q40956    MRRTVRSAPE SIWYGPDRPK YLGPFSEQTP SYLTGEFPGD YGWDTAGLSA
Q40934    MRRTVRSAPE SIWYGPDRPK YLGPFSEQTP SYLTGEFPGD YGWDTAGLSA
Q00984    MRRTVRSAPE SIWYGPDRPK YLGPFSEGTP SYLTGEFPGD YGWDTAGLSA
P10049    MRRTVRSAPE SIWYGPDRPK YLGPFSEGTP SYLTGEFPGD YGWDTAAVSA
P15193    GKKSVAASID SPWYGPDRVL YLGPFSGEPP SYLTGEFPGD YGWDTAGLSA
PrCAB1    MRRTVRSAPE SIWYGPDRPK YLGPFSEQTP SYLTGEFPGD YGWDTAGLSA

          101                                                 150
Q40956    DPETFAKNRE LEVIHSRWAM LGALGCVFPE LLAKNGVKFG EAVWFKAGAQ
Q40934    DPETFAKNRE LEVIHSRWAM LGALGCVFPE LLAKNGVKFG EAVWFKAGAQ
Q00984    DPETFAKNRE LEVIHCRWAM LGALGCLFPE LLAKNGVKFG EAVWFKAGAQ
P10049    DPETFAKNRE LEVIHCRWAM LGALGCVFPE LLAKNGLKFG EAVWFKAGAQ
P15193    DPETFAKNRE LEVIHSRWAM LGALGCVFPE LLARNGVKFG EAVWFKAGAQ
PrCAB1    DPETFAKNRE LEVIHCRWAM LGALGCVFPE LLAKNGVEIW GSCVVQGRGA

          151                                                 200
Q40956    IFSEGGLDYL GNPNLIHAQS ILAIWACQVV LMGLIEGYRV GGGPLGEGLD
Q40934    IFSEGGLDYL GNPNLIHAQS ILAIWAFQVV LMGLIEGYRV GGGPLGEGLD
Q00984    IFSEGGLDYL GNPNLIHAQS ILAIWACQVV LMGLIEGYRV GGGPLGEGLD
P10049    IFSEGGLDYA GNPNLIHAQS ILAIWACQVV LMGLIEGYRV GGGTLGEGLD
P15193    IFSEGGLDYL GSPQLIHAQS ILAIWACQVI LMGAIEGYRV AGGPLGEVTD
PrCAB1    DILRGRLDYL GNPNLIHAQS ILAIWACQVV LMGLIEG.EW EEDHLERGWT

          201                                                 250
Q40956    PLYPGGAFDP LGLADDPEAF AELKVKEIKN GRLAMFSMFG FFVQAIVTGK
Q40934    PLYPGGAFDP LGLADDPEAF AELKVKELKN GRLAMFSMFG FFVQAIVTGK
Q00984    PLYPGGAFDP LGLADDPEAF AELKVKEIKN GRLAMFSMFG FFVQAIVTGK
P10049    PLLPGGAFDP LGLADDPEAC AELKVKEIKN GRLAMFSMFG FFVQAIVTGK
P15193    PIYPGGNFDP LGLADDPDAF AELKVKEIKN GRLAMFSMFG FFVQAIVTGK
PrCAB1    LCTQGVPLTH WGWPTTQRPS RS~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          251                       279
Q40956    GPIENLYDHL ADPVANNAWA YATNFVPGK
Q40934    GPIENLFDHL ADPTANNAWA YATNFVPGK
Q00984    GPIENLYDHL ADPVANNAWA YATNFVPGK
P10049    GPIENLYDHL ADPVANNAWA YATNFVPGK
P15193    GPIENLADHL ADPVNNNAWA YATNFVPGK
PrCAB1    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~
```

Figure 4.14 Comparison of conserved amino acids between deduced PrCAB and other chlorophyll a/b binding proteins of conifers. The multiple alignment was achieved using PILEUP of the GCG package with a gap weight of 12 and a gap length weight of 4. The conserved amino acids are highlighted as bold letters. Accession numbers Q40956, Q40934, Q00984, P10049 and P15193 represent different chlorophyll a/b binding protein sequences derived from *Pinus palustris*, *Pseudotsuga menziesii*, *Pinus thunbergii*, *Pinus thunbergii* and *Pinus sylvestris* respectively.

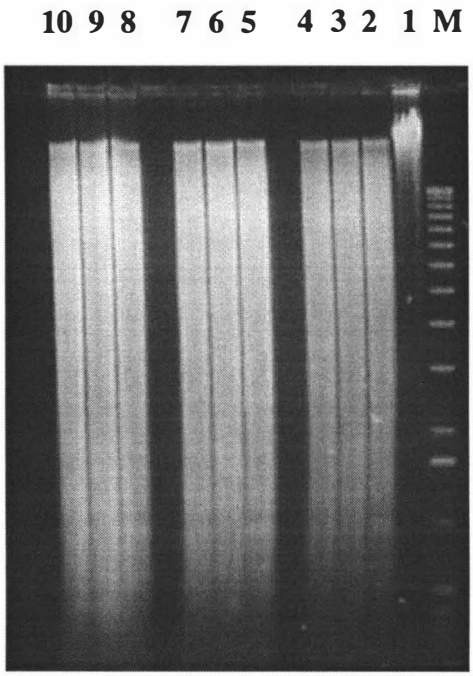


Figure 4.15 Restriction enzyme digestion of genomic DNA of *P. radiata*. 30 µg of genomic DNA was digested with *Eco*RI, *Hind*III or *Xba*I (Section 4.2.12.1). The digestions were run overnight at 25 V and subsequently stained with ethidium bromide. Lane M, standard size marker. Lane 1, uncut control of genomic DNA. Lane 2 - 7, digestion of genomic DNA from 9-year-old mature buds. Lane 8 - 10, digestion of genomic DNA isolated from the buds of G27 clone tree. Lane 2, 5 and 8, DNA was digested with *Eco*RI. Lane 3, 6 and 9, with *Hind*III. Lane 4, 7 and 10, DNA was digested with *Xba*I.

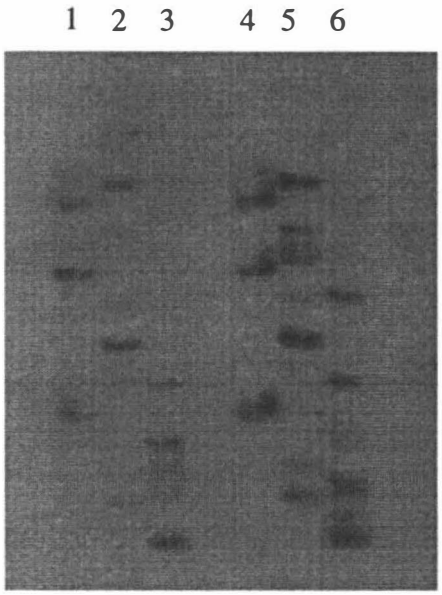


Figure 4.16 Southern analysis of *Prcr5* gene arrangment in genomic DNA in *P. radiata*. 30 µg genomic DNA extracted from an 9-year-old tree (Lanes 1, 2 and 3) and a four-year-old tree (Lanes 4, 5 and 6) was digested with *Xba*I (Lanes 1 and 4), *Hind*III (Lanes 2 and 5) and *Eco*R1 (Lanes 3 and 6). The digested DNA was fractionated on a 1% agarose gel and blotted onto Hybond^{+} nylon membrane. The membrane was probed with ^{32}P-labelled *Prcr5* cDNA according to the conditions described in Section 4.2.11.

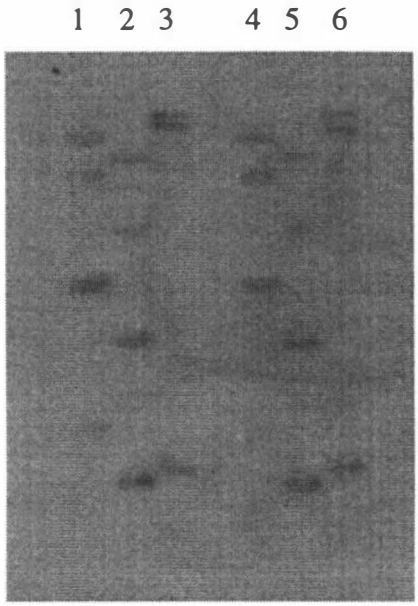


Figure 4.17 Southern analysis of *Prcab* gene arrangement in genomic DNA in *P. radiata*. 30 µg genomic DNA extracted from a 9-year-old tree (Lanes 1, 2 and 3) and a four-year-old tree (Lanes 4, 5 and 6) was digested with *Xba*I (Lanes 1 and 4), *Hind*III (Lanes 2 and 5) and *Eco*R1 (Lanes 3 and 6). The digested DNA was fractionated on a 1% agarose gel and blotted onto Hybond+ nylon membrane. The membrane was probed with ^{32}P-labelled *Prcab* cDNA according to the conditions described in Section 4.2.11.

used in performing the Southern analyses (could be a tandem gene cluster).

To control the loading in Northern analyses, a 28S rRNA gene cDNA was also cloned in this thesis. In contrast, when the digested genomic DNA was probed with ^{32}P-cDNA of 28S rRNA, a single hybridisation band was generated from every restriction enzyme digestion lane of genomic DNA obtained from all trees used (Fig. 4.18). The results revealed that the cloned 28S rRNA gene may be a single-copy gene in the genome of *P. radiata* with no significant changes of copy number among the individual trees, indicating that this is ideal gene to be used in loading control.

4.3.8 Northern analyses of gene transcription

4.3.8.1 Exogenous cytokinin treatment promotes and maintains the transcription of the Prcr5 gene: To assess how exogenous cytokinin treatments affect the transcription of the *Prcr5* gene over time, kinetic analyses of gene expression were performed. Figure 4.19 shows representative results of Northern analyses of *Prcr5* gene expression in response to cytokinin over a 7 d period.

Under both light/dark cycles and complete dark conditions, exogenous cytokinin treatment promoted similar kinetic patterns of gene expression of the *Prcr5* (Fig. 4.19). No transcription of the *Prcr5* gene was observed in the field mature buds (Lane C, Fig. 4.19) and the buds that had been cultured on cytokinin for 12 h (Fig. 4.19). Gradually, the *Prcr5* mRNA in the buds started to accumulate after 24 h culture with exogenous cytokinin and continued to rise to about the maximum level after 72 h under both light/dark cycles and dark culture conditions. In the absence of exogenous cytokinin, under both light/dark cycle and complete dark culture conditions, the *Prcr5* mRNA accumulation was also noticed after 24 h and kept rising until 72 h. However, the maximum level of the *Prcr5* gene expression was much lower in the buds without the treatment of exogenous cytokinin (Fig. 4.19).

The presence of exogenous cytokinin maintained *Prcr5* expression at a high level for a long period of time. Fig. 4.20 shows that, after 25 d treatment with exogenous cytokinin, the *Prcr5* mRNA accumulation in the cultured buds either from the

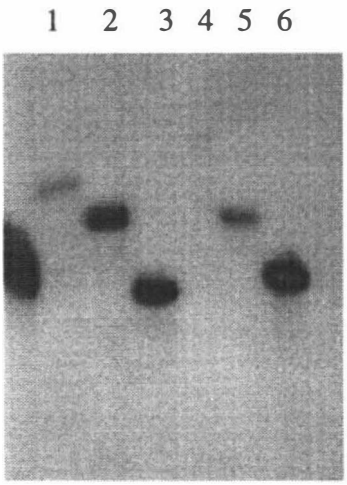


Figure 4.18 Southern analysis of 28 S rRNA gene arrangement in genomic DNA in *P. radiata*. 30 µg genomic DNA extracted from a 9-year-old tree (Lanes 1, 2 and 3) and a four-year-old tree (Lanes 4, 5 and 6) was digested with *Xba*I (Lanes 1 and 4), *Hind*III (Lanes 2 and 5) and *Eco*R1 (Lanes 3 and 6). The digested DNA was fractionated on a 1% agarose gel and blotted onto Hybond+ nylon membrane. The membrane was probed with ^{32}P-labelled 28S rRNA cDNA according to the conditions described in Section 4.2.11.

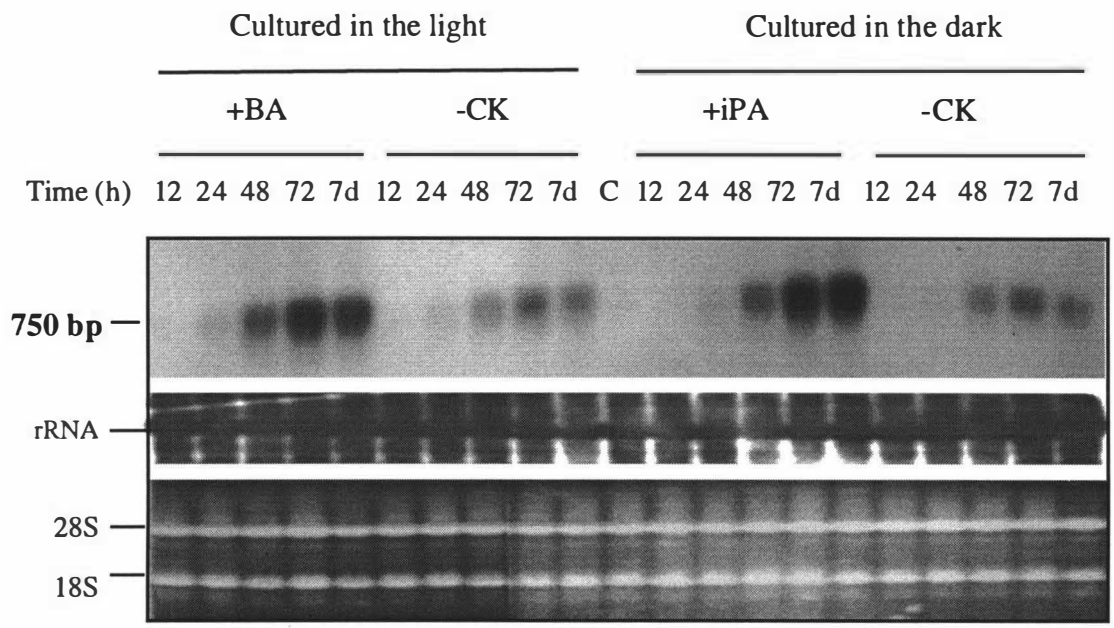


Figure 4.19 Northern analysis of *Prcr5* gene expression in the mature *P. radiata* buds cultured over a period of 7 days in the presence or absence of exogenous cytokinin. The 'light' conditions were a 16/8 light/dark cycle. 25 µg total RNA was loaded in each lane and fractionated on 1.2% denaturing agarose gel. The fractionated total RNA was blotted onto Hybond^{+} nylon membrane and probed with ^{32}P-labelled *Prcr5* cDNA according to the condition described in Section 4.2.11. A loading control was included by probing the membrane using ^{32}P-labelled 28S rRNA cDNA and ethidium bromide staining. C represents field mature buds.

9-year-old tree or the 4 year-old tree (clone G27) remained very high while its level stayed low in the buds from the same trees that had not been treated with exogenous cytokinin (Fig. 20A and B).

4.3.8.2. Prcr5 gene transcription responds to cytokinin in a dose-dependent manner: To gain more information on the promotion of *Prcr5* expression by cytokinin, dose response experiments were performed. Fig. 4.21 shows the results of the *Prcr5* mRNA accumulation in the buds cultured on media containing a range of concentrations of 0, 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M of either BA or isopentenyl adenosine (iPA). As indicated in the Fig. 4.21, it is clear that the natural cytokinin form, iPA, is more effective (the effective concentration was one order lower) than the recently accepted naturally-occurring form, BA, in promoting the *Prcr5* mRNA transcription.

4.3.8.3. High level gene transcription of Prcr5 lasts longer in originally more juvenile buds after removal of cytokinin treatment: To understand how the withdrawal of exogenous cytokinin from culture media impacted on the *Prcr5* mRNA level in the buds whose *Prcr5* mRNA level had already reached a very high level through cytokinin treatment, the buds which had already been cultured with cytokinin for 25 d were transferred to media without exogenous cytokinin and harvested after 20 d. The Northern analyses showed that the more juvenile buds from the 4 year-old tree were able to maintain the high level of the *Prcr5* mRNA transcription (Fig. 4.20A) while the originally completely mature buds from the 9 year-old tree had reduced the mRNA accumulation (Fig. 4.20B) significantly 20 days after the removal of exogenous cytokinin from the culture media.

4.3.8.4. Prcr5 mRNA does not accumulate in fully developed leaves: To investigate if *Prcr5* mRNA is expressed in other organs, the primary and secondary needles from seedling and 2.5 year old trees were collected for Northern analysis. No *Prcr5* mRNA accumulation was observed in either leaf type (Fig. 4.22B).

4.3.8.5. Prcab gene expression occurs without the requirements of light and exogenous cytokinin treatments: It has been reported in other species that light regulates the expression of the chlorophyll a/b binding protein gene (Chin-Atkins et al., 1996) and

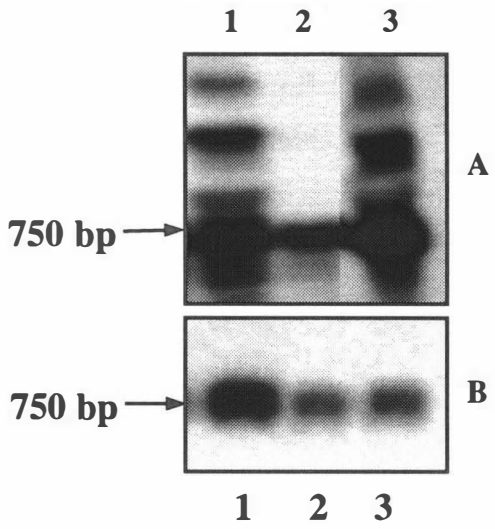


Figure 4.20 Northern analysis of the effect of exogenous cytokinin on *Prcr5* gene expression. Lane 1, mature bud fragments cultured on cytokinin-containing media for 25 d. Lane 2, mature bud fragments cultured on cytokinin-free media for 25 d. Lane 3, bud fragments, which had been cultured for 25 d on cytokinin, transferred to and cultured on cytokinin-containing media for another 20 d. A: Bud fragments of 4-year-old tree (clone G27), 30 µg total RNA per lane. B: bud fragments of 9-year-old tree, 5 µg total RNA per lane. The *Prcr5* cDNA labelled with ^{32}P was used to probe the gene expression according to the conditions described in Section 4.2.11..

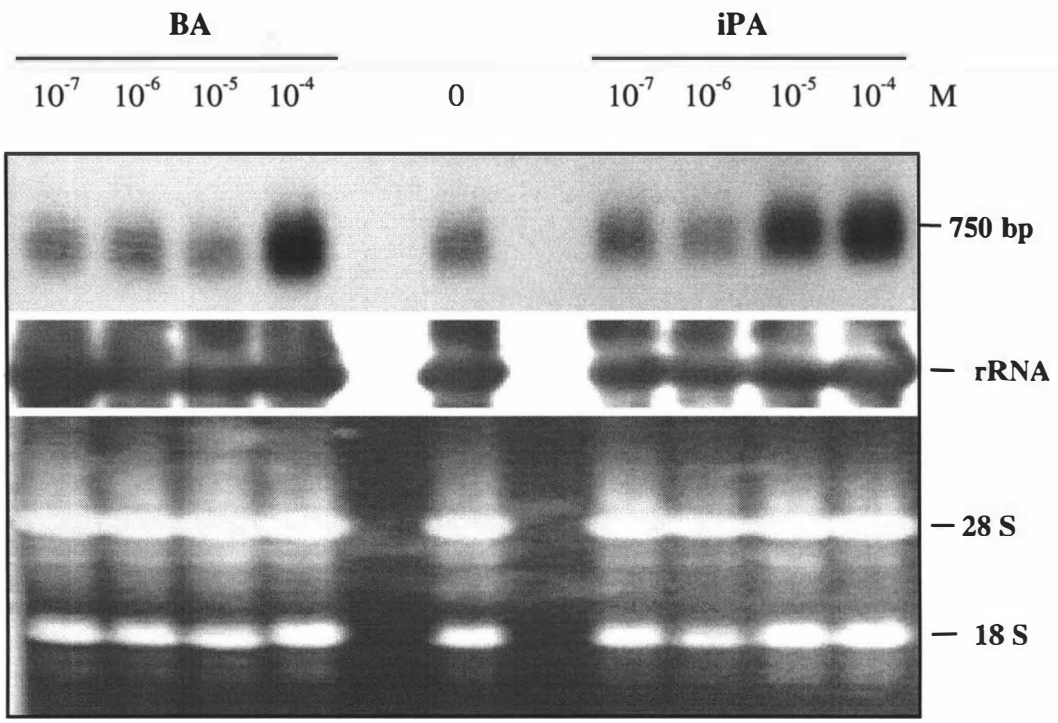


Figure 4.21 Northern analysis of the effect of exogenous cytokinin dose on the *Prcr5* gene expression. Field mature buds collected from the 4-year-old tree (clone G27) were cut into fragments and cultured on cytokinin-free media for 48 h in the complete dark. Subsequently, the fragments were transferred to cytokinin-containing media and cultured for 24 h in the complete dark. Cytokinins BA and iPA were used at a series of concentration as indicated on the top panel. 25 μg total RNA loading per lane. Total RNA was fractionated on 1.2% denaturing agarose gel and blotted onto Hybond^{+} nylon membrane and hybridisd with ^{32}P-labelled *Prcr5* cDNA.

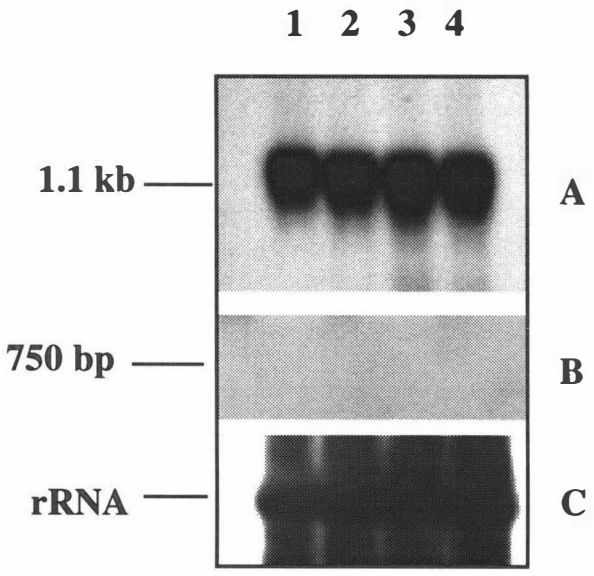


Figure 4.22 Northern analysis of *Prcr5* and *Prcab* gene expression in primary and secondary needls from *Pinus radiata* trees of different maturation states. Lane 1: primary needles of 1-year-old seedlings; Lane 2: primary needles of 2.5-year-old trees; Lane 3: secondary needles of 1-year-old seedlings; Lane 4: secondary needles of 2.5-year-old trees. Total RNA was fractionated on 1.2% denaturing agarose gel and transferrred to Hybond^{+} nylon membrane. The membrane was probed with ^{32}P-labelled *Prcab* (A), *Prcr5* (B) and 28S rRNA cDNA (as loading control) (C).

in the dark cytokinin can replace the light effect (Kusnetrov et al., 1994; Chin-Atkins et al., 1996). To investigate *Prcab* gene expression behaviour in relation to cytokinin and light factors, the Northern blotting membranes used in Fig. 4.19 were stripped of their ^{32}P-*Prcr5* probes and were re-probed with ^{32}P-labelled *Prcab* cDNA. Figure. 4.23 shows that there is a degree of the *Prcab* expression in the mature buds from the 9 year-old field-grown tree, but that its expression level was greatly reduced in the buds which had been cultured for 12 and 24 h under all culture conditions. From 48 h on, the buds cultured on every treatment started accumulating *Prcab* mRNA and continued its accumulation up to 7 d (Fig. 4.23). Unlike in *Lupinus* and *Arabidopsis* (Kusnetsov et al., 1994; Chin-Atkins et al., 1996), exogenous cytokinin had little effect on *Prcab* gene expression in *P. radiata* (Figs. 4.23). This was further confirmed by the dose response experiment as shown in Fig. 4.24. In fact, cytokinin at the concentration used in this experiment slightly reduced the *Prcab* gene expression (Fig. 4.25). Also, the regulation effect of light on the *Prcab* gene expression was very limited (Fig. 4.23).

4.3.8.6. No significant difference in the expression of the Prcab gene exists between the primary and secondary needles of different maturation states: It has been reported that Chlorophyll a/b binding protein gene expressed differentially during phase change in Larch (Hutchison et al., 1990) and English ivy (Woo et al., 1994). To examine if this is the case during phase change in *Pinus radiata*, the mRNA levels in the primary and secondary needles from seedlings and 2.5 year old trees were investigated using Northern analysis. Figure. 4.22A shows that there was no significant difference in *Prcab* gene expression between the needle types or source trees with different maturity.

4.3.8.7. There is a difference in chlorophyll a/b contents between needle types and between the same type of needles from trees with different maturity: Although no difference in *Prcab* gene expression was found between needle types (Section 4.3.8.6), a significant difference in chlorophyll a and b was measured (Table 4.3). The amount of chlorophyll a and b in the primary needles from seedlings was twice as high as in the primary needles of 2.5 year old trees. In the case of seedlings, the chlorophyll level in the primary needles was higher than that in the secondary needles while the opposite pattern was the case in the 2.5 years old trees.

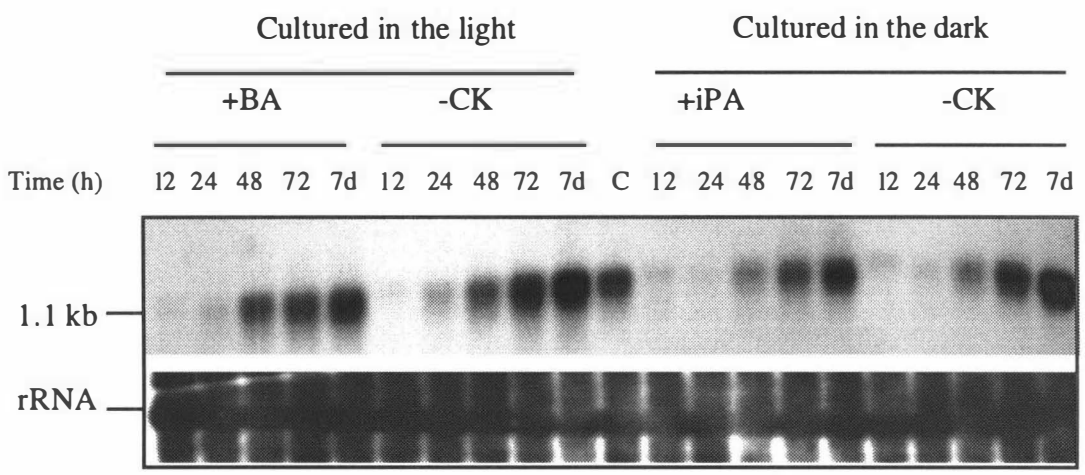


Figure 4.23 Northern analysis of *Prcab* gene expression in the mature *P. radiata* buds cultured over a period of 7 days in the presence (5.0 mg/L) or absence of exogenous cytokinin. 25 µg total RNA was loaded in each lane and fractionated on 1.2% denaturing agarose gel. The fractionated total RNA was blotted onto Hybond^{+} nylon membrane and probed with ^{32}P-labelled *Prcab* cDNA according to the conditions described in Section 4.2.11. ^{32}P-labelled 28S rRNA cDNA was used as loading control probe and ethidium bromide staining was also used as loading control.

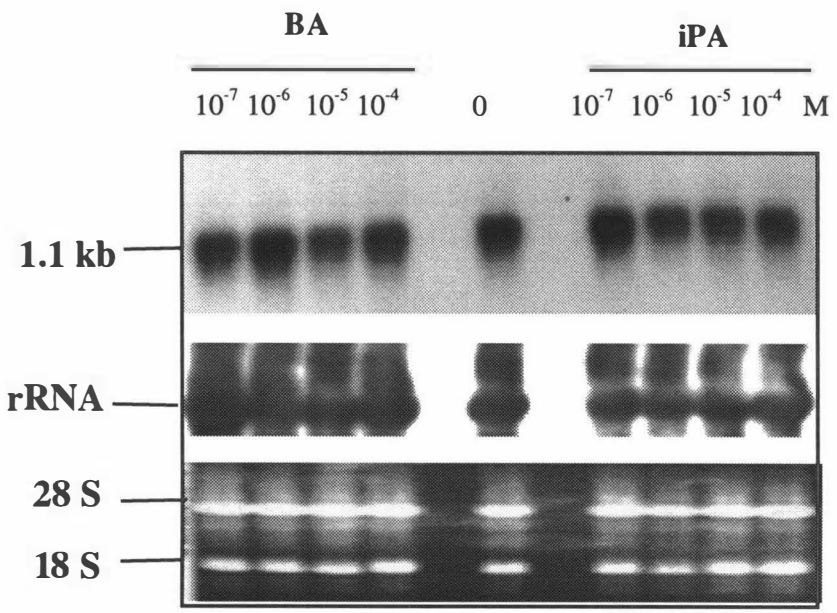


Figure 4.24 Northern analysis of the effect of exogenous cytokinin dose on the *Prcab* gene expression. Field mature buds collected from 4-year-old G27 tree were cut into fragments and cultured on cytokinin-free media for 48 h in the complete dark. Subsequently, the fragments were transferred to cytokinin-containing media and cultured for 24 h in the complete dark. Cytokinins BA and iPA were used at a series of concentrations as indicated on the top panel. 25 μg total RNA was fractionated on 1.2% denaturing agarose gel and transferred to Hybond^{+} nylon membrane. The membrane was probed with ^{32}P-labelled *Prcab* according to the conditions described in Section 4.2.11.

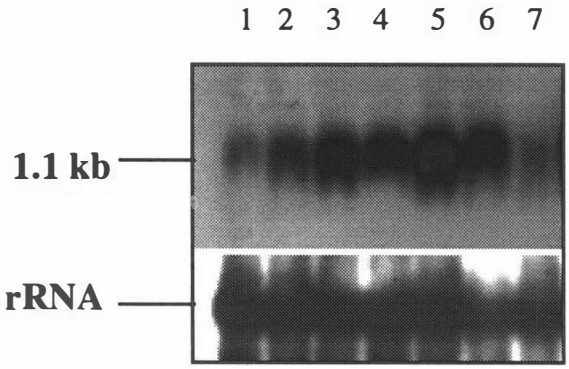


Figure 4.25 Nothern analysis of *Prcab* gene expression in different buds of *P.radiata* under different cytokinin regimes. 25 µg total RNA per lane was fractionated on 1.2% denaturing agarose gel and transferred to Hybond^{+} nylon membrane. The membrane was probed with ^{32}P-labelled *Prcab* cDNA according to the conditions described in Section 4.2.11. Lane 1: 9-year-old field buds; Lane 2: the 9-year-old buds cultured on BA-containing medium for 3 d: Lane 3: the 9-year-old field buds cultured on cytokinin-free medium for 3 d; Lane 4: the 9-year-old field buds cultured on BA-containing medium for 25 d; Lane 5: the 9-year-old field buds cultured on cytokinin-free medium for 25 d; Lane 6: the 9-year-old field buds cultured on cytokinin-free medium for 20 d following 25-d culture on BA-containing medium; Lane 7: 1-year-old seedling buds.

Table 4.3. Chlorophyll contents in different types of needles of *P. radiata*. Fully developed primary needles were collected from shoot regions below terminal buds and the fully developed secondary needles were collected from the axils of these primary needles. Chlorophyll was determined by the method described by Moran and Porath (1980) and Moran (1982). The figures shown in the table were derived from triplicate samples (mg/kg FW).

Needle type	Chla	Chlb	Total chlorophyll
Seedling primary needles	1034.1	351.0	1384.7±106.9
Primary needles from 2.5-yr trees	507.0	174.6	681.4±140.5
Seedling secondary needles	711.5	232.5	943.8±128.0
Secondary needles from 2.5-yr trees	955.2	315.1	1269.9±114.1

4.4 Discussion

4.4.1. Nucleic acid isolation

4.4.1.1 RNA extraction: RNA extraction from coniferous species such as *Pinus radiata* has always been found to be difficult due to the high levels of polyphenols, polysaccharides and other unidentified compounds (Chang et al., 1993; Dong and Dunstan, 1996). Many successful methods used for RNA isolation from other species have been shown to be unsuitable for *Pinus* species (Baker et al, 1990; Chang et al., 1993; Bahloul and Burcard, 1993). Further, the levels of polysaccarides and polyphenolics, as well as RNase activity are even higher in cultured tissues (Dong and Dunstan, 1996). Initially, the protocol published by Chang et al. (1993) was evaluated for isolating total RNA from field and tissue-cultured buds of *P. radiata* in this work. Unfortunately, both yield and quality were unacceptable (Section 4.3.1). Consequently, an extraction protocol based on the method described by Chang et al. (1993) was further developed. The criteria of (1) removing polysaccharides, (2) eliminating polyphenolics, (3) destroying endogenous RNase activities, as well as (4) maintaining intact and functional mRNA molecules were always considered when the RNA extraction strategy was designed in this project. Quick and multiple sample preparation capacity was also a priority in developing the RNA isolation procedure.

It was found that modification to the original extraction buffer (Chang et al., 1993), by addition of extra 2.0% SDS, reduction of soluble PVP from 2% to 1% and elimination of spermidine, gave reproducibly good RNA quantity and quality (Section 4.3.1, Table 4.1, Figure 4.1 and 4.2) compared to other extraction buffers tested. It was vital that β-mercaptoethanol was added in the extraction buffer just before use. β-mercaptoethanol worked as an antioxidant to prevent the oxidation of phenolics and other compounds which, after oxidation, can irreversibly bind to and co-precipitate with RNA (Katterman and Shuttack, 1983; Schneiderbauer et al., 1991).

The high concentration of the detergents hexadecyltrimethylammonium bromide (CTAB) and sodium dodecyl sulfate (SDS) in the extraction buffer (Section 4.2.2) obviously denatured all proteins including RNases, which were then removed by chloroform partitioning. The use of aurintricarboxylic acid, an RNase inhibitor (Lewinsohn et al., 1994), in the extraction buffer led to very similar results in terms of quality or quantity of RNA. This indicated that RNase activity was destroyed by the optimised extraction buffer alone. In the meantime, the high concentration of salts contained in the extraction buffer effectively eliminated the contamination of the RNA preparation by polysaccharides. This observation was in agreement with other work published previously ((Fang et al., 1992).

Polyvinylpyrrolidone (PVP) has the capacity to retain phenolics and has been used to remove polyphenolic compounds during RNA and DNA extractions (Chairgwin and John, 1992; Pich and Schubert, 1993). However, Dong and Dunstan (1996) found that the yield of total RNA might be dramatically reduced if the PVP content in the extraction buffer exceeded 1.0%. Therefore, 1.0 % soluble PVP was used in the modified RNA extraction to maximise the PVP-binding ability and minimise the negative impact on the yield of total RNA.

Heat treatment was found to be an important step in increasing the yield of total RNA. This may be due to the combined effect of heat (55°C), increased detergents as well as chloroform, which was harsh enough to both kill RNases and to release RNA molecules from any complexes containing RNA molecules.

Obviously, the critical point of isolating total RNA is to gain functional mRNA. The results obtained from reverse transcription (Section 4.3.2) and Northern analyses (Section 4.3.9) indicate that the total RNA isolated with the optimised procedure contained functional mRNA molecules.

It was found unnecessary to use diethylpyrocarbonate (DEPC), a mutagen, to eliminate RNAase from solutions as long as the water used was collected directly from the Milli Q water system and autoclaved for 20 min. It was possible that the multiple ion exchange columns used in the Milli Q system efficiently removed most, if not all, RNase from the passing water.

4.4.1.2 DNA extraction: As the problems associated with DNA extraction from pine species are similar to those for RNA extraction, the RNA extraction buffer was also used to isolate genomic DNA (Section 4.2.3). To avoid mechanical shearing of long DNA molecules, violent vortexing was never applied during DNA extraction. After partitioning against chloroform, the genomic DNA was easily precipitated with 0.5 volumes of 100% ethanol from the aqueous phase. The quality of the DNA isolated with such simple steps was excellent, having a typical UV light absorbance spectrum identical to that of pure nucleic acid and good solubility in water. The DNA isolated was easily digested with restriction enzymes (Fig. 4.15) and hybridised with DNA probes (Figs. 4.16, 4.17 and 4.18).

Although it was recommended to use 0.35 volumes of 100% ethanol to remove polysaccharides in nucleic acid extraction (Dong and Dunstan, 1996), extreme care should be taken as different components of the extraction buffer can significantly alter the precipitation behaviour of nucleic acid in a solution. For instance, the DNA in our extraction buffer, which contains high levels of organic and inorganic salts, can be precipitated by adding as little as 0.3 volumes of 100% ethanol. On the other hand, if too much ethanol is used, for example 1 volume, salts in the solution start to precipitate. It was found in this thesis that 0.5 volume of 100% ethanol gave high quality of genomic DNA preparation using the extraction buffer described in this thesis.

4.4.1.3 Simultaneous isolation of total RNA and genomic DNA: It is quite often that plant samples are very limited, so the simultaneous isolation of RNA and DNA is sometimes required. In this thesis, the simultaneous isolation of RNA and DNA can be achieved by lithium chloride precipitation for RNA, followed by 0.5 volume of 100% ethanol precipitation for DNA from the supernatant left by the RNA extraction. In this case, vigorous shaking must be avoided when RNA extraction steps were carried out to prevent mechanical shearing of DNA.

4.4.2 Strategy for isolating differentially expressed gene cDNAs

A lack of suitable techniques has been the major barrier in studying the regulatory mechanisms of cytokinins on plant development. Although cytokinins have been widely used in plant proliferation and regeneration since the first demonstration that cytokinins, along with auxin, control the proliferation and regeneration of stem-pith tissue from *Nicotiana tabacum* in culture (Skoog and Miller 1957), our knowledge on how cytokinins regulate this process is still not resolved. Therefore, development and/or application of new techniques will be a great help in advancing our understanding of the regulatory effects of cytokinins.

In order to investigate how cytokinins regulate the "rejuvenation" process of mature buds induced *in vitro* by exogenous cytokinin, it is crucial to identify and isolate those genes whose expression is responsible for the changes in morphological development and which are also responsive to applied cytokinins. To try to explore a way to reach this goal, a modified procedure was developed based on the principles of the mRNA differential display technique which was developed to detect and characterise altered gene expression in eukaryotic cells (Liang and Pardee, 1992, Liang et al., 1993). Instead of using 10-mer random primers during PCR and a sequencing gel to separate amplified cDNA species, in this thesis, a 14-mer degenerate primer designed from the conserved region of MADS box genes was used as the random primer which resulted in substantially fewer bands than using 10-mer primers. The PCR was followed by 1.5% agarose gel electrophoresis which can fractionate much longer sequences (636 bp, Fig.

4.3 and 923 bp, Fig. 4.4) than a denaturing polyacrylamide gel does (below 500 bp, Liang and Pardee, 1992). Thus, the cDNA sequences isolated using the modified protocol gave a greater chance of isolating cDNA sequences having much longer coding regions and entire 3′ untranslated regions of the corresponding genes. In contrast, cDNAs isolated from sequencing gels usually have much less information on coding regions, which increases the difficulty of identifying a cloned sequence.

By using the modified technique, RNA extracted from plant samples which had been subjected to five different treatments (Section 4.2.1.2 (I)) was used to amplify cDNA sequences. Because of the relatively high endogenous cytokinin contents in the bud tissues of *P. radiata* (see Chapter 3), the bands displaying different of brightness intensities among the different cytokinin treatments were selected.

The advantage of the differential display technique is that it allows simultaneous identification of genes that are up- or down-regulated under different developmental or environmental conditions. However, the technique also suffers from two main drawbacks (Shoham et al., 1996). First, the bands excised from the display gel are often associated with non-specific background and the second drawback stems from the fact that quite often more than one cDNA species is present in one chosen band. Therefore, while those clones containing contaminated cDNA inserts can be easily discriminated by comparing their sizes (Figs. 4.6 and 4.7), the clones containing cDNA inserts of the same size shown in Fig. 4.6 or Fig. 4.7 could be different in sequences. To avoid missing out some cDNA species by randomly picking up clones, Shoham et al. (1996) recommended all clones be grouped by their fingerprinting profiles of digested plasmid DNA by a tetra-nucleotide recognition site enzyme, *Sau*3AI. In an attempt to increase the sensitivities in this thesis, the clones having the same cDNA insert sizes were treated with two restriction enzymes (Section 4.3.4). As shown in figure 4.8 A, all *Prcr5* cDNA sequences cloned are very likely to have the same sequence while the profiles of the clones from a unidentified band displayed in Fig. 4.8B had different groups of sequences. This indicates that all clones from the *Prcr5* band had the same sequence and that the clones from the unidentified band belonged to different sequence groups. This was later confirmed by sequencing data.

4.4.3 *Prcr5* gene

4.4.3.1 Prcr5 gene may code a protein relating to RNases: Originally, a degenerate primer coding for the RQVTF amino acid motif in MADS box genes (Wang, 1995; Tandre et al., 1995) was used to try to increase the chance of picking up MADS box related gene cDNAs that were responsive to cytokinin treatment. However, no MADS box genes were obtained. This may be because mRNA differential displays were carried out only using a combination of two sets of the random primer and the polyT anchor primer. Nevertheless, it was also the goal of this part of the project to clone other gene cDNA species responsive to cytokinin treatment. To achieve this, advantage was taken of the fact that the oligonucleotide coding for RQVTF was short enough to work as a random primer, especially when used in combination with a non-specific poly(T_{12}) primer. As a result, one of the cDNAs cloned (*Prcr5*) coded for a transcript with a predicted gene product (Fig. 4.9) homologous to a group of pathogenesis-related proteins (Table 4.2; Fig. 4.11). These proteins have been found to share a degree of structural similarity with gingseng RNases and the pollen allergen Bet v1 (Moieyev et al., 1994, 1997 and Fig. 4.11). According to the nomenclature for PR proteins suggested by van Loon et al. (1994), this class of proteins is now referred to as PR-10 (Pinto and Ricardo, 1995). Based on evolutionary analysis (Moiseyev et al., 1997) and the fact that the PR-10 proteins have been found in both dicotyledonous and monocotyledonous species, PR-10 proteins were thought to have evolved from a common ancestor and to have similar function (Pinto and Ricardo, 1995, Moiseyev et al., 1997). According to the results of searching the GenBank, SwissProt and PIR protein databases carried out on November 12 1997, *Prcr5* is the first cDNA cloned from a gymnosperm that encodes a protein structurally homologous to the PR-10 proteins, although its isoelectronic point is more basic (PI 8.08) than those of the related PR-10 proteins. However, to support the hypothesis that PR-10 proteins have a common function as a RNase, there needs as yet to be experimental evidence to demonstrate that the other members of the PR-10 proteins do show the ribonuclease activity displayed by the Ginseng RNases.

Structurally, the members of PR-10, PrCR5 and Bet v1 shown in Fig 4.11 have no similarity with the three-dimensional structures of other groups of ribonucleases from higher plants (Green, 1994). However, it has recently been demonstrated that purified Bet v1 allergen extracts from pollens and recombinant Bet v1 protein from *E.coli* do have ribonuclease activity, but no activity on single or double DNA substrates (Bufe et al., 1996; Swoboda et al., 1996).

The recently published structures of Bet v1 protein determined with X-ray and NMR shows that a P-loop structure with the sequence GXGGXGXXK (Gajhede et al., 1996) is present in the Bet v1 three-dimensional structure, which (nucleotide binding motif) may be a site for binding a phosphate group of RNA (Moiseyev, et al., 1997). From the primary structures of the peptides shown in Figure 4.11, it can be deduced that, in common with the other proteins, PrCR5 also has a conserved GXGGXGXIK common three-dimensional loop site. In contrast to Bet v1, lysine is replaced by an arginine residue in the putative P-loop of PrCR5 and as well as in related proteins from the monocotyledonous species *A. officinalis*. In addition, other (conserved) amino acid residues also exist along the PrCR5 sequence and other related sequences in a conserved pattern (Fig. 4.11). These common features of the protein sequences make it very likely that the PrCR5 protein and the other PR-10 proteins (Moiseyev, et al., 1997) have RNase activity.

PR-10 gene members belong to a large gene family (Moiseyev et al, 1997) and multigene copies are usually present in an individual plant. The *Prcr5* gene also belongs to multigene family (Fig. 4.16). Interestingly, it was found in this thesis that gene copy number differs among individual trees.

4.4.3.2 The expression of Prcr5 gene is regulated by cytokinins: The induction of the gene expression of PR-10 proteins is usually associated with fungus infections (Somssich et al., 1988; Walter et al., 1990; Pinto and Ricardo, 1995) or wounding responses (Matton and Brisson, 1989; Warner et al., 1992, 1993). Therefore, these genes are believed to be involved in plant defence against pathogen attacks. However, more detailed research revealed that these genes are also developmentally regulated

(Warner et al., 1994; Walter et al., 1996), as are other PR proteins (Mayer et al., 1996). Through the analyses of endogenous AoPR1 (PR-10 protein) gene expression in asparagus and AoPR1 transgenic tobacco, Warner et al. (1994) found that AoPR1 promoter activity was localised to cells producing phenylpropanoid derivatives in developing flowers and the AoPR1-GUS expression was greatly up-regulated in developing seeds. Very recently, Yeo et al. (1996) reported that a 17 kDa acidic protein (PR-10), to which PrCR5 has the highest sequence similarity, was highly associated with induced early flowering in *Asparagus* seedlings. High transcript expression of PR-10 protein was also observed in developing pea seeds (Barratt and Clark, 1991) and during the development of xylem and phloem as well as the onset of leave senescence in bean (Walter et al., 1996). These experiments clearly indicated that PR-10 proteins may have a role to play in normal plant development and in response to environmental stimuli.

The investigation on the relationship between PR-10 gene expression and cytokinins has not been reported yet, while the gene expression of the other types of PR proteins has been observed to be enhanced by both exogenous cytokinin (Sano et al., 1996) and elevated endogenous cytokinins (Memelink et al., 1987). Futher, it has been reported that a soybean pollen allergen gene mRNA accumulation was greatly up-regulated by cytokinin in suspension-cultured soybean cells (Crowell et al., 1990; Crowell, 1994).

In this thesis, the transcription of the *Prcr5* gene has been found to be promoted by exogenous cytokinin. The *Prcr5* gene transcription enhancement by cytokinin treatment was in a dose-dependent manner and iPA was more effective than BA (Fig. 4.21). This indicates that the *Prcr5* gene expression may be modulated by cytokinin in the same way as cytokinin regulates mRNA accumulation of other PR protein genes (Sano et al, 1996), nitrate reductase in etiolated barley leaves (Lu et al., 1990, 1992), hydroxypyruvate reductase (HPR) gene expression in etiolated pumpkin cotyledons (Anderson et al., 1996) and a gene (related to ATP-synthase) specific mRNA (L-22) accumulation in pea leaves (McDaniel and Lightfoot, 1997). It was noted, in this thesis, that the same accumulation patterns but different accumulation strength obtained from cytokinin-treated and non-cytokinin treated mature buds in culture during time-course

studies (Fig. 4.19 and 4.20) clearly indicated that the modulation by exogenous cytokinin of *Prcr5* gene expression is simply to increase the net accumulation of the *Prcr5* mRNA. The low expression of *Prcr5* gene expression in the mature buds cultured on cytokinin-free media may be due to the lower endogenous cytokinin content of the buds. However, the endogenous cytokinins alone in the mature buds seem not effective enough to raise the *Prcr5* gene expression to a high enough level while the exogenous cytokinin (20 μM) not only promotes but also maintains the gene expression over a long period of time (Fig. 4.19 and 4.20). This long-term expression of the *Prcr5* gene differs from the other cytokinin-inducible gene expression reported in angiosperm plants. In pea leaves, the expression level of the cytokinin-inducible gene (L-22) returned to the level of non-treated leaves after 24 h (McDaniel and Lightfoot, 1997) and after 35 h the HPR transcripts in pumpkin cotyledons enhanced by 10^{-4} M BA began to decrease (Anderson et al., 1996). The authors reasoned that was due a decreasing amount of available BA as a result of catabolism although no cytokinin analyses were carried out (Anderson et al., 1996; McDaniel and Lightfoot, 1997). However, the data on BA metabolism in this thesis showed that the cultured bud fragments of *P. radiata* still maintain very high level of BA and BA riboside even after 25 d culture on cytokinin-containing media (Fig. 3.13, Chaper 3). This is in agreement with the *Prcr5* gene expression time course, supporting that the *Prcr5* gene expression is regulated by cytokinin.

The cytokinin-promoted increase in steady-state levels of mRNA could be the result of transcriptional and/or post-transcriptional events (Anderson et al., 1996). However, the two extra bands with higher molecular masses revealed by the Northern analysis as shown in Fig. 4.20A indicate that the more likely effect of the cytokinin treatment is to increase the *Prcr5* gene expression activity at the transcriptional level. The two extra bands may be pre-mRNAs of *Prcr5* gene transcripts.

Interestingly, the *Prcr5* gene expression level remained very high in the 4-year-old bud fragments 20 d after the withdrawal of cytokinin from the media while the expression level in the 9-year-old bud fragments decreased dramatically when cytokinin was withdrawn. Moreover, the endogenous cytokinin analyses showed that although BA and

BA riboside contents in cultured 9-year-old buds had started to decline 20 d after withdrawal of exogenous cytokinin, they still had relatively high concentrations. Putting these two lines of evidence together, an interpretation of the data might be that the more juvenile buds have higher sensitivity to cytokinin in the regulation of *Prcr5* gene transcription than the more mature buds. However, it is also possible that the different sensitivity to cytokinin is due to the different genotypes of the individual trees used for juvenile and mature materials.

4.4.3.3. Possible function of Prcr5 gene in the resetting of needle development in the buds of P radiata during culture: The requirement of cytokinin for the mature bud fragments to be rejuvenated into juvenile-looking buds producing primary needles is about 14 d (Horgan, 1987). This is in accordance with the finding that cytokinin must be present during the first 3 d in culture for any shoot formation to occur; however, it is needed for 21 d for optimum shoot formation (Biondi and Thorpe 1982; Villalobos et al., 1984). This morphological time course fits well with the *Prcr5* gene expression time course and its ability to remain high over a long time period in the presence of exogenous cytokinin. If the product of *Prcr5* gene indeed harbours RNase activity, as shown for the ginseng RNases and Bet v1 allergens, this gene family may act as a down stream step of signal sensing and transduction systems as well as a global regulator of RNA metabolism (Glund and Goldstein 1993), which would lead to an alteration at the molecular and physiological levels of the plant cell. As a result, morphological changes would follow.

Anatomically, when the mature bud fragments were cultured without cytokinin treatment, the secondary needle primordia developed very rapidly to form bundles of three secondary needles. Meanwhile, the fascicle meristem sitting in the base of the three secondary needle primordia were not able to enlarge during the period of the growth and development of the secondary needles (Fig.3.3B, D, F,H and J). However, when the bud fragments were cultured with exogenous cytokinin (20 μM), the development of the secondary needle primordia was inhibited and the primordia gradually degraded. Instead, the fascicle meristems underwent enlargement with primary needle primordia being initiated (Fig.3.3A, C, E, G and I). Over this

corresponding period, the *Prcr5*-coded protein may be working as a RNase to specifically destroy the existing "state" of some RNA populations which are crucial to determine certain features in the mature meristem such as secondary needle development and/or bud scales development in *P. radiata*. The *Prcr5* gene product, therefore, could be involved in resetting the development of mature meristems and/or the primordia to that of the juvenile developmental programme in terms of primary needle production. In particular, high levels of the protein encoded by *Prcr5* gene may be toxic to secondary needle primordia but not to the primary needle primordia, because, when exogenous cytokinin is present (20 μM) - a condition leading to high *Prcr5* gene expression - all the secondary needle primordia underwent degradation while primary needle primordia were being vigorously initiated (Fig. 3.2). As a low level of *Prcr5* expression was also observed in the cultured mature buds, the regulation of the apical meristem and/or its flanking primordia by the *Prcr5* gene product during "rejuvenation" appears to be a quantitative response, a threshold of *Prcr5* expression may be required for "rejuvenating" the mature meristem in culture. It has recently been reported that the *LEAFY* gene regulates the transition from the vegetative to the floral phase in a quantitative way in some species (Blazquez et al., 1997; Hempel et al., 1997).

Although there has not been sufficient experimental evidence to draw a conclusive picture, this hypothesis may be endorsed by some other lines of evidence on the biological function of RNases in normal plant development. Firstly, RNases, similar to fungal RNase T2, has been found to be responsible for self-incompatibility (McClure et al., 1989; Kao and Huang, 1994; Kao and McCubbin 1997). The products of the S-locus in pistils are basic proteins with RNase activity (Kao and McCubbin, 1997; Kao and Huang, 1994). Molecular and physiological analyses with mutants revealed that deletion of RNase activity from S-proteins led to failure to reject self pollen (Sassa et al., 1997; Huang et al, 1994). More recently, it was found that exchanging sequence domains without reducing RNase activity also disrupted pollen recognition (Zurek et al., 1997), indicating that the specificity and the RNase activity in S-proteins are both essential for the recognition function of S-proteins. Secondly, Bet v1 allergen also possesses RNase activity and may also be involved in the recognition interaction between pollen tube growth and female reproductive tissue (Swoboda et al., 1996).

Thirdly, the expression of some PR-10 genes is associated with special developmental events such as EFP (PR-10 protein) in *Asparagus* with flowering (Yeo et al., 1996), *Asparagus* AoPR1 (PR-10 protein) with developing seeds and pollen (Warner et al., 1993, 1994) and potato wun1 (PR-10) with anther and pollen (Siebertz et al, 1989). A recent study showed that the expression of a bean ribonuclease-like PR-10 was developmentaliy associated with developing xylem, phloem, flowers and the onset of leaf senescence (Walter et al., 1996).

Interestingly in *P. radiata*, the effect of cytokinin on rejuvenation is not permanent and shoots displaying the juvenile form are able to revert to the mature form after they are rooted and placed out in the nursery bed (Horgan, 1987). This fits well with the finding in this thesis that the concentration of cytokinins and the expression activity of *Prcr5* in the rejuvenated buds both gradually declined after the buds were removed from cytokinin-containing medium (Fig. 3.13B and Fig. 4.20B). All of these lines of information point to the fact that, if PR-10 proteins indeed have RNase activity as their structures indicate, RNase activity may be the general biological function for these proteins. With their respective structural specificity (both primary and secondary structures) and tissue or cell-specific expression, these proteins, encoded by the PR-10 homologous genes, may then regulate different developmental processes.

4.4.4 *Prcab* gene

4.4.4.1 The cloned sequences are chlorophyll a/b binding protein cDNA: The cloned cDNA sequences of *Prcab* from *P. radiata* have a very high homology with other chlorophyll a and b binding protein genes (*cab*) (Fig. 4.14). Southern analyses indicated that the *Prcab* gene may belong to a multigene family in *P. radiata*.

4.4.4.2 Prcab gene can be expressed in the cultured buds of P. radiata in the complete dark without requirement of exogenous cytokinin: Through the comparison of cDNA libraries made from RNA extracted from juvenile and mature phase foliage, Hutchison et al. (1988) and Hackett et al. (1992) found that the expression of most genes studied is mostly same between juvenile and mature phase foliage in both larch and English ivy. However, in both larch and English ivy, they found that the expression of the

chlorophyll a/b binding protein (*cab*) gene decreased in mature leaves (Hutchison et al., 1990; Woo et al, 1994).

The expression of the *cab* gene in most angiosperms is normally up-regulated by light (Chory et al., 1994). Recently, cytokinins have also been reported to modulate the expression of the *cab* gene in angiosperm species (Teyssendier de la Serve et al.1985; Flores and Tobin 1988; Kusnetsov et al. 1994; Chin-Atkins et al. 1996). Therefore, the cloned partial cDNA of *cab* gene from *P. radiata* (designated as *Prcab*) was used as a probe to investigate how cytokinin modulates *Prcab* gene expression and how the expression of the gene is related to phase change in field grown trees and in rejuvenated and mature buds of *P. radiata* cultured *in vitro*.

In contrast to the angiosperm species studied, increasing concentrations of either BA or iPA had little effect on the steady state accumulation of *Prcab* gene transcripts in the cultured bud fragments either in the light or in the dark (Fig. 4.23 and 4.24). All applied concentrations of cytokinin gave similar *Prcab* transcript accumulation as did the control. The results indicate that, in *P. radiata* bud fragments, the accumulation of *Prcab* transcripts is light-independent and does not require an exogenous supply of cytokinin. This is further supported by the time-course analyses (Fig. 4.23). In figure 4.23, it can be clearly seen that light treatments did not increase the accumulation of *Prcab* transcripts. Moreover, cytokinin treatments did not enhance gene expression at all, if anything expression was reduced (Fig. 4.25). This is completely different from what has been reported in other species (Flores and Tobin, 1986, Kusnetsov et al., 1994), but in agreement with the phenomena that pine seedlings growing in complete darkness have normal morphology and synthesise chlorophyll (Drumm-Herrel and Mohr, 1994; Ou and Adomson; 1995, Raskin and Marder, 1997). Therefore, some endogenous factor(s) must be responsible for triggering and maintaining the gene expression in the cultured bud fragments in *P. radiata*. Flores and Tobin (1986) found that application of BA to *Lemna gibba* plants kept in total darkness resulted in an increase in level of *cab* mRNA. Strong induction by cytokinin of *cab* gene steady state mRNA accumulation has also been observed in *Lupinus luteus* cotyledons in complete darkness (Kusnetsov et al., 1994). Therefore, it seems that cytokinin can replace the

effect of light to activate *cab* gene expression. Indeed, this is strongly supported by a recent finding that an *Arapidopsis* mutant (*amp1-1*) which has raised level of endogenous cytokinin also accumulate *cab* gene transcripts in darkness (Chin-Atkins et al., 1996). Therefore, the high expression of *Prcab* gene in the cultured bud fragments in darkness is very likely due to the relatively high concentration of active endogenous cytokinins shown to be in the buds (Fig. 3.4). The fact that the *Prcr5* gene maintains a low level of expression in mature buds in culture in the absence of exogenous cytokinin endorses this explanation.

Furthermore, *Prcab* and *Prcr5* genes may have different sensitivities to cytokinins, because an increase in exogenous cytokinin supply promoted the expression of the *Prcr5* gene but did not impact on *Prcab* gene expression, meaning that endogenous cytokinins might already have reached a favourable level for *Prcab* gene expression but not for the expression of *Prcr5* gene in mature buds of *P. radiata*.

It has been observed in this thesis that the *Prcab* was constitutively expressed in field grown mature buds (Fig. 4.23). However, when the buds were first put on tissue culture media, the *Prcab* gene expression was down-regulated to a extremely low level within 12 h but then quickly up-regulated after 24 h (Fig. 4.23). This fluctuation may be due to the circadian rhythm that has been reported in *Arabidopsis* (Millar and Kay, 1996) and in *Chlamydomonas reinhardtii* (Jacobshagen et al., 1996). Such oscillations might have remained valid for a period of time after the buds were excised from the trees and put on culture media.

4.4.4.3 Prcab gene is not differentially expressed between mature and juvenile tissues: Seedling trees and trees of 2.5 years of age were used to provide both primary and secondary needles in this thesis. Although trees of 2.5 years of age were believed still to be at a juvenile stage, their terminal buds were developing mature characteristics at the time of sampling such as sealed buds, but the branches were still carrying both primary and secondary needles. From both the seedling trees and the trees of 2.5 years of age, fully developed primary needles and the fully developed secondary needles which were produced in the axils of the primary needles on the stem below the terminal buds were

collected separately and used in comparison of chlorophyll levels and the *Prcab* gene expression.

The measurements of chlorophyll content showed that on seedling plants chlorophyll a and b levels were much higher in the primary needles than in the secondary needles (Table 4.3). However, when the trees reached only 2.5-year-old, the chlorophyll distribution patterns between the primary and secondary needles of the trees have been reversed, with the secondary needles having twice the level of chlorophyll a and chlorophyll b as than the primary needles in the same trees (Table 4.3). Therefore, the data indicate that primary needles are very important for seedlings in term of photosynthesis, but that their importance rapidly declines as trees mature because their chlorophyll content decreases from 1384.7 mg/kg in seedlings to 681.4 mg/kg in 2.5-year-old trees. Furthermore, when *P. radiata* trees reached full maturity, such as the 9-year-old trees, the trees do not produce any primary needles at all. In contrast to the primary needles, as trees mature, secondary needles become more important in photosynthesis and finally become the sole photosynthetic organs when trees have completely matured. This is in agreement with the chlorophyll levels measured in secondary needles in this thesis and the chlorophyll data obtained in larch by Greenwood et al. (1989) and Hutchison et al. (1990). This indicates that, as the trees mature, the secondary needles gradually replace the role which primary needles played in terms of photosynthesis.

Surprisingly, unlike chlorophyll a and b, *Prcab* gene expression did not show any significant difference between the primary and secondary needles in both seedlings and 2.5-year-old trees (Fig. 4.22). Even though the primary needles from the 2.5-year-old trees had only half the content of chlorophyll a and b as that in the primary needles of seedlings, they expressed the *Prcab* gene as strongly as seedling primary needles did. In addition, there was no difference of *Prcab* gene expression observed between the 9 year-old field grown mature buds and seedling buds (Fig. 4.25). These results also differed from the results reported in other species. In both larch and English ivy, the expression of the *cab* gene was higher in juvenile foliage than in mature (Hutchison et al., 1990; Woo et al., 1994).

On tissue culture, the mature buds undergoing "rejuvenation" expressed slightly less *Prcab* than the buds continuing mature phase development (Fig. 4.25). However, when the rejuvenated buds were transplanted onto cytokinin-free culture media, the *Prcab* gene expression in rejuvenated buds started to catch up with the mature buds in culture and the rejuvenated plantlets had expression similar to the mature buds in culture (Fig 4.25). A slightly lower expression of the *Prcab* gene in the cultured buds which were undergoing rejuvenation may be due to the inhibition of the secondary needle development by exogenous cytokinin.

4.4.5 Summary

The molecular work described in this chapter has shown that the protocols established in this thesis for isolating RNA and DNA from tissue-cultured materials and field buds of *P. radiata* is efficient and reliable, producing functional RNA and DNA readily cut by restriction enzymes. The mRNA differential display technique is a fast and efficient approach to identifying and cloning plant hormone responsive- and developmental event-related genes.

The cloned *Prcr5* cDNA sequences may belong to a multigene family in *P. radiata* according to Southern analyses. The coding regions of the *Prcr5* cDNA sequences are homologous to PR-10 genes whose translation products may harbour RNase activity. The *Prcr5* gene may act as a RNase to participate in the resetting of the mature bud meristems by cytokinin *in vitro* through modifying existing RNA species in the mature buds. Northern analyses confirmed that cytokinin strongly promotes *Prcr5* gene transcription.

In contrast to the situation in angiosperm species, the expression of chlorophyll a and b binding gene in *P. radiata* (*Prcab*) can occur in complete darkness; light and exogenous cytokinin do not promote its expression *in vitro*. There was no significant difference in the *Prcab* gene expression in the primary and secondary needles obtained from the trees of different maturation state, although the content of chlorophyll a and b in these needles is quite different. The down regulation of the chlorophyll level in primary needles as the

tree matures may be responsible for the elimination of the juvenile feature - primary needles - during natural maturation.

Chapter 5 Final Discussion and Conclusion

In this study a multiple-faceted approach, utilising morphological, physiological and molecular techniques, was taken to investigate the involvement of cytokinins in phase change in *Pinus radiata*.

The basis for the study was the observation by Horgan (1987) that, *in vitro*, the cytokinin, 6-benzylaminopurine, caused an apparent "rejuvenation" of mature buds.

Morphologically, the most striking feature during phase change in *Pinus radiata* is the change in bud morphology. The production of photosynthetically functional primary needles is confined to the juvenile phase while dried membranous bud scales are characteristic of mature buds (Fig. 3.1A, B, C and D). Therefore, the primary needles can be used as a morphological marker for the juvenile phase. Critical to this choice of marker is the fact that both the primary needles and bud scales derive from the same source foliar primordia. This conversion from the development of primary needles to the production of bud scales during phase change is likely to be a common feature in *Pinus* - a similar conversion of bud morphology was also reported recently in *Pinus banksiana* (Browne, 1995).

While the mature characteristics in *P. radiata* are very stable under both *in vivo* and *in vitro* (without exogenous cytokinin) conditions (Fig. 3.1E and 3.2C), the cytokinin BA can effectively induce mature buds in culture to initiate primary needles (Horgan, 1987; Fig. 3.2A, B and D). Detailed examination using light microscopy revealed that cytokinin may induce the transition from mature to juvenile morphology through resetting the fate of meristems and/or foliar primordia. Evidence to support this supposition is the fact that cytokinin inhibits the development of the secondary needles while inducing the initiation of primary needles (Fig. 3.1A and C). Secondly, the apical meristems of mature buds treated with BA are morphologically similar to the apical meristems of seedling shoots (Fig. 3.3I; Riding, 1972), while the mature buds cultured *in vitro* in the absence of exogenous cytokinin eventually produce only very small meristems (Fig. 3.3J). Thirdly, cytokinin treatment of mature buds prevented

accumulation of safranine-stainable substances in the epidermal cells of the foliar primordia (Fig. 3.3I) which subsequently developed into primary needles. In contrast, without cytokinin treatment *in vitro*, the epidermal cells of the primordia initiated from mature buds accumulated safranine-stainable substances. These primordia eventually developed into bud scales.

In summary, the morphological analyses support the contention that cytokinins may play a key role in the regulation of phase change. Thus, detailed studies on the changes in endogenous cytokinins in buds during phase change, and on the metabolism of exogenous cytokinin and the impact of exogenous cytokinin on the endogenous cytokinin in the buds undergoing "rejuvenation" was undertaken.

To analyse the cytokinins, extracts from buds, both from the field and those *in vitro* were subjected to extensive purification and separation using PVPP, DE52, C_{18} columns linked in series, as well as normal and reverse phase HPLC, prior to quantification using RIAs (Fig. 2.1). A fast and efficient immunoaffinity purification procedure was also developed to purify cytokinin samples before ES MS/MS (Fig. 2.2). As a result of using these techniques, a wide range of endogenous cytokinins was detected from the buds of *P. radiata*, including not only the traditional free base, riboside and nucleotide forms of cytokinins (Fig. 2.11A and B), but also several novel cytokinin glucosides and their corresponding phosphorylated forms (Fig. 2.11B and C). Although ZR and the ZR-G form had been identified previously in the buds of *P. radiata* (Taylor et al., 1984), this is the first report of the presence of the traditional cytokinins iP, iPA, DZR, iPNT, ZNT, DZNT in the tissues of *P. radiata* and the first report of the novel cytokinin glucosides iPA-G and DZR-G (Fig. 2.17), and of the novel phosphorylated cytokinin glucosides iPA-G-P, ZR-G-P and DZR-G-P (Fig. 2.17) in any species.

The identification of the metabolites of exogenous cytokinin from the buds cultured on the medium containing BA revealed a wide spectrum of BA metabolites, including BA, BAR, BA9G, BAR-G, BANT and BAR-G-P (Figs. 3.13 and 3.11). A lack of the 9-glucosides of endogenous cytokinin in the buds of *P. radiata* implies that the BA9G may be an inactivation or detoxification form when a high concentration of exogenous

BA is present (Van Staden and Crouch, 1996). The novel glucoside of BA (BAR-G) isolated in this thesis is most likely to be the same compound as identified previously by other researchers (Horgan, 1985; Blakesley et al., 1990; Auer and Cohen, 1993; Auer et al., 1992). However, the phosphorylated form of the novel glucoside (BAR-G-P) has not been reported before in any species. Although the novel glucosides were relatively abundant in both the field and tissue-cultured buds, no traditional *O*-glucosides were detected in the buds of *P. radiata*. This indicates that the enzymes responsible for the formation of the novel glucosides are different from those responsible for the formation of *O*-glucosides.

Although qualitative analyses revealed no difference in the cytokinin species between the mature and the juvenile buds, differences in cytokinin concentration were detected between the juvenile and mature buds of the field trees. A general trend of seedling buds > juvenile (J4) buds > mature (M4) buds > mature (M8) buds for the combined concentration of free base and riboside cytokinins was revealed in *P. radiata* (Fig. 3.4A). This trend is in accordance with the results obtained in other species (Hendry et al., 1982; Galoch, 1985; Day et al., 1995; Perrin et al., 1997). For *P. radiata*, within the free base and ribosides, maintaining a threshold concentration of iP appears critical for the development of juvenile buds (Fig. 3.4D).

A notable feature with respect to the phosphorylated cytokinins is the difference in concentration between the juvenile and mature buds (Fig. 3.5). The concentrations of all the phosphorylated cytokinins detected were significantly lower in seedling and juvenile buds (J4) than in the mature buds (M4 and M8). The results here strongly demonstrate the importance of including phosphorylated cytokinins in cytokinin analysis. Despite the significant differences in the concentrations, the relative levels of the individual phosphorylated cytokinins in the juvenile and mature buds were similar, indicating that metabolism pathways for these cytokinins in the juvenile and mature buds may be similar but that the general rates are very different. Although it shows that a low concentration of phosphorylated cytokinins is associated with juvenile buds and a high concentration with mature buds *in vivo*, the actively growing mature buds in culture also displayed an extremely low concentration of phosphorylated cytokinins,

indicating that a low level of phosphorylated cytokinins alone may be more associated with growth processes regardless of the phase of the material.

The novel glucosides ZR-G, DZR-G and iPA-G were the most abundant cytokinins in both juvenile and mature buds. It appears that a lower concentration of ZR-G and DZR-G and a higher concentration of iPA-G are associated with juvenile buds from the field trees (Table 3.1). However, the actively growing mature buds *in vitro* also displayed similar patterns (Fig. 3.16), again indicating a possible involvement in the growth process.

Overall, the balance among the different forms of cytokinins may be more important than individual cytokinins in the regulation of phase change *in vivo* in *P. radiata.* A high concentration of the free base and ribosides (particularly iP) and iPA-G, and a low concentration of phosphorylated cytokinins, ZR-G and DZR-G is positively correlated with juvenile bud morphology: the opposite pattern is true for the mature buds.

The investigation into BA metabolism revealed that the buds which underwent "rejuvenation" contained a relatively high abundance of BA, BAR and BAR-G and a relatively low abundance of phosphorylated BAR and BAR-G (Fig. 3.13). Although previous reports in other species showed exogenous cytokinin induces increases in endogenous cytokinin concentration (Kuiper et al., 1989; Kataeva et al., 1991; Vankova et al., 1991; Feito etal., 1994), the data (Fig. 3.14) obtained from this study shows a decline in endogenous cytokinin concentration in the buds treated with BA, indicating that the BA acts 'per se' and not through endogenous cytokinins. This finding is supported by the data obtained from kiwifruit treated with CPPU (Lewis et al. 1996).

Since different lines of information all point to an involvement of cytokinin in the regulation of phase change, the next phase of the project was an attempt to isolate and identify cytokinin-responsive genes in order to investigate the role of cytokinins in phase change at the molecular level. To achieve this goal, an efficient and fast protocol for simultaneous isolation of RNA and DNA from pine tree material and a modified mRNA differential display approach were developed in this study. The modified

approach allowed me to quickly identify and isolate differentially expressed gene cDNA sequences with their coding regions. The traditional differential display technique often leads to the isolation of cDNA sequences without their coding regions, which can make gene identification and characterisation difficult.

The deduced amino sequence of one of the isolated cDNA sequences in this study, designated as *Prcr5* (Fig. 4.9), was found to be homologous to ginseng RNases, pollen allergen Bet v1 (which has been shown to possess RNase activity) and some PR-10 proteins. In common with all the homologous sequences obtained from GenBank, PrCR5 has a P-loop phosphate-binding motif GxGGXGXXR. Therefore, the *Prcr5* gene most likely encodes a RNase. Southern analyses revealed that this gene may belong to a small gene family (Fig. 4.16). Northern analyses showed that the expression of the *Prcr5* gene in the cultured buds is highly regulated by cytokinins (Fig. 4.20, 4.21 and 4.22), and that the regulation most likely occurs at the transcriptional level (Fig. 4.21). As gene expression was not observed in the fully developed primary or secondary needles, I deduce that *Prcr5* must be expressing in the meristem and/or its flanking foliar primordia promoted by cytokinin. It is proposed that, if *Prcr5* is indeed a RNase, high level expression of *Prcr5* induced by cytokinin may be involved in resetting the fate of the meristems and/or primordia during rejuvenation of the mature buds in culture.

Another isolated cDNA sequence, designated as *Prcab*, encodes a chlorophyll a/b binding protein. Although expression of the *cab* gene decreases in the mature leaves in other species (Hutchison et al., 1990; Woo et al., 1994), no such changes were observed in *P. radiata* (Fig. 4.23).

Generally, the results obtained in this thesis support the models of phase change proposed by Poethig (1990) and Hackett and Murray (1996). Poethig (1990) proposed that phase-related characteristics are specified by independently regulated programmes in shoot apical meristem. The results from the morphological, cytokinin and molecular analyses in this thesis indicate that the effects of cytokinins on phase-related characteristics in *P. radiata* may be through modulating independently regulated

programmes which determine the fate of the foliar primordia and/or meristem. This explanation is also in agreement with the model suggested by Hackett and Murray (1996) which emphasises that phase change is not a single 'master-switch' process but a composite of a number of processes or programmes (Fig. 1.2). These models fit well with the fact that cytokinin treatment does not affect all phase-related traits equally, with primary needles being the most sensitive characteristic.

Greenwood (1995) suggested that rejuvenation of woody plants derives from juvenile cells conserved within the meristems of mature buds. However, this does not appear to be the case in *P. radiata,* because rejuvenated shoots tend to revert to the mature form more quickly than true juvenile trees following rooting and placement in the nursery bed (Horgan, 1987). This again indicates that cytokinin regulates particular programmes, such as those determining primary needles, but not all programmes. Therefore, in this context, rejuvenated characteristics are able to mature more quickly when the influence of the cytokinin is removed.

Overall, this project has provided new information on the relationship between cytokinins and phase change in *P. radiata*. Investigations of the biological function(s) of the novel cytokinins is required to clarify the role of cytokinin metabolism in the regulation of phase change. Molecular approaches such as using the modified mRNA differential display will be useful tools in further searches for cytokinin-regulated genes which may be involved in phase change. Further study of the *Prcr5* gene using *in situ* hybridisation to determine the spatial pattern of its expression, and gene transformation techniques to elucidate its function will help towards an understanding of the role of cytokinins in phase change.

References

ABO EL-NIL,MM (1982): Method for asexual reproduction of coniferous trees. US Patent 4353184, issued October 12.

Adams,P; Nelson,DE; Yamada,S; Chmara,W; Jensen,RG; Bohnert,HJ; Griffiths,H (1998): Growth and development of *Mesembryanthemum crystallinum* (Aizoaceae). *New Phytol.* 138: 171-190.

Amasino,RM (1996): Control of flowering time in plants. *Current Opinion in Genetics and Development* 6: 480-487.

Amo-Marco,JB; Vidal,N; Vieitez,AM; Ballester,A (1993): Polypeptide markers differentiating juvenile and adult tissues in Chestnut. *FEBS Lett.* 142: 117-119.

Andersen,BR; Jin,G; Chen,R; Ertl,JR; Chen,CM (1996): Transcriptional regulation of hydroxypyruvate reductase gene expression by cytokinin in etiolated pumpkin cotyledons. *Planta* 198: 1-5.

Armstrong,DJ (1994): Cytokinin oxidase an the regulation of cytokinin degradation. In: Cytokinins: Chemistry, activity, and Function. (Eds: Mok,DWS; Mok,MC) CRC Press, Boca Raton, 139-154.

Auer,CA (1997): Cytokinin conjugation: recent advances and patterns in plant evolution. *Plant Growth Regul.* 23: 17-32.

Auer,CA; Cohen,JD (1993): Identification of a benzyladenine disaccharide conjugate produced during shoot organogenesis in *Petunia* leaf explants. *Plant Physiol.* 102: 541-545.

Auer,CA; Cohen,JD; Laloue,M; Cooke,TJ (1992a): Comparison of benzyladenine metabolism in two Petunia hybrida lines differing in shoot organogenesis. *Plant Physiol.* 98: 1035-1041.

Auer,CA; Laloue,M; Cohen,JD; Cooke,TJ (1992b): Uptake and metabolism of benzyladenine during shoot organogenesis in Petunia leaf explants. *Plant Growth Regul.* 11: 105-114.

Badenoch-Jones,J; Letham,DS; Parker,CW; Rofle,BG (1984): Quantitation of cytokinins in biological samples using antibodies against zeatin riboside. *FEBS Lett.* 75: 1117-1125. (N)

Badenoch-Jones,J; Parker,CW; Letham,DS (1987): Use of isopentenyladenosine and dihydrozeatin riboside antibodies for the quantification of cytokinins. *J. Plant Growth Regul.* 6: 159-182.

Bahloul,M; Burcard,G (1993): An improved method for the isolation of total RNA from spruce tissues. *Plant Mol. Biol. Rep.* 11: 212-215.

Bai,S; Sung,ZR (1995): The role of *EMF1* in regulating the vegetative and reproductive transition in *Arabidopsis thaliana* (Brassicaceae). *Am. J. Bot.* 82: 1095-1103.

Baker,SS; Rugh,CL; Kamlay,JC (1990): RNA and DNA isolation from recalcitrant plant tissues. *BioTechniques* 9: 268-272.

Banks,MS (1979): Plant regeneration from callus from two growth phases of English ivy *Hedera helix* L. *Z. Pflanzenphysiol* 92: 349-353.

Banowetz,GM (1994): Imunoanalysis of cytokinins. In: Cytokinins: Chemistry, Activity, and Function. (Eds: Mok,DWS; Mok,MC) CRC Press, Boca Raton, 305-316.

Barratt,DHP; Clark,JA (1991): Proteins arising during the late stages of embryogenesis in *Pisum sativum* L. *Planta* 184: 14-23.

Bassil,NV; Mok,DWS; Mok,MC (1993): Partial purification of a ***cis-trans***-isomerase of zeatin from immature seed of Phaseolus vulgaris L. *Plant Physiol.* 102: 867-872.

Bassiri,A; Irish,EE; Poethig,RS (1992): Heterochronic effects of *Teopod2* on the growth and photosensitivity of the maize shoot. *The Plant Cell* 4: 497-504.

Bernier,G (1988): The control of floral evocation and morphogenesis. *Ann. Rev. Plant Physiol.Plant Mol.Biol.* 39: 175-219.

Bestford,RT; Hand,P; Peppitt,SD; Richardson,CM; Thomas,B (1996): Phase change in *Prunus avium*: Differences between juvenile and mature shoots identified by 2-dimensional protein separation and *in vitro* translation of mRNA. *FEBS Lett.* 147: 534-538.

Biddington,NL; Thomas,TH (1976): Effect of pH on the elution of cytokinins from polyvinylpyrrolidone columns. *J. Chromatogr.* 121: 107-109.

Bieleski,RL (1964): The problem of halting enzyme action when extracting plant tissues. *Analytical Biochemistry* 9: 431-442.

Bieman,K; Tsunakawa,S; Sonnenbickler,J; Feildmann,H; Dutting,D; Zachau,HG (1966): Structure of an odd nucleoside from serine-specific transfer ribonucleic acid. *Angew. Chem.* 78: 600.

Binns,AN (1994): Cytokinin accumulation and action: biochemical, genetic and molecular approches. *Ann. Rev. Plant Physiol. Plant. Mol. Biol.* 45: 173-196.

Biondi,S; Canciani,L (1984): Uptake and translocation of bezyladenine by elm shoots cultured in vitro. *Can J. Bot.* 62: 2385-2390.

Biondi,S; Thorpe,TA (1982): Growth regulator effects, metabolite changes and respiration during shoot initiation in cultured cotyledon explants of *Pinus radiata. Botanical Gazette* 143: 20-25.

Bisgrove,SR; Crouch,ML; Fernandez,DF (1995): Chimeric nature of pecociously germinating *Brassica napus* embryos: mRNA accumulation patterns. *J. Exp. Bot.* 46: 27-33.

Blackwell,JR; Horgan,R (1994): Cytokinin biosynthesis by extracts of *Zea mays*. *Phytochemistry* 35: 339-342.

Blakesley,D; Constantine,D (1992): Uptake and metabolism of 6-benzyladenine in shoot culture of a range of species. *Plant Cell, Tissue and Organ Culture* 28: 183-186.

Blakesley,D; Lenton,JR; Horgan,R (1990): Benzyladenine ribosylglucoside: a metabolite of benzyladenine in *Gerbera jamesonii*. *Phytochemistry* 30: 387-388.

Blazquez,MA; Soowal,LN; Lee,I; Weigel,D (1997): *LEAFY* expression and flower initiation in *Arabidopsis*. *Development* 124: 3835-3844.

Bollmann,MP; Sweet,GB (1976): Bud morphogenesis of *Pinus radiata* in New Zealand I: The initiation and extension of the leading shoot of one clone at two sites. *NZJ. For. Sci.* 6: 376-392.

Bollmann,MP; Sweet,GB (1979): Bud morphogenesis of *Pinus radiata* in New Zealand II. The seasonal shoot growth pattern of seven clones at four sites. *NZJ. For. Sci.* 9: 153-165.

Bollmark,M; Chen,HJ; Moritz,T; Eliason,L (1995): Relations between cytokinin level, bud development and apical control in Norway spruce, *Picea abies*. *Physiol. Plant.* 95: 563-568.

Bon,MC (1988): J16: an apex protein associated with the juvenility of *Sequoiadendron giganteum*. *Tree Physiology* 4: 381-387.

Bon,MC; Riccardi,F; Monteuuis,O (1994): Influence of phase change in 90-year-old Sequoia sempervirens on its in vitro organogenic capacity and protein patterns. *Trees* 8: 283-287.

Bowman.JL; Alvarez.J; Weigel,D; Meyerowitz,EM; Smyth,DR (1993): Control of flower development in *Arabidopsis thaliana* by *APETALA l* and interacting genes. *Development* 119: 721-743.

Brand,MH; Lineberger,RD (1992): *In vitro* rejuvenation of Betulaceae: morphological evaluation. *Am. J. Bot.* 79: 618-625.

Brink,RA (1962). Phase change in higher plants and somatic cell heredity. *Quart. Rev. Biol.* 37: 1-22.

Browne,RD (1995): Maturation and rejuvenation in Jack pine (*Pinus banksiana* Lamb): Aspects of shoo morphology, anatomy and adventitious rooting of cuttings. Ph.D. Dissertation, University of Saskatchewan, Saskatoon.

Brzobohaty,B; Moore,I; Kristoffersen,P; Bako,L; Campos,N; Schell,J; Palme,K (1993): Release of active cytokinin by β-glucosidase localized to the maize root meristem. *Science* 262: 1051-1054.

Brzobohaty,B; Moore,I; Palme,K (1994): Cytokinin metabolism: Implications for regulation of plant growth and development. *Plant Mol. Biol.* 26: 1483-1497.

Bufe,A; Spangfort,MD; Kahlert,H; Schlaak,M; Becker,W-M (1996): The major birch pollen allergen, Bet v 1, shows ribonuclease activity. *Planta* 199: 413-415.

Burch,LR; Stuchbury,T (1986): Purification and properties of adenosine nucleosidases from tomato (*Lycopersicon esculentum*) roots and leaves. *FEBS Lett.* 125: 267-273.

Burch,LR; Stuchbury,T (1987): Activity and distribution of enzymes that interconvert purine bases, ribosides for cytokinin metabolism. *Physiol. Plant.* 69: 283-288.

Cahill,DM; Weste,GM; Grant,BR (1986): Changes in cytokinin concentrations in xylem extrudate following infection of *Eucalyptus marginata* Donn ex Sm with *Phytophthora cinnamomi*. *Plant Physiol.* 81: 1103-1109.

Cappiello,PE; Kling,GJ (1990): Determination of zeatin and zeatin riboside in plant tissue by solid-phase extraction and ion-exchange chromatography. *J. Chromatogr.* 504: 197-201.

Carswell,FE; Day,JS; Gould,KS; Jameson,PE (1996): Cytokinins and the regulation of plant form in three species of *Sophora*. *NZ J. Bot.* 34: 123-130.

Carter,P (1989): Opening address for Clonal Forestry Worshop. FRI Bulletin No. 160 (Proceedings. FRI/NZFP Ltd Clonal Forestry Workshop) 1-2 May, 1989, 7-9.

Centeno,ML; Rodriguez,A; Feito,I; Fernandez,B (1996): Relationship between endogenous auxin and cytokinin levels and morphogenic responses in *Actinidia deliciosa* tissue cultures. *Plant Cell Reports* 16: 58-62.

Chairgwin,JM; John,ME (1992): An efficient method for isolation of RNA and DNA from plants containing polyphenolics. *Nucleic Acids Res.* 20: 2381.

Chalupka,W; Giertych,M; Kopcewicz,J (1982): Effects of polyethylene covers, a flower inducing treatment, on the content of endogenous gibberellin-like substances in grafts of Norway spruce. *Physiol. Plant.* 54, 79-82.

Chang,SJ; Puryear,J; Careney,J (1993): A simple and efficient method for isolating RNA from pine trees. *Plant Mol. Biol. Rep.* 11: 113-116.

Chaperon,H (1989): Open-pollinated seed orchards: tested families or tested clones for tomorrow's forests? FRI Bulletin N0.160 (Proceedings. FRI/NZFP Forests Ltd Clonal Forestry Workshop) 1-2 May, 1989, 15-24.

Chatfield,JM; Armstrong,DJ (1986): Regulation of cytokinin oxidase activity in callus tissues of *Phaseolus vulgaris* L. cv. Great Northern. *Plant Physiol.* 80: 493-499.

Chaudhury,AM; Letham,DS; Graig,S; Dennis,ES (1993): *amp1* - a mutant with high cytokinin levels and altered embryonic pattern, faster vegetative growth, constitutive photomorphogenesis and precocious flowering. *The Plant Journal* 4: 907-916.

Chen,CM (1982): Cytokinin biosynthesis in cell-free systems. In: Plant Growth Substances. (Ed: Wareing,FP) Academic Press, New York, 155-163.

Chen,CM (1997): Cytokinin biosynthesis and interconversion. *Physiol. Plant.* 101: 665-673.

Chen,CM; Eckert,RL (1977): Phosphorylation of cytokinin by adenosine kinase from wheat germ. *Plant Physiol.* 59: 443-447.

Chen,CM; Eckert,RL; McChesney,JD (1976): Evidence for biosynthesis of transfer RNA-free cytokinins. *FEBS Lett.* 64: 429-434.

Chen,CM; Kristopeit,SM (1981a): Dephosphorylation of cytokinin ribonucleotide by 5'-nucleotidases from wheat germ cytosol. *Plant Physiol.* 67: 494-498.

Chen,CM; Kristopeit,SM (1981b): Deribosylation of cytokinin ribonucleoside by adenosine nucleosidase from wheat germ cells. *Plant Physiol.* 68: 1020-1023.

Chen,CM; Leisner,SM (1984): Modification of cytokinins by cauliflowr microsomal enzymes. *Plant Physiol.* 75: 442-446.

Chen,CM; Melitz,DK (1979): Cytokinin biosynthesis in a cell-free system from cytokinin-autotrophic tobaco tissue cultures. *FEBS Lett.* 107: 15-20.

Chen,CM; Melitz,DK; Clough,FW (1982): Metabolism of cytokinins: Phosphoribosylation of cytokinin bases by adenine phosphoribosyltransferase from wheat germ. *Arch. Biochem. Biophys.* 214: 634-641.

Chen,CM; Petschow,B (1978): Metabolism of cytokinin. Ribosylation of cytokinin base by adenosine phosphorylase from wheat germ. *Plant Physiol.* 62: 872-874.

Chen,HJ; Bollmark,M; Eliason,L (1996): Evidence that cytokinin controls bud size and branch form in Norway spruce. *Physiol. Plant.* 98, 612-618.

Chien,JC; Sussex,IM (1996): Differential regulation of trichome formation on the adaxial and abaxial leaf surfaces by gibberellins and photoperiod in *Arabidopsis thaliana* (L.) Heynh. *Plant Physiol.* 111: 1321-1328.

Chin-Atkins,AN; Craig,S; Hocart,CH; Dennis,ES; Chaudhury,AM (1996): Increased endogenous cytokinin in the *Arabidopsis amp1* mutant corresponds with de-etiolation responses. *Planta* 198: 549-556.

Chomczynski,P (1992): One-hour downward alaline capillary transfer for blotting of DNA and RNA. *Analytical Biochemistry* 201: 134-139.

Chory,J; Reinecke,D; Sim,S; Washburn,T; Brenner,M (1994): A role for cytokinin in de-etiolation in Arabidopsis: *det* mutants have an altered response to cytokinins. *Plant Physiol.* 104: 339-347.

Clemens,J; Jameson,PE; Bannister,P; Pharis,RP (1995): Gibberellins and bud break, vegetative shoot growth and flowering in *Metrosideros collina* cv. Tahiti. *Plant Growth Regul.* 16: 161-171.

Coen,ES; Meyerowitz,EM (1991): The war of the whorls: genetic interactions controlling flower development. *Nature* 353: 31-37.

Conway,LJ; Poethig,RS (1997): Mutations of *Arabiopsis* thaliana that transform leaves into cotyledons. *PNAS USA* 94: 10209-10214.

Crowell,DN (1994): Cytokinin regulation of a soybean pollen allergen gene. *Plant Mol. Biol.* 25: 829-835.

Crowell,DN; Kadlecek,AT; John,MC; Amasino,RM (1990): Cytokinin-induced mRNAs in cultured soybean cells. *PNAS USA* 87: 8815-8819.

Davis,GC; Hein,MB; Chapman,DA; Neely,BC; Sharp,CR; Durley,RC; Biest,DK; Heyde,BR; Carnes,MG (1986): Immunoaffinity columns for the isolation and analysis of plant hormones. In: Plant Growth Substances 1985. (Ed: Bopp,M) Springer-Verlag, Berlin, 44-51.

Dawson,RMC; Elliott,DC; Elliott,WH; JonesKM (1986): Data for Biochemical Research. Third Edition ed. Oxford Science Publications, Oxford.

Day,JS; Jameson,PE; Gould,KS (1995): Cytokinins associated with metamorphic vegetative growth in *Elaeocarpus hookerianus*. *Aust. J. Plant Physiol.* 22: 67-73.

de Nys,R; Jameson,PE; Chin,N; Brown,MT; Sanderson,KJ (1990) The cytokinins as endogenous growth regulators in *Macryocystis pyritera* (L.) C. Ag. (Phaeophyceae). *Botanica Marina* 33: 467-475.

Deikman,J (1997): Elucidating cytokinin response mechanisms using mutants. *Plant Growth Regul.* 23: 33-40.

Deikman,J; Hammer,PE (1995): Induction of anthocyanin accumulation by cytokinins in *Arabidopsis thaliana*. *Plant Physiol.* 108: 47-57.

Deikman,J; Ulrich,M (1995): A novel cytokinin-resistant mutant of *Arabidopsis* with abbreviated shoot development. *Planta* 195: 440-449.

Dieleman,JA; Verstappen,FWA; Nicander,B; Kuiper,D; Tillberg,E; Tromp,J (1997): Cytokinins in *Rosa* hybrida in relation to bud break. *Physiol. Plant.* 99: 456-464.

Dietrich,JT; Kamineck,M; Blevins,DG; Reinbott,TM; Morris,RO (1995): Changes in cytokinins and cytokinin oxidase activity in developing maize kernels and the effects of exogenous cytokinin on kernel development. *Plant Physiol. Biochem.* 33: 327-336.

Dixon,SC; Martin,RC; Mok,MC; Shaw,G; Mok,DWS (1989): Zeatin glucosylation enzymes in *Phaseolus*. Isolation of O-glucosyltransferase from *P. lunatus* and comparison to O-xylosyltransferase from *P. vulgaris*. *Plant Physiol.* 90: 1316-1321.

Dominov,JA; Stenzler,L; Lee,S; Schwarz,JJ; Leisner,S; Howell,SH (1992): Cytokinin and auxins control the expression of a gene in *Nicotiana* plumbaginifolia cells by feedback regulation. *The Plant Cell* 4: 451-461.

Dong,J-Z; Dunstan,DI (1996): A reliable method for extraction of RNA from various conifer tissues. *Plant Cell Reports* 15: 516-521.

Doorenbos,J (1954): "Rejuvenation" of *Hedera helix* in graft combinations. *Ned Akad Wetensch Amsterdam Pro* 57: 99-102.

Doree,M; Guern,J (1973): Short-time metabolism of some exogenous cytokinins in *Acer pseudoplatanus* cells. *Biochim. Biophys. Acta* 304: 611-622.

Doumas,P; Bonnet-Masimbert,M; Zaerr,JB (1989): Evidence of cytokinin bases, ribosides and glucosides in roots of Douglas-fir, *Pseudotsuga menziesii*. *Tree Physiology* 5: 63-72.

Doumas,P; Morris,JW; Chien,C; Bonnet-Masimbert,M; Zaerr,JB (1986): A possible relationship between a cytokinin conjugate and flowering in Douglas-fir. Proceedings of 9th North American Forest Biology Workshop Stillwater, Oklahoma, 285-296.

Doumas,P; Zaerr,JB (1988): Seasonal changes in levels of cytokinin-like compounds from Douglas-fir xylem extrudate. *Tree Physiology* 4: 108.

Drumm-Herrel,H; Mohr,H (1994): Regulation by light of chlorophyll synthsis in the cotyledons of Scots pine (*Pinus sylvestris*) seedlings. *Physiol. Plant.* 91: 300-306.

Dudley,M; Poethig,RS (1993): The heterochronic *Teopod1* and *Teopod2* mutations of maize are expressed non-cell-autonomously. *Genetics* 133: 389-399.

Dunberg,A; Malmberg,G; Sassa,T; Pharis,RP (1983): Metabolism of tritiated gibberellins A4 and A9 in Norway spruce, *Picea abies* (L.) Karst. *Plant Physiol.* 71: 257-262.

Eason, JR; Morris,RO; Jameson,PE (1996) The relationship between virulence and cytokinin production by Rhodococcus fascians (Tillford 1936) Goodfellow 1984. *Plant Pathology* 45: 323-331.

Entsch,B; Letham,DS (1979): Enzymic glucosylation of the cyokinin, 6-benzylaminopurine. *Plant Sci. Lett.* 14: 205-212.

Entsch,B; Parker,CW; Letham,DS; Summons,RE (1979): Preparation and characterisation using HPLC of an enzyme forming glucosides of cytokinins. *Biochim. Biophys. Acta* 570: 124-139.

Ernst,D; Sch„fer,W; Oesterholt,D (1983): Isolation and identification of a new, naturally occurring cytokinin (6-benzylaminopyrine riboside) from an anise cell culture (*Pimpinella anisum* L.). *Planta* 159: 222-225.

Estruch,JJ; Granell,A; Hansen,G; Prinsen,E; Redig,P; Van Onckelen,H; Schwarz-Sommer,Z; Sommer,H; Spena,A (1993): Floral development and expression of floral homeotic genes are influenced by cytokinins. *The Plant Journal* 14: 379-384.

Evans,MMS; Passas,HJ; Poethig,RS (1994): Heterochronic effects of *glossy15* mutations on epidermal cell identity in maize. *Development* 120: 1971-1981:.

Evans,MMS; Poethig,RS (1995): Gibberellins promote vegetative phase change and reproductive maturity in maize. *Plant Physiol.* 108: 475-487.

Evans,MMS; Poethig,RS (1997): The *viviparous8* mutation delays vegetative phase change and accelerates the rate of seedling growth in maize. *The Plant Journal* 12: 769-779.

Eysteinsson,T; Greenwood,MS (1990): Promotion f flowering in young *Larix laricina* grafts by gibberellin GA4/7 and root pruning. *Can. J. For. Res.* 20: 1448-1452.

Eysteinsson,T; Greenwood,MS (1995): Flowering on long and short shoots of *Larix laricina* in response to differential timing of GA4/7 applications. *Tree Physiology* 15: 467-469.

Faiss,M; Zalubilova,J; Strnad,M; Schm•lling,T (1997): Conditional transgenic expression of the *ipt* gene indicates a function for cytokinins in paracrine signaling in whole tobacco plants. *The Plant Journal* 12: 401-415.

Falk,A; Rask,L (1995): Expression of a zeatin-*O*-glucoside-degrading β-glucosidase in *Brassica napus*. *Plant Physiol.* 108: 1369-1377.

Fang,G; Hammar,S; Grumet,R (1992): A quick and inexpensive method for removing polysaccharides from plant genomic DNA. *BioTechniques* 13: 52-55.

Feito,I; Rodriguez,A; Centeno,ML; Sanchez-Tames,R; Fernandez,B (1994): Effect of the physical nature of the culture medium on the metabolism of benzyladenine and

endogenous cytokinins in Actinidia deliciosa tissues cultured in vitro. *Physiol. Plant.* 91: 449-453.

Feito,I; Rodriguez,A; Centeno,ML; Sanchez-Tames,R; Fernandez,B (1995): Effect of applied benzyladenine on endogenous cytokinin content during the early stages of bud development of kiwifruit. *Physiol. Plant.* 95: 241-246.

Fernandez,DE (1997): Developmental basis of homeosis in precociously germinating *Brassica napus* embryos: Phase change at the shoot apex. *Development* 124: 1149-1157.

Finkelstein,RR; Crouch,ML (1984): Precociously germinating rapeseed embryos retain characteristics of embryogeny. *Planta* 162: 125-131.

Flores,S; Tobin,EM (1986): Benzyladenine modulation of the expression of two genes for nuclear-encoded chloroplast proteins in *Lemna gibba*: Apparent post-transcriptional regulation. *Planta* 168: 340-349.

Flores,S; Tobin,EM (1988): Cytokinin modulation of LHCP mRNA levels: the involvement of posttranscriptional regulation. *Plant Mol. Biol.* 11: 409-415.

Fortanier,EJ; Jonkers,H (1976): Juvenility and maturity of plants as influenced by their ontogenetical and physiological aging. *Acta Hort.* 56: 37-44.

Fox,JE; Cornette,J; Deleuze,G; Dyson,W; Giersak,C; McChesney,J (1973): The formation, isolation and biological activity of a cytokinin 7-glucoside. *Plant Physiol.* 52: 627-632.

Fox,JE; Sood,CK; Buckwalter,B; McChesney,JD (1971): Metabolism and biological activity of 9-substituted cytokinins. *Plant Physiol.* 47: 275-281.

Franclet,A (1981): Ajeunissement et propagation vegetative de lignieux. *Annales Afocel* 1980, 12-39.

Frydman,VM; Wareing,PF (1973a): Phase change in *Hedera helix* L. I. Gibberellin-like substances in the two growth phases. *J. Exp. Bot.* 24: 1131-1138.

Frydman,VM; Wareing,PF (1973b): Phase change in *Hedera helix* L. II. The possible role of roots as a source of shoot gibberellin-like substances. *J. Exp. Bot.* 24: 1139-1148.

Frydman,VM; Wareing,PE (1974): Phase change in *Hedera helix* L. III. The effects of gibberellins, abscisic acid and growth retardants on juvenile and adult ivy. *J. Exp. Bot.* 25: 420-429.

Gajhede,M; Osmark,P; Poulsen,FM; Ipsen,H; Larsen,JN; Vanneerven,BJJ; Schou,C; Lowenstein,H; Spangfort,MD (1996): X-ray and NMR structure of Bet v 1, the origin of birch pollen allergy. *Nature Struct. Biol.* 3: 1040-1045.

Galoch,E (1985): Comparison of the content of growth regulators in juvenile and adult plants of birch (*Betula verrucoca* Ehrh). *Acta Physiol. Plant.* 7: 205-215.

Gawer,M; Laloue,M; Terrine,C; Guern,J (1977): Metabolism and biological significance of benzyladenine-7-glucoside. *Plant Sci. Lett.* 8: 267-274.

Gianfagna,TJ; Merritt,RH (1998): GA4/7 promotes stem growth and flowering in a genetic line of *Aquilegia* X hybrida Sims. *Plant Growth Regul.* 24: 1-5.

Glund,K; Goldstein,AH (1993): Regulation, synthesis, and excretion of a phosphate starvation inducible RNase by plant cells. In: Control of Plant Gene Expression. (Ed: Verma,DPS) CRC Press, Boca Raton, FL., 311-323.

Goethe,JW (1790): Versuch die Metamorphose der Pflanzen zu erkl„ren. (Gotha, Saxe-Gotha: C.W. Ettinger), .

Grayling,A; Hanke,DE (1992): Cytokinins in exudates from leaves and roots of red *Perilla*. *Phytochemistry* 31: 1863.

Green,P (1994): The ribonucleases of higher plants. *Ann. Rev. Plant Physiol.Plant Mol. Biol.* 45: 421-445.

Greene,EM (1980): Cytokinin production by microorganisms. *Bot. Rev.* 46: 25-74.

Greenwood,MS (1987): Rejuvenation of forest trees. *Plant Growth Regul.* 6: 1-12.

Greenwood,MS (1992): Theoretical aspects of juvenility and maturation. In: A symposium in IUFRO'S centennial Year. (:) Bordeaux-France, 14-18 September 1992,, .

Greenwood,MS (1995): Juvenility and maturation in conifers: current concepts. *Tree Physiology* 15: 433-438.

Greenwood,MS; Hopper,ĊA; Hutchison,KW (1989): Maturation in larch. I. Effect of age on shoot growth, foliar characteristics, and DNA methylation. *Plant Physiol.* 90: 406-412.

Greenwood,MS; Hutchison,KW (1993): maturation as developmental process. In: Clonal Forestry: Genetics, Biotechnology and Application. (Eds: Ahuja,MR; Libby,WJ) Springer-Verlag, New York, 14-33.

Guinn,G (1973): Purification of leaf nucleotides and nucleosides on insoluble polyvinylpyrrolidone. *Analytical Biochemistry* 54: 276-282.

Guinn,G; Brummett,DL (1990): Solid phase extraction of cytokinins from aqueous solutions with C18 cartridge and their use in a rapid purification procedure. *Plant Growth Regul.* 9: 305-314.

Hackett,WP (1985): Juvenility, maturation, and rejuvenation in woody plants. *Horticultural Reviews* 7: 109-155.

Hackett,WP; Cordero,RE; Srinivasan,C (1987): Apical meristem characteristics and activity in relation to juvenility in *Hedera*. In: Manipulation of Flowering. (Ed: Atherton,JG) Butterworths, London, 92-99.

Hackett,WP; Murray,JR (1993): Maturation and rejuvenation in woody species. In: Micropropagation of Woody Plants. (Ed: Ahuja,MR) Kluwer Academic Publishers, Dordrecht, Netherlands, 93-105.

Hackett,WP; Murray,JR (1996): Maturation or Phase Change. In: Molecular and Morphological Markers for Juvenility, Maturity, Rejuvenation and Somatic Embryogenesis in Woody

Plant Species - The Biotechnological Approach. (Eds: Thompson,D; Welander,M) European Commission Directorate-General for Science, Research and Development DG XII-E,, 7-22.

Hackett,WP; Murray,JR; Smith,A (1992): Control of maturation in woody species. In: Mass Production Technology for Genetically Improved Fast Growing Forest Tree Species, Syntheses, AFOCEL, Paris, France, 45-50.

Hall,RH (1970): N6-(-isopentenyl)adenosine: chemical reactions, biosynthesis, metabolism, and significance to the structure and function of tRNA. *Prog. Nucleic. Acid Res. Mol. Biol.* 10: 57-86.

Hall,RH (1973): Cytokinin as a probe of developmental processes. *Ann. Rev. Plant Physiol.* 24: 425-444.

Hammerton,RD; Nicander,B; Tillberg,E (1996): Identification of some major cytokinins in *Phaseolus vulgaris* and their distribution. *Physiol. Plant.* 96: 77-84.

Hand,P; Bestford,RT; Richardson,CM; Peppitt,SD (1996): Antibodies to phase related proteins in juvenile and mature *Prunus avium*. *Plant Growth Regul.* 20: 25-29.

Hare,PD; van Staden,J (1994): Cytokinin oxidase: Biochemical features and physiological significance. *Physiol. Plant.* 91: 128-136.

Hare,PD; van Staden,J (1997): The molecular basis of cytokinin action. *Plant Growth Regul.* 23: 41-78.

Haughn,GW; Schultz,EA; Martinez-Zapater,JM (1995): The regulation of flowering in *Arabidopsis thaliana*: meristems, morphogenesis, and mutants. *Can J. Bot.* 73: 959-981.

Hautala,E; Stafford,A; Corse,J; Barker,PA (1986): Cytokinin variation in the sap of male and female *Gymnocladus dioica*. *J. Chromatogr.* 351: 560.

Hedden,P (1993): Modern methods for the quantitative analysis of plant hormones. *Ann. Rev. Plant Physiol.Plant Mol. Biol.* 44: 107-129.

Hempel,FD; Weigel,D; Mandel,MA; Ditta,G; Zambryski,PC; Feldman,LJ; Yanofsky,MF (1997): Floral determination and expression of floral regulatory genes in *Arabidopsis*. *Development* 124: 3845-3853.

Hendry,NA; Van Staden,J; Allan,P (1982): Cytokinins in citrus II. Fluctuations during growth in juvenile and adult plants. *Scietia Horticulturae* 17: 247-256.

Henson,IE; Wareing,PF (1977a): Cytokinins in *Xanthium strumarium* L.: the metabolism of cytokinins in detached leaves and buds in relation to photoperiod. *New Phytol.*. 78: 27-33.

Henson,IE; Wareing,PF (1977b): Cytokinins in *Xanthium strumarium* L.: some aspects of the photoperiodic control of endogenous levels. *New Phytol.*. 78: 35-45.

Hicks,KA; Sundas,A; Meeks-Wagner,DR (1996): *Arabidopsis* early-flowering mutants reveal multiple levels of regulation in the vegetative-to-floral transition. *Semin. Cell Dev. Biol.* 7: 409-418.

Hocart,CH; Letham,DS; Parker,CW (1990): Metabolism and translocation of exogenous zeatin riboside in germinating seeds and seedlings of *Zea mays*. *J. Exp. Bot.* 41: 1517-1524.

Holtz,J; Klambt,D (1975): tRNA-isopentenyltransferase from *Lactobacillus acidophilus* ATCC4963. *Hoppe-Seyler's Z Physiol. Chem.* 356: 1459-1464.

Holtz,J; Klambt,D (1978): tRNA-isopentenyltransferase from *Zea mays* L. Characterisation of the isopentylation reacrion of tRNA, Oligo(A) and other nucleic acids. *Hoppe-Seyler's Z Physiol. Chem.* 359: 89-101.

Horgan,K (1987): . In: Tissue culture in forestry. Vol. 3. (Eds: Bonga,JM; Durzan,DJ) Martinus Nijhalf Publishers,, 128-145.

Horgan,K; Skudder,D; Holden,G (1997): Clonal storage and rejuvenation. In: Proceedings of IUFRO'97 Genetics of Radiata Pine, Rotorua, New Zealand. FRI Bulletin No. 203 1-4 December, 1997, 273-280.

Horgan,R (1984): Cytokinins. In: Advanced *Plant Physiol.*. (Ed: Wilkins,MB) Pitman, London, 53-75.

Horgan,R (1985): Cytokinin biosynthesis and metabolism. In: Plant Growth Substances 1985. (Ed: Bopp,M) Springer-Verlag, Berlin, 92-98.

Horgan,R (1992): Present and future prospects for cytokinin research. In: Physiology and Biochemistry of Cytokinins in Plants. (Eds: Kamínek,M; Mok,DWS; Zazimalova,E) SPB Academic Publishing, The Hague, 3-13.

Horgan,R (1995): . Instrumental methods of plant hormone analysis. In: Plant Hormones. (Ed: Davies,PJ) Kluwer Academic Publishers, Boston, 415-432.

Horgan,R; Scott,IM (1987): Cytokinins. In: Principles and practices of plant hormone analysis. (Eds: Rivier,L; Crozier,A) Academic Press, New York, 303-365.

Horrell,BA; Jameson,PE; Bannister,P (1990): Growth regulation and phase change in some New Zealand heteroblastic plants. *NZ J. Bot.* 28: 187-193.

Huala,E; Sussex,IM (1992): LEAFY interacts with floral homeotic genes to regulate *Arabidopsis* floral development. *The Plant Cell* 4: 901-913.

Huang,HJ; Chen,Y; Kuo,JL; Kuo,TT; Tzeng,CC; Huang,BL; Chen,CM; Huang,LC (1996): Rejuvenation of *Sequoia semperviens in vitro*: Change in isoesterases and isoperoxidases. *Plant Cell Physiol.* 37: 77-80.

Huang,LC; Lin,LY; Chen,CM; Chen,LJ; Huang,BL; Murashige,T (1995): Phase reversal in Sequoia sempervirens in relation to mtDNA. *Physiol. Plant.* 94: 379-383.

Huang,LC; Lius,S; Huang,BL; Murashige,T; Mahdi,EFM; Van Gundy,R (1992): Rejuvenation of Sequoia sempervirens by repeated grafting of shoot tips onto juvenile rootstocks *in vitro*: Model for phase reversal of trees. *Plant Physiol.* 98: 166-173.

Huang,S; Lee,HS; Karunanandaa,B; Kao,TH (1994): Ribonuclease activity of *Petunia inflata* S proteins is essential for rejection of self-pollen. *The Plant Cell* 6: 1021-1028.

Hutchison,KW; Sherman,CD; Smith,SS; Singer,PB; Greenwood,MS (1990): Maturation in Larch. II. Effects of age on photosynthesis and gene expression in developing foliage. *Plant Physiol.* 94: 1308-1315.

Hutchison,KW; Singer,PB; Greenwood,MS (1988): Molecular genetic analysis of development and maturation in larch. In: Molecular Genetics of Forest Trees. Vol. No.P1-X-80. (:) Petawawa National Forest Research Institute, Chalk River, Ontario, Canada, 26-33.

Imbault,N; Moritz,T; Nilsson,O; Chen,H-J; Bollmark,M; Sandberg,G (1993): Separation and identification of cytokinins using combined capillary liquid chromatography/mass spectrometry. *Biological Mass Spectrometry* 22: 201-210.

Imbault,N; Tardieu,I; Zaerr,JB; Bonnet-Masimbert,M (1988): Possible role of isopentenyladenine and isopentenyladenosine in flowering of *Pseudotsuga menziesii*: endogenous variations and exogenous applications. *Plant Physiol. Biochem.* 26: 291-296.

Irish,EE; Nelson,TM (1988): Development of maize plants from cultured shoot apices. *Planta* 175: 9-12.

Irish,VF; Sussex,IM (1990): Function of the *apetala*-1 gene during *Arabidopsis* floral development. *The Plant Cell* 2: 741-753.

Jacobs,WP (1979): Plant Hormones and Plant Development. Cambridge University Press, London.

Jacobshagen,S; Kindle,KL; Johnson,CH (1996): Transcription of CABII is regulated by the biological clock in *Chlamydomonas reinhardtii*. *Plant Mol. Biol.* 31: 1173-1184.

Jameson,PE (1994): Cytokinin metabolism and compartmentation. In: Cytokinins - Chemistry, Activity, and Function. (Eds: Mok,DWS; Mok,MC) CRC Press, Boca Raton, 113-128.

Jameson,PE; Letham,DS; Zhang,R; Parker,CW; Badnoch-Jones,J (1987): Cytokinin translocation and metabolism in *Lupin* species. I. Zeatin riboside introduced into the xylem at the base of *Lupinus angustifolius* stems. *Aust. J. Plant Physiol.* 14: 695-718.

Jameson,PE; McWha,JA; Wright,GJ (1982): Cytokinins and changes in their activity during the development of grains of wheat (*Triticum aestivum* L.). *Zeitschrift Pflanzenphysiologie* 106: 27-36.

Jameson,PE; Morris,RO (1989): Zeatin-like cytokinins in yeast: Detection by immunological methods. *FEBS Lett.* 135: 385-390.

Johansen,DA (1940): Plant Microtechnique. McGraw-Hill, New York.

Jones,RJ; Schreiber,BMN (1997): Role and function of cytokinin oxidase in plants. *Plant Growth Regul.* 23: 123-134.

Kamínek,M (1992): Progress in cytokinin research. *Trends in Biotechnology* 10: 159-164.

Kamínek,M; Armstrong,DJ (1990): Genotypic variation in cytokinin oxidase from *Phaseolus* callus cultures. *Plant Physiol.* 93: 1530-1538.

Kamínek,M; Motyka,V; Vankova,R (1997): Regulation of cytokinin content in plant cells. *Physiol. Plant.* 101: 689-700.

Kania,T; Russenberger,D; Peng,S; Apel,K; Melzer,S (1997): FPF1 promotes flowering in *Arabidopsis*. *The Plant Cell* 9: 1327-1338.

Kao,TH; Huang,S (1994): Gametophytic self-incompatiblility: A mechanism for self/nonself discrimination during sexual reproduction. *Plant Physiol.* 105: 461-466.

Kao,TH; McCubbin,AG (1997): Molecular and biochemical bases of gametophytic self-incompatibility in *Solanaceae*. *Plant Physiol. and Biochem.* 35: 171-176.

Kataeva,NV; Alexandrova,IG; Butenko,RG; Dragavteeva,EV (1991): Effect of applied and internal hormones on vitrification and apical necrosis of different plants cultured in vitro. *Plant Cell, Tissue and Organ Culture* 27: 149-154.

Katterman,FRH; Shattuck,VI (1983): An effective method of DNA isolation from the mature leaves of *Gossypium* species that contain large amounts of phenolic terpenoids and tannins. *Prep. Biochem.* 13: 347-359.

Kester,DE (1976): The relationship of juvenility to plant propagation. *Proc. Int. Plant Prop. Soc.* 26: 71-84.

Kinet,JM; Lejeune,P; Bernier,G (1993): Shoot-root interactions during floweral transition: a possible role for cytokinins. *Environ. Exp. Bot.* 33: 459-469.

King,RW; Pharis,RP; Mander,LN (1987): Gibberellin in relation to growth and flowering in *Pharbitis nil* Chois. *Plant Physiol.* 84: 1126-1131.

Klemen,F; Klambt,D (1974): Half life of sRNA from primary roots of *Zea mays*. A contribution to the cytokinin production. *Physiol. Plant.* 31: 186-188.

Knypl,JS; Letham,DS; Palni,LMS (1985): Cytokinins in maturing and germinating *Lupinus luteus* L. seeds. *Biol. Plant.* 27: 188-195.

Kobayashi,H; Morisaki,N; Tago,Y; Hashimoto,Y; Iwasaki,S; Kawachi,E; Nagata,R; Shudo,K (1997): Structural identification of a major cytokinin in coconut milk as 14-O-3-O-[beta-D-galactopyranosyl-(1→2)-alpha-D-galactopyranosyl-(1→3)-alpha-L-arabinofuranosyl]-4-O-(alpha-L-arabinofuranosyl)-beta-D-galactopyranosyl-trans-zeatin riboside. *Chemical and Pharmaceutical Bulletin* 45: 260-264.

Kossuth,SV (1978): Induction of fascicular bud development in *Pinus sylvestris* L. *HortSci.* 13: 174-176.

Kuiper,D; Kuiper,PJC; Lambers,H; Schuit,J; Staal,M (1989): Cytokinin concentration in relation to mineral nutrition and benzyladenine treatment in *Plantago* major ssp. Pleiosperma. *Physiol. Plant.* 75: 511-517.

Kusnetsov,VV; Oelmuller,R; Sarwat,MI; Porfirova,SA; Cherepneva,GN; Herrmann,RG; Kulaeva,ON (1994): Cytokinins, abscisic acid and light affect accumulation of chloroplast proteins in *Lupinus luteus* cotyledons without notable effect on steady-state mRNA levels. *Planta* 194: 318-327.

Lakshmanan,KK; Ambegaokar,KB (1984): Polyembryony. In: Embryology of angiosperms. (Ed: Johri,BM) Springer, Berlin, Heidelberg New York, 445-474.

Laloue,M; Pethe,C (1982): Dynamics of cytokinin metabolism in tobacco cells. In: Plant Growth Substance 1982. (Ed: Wareing,PF) Academic Press, London, 185-195.

Laloue,M; Pethe-Terrine,C; Guern,J (1981): Uptake and metabolism in tobacco cells: studies in relation to the expression of their biological activities. In: Metabolism and Molecular Activities of cytokinins. (Eds: Guern,J; Peaud-Lenoel,C) Springer-Verlag, Berlin, 80-96.

Langridge,J (1957): Effect of day-length and gibberellic acid on the flowering of Arabidopsis. *Nature* 180: 36-37.

Lawson,EJR; Poethig,RS (1995): Shoot development in plants: time for a change. Trends in Genetics 11: 263-268.

Letham,DS (1963): Zeatin, a factor inducing cell division from *Zea mays*. Life Sci. 8: 569-573.

Letham,DS (1978): Cytokinins. In: Phytohormones and Related Compounds - A Comprehensive Treatise. Vol. 1. (Eds: Letham,DS; Goodwin,PB; Higgins,TJV) Elsevier/North-Holland, Amsterdam, 641.

Letham,DS (1994): Cytokinins as phytohormones - Sites of biosynthesis, translocation, and function of translocated cytokinin. In: Cytokinins: Chemistry, Activity and Function. (Eds: Mok,DWS; Mok,MC) CRC Press, Boca Raton, FL, 57-80.

Letham,DS; Gollnow,BI (1985): Regulators of cell division in plant tissues. XXX. Cytokinin metabolism in relation to radish cotyledon expansion and senescence. *J. Plant Growth Regul.* 4: 129-145.

Letham,DS; Palni,LMS (1983): The biosynthesis and metabolism of cytokinins. *Ann. Rev. Plant Physiol.* 34: 163-197.

Letham,DS; Palni,LMS; Tao,GQ; Gollnow,BI; Bates,CM (1983): Regulators of cell division in plant tissues. XXIX. The activities of cytokinin glucosides and alanine conjugates in cytokinin bioassays. *J. Plant Growth Regul.* 2: 103-115.

Letham,DS; Shannon,JC; MacDonald,IRC (1964): The structure of zeatin, a kinetin-like factor inducing cell division. *Proc. Chem. Soc. London*, 230.

Letham,DS; Singh,S; Wong,OC (1991): Mass spectrometric analysis of cytokinin in plant tissue. VII. Quantification of cytokinin bases by negative ion mass spectrometry. *Plant Growth Regul.* 10: 107-113.

Letham,DS; Tao,GQ; Parker,CW (1982): An overview of cytokinin metabolism. In: Plant Growth Substances 1982. (Ed: Wareing,PF) Academic Press, London, 143-153.

Letham,DS; Wilson,MM; Parker,CW; Jenkins,ID; MacLeod,JK; Summons,RE (1975): The identity of an unusual metabolite of 6-benzylaminopurine. *Biochim. Biophys. Acta* 399: 61-70.

Letham,DS; Zhang,R (1989): Cytokinin translocation and metabolism in *lupin* species II. New nucleotide metabolites of cytokinins. *Plant Sci.* 64: 161-165.

Lewinsohn,E; Steele,CL; Croteau,R (1994): Simple isolation of functional RNA from woody stems of gymnosperms. *Plant Mol. Biol. Rep.* 12: 20-25.

Lewis,DH; Burge,GK; Hopping,ME; Jameson,PE (1996): Cytokinins and fruit development in the kiwifruit (Actinidia deliciosa). II. Effects of reduced pollination and CPPU application. *Physiol.Plant.* 98: 187-195.

Lewis, DH (1994): Cytokinins and Fruit growth in kiwifruit. Ph.D. Dissertation, The University of Otago, Dunedin, New Zealand.

Liang,P; Averboukh,L; Pardee,A (1993): Distribution and cloning of eukaryotic mRNAs by means of differential display: refinements and optimization. Nucleic Acid Research 21: 3269-3275.

Liang,P; Pardee,AB (1992): Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* 257: 967-971.

Libby,WJ; Ahuja,MR (1993): The genetics of clones. In: Clonal Forestry I. Genetics and Biotechnology. (Eds: Ahuja,MR; Libby,WJ) Springer-Verlag, Berlin, 5-13.

Lorenzi,R; Horgan,R; Wareing,PF (1975): Cytokinins in *Picea sitchensis* Carriere: identification and relation to growth. *Biochemie und Physiologie der Pflanzen* 168: 333-339.

Lu,JL; Ertl,J; Chen,CM (1990): Cytokinin enhancement of the light induction of nitrate reductase transcript levels in etiolated barley leaves. *Plant Mol. Biol.* 14: 585-594.

Lu,J-I; Ertl,JR; Chen,CM (1992): Transcriptional regulation of nitrate reductase mRNA levels by cytokinin-abscisic acid interactions in etiolated barley leaves. *Plant Physiol.* 98: 1255-1260.

MacDonald,EMS; Morris,RO (1981): Combined high-performance liquid chromatography-radioimmunoassay for cytokinins. *J. Chromatogr.* 214: 101-109.

MacDonald,MS; Morris,RO (1985): Isolation of cytokinins by immunoaffinity chromatography and analysis by high-performance liquid chromatography radioimmunoassay. *Methods Enzymol.* 110: 347-358.

Mandel,MA; Gustafson-Brown,C; Savidge,B; Yanofsky,MF (1992): Molecular characterisation of the *Arabidopsis* floral homeotic gene *APETALA1*. *Nature* 360: 273-277.

Mandel,MA; Yanofsky,MF (1995): A gene triggering flower formation in Arabidopsis. *Nature* 377: 522-524.

Marc,J; Hackett,WP (1991): Gibberellin-induced reorganization of spatial relationships of emerging leaf primordia at the shoot apical meristem in *Hedera helix* L. *Planta* 185: 171-178.

Marc,J; Hackett,WP (1992): Changes in the pattern of cell arrangement at the surface of the shoot apical meristem in *Hedera helix* L. following gibberellin treatment. *Planta* 186: 503-510.

Martin,RC; Mok,MC; Mok,DWS (1997): Protein processing and auxin response in transgenic tobacco harbouring a putative cDNA of zeatin O-xylosyltransferase from *Phaseolus vulgaris*. *The Plant Journal* 12: 305-312.

Martin,RC; Mok,MC; Shaw,G; Mok,DWS (1989): an enzyme mediating the conversion of zeatin to dihydrozeatin in *Phaseolus* embryos. *Plant Physiol.* 90: 1630-1635.

Martinez-Zapater,JM; Coupland,G; Dean,C; Koornneef,MK (1994): The transition to flowering in Arabidopsis. In: Arabidopsis. (Eds: Meyerowitz,EM; Somerville,CR) Cold Spring Harbor Press, Cold Spring Harbor, New York, .

Matton,DP; Brisson,N (1989): Cloning, expression, and sequence conservation of pathogenesis-related gene transcripts of potato. *Mol. Plant Microbe Interact.* 2: 325-331.

Mayer,R; Raventos,D; Chua,N-H (1996): *det1*, *cop1*, and *cop9* mutations cause inappropriate expression of several gene sets. *The Plant Cell* 8: 1951-1959.

McClure,BA; Haring,V; Ebert,PR; Anderson,MA; Simpson,RJ; Sakiyama,F; Clarke,AE (1989): Style self-incompatibility gene products of *Nicotiana alata* are ribonucleases. *Nature* 342: 955-957.

McDaniel,KL; Lightfoot,DA (1997): Accumulation of a gene specific mRNA in response to cytokinin treatment of leaves in *Phaseolus vulgaris*. *Plant Physiol. Biochem.* 35: 373-380.

McGaw,BA; Burch,LR (1995): Cytokinin biosynthesis and metabolism. In: Plant Hormones: Physiology, Biochemistry and Molecular Biology. 2nd ed. (Ed: Davies,PJ) Kluwer Academic Publishers, Dordrecht, The Netherlands, 98-117.

McGaw,BA; Horgan,R (1983): Cytokinin catabolism and cytokinin oxidase. *Phytochemistry* 22: 1103-1105.

McGaw,BA; Horgan,R; Heald,JK (1985): Cytokinin metabolism and the modification of cytokinin activity in radish. *Phytochemistry* 24: 9-13.

McGaw,BA; Scott,IM; Horgan,R (1984): Cytokinin biosynthesis and metabolism. In: The Biosynthesis and Metabolism of Plant Hormones. (Eds: Crozier,A; Hillman,JR) Cambridge University Press,, Cambridge, 105-133.

McKenzie,MJ (1996): Controlled synthesis of cytokinins in transgenic plants. Ph.D. Dissertation, The University of Otago, Dunedin, New Zealand.

McKenzie,MJ; Jameson,PE; Poulter,RTM (1994): Cloning an *ipt* gene from *Agrobacterium tumefaciens*: characterisation of cytokinins in derivative transgenic plant tissue. *Plant Growth Regul.* 14: 217-228.

McKenzie,MJ; Mett,V; Reynolds,PHS; Jameson,PE (1998): Controlled cytokinin production in transgenic tobacco using a copper-inducible promoter. *Plant Physiol.* 116: 969-977.

McMullan,EE (1980): Effect of applied growth regulators on cone production in Douglas-fir, and relation of endogenous growth regulators in cone production capacity. *Can. J. For. Res.* 10: 405-422.

Medford,JI; Horgan,R; Ei-Sawi,Z; Klee,HJ (1989): Alterations of endogenous cytokinins in transgenic plants using a chimeric isopentenyl transferase gene. *The Plant Cell* 1: 403-413.

Meier-Dinkel,A; Kleinschmit,J (1990): Aging in tree species: Present Knowledge. In: Plant aging: Basic and applied approches. (Eds: Rodriguez,RR; Sanchez,T; Durzan,DJ) Plenum Press, New York, 51-63.

Meilan,R; Horgan,R; Heald,JK; LaMotte,CE; Schultz,RC (1993): Identification of cytokinins in red pine seedlings. *Plant Growth Regul.* 13: 169-178.

Meinke,DW (1992): A homeotic mutant of *Arabidopsis thaliana* with leafy cotyledons. *Science* 258: 1647-1650.

Meinke,DW; Franzman,LH; Nickle,TC; Yeung,EC (1994): Leafy cotyledon mutants of Arabidopsis. *The Plant Cell* 6: 1049-1064.

Memelink,J; Hoge,JHC; Schilperoort,RA (1987): Cytokinin stress changes the developmental regulation of several defence-related genes in tobacco. *EMBO J.* 6: 3579-3583.

Millar,AJ; Kay,SA (1996): Integration of circadian and phototransduction pathways in the network controlling CAB gene transcription in Arabidopsis. *PNAS USA* 93: 15491-15496.

Miller,CO (1963): Kinetin and kinetin-like compounds. In: Modern methods of plant analysis. Vol. VI. (Eds: Linskens,HF; Tracey,MV) Springer-Verlag, Berlin, 194-202.

Miller,CO; Skoog,F; van Saltza,MH; Strong,FM (1955): Kinetin, a cell division factor from deoxyribonucleic acid. *J. Amer. Chem. Soc.* 77: 1329-1334.

Miller,CO; Strong,F; Okomura,FS; van Saltza,MH; Strong,FM (1956): Isolation, structure and synthesis of kinetin, a substance promoting cell division. *J. Amer. Chem. Soc.* 78: 1345-1350.

Minocha,SC; Nissen,P (1982): Uptake of benzyladenine by tube slices of Jerusalem artichoke (Helianthus tuberosus L.) over a wide concentrationrange. *Plant Physiol.* 70: 528-531.

Moffatt,B; Pethe,C; Laloue,M (1991): Metabolism of benzyladenine is impaired in a mutant of *Arabidopsis thaliana* lacking adenine phosphoribosyltransferase activity. *Plant Physiol.* 95: 900-908.

Moiseyev,GP; Beintema,JJ; Fedoreyeva,LI; Yakovlev,GI (1994): High sequence similarity between a ribonuclease from ginseng calluses and fungus-elicited proteins from parsley indicates that intracellular pathogenesis-related proteins are ribonucleases. *Planta* 193: 470-472.

Moiseyev,GP; Fedoreyeva,LI; Zhuravlev,YN; Yasnetskaya,E; Jekel,PA; Beintema,JJ (1997): Primary structures of two ribonucleases from ginseng calluses New members of the PR-10 family of intracellular pathogenesis-related plant protein. *FEBS Lett.* 407: 207-210.

Mok, MC (1994): Cytokinins and plant development - an overview. In: Cytokinins: Chemistry, Activity and Function. (Eds: MoK,DWS; Mok,MC) CRC Press, Florida, 155-166.

Mok,DWS; Mok,MC; Shaw,G; Dixon,S; Martin,RC (1988): Genetic differences in the enzymatic regulation of zeatin metabolism in *Phaseolus* embryos. In: Plant Growth Substances. (Eds: Pharis,RP; Rood,SB) Springer-Verlag, Berlin, 267-274.

Monteuuis,O (1991): Rejuvenation of a 100-year-old *Sequoiadendron giganteum* through in vitro meristem culture. I. Organogenic and morphological arguments. *Physiol. Plant.* 81: 111-115.

Moose,SP; Sisco,PH (1994): Glossy15 controls the epidermal juvenile-to-adult phase transition in maize. *The Plant Cell* 6: 1343-1355.

Moose,SP; Sisco,PH (1996): Glossy15, an *APETALA2*-like gene from maize that regulates leaf epidermal cell identity. *Genes & Development* 10: 3018-3027.

Moran,R (1982): Formulae for determination of chlorophyllous pigments extracted with N,N-dimethylformamide. *Plant Physiol.* 69: 1376-1381.

Moran,R; Porath,D (1980): Chlorophyll determination in intact tissues using N,N-dimethylformamide. *Plant Physiol.* 65: 478-479.

Moritz,T (1996): Mass spectrometry of plant hormones. In: Applications of Modern Mass Spectrometry in Plant Science Research. (Eds: Newton,RP; Walton,TJ),, 139-158.

Moritz,T; Oden,PC (1990): Metabolism of tritiated and deuterated gibberellin A9 in Norway spruce (*Picea abies*) shoots during the period of cone-bud differentiation. *Physiol. Plant.* 79: 242-249.

Moritz,T; Philipson,JJ; Oden,PC (1989): Metabolism of tritiated and deuterated gibberellin A1, A4 and A9 in Sitka spruce (*Picea sitchensis*) shoots during the period of cone-bud differentiation. *Physiol. Plant.* 77: 39-45.

Moritz,T; Sundberg,B (1996): Endogenous cytokinins in the vascular cambial region of *Pinus sylvestris* during activity and dormancy. *Physiol. Plant.* 98: 693-698.

Morris,JW; Doumas,P; Morris,RO; Zaerr,JB (1990): Cytokinins in vegetative and reproductive buds of *Pseudotsuga menziesii*. *Plant Physiol.* 93: 67-71.

Morris,RO (1977): Mass spectroscopic identification of cytokinins. *Plant Physiol.* 59: 1029-1033.

Morris,RO (1995): Genes specifying auxin and cytokinin biosynthesis in prokaryotes. In: Plant Hormones. Physiology, Biochemistry and Molecular Biology. (Ed: Davies,PJ) Kluwer Academic Publishers, Dordrecht, 318-339.

Morris,RO; Jameson,PE; Laloue,M; Morris,JW (1991): Rapid identification of cytokinins by an immunological method. *Plant Physiol.* 95: 1156-1161.

Morris,RO; Zaerr,JB; Chapman,RW (1976): Trace enrichment of cytokinins from Douglas-fir xylem extrudate. *Planta* 131: 271-274.

Mousdale,DMA; Knee,M (1979): Poly-N-vinylpyrrolidone column chromatography of plant hormones with methanol as eluent. *J. Chromatogr.* 177: 398-400.

Murai,N (1994): Cytokinin biosynthesis in tRNA and cytokinin incorporation into plant RNA. In: Cytokinins: Chemistry, Activity and Function. (Eds: MoK,DWS; Mok,MC) CRC Press, Florida, 87-99.

Murashige,T; Skoog,F (1962): A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.* 15: 473-497.

Murray,JR (1988): Flavonoid metabolism in relation to differential anthocyanin accumulation in juvenile and mature phase *Hedera helix* L. Ph.D. Dissertation, University of Minnesota.

Murray,JR; Smith,AG; Hackett,WP (1994): Differential dihydroflavonol reductase transcription and anthocyanin pigmentation in the juvenile and mature phases of ivy (*Hedera helix* L.). *Planta* 194: 102-109.

Nandi,SK; Letam,DS; Palni,LMS; Wong,OC; Summons,RE (1989a): 6-Benzylaminopurine and its glycosides as naturally occurring cytokinins. *Plant Sci.* 61: 189-196.

Nandi,SK; Palni,LMS; Letham,DS; Wong,OC (1989b): Identification of cytokinins in primary crown gall tumours of tomato. *Plant Cell Environment* 12: 273-283.

Navarro,L; Roistacher,CN; Murashige,T (1975): improvement of shoot tip grafting in vitro gor virus-free Citrus. *J. Amer. Soc. Hort. Sci.* 100: 471-479.

Nicander,B; Stahl,U; Bjorkman,PO; Yillberg,E (1993): Immunoaffinity co-purification of cytokinins and analysis by high-performance liquid chromatography with ultraviolet-spectrum detection. *Planta* 189: 312-320.

Nishinari,N; Syono,K (1980): Cell-free biosynthesis of cytokinins in cultured tobacco cells. *Z. Pflanzenphysiol* 99: 383-392.

Nooden,LD; Letham,DS (1993): Cytokinin metabolism and signalling in the soybean plant. *Aust. J. Plant Physiol.* 20: 639-653.

Oh,B-J; Balint,DE; Giovannoni,JJ (1995): A modified procedure for PCR-based differential display and demonstration of use in plants for isolation of genes related to fruit ripening. *Plant Mol. Biol. Rep.* 13: 70-81.

Olson,JW (1954): A comparison of leaf number, internode and mesocotyl lengths in dwarf1 and normal *Zea mays* L. *Proc Minn Acad Sci.* 22: 99-104.

Ou,K; Adamson,H (1995): Chlorophyll accumulation in cotyledons, hypocotyls and primary needles of *Pinus pinea* seedling in light and dark. *Physiol. Plant.* 93: 719-724.

Owens,JN; Phillipson,JJ; Harrison,DLS (1992): The effects of the duration and timing of drought plus heat plus gibberellin A4/7 on apical meristem development and coning in Sitka spruce (*Picea sitchensis* (Bong.). *New Phytol.* 122: 515-518.

Owens,JN; Simpson,SJ (1988): Bud and shoot development in *Picea engelmannii* in response to cone induction treatments. *Can. J. For. Res.* 18: 231-241.

Owens,JN; Webber,JE; Ross,SD; Pharis,RP (1985): Interaction between gibberellin A4/7 and root pruning on the reproductive and vegetative processes n Douglas-fir. III. Effect on anatomy of shoot elongation and terminal bud development. *Can. J. For. Res.* 15: 354-364.

Palni,LM; Sumons,RE; Letham,DS (1983): Mass spectrometric analysis of cytokinins in plant tissues. *Plant Physiol.* 72: 858-863.

Palni,LMS; Horgan,R (1983): Cytokinin biosynthesis in crown-gall tissue of *Vinca rosea*: metabolism of isopentenyladenine. *Phytochemistry* 22: 1597-1601.

Parker,CW; Letham,DS (1973): Regulators of cell division in plant tissues XVI. Metabolism of zeatin by radish cotyledons and hypocotyls. *Planta* 114: 199-218.

Parker,CW; Letham,DS (1974): Regulators of cell division in plant tissues XVII. Metabolism of zeatin in *Zea mays* seedlings. *Planta* 115: 337-344.

Parker,CW; Letham,DS; Gollnow,BI; Summons,RE; Duke,CC; MacLeod,JK (1978): Metabolism of zeatin by lupin seedlings. *Planta* 142: 239-251.

Parker,CW; Letham,DS; Wilson,MM; Jenkins,ID; MacLeod,JK; Summons,RE (1975): The identity of two new cytokinin metabolites. *Ann. Bot.* 39: 375-376.

Pelese,F; Megnegneau,B; Scotta,B; Sossountzov,L; Caboche,M; Miginiac,E (1989): Hormonal characterisation of a non-rooting nophthalene-acetic acid tolerant tobacco mutant by an immunoenzymic method. *Plant Physiol.* 89: 86-92.

Perrin,Y; Doumas,P; Lardet,L; Carron,MP (1997): Endogenous cytokinins as biochemical markers of rubber-tree (*Hevea Brasiliensis*) clone rejuvenation. *Plant Cell, Tissue and Organ Culture* 47: 239-245.

Peters,W; Beck,E (1992): Endogenous cytokinins in suspension cultured cells of *Chenopodium rubrum* at different growth stages. In: Physiology and Biochemistry of Cytokinins in Plants. (Eds: Kamínek,M; Mok,DWS; Zazimalova,E) SPB Academic Publishing, The Hague, The Netherlands, 71-73.

Pharis,RP; Evans,LT; King,RW; Mander,LN (1989): Gibberellins and flowering in higher plants: differing structures yield highly specific effects. In: Plant Reproduction: From Floral Induction to Pillination. Vol. 1. (Eds: Lord,E; Bernier,G) Am. Soc. Plant Physiologists Symp. Series,, 29-41.

Pharis,RP; King,RW (1985): Gibberellins and reproductive development in seed plants. *Ann. Rev. Plant Physiol.* 36: 517-568.

Pharis,RP; Morf,W (1968): Physiology of gibberellin-induced flowering in conifers. In: Biochemistry and Physiology of Plant Growth Substances. (Eds: Wightman,F; Setterfield,G) Runge,, Ottawa, 1341-1356.

Pharis,RP; Owens,JN (1966): Hormonal induction of flowering in conifers. Yale Scientific Mag XLI 2: 10-19.

Pharis,RP; Ross,SD (1986): Hormonal promotion of flowering in Pinaceae family conifers. In: Handbook of Flowering. Vol. 5. (Ed: Halevy,A) CRC Press, Boca Raton, FL, 269-286.

Pich,U; Schubert,I (1993): Midiprep method for isolation of DNA from plants with a high content of polyphenols. *Nucleic Acids Res.* 21: 3328.

Pinto,MP; Ricardo,PPC (1995): *Lupinus albus* L. pathogenesis-related proteins that show similarity to PR-10 proteins. *Plant Physiol.* 109: 1345-1351.

Podolnyi,VZ; Josefusova,Z; Machackova,I; Krekule,J; Chailakhyan,M Kh (1988): Phytohormone signals in the juvenile-mediated control of flowering in *Xanthium*

strumarium. In: Signals in plant development. (Eds: Krekule,J; Seidlova,F) SPB Academic Publishing bv, The Hague, The Netherlands, 129-143.

Poethig,RS (1985): Homeotic mutations in maize. In: Plant Genetics. (Ed: Freeling,M) Alan R Liss, New York, 33-43.

Poethig,RS (1988a): Heterochronic mutations affecting shoot development in maize. *Genetics* 119: 959-973.

Poethig,RS (1988b): A non-cell-autonomous utation regulating juvenility in maize. *Nature* 336: 82-83.

Poethig,RS (1990): Phase change and regulation of shoot morphogenesis in plants. *Science* 250: 923-930.

Poethig,RS (1997): Leaf morphogenesis in flowering plants. *The Plant Cell* 9: 1077-1087.

Prinsen,E; Chriqui,D; Vilaine,F; Tepfer,M; Van Onckelen,H (1994): Endogenous phytohormones in tobacco plants transformed with *Agrobacterium rhizogenes* pRi TL-DNA genes. *FEBS Lett.* 144: 80-85.

Prinsen,E; Kamínek,M; van Onckelen,HA (1997): Cytokinin biosynthesis: a black box? *Plant Growth Regul.* 23: 3-15.

Prinsen,E; Redig,P; Van Dongen,W; Esmans,EL; Van Onckelen,HA (1995): Quantitative analysis of cytokinins by electrospray tandem mass spectrometry. *Rapid Communication in Mass Spectrometry* 9: 948-953.

Raskin,VI; Marder,JB (1997): Chlorophyll organisation in dark-grown and light-grown pine (*Pinus brutia*) and Barley (*Hordeum vulgare*). *Physiol. Plant.* 101: 620-626.

Rebbeck,J; Jensen,KF; Greenwood,MS (1993): Ozone effects of grafted mature and juvenile red spruce: photosynthesis, stomatal conductance and chlorophyll concentration. *Can. J. For. Res.* 23: 450-456.

Redig,P; Schmülling,T; Van Onckelen,H (1996): Analysis of cytokinin metabolism in *ipt* transgenic tobacco by liquid chromatography-tandem mass spectrometry. *Plant Physiol.* 112: 141-148.

Reeve,DR; Crozier,A (1980): Quantitative analysis of plant hormones. In: Hormonal Regulation f Development. I. Molecular Aspects of Plant Hormones. Encycl. Plant Physiol. New Ser. Vol. 9. (Ed: MacMillan,J) Sprnger-Verlag, Berlin, 203-280.

Reid,JB; Dalton,PJ; Murfet,IC (1977): Flowering in *Pisum*: does gibberellic acid directly influence the flowering process. *Aust. J. Plant Physiol.* 4: 479-483.

Reinecke,DM; Brenner,ML; Rubenstein,I (1991): Cytokinin biosynthesis in developing corn kernels. *Plant Physiol.* 112: 141-148.

Reinecke,DM; Brenner,ML; Rubenstein,I (1992): Cytokinin biosynthesis in developing *Zea mays* kernels. *Plant Physiol.* 96 (Suppl.): 77.

Riding,RT (1972): Early ontogeny of seedlings of *Pinus radiata*. *Can J. Bot.* 50: 2381-2387.

Robbins,WJ (1957): Gibberellic acid and the reversal of adult *Hedera* to a juvenile state. *Am. J. Bot.* 44: 743-746.

Robertson, DS (1955): The genetics of vivipary in maize. *Genetics* 40: 745-760.

Rogler,CE; Hackett,WP (1975): Phase change in *Hedera helix*: Induction of the mature to juvenile phase change by gibberellin A3. *Physiol. Plant.* 34: 141-147.

Ross,SD (1988): Effects of temperature, drought, and gibberellin A4/7 and timing of treatment, on flowering in potted *Picea engelmannii* and *Picea glauca* grafts. *Can. J. For. Res.* 18: 163-171.

Ross,SD; Pharis,RP (1976): Promotion of flowering in the Pinaceae by gibberellins. I. Sexually mature, non-flowering grafts of Douglas-fir. *Physiol. Plant.* 36: 182-186.

Ruiz-Garcia,L; Madueno,F; Wilkinson,M; Haughn,G; Salinas,J; Martinez-Zapater (1997): Different roles of flowering-time genes in the activation of floral initiation genes in Arabidopsis. *The Plant Cell* 9: 1921-1934.

Sambrook,J; Fritch,EF; Maniatis,T (1989): Molecular cloning: A laboratory manual. 2nd ed. Vol. I: 1.25-1.28. Cold Spring Harbor Laboratory Press, New York.

Sano,H; Seo,S; Koizumi,N; Niki,T; Iwamura,H; Ohashi,Y (1996): Regulation by cytokinins of endogenous levels of jasmonic and salicylic acids in mechanically wounded tobacco plants. *Plant Cell Physiol.* 37: 762-769.

Sassa,H; Hirano,H; Nishio,T; Koba,T (1997): Style-specific self-compatible mutation caused by deletion of the S-RNase gene in Japanese pear (*Pyrus serotina*). *The Plant Journal* 12: 223-227.

Schaffalitzky de Muckadell,M (1954): Juvenile stages in woody plants. *Physiol. Plant.* 7: 782-796.

Schmülling,T; Beinsberger,S; De Greef,J; Schell,J; Van Onckelen,H; Spena,A (1989): Construction of a heat-inducible chimaeric gene to increase the cytokinin content in transgenic plant tissue. *FEBS Lett.* 249: 401-406.

Schmülling,T; Schafer,S; Romanov,G (1997): Cytokinins as regulators of gene expression. *Physiol. Plant.* 100: 505-519.

Schneiderbauer,A; Sandermann Jr.,H; Ernst,D (1991): Isolation of functional RNA from plant tissues rich in phenolic compounds. *Analytical Biochemistry* 197: 91-95.

Schnorr,KM; Gaillard,C; Biget,E; Nygaard,P; Laloue,M (1996): A second form of asenine phosphoribosyltransferase in *Arabidopsis thaliana* with relative specificity towards cytokinis. *The Plant Journal* 9: 891-898.

Schreiber-BMN (1990): The role of cytokinins in maize kernel development. Ph.D. Dissertation, University of Minnesota.

Schultz,EA; Haughn,GW (1991): Leafy, a homeotic gene that regulates inflorescence development in Arabidopsis. *The Plant Cell* 3: 77-781.

Scorza,R; Janick,J (1980): In vitro flowering of *Passiflora suberosa* L. *J. Am. Soc. Hort. Sci.* 105: 892-897.

Scott,CW (1960): FAO Forestry and Forest Products Studies No.14 (*Pinus radiata*). Food and Agricultural Organisation of the United Nations, Rome.

Scott,IM; Horgan,R (1984): Mass-spectrometric quantification of cytokinin nucleotides and glucosides in tobacco crown-gall tissue. *Planta* 161: 345-354.

Scott,IM; Horgan,R; McGaw,BA (1980): Zeatin-9-glucoside, a major endogenous cytokinin of *Vinca rosea* L. crown gall tissue. *Planta* 149: 472-475.

Shantz,EM; Steward,FC (1955): The identification of compound A from coconut milk as 1, 3-diphenylurea. *J. Amer. Chem. Soc.* 74: 6133.

Shaw,G; Smallwood,BM; Steward,FC (1971): The structure and physiological activity of some N6-substituted adenines and related compounds. *Phytochemistry* 10: 2329.

Shaw,G; Smallwood,BM; Wilson,DV (1966): Purines, pyrimidines and imidazoles. XXIV. Synthesis of zeatin, a naturally occurring adenine derivative with plant cell division promoting activity and its 9-B-D-ribofuranoside. *J. Chem. Soc.* (C): 921.

Shaw,G; Wilson,DV (1964): A synthesis of zeatin. *Proc. Chem. Soc. London*, 231.

Shoham,NG; Arad,T; Robin-Abersfeld,R; Mashiah,P; Gazit,A; Yaniv,A (1996): Differential display assay and analysis. *BioTechniques* 20: 182-184.

Siebertz,B; Logemann,J; Willimitzer,L; Schell,J (1989): *Cis* analysis of the wound-inducible promoter *wun* 1 in transgenic tobacco plants and histochemical localisation of its expression. *The Plant Cell* 1: 961-968.

Silver,DL; Pinaev,A; Chen,R; de Bruijn,FJ (1996): Post-transcriptional regulation of specific genes by phytochrome. *PNAS USA* 81: 1112-1116.

Singh,S; Letham,DS; Jameson,PE; Zhang,R; Parker,CW; Badenoch-Jones,J; Nooden,LD (1988): Cytokinin biochemistry in relation to leaf senescence. IV. Cytokinin metabolism in soybean explants. *Plant Physiol.* 88: 788-794.

Singh,S; Letham,DS; Palni,LMS (1992): Cytokinin biochemistry in relation to leaf senescence. VIII. Translocation, metabolism and biosynthesis of cytokinins in relation to sequential leaf senescence of tobacco. *Physiol. Plant.* 86: 398-406.

Skoog,F; Armstrong,DJ (1970): Cytokinins. *Ann. Rev. Plant Physiol.* 21: 359.

Skoog,F; Miller,C (1957): Chemical regulation of growth and organ formation in plant tissues cultured in vitro. *Symp. Soc. Exp. Biol.* 11: 118-131.

Smart,CM; Scofield,SR; Bevan,MW; Dyer,TA (1991): Delayed leaf senescene in tobacco plants transformed with *tmr*, a gene for cytokinin production in *Agrobacterium. The Plant Cell* 3: 647-656.

Smigocki,AC (1991): Cytokinin content and tissue distribution in plants transformed by a restructured isopetenyltransferase gene. *Plant Mol. Biol.* 16: 106-115.

Smigocki,AC; Owens,LD (1988): Cytokinin gene fused with a strong promoter enhances shoot organogenesis and zeatin levels in transformed plant cells. *PNAS USA* 85: 5131-5135.

Smith,AR; van Staden,J (1978): Changes in endogenous cytokinin levels in kernels of *Zea mays* L. during imbibition and germination. *J. Exp. Bot.* 29: 1067-1075.

Smith,R; Greenwood,MS (1995): Effects of gibberellin A4/7, root pruning and cytokinins on seed and pollen cone production in black spruce (*Picea mariana*). *Tree Physiology* 15: 457-465.

Snowball,AM; Zeman,AM; Tchan,YT; Mullins,MG; Goodwin,PB (1991): Phase change in citrus: Immunologically detectable differences between juvenile and mature plants. *Aust. J. Plant Physiol.* 18: 385-396.

Soejima,H; Sugiyama,T; Ishihara,K (1992): Changes in cytokinin activities and mass spectrometric analysis of cytokinins in root exudates of rice plant (*Oryza sativa* L.): comparison between cultivars Nipponbare and Akenohoshi. *Plant Physiol.* 100: 1724-1729.

Soejima,H; Sugiyama,T; Ishihara,K (1995): Changes in the chlorophyll contents of leaves and in levels of cytokinins in root exudates during ripening of rice cultivars Nipponbare and Akennohoshi. *Plant Cell Physiol.* 36: 1105-1114.

Somssich,IE; Schmelzer,E; Kawalleck,P; Halbrock,K (1988): Gene structure and in situ transcript localization of pathogenesis-related protein 1 in parsley. *Mol. Gen. Genet.* 213: 93-98.

Sprinzl,M; Dank,N; Nock,S; Schon,A (1991): Compilation of tRNA sequences and sequences of tRNA genes. *Nucleic Acids Res.* 19: 2127-2171.

Steele,MJ; Coutts,MP; Yeoman,MM (1989): Developmental changes in Sitka spruce as indices of physiological age. I. Changes in needle morphology. *New Phytol.* 113: 367-375.

Stein,OL (1955): Rates of leaf initiation in two mutants of *Zea mays*, dwarf1 and brachytic2. *Am. J. Bot.* 42: 885-892.

Stein,OL; Fosket,EB (1969): Comparative developmental anatomy of shoots of juvenile and adult *Hedera helix*. *Am. J. Bot.* 56: 546-551.

Strnad, M. (1997): The aromatic cytokinins. *Physiol. Plant.* 101: 674-688.

Strnad,M; Hanus,J; Vanek,T; Kamínek,M; Ballantine,JA; Fussell,B; Hanke,DE (1997): Meta-topolin, a highly active aramatic cytokinin from poplar leaves (*Populus* X *canadensis* Moench,cv.Robasta). *Phytochemistry* 45: 213-218.

Stuchbury,T; Palni,LM; Horgan,R; Wareing,PF (1979): The biosynthesis of cytokinins in crown-gall tissue of *Vinca rosea*. *Planta* 147: 97-102.

Summons,RE; Palni,LMS; Letham,DS (1983): Determination of intact zeatin nucleotide by direct chemical ionisation mass spectrometry. *FEBS Lett.* 151: 122-126.

Sung,ZR; Belachew,A; Shunong,B; Bertrand-Garcia,R (1992): *EFM*, an *Arabidopsis* gene required for vegetative shoot development. *Science* 258: 1645-1647.

Sweet,GB (1979): A physiological study of seed cone production in *Pinus radiata*. *NZ J. For. Sci.* 9: 20-33.

Swoboda,I; Hoffmann-Sommergruber,K; O'Riordain,G; Scheiner,O; Heberle-Bors,E; Vicente,O (1996): Bet v 1 proteins, the major birch pollen allergens and members of a family of conserved pathogenesis-related proteins, show ribonuclease activity in vitro. *Physiol. Plant.* 96: 433-438.

Taller,BJ (1994): Distribution, Biosynthesis, and Function of Cytokinins in tRNA. In: Cytokinins: Chemistry, Activity, and Function. (Eds: Mok,DWS; Mok,MC) CRC Press, Boca Raton, FL, 101-112.

Taller,BJ; Sturtevant,DB (1991): Cytokinin production by *Rhizobia*. In: Advances in molecular genetics of plant-microbe interactions. Vol. 1. Kluwer Academic Publisher, 215-222.

Tandre,K; Albert,VA; Sundas,A; Engstrom,P (1995): Conifer homologues to genes that control floral development in angiosperms. *Plant Mol. Biol. Rep.* 27: 69-78.

Taylor,JS; Koshioka,M; Pharis,RP; Sweet,GB (1984): Changes in cytokinins and gibberellin-like substances in *Pinus radiata* buds during lateral shoot initiation and the characterisation of ribosyl zeatin and a novel ribosyl zeatin glycoside. *Plant Physiol.* 74: 626-631.

Telfer,A; Bollman,KM; Poethig,RS (1997): Phase change and the regulation of trichome distribution in *Arabidopsis thaliana*. *Development* 124: 645-654.

Teramoto,H; Momotani,E; Takeba,G; Tsuji,H (1994): Isolation of a cDNA clone for a cytokinin-repressed gene in excised cucumber cotyledons. *Planta* 193: 573-579.

Teramoto,H; Toyama,T; Takeba,G; Tsuji,H (1995): Changes in expression of two cytokinin-repressed genes, *CR9* and *CR20*, in relation to aging, greening and wounding in cucumber. *Planta* 196: 387-395.

Teyssendier de la Serve,B; Axelos,M; Peaud-Lenoel,C (1985): Cytokinins modulate the expression of genes encoding he protein of the light-harvesting chlorophyll a/b complex. *Plant Mol. Biol.* 5: 155-163.

Thomas,JC; Smigocki,AC; Bohnert,HJ (1995): Light-induced expression of *ipt* from *Agrobacterium tumefaciens* results in cytokinin accumulation and osmotic stress symptoms in transgenic tobacco. *Plant Mol. Biol.* 27: 225-235.

Tokota,T; Murofushi,N; Takahashi,N (1980): Extraction, purification and identification of cytokinins. In: Encyclopedia of PlantPhysiology New Series: Horonal Regulation of Development. I. Molecular Aspects of Plant Hormones. Vol. 9. (Ed: MacMillan,J) Springer-Verlag, Berlin, 113-210.

Tompsett,PB (1977): Studies of growth and flowering in *Picea sitchensis* (Bong.) Carr. I. Effect of growth regulator applications to mature scions on seedling rootstocks. *Ann. Bot.* 41: 1171-1178.

Trewavas,A (1970): The turnover of nucleic acids in *Lemna minor*. *Plant Physiol.* 45: 742-751.

Trione,EJ; Sayavedra-Soto,LA (1988): Wheat development enhanced by hormone syndrome. *Botanical Gazette* 149: 317-324.

Trione,EJ; Krygier,BB; Banowetz,GM; Kathrein, JM (1985): The development of monoclonal antibodies against the cytokinin zeatin riboside. *J. Plant Growth Regul.* 4: 101-109.

Turner,JE; Mok,DWS; Mok,MC; Shaw,G (1987): Isolation and partial purification of an enzyme catalysing the formation of O-xylosylzeatin in *Phaseolus vulgaris* embryos. *PNAS USA* 84: 3714-3717.

Vahala,T; Eriksson,T; Tillberg,E; Nicander,B (1993): Expression of a cytokinin synthesis gene from *Agrobacterium tumefaciens* T-DNA in basket willow (Salix viminalis). *Physiol. Plant.* 88: 439-445.

Van der Krieken,WM; Croes,AF; Barendse,GWM; Wullems,GJ (1988): Uptake and metabolism of benzyladenine in the early stage of flower bud development in vitro in tobacco. *Physiol. Plant.* 74: 113-118.

Van der Krieken,WM; Croes,AF; Smulders,MJM; Wullems,GJ (1990): Cytokinins and flower bud formation in vitro in tobacco: role of metabolites. *Plant Physiol.* 92: 565-569.

Vankova,R; Hsiao,KC; Bornman,CH; Gaudinova,A (1991): Effects of synthetic cytokinins on levels of endogenous cytokinins and respiration patterns of Beta vulgaris cells in suspension. *J. Plant Growth Regul.* 10: 197-199.

Van Lijsebettens,M; Clarke,J (1998): Leaf development in Arabidopsis. *Plant Physiol. Biochem.* 36: 47-60.

van Loon,LC; Pierpoint,WS; Boller,Th; Conejero,V (1994): Recommendations for naming plant pathogenesis-related proteins. *Plant Mol. Biol. Rep.* 12: 245-264.

Van Schwartzenberg,K; Bonnet-Masimbert,M; Doumas,P (1994): Isolation of two cytokinin metabolites from the rhizosphere of Norway spruce seedlings (*Picea abies* L. Karst). *Plant Growth Regul.* 15: 117-124.

van Staden,J (1978): A comparison of the endogenous cytokinins in the leaves of four gymnosperms. *Botanical Gazette* 139: 32-35.

van Staden,J; Bayley,AD; Upfold,SJ; Drewes,FE (1990): Cytokinins in cut carnation flowers. VIII. Uptake, transport and metabolism of benzyladenine and the effect of benzyladenine derivatives on flower longevity. *FEBS Lett.* 135: 703-707.

van Staden,J; Crouch,NR (1996): Benzyladenine and derivatives - their significance and interconversion in plants. *Plant Growth Regul.* 19: 153-175.

van Staden,J; Dimalla,GG (1978): Endogenous cytokinins and the breaking of dormancy and apical dominance in potato tubers. *J. Exp. Bot.* 29: 1077-1084.

van Staden,J; Drewes,FE (1992): The stability and metabolism of benzyladenine glucosides in soybean callus. *FEBS Lett.* 140: 92-95.

Venglat,SP; Sawhney,VK (1996): Benzylaminopurine induces phenocopies of floral meristem and organ identity mutants in wild-type *Arabidopsis* plants. *Planta* 198: 480-487.

Villalobos,VM; Leung,DWM; Thorpe,TA (1984): Light-cytokinin interaction in shoot formation in cultured cotyledon explants of radiata pine. *Physiol. Plant.* 61: 497-504.

Vogelmann,TC; Bomman,CH; Nissen,P (1984): Uptake of benzyladenine in explants of *Picea abies* and *Pinus sylvestris*. *Physiol. Plant.* 61: 513-517.

Wakana,A; Uemoto,S (1987): Adventive embryogenesis in Citrus. I. The occurrence of adventive embryos withput pollination or fertilization. *Am. J. Bot.* 74: 517-530.

Wakana,A; Uemoto,S (1988): Adventive embryogenesis in Citrus (Rutaceae). II. Postfertilization development. *Am. J. Bot.* 75: 1033-1047.

Walter,C; Smith,DR (1995): Transformed *Pinus radiata* now growing in greenhouse at the New Zealand Forest Research Institute (NZ FRI). *Dendrom: Forest Tree Genome Research Updates.* 2: No.2.

Walter,MH; Liu,JW; Wunn,J; Hess,D (1996): Bean ribonucease-like pathogenesis-related protein genes (YPR10) display complex patterns of developmental, dark-induced and exogenous-stimulus-dependent expression. *European Journal of Biochemistry* 239: 281-293.

Walter,MH; Liu,J-W; Grand,C; Lamb,CG; Hess,D (1990): Bean pathogenesis-related (PR) proteins deduced from elicitor-induced transcripts are members of a ubiquitous new class of conserved PR proteins including pollen allergens. *Mol. Gen. Genet.* 222: 353-360.

Wang,J; Letham,DS; Taverner,E; Badenoch-Jones,J; Hocart,CH (1995): A procedure for quantification of cytokinins as free bases involving scintillation proximity immunology. *Physiol. Plant.* 95: 91-98.

Wang,TL; Thompson,AG; Horgan,R (1977): A cytokinin glucoside from the leaves of *Phaseolus vulgaris* L. *Planta* 135: 285-288.

Wang,YD (1995): Male cone development in *Pinus radiata*. Ph.D. Dissertation, Massey University.

Wareing,PE; Frydman,VM (1976): General aspects of phase change, with special reference to *Hedera helix* L. *Acta Hort.* 56: 57-69.

Wareing,PF; Phillips,IDJ (1978): The control of growth and differentiation in plants. 2nd ed. Pergamon Press, Oxford.

Wareing,PE (1959): Problems of juvenility and flowering in trees. *J. Linn. Soc.* (London) 56: 282-289.

Warner,SAJ; Gill,A; Draper,J (1994): The developmental expression of the asparagus intracellular PR protein (AoPR1) gene correlates with sites of phenylpropanoid biosynthesis. *The Plant Journal* 6: 31-43.

Warner,SAJ; Scott,R; Draper,J (1992): Characterisation of a wound-induced transcript from the monocot asparagus that shares similarity with a class of intracellular pathogenesis-related

(PR) proteins. *Plant Mol. Biol.* 19: 555-561.

Warner,SAJ; Scott,R; Draper,J (1993): Isolation of an asparagus intracellular PR gene (AoPR1) wound-responsive promoter by the inverse polymerase chain reaction and its characterization in transgenic tobacco. *The Plant Journal* 3: 191-201.

Weigel,D; Alvarez,J; Smyth,DR; Yanofsky,MF; Meyerowitz,EM (1992): LEAFY controls floral meristem identity in Arabidopsis. *Cell* 69: 843-859.

Weigel,D; Meyerowitz,EM (1993): Activation of floral homeotic genes in *Arabidopsis. Science* 261: 1723-1726.

Weigel,D; Meyerowitz,EM (1994): The ABCs of floral homeoyic genes. *Cell* 78: 203-209.

Weigel,D; Nilsson,O (1995): A developmental switch sufficient for flower initiation in diverse plants. *Nature* 377: 495-500.

Weiler,EW (1979): Radioimmunoassay for the determination of free and conjugated abcisic acid. *Planta* 144: 255-263.

Weiler,EW (1980): Radioimmunoassay for trans-Zeatin and related cytokinins. *Planta* 149: 155-162.

Weiler,EW; Spanier,K (1981): Phytohormones in the formation of crown gall tumors. *Planta* 153, 326-337.

Werbrouck,SPO; Van der Jeugt,B; Dewitte,W; Prinsen,E; Van Onckelen,HA; Debergh,PC (1995): The metabolism of benzyladenine in *Spathiphyllum floribundum* 'Schott Petite' in relation to acclimatisation problems. *Plant Cell Reports* 14: 662-665.

West,MAL; Yee,KM; Danao,J; Zimmerman,JL; Fischer,RL; Goldberg,RB; Harada,JJ (1994): LEAFY COTYLEDON1 is an essential regulator of late embryogenesis and cotyledon identity in Arabidopsis. *The Plant Cell* 6: 1731-1745.

Weston,GC (1957): "Exotic Forest Trees in New Zealand". Govement Printer, Wellinton, New Zealand, .

Whitty,CD; Hall,RH (1974): A cytokinin oxidase in *Zea mays. Can. J. Biochem.* 52: 781-799.

Wilson,MM; Gordon,ME; Letham,DW; Parker,CW (1974): Regulators of cell division in plant tissues (XIX). The metabolism of 6-benzylaminopuine in radish cotyledons and seedlings. *J. Exp. Bot.* 25: 725-732.

Wilson,RN; Heckman,JW; Somerville,CR (1992): Gibberellin is required for flowering in *Arabidopsis thaliana* under short days. *Plant Physiol.* 100: 403-408.

Woo,H-H; Hackett,WP; Das,A (1994): Differential expression of a chlorophyll a/b binding protein gene and a proline rich protein gene in juvenile and mature phase English ivy (*Hedera helix*). *Physiol. Plant.* 92: 69-78.

Yahalom,E; Okon,Y; Dovrat,A (1990): Possible mode of action of *Azospirillum brasilense* strain Cd on the root morphology and nodule formation in blurr medic (*Medicago polymorpha*). *Can. J. Microbiol.* 36: 10-14.

Yang,CH; Chen,LJ; Sung,ZR (1995): Genetic regulation of shoot development in Arabidopsis: Role of the *EMF* genes. *Developmental Biology* 169: 421-435.

Yang,YY; Yamaguchi,I; Kato,Y; Weiler,EW; Murofushi,N; Takahashi,N (1993): Qualitative and semi-quantitative analysis of cytokinins using LC/APCI-MS in combination with ELISA. *J. Plant Growth Regul.* 12: 21-25.

Yeo,D; Abe,T; Abe,H; Sakurai,A; Takio,K; Dohmae,N; Takahashi,N; Yoshida,S (1996): Partial characterization of a 17 kDa acidic protein, EFP, induced by thiocarbamate in the early flowering phase in Asparagus seedlings. *Plant Cell Physiol.* 37: 935-940.

Zachau,HG; Dutting,DJ; Feildmann,H (1966): Nucleotide sequence of two serine-specific transfer ribonucleic acids. *Angew. Chem.* 78: 392.

Zaerr,JB; Bonnet-Masimbert,M (1987): Cytokinin level and flowering in Douglas-fir. *For. Ecol. Manage.* 19: 115-120.

Zagotta,MT; Hicks,KA; Jacobs,CI; Young,JC; Hangarter,RP; Meeks-Wagner,DR (1996): The *Arabidopsis ELF3* gene regulates vegetative photomorphogenesis and the photoperiodic induction of flowering. *The Plant Journal* 10: 691-702.

Zagotta,MT; Shannon,S; Jacobs,C; Meeks-Wagner,DR (1992): Early-flowering mutants of *Arabidopsis thaliana*. *Aust. J. Genet.* 19: 411-418.

Zeevaart,JAD (1983): Gibberellins and flowering. In: The biochemistry and Physiology of Gibberellins. (Ed: Crozier,A) Praeger, New York, 333-374.

Zhang,R; Letham,DS; Wong,OC; Nooden,LD; Parker,CW (1987): The metabolism of 6-benzylaminopurine in soybean leaves and the inhibition of its conjugation. *Plant Physiol.* 83: 334-340.

Zimmerman,RH; Hackett,WP; Pharis,RP (1985): Hormonal aspects of phase change and precocious flowering. In: Encyclopedia of Plant Physiology. Vol. 11/IV. (Ed: Pirson,A) Springer-Verlag, Berlin and New York, 79-115.

Zurek,DM; Mou,B; Beecher,B; McClure,B (1997): Exchanging sequence domains between S-RNases from *Nicotiana alata* disrupts pollen recognition. *The Plant Journal* 11: 797-808.

Appendix A - Cross reactivity of antibodies with cytokinins

Cross reactivity of the clone 12 and the clone 16 used in this thesis, with various forms of cytokinins are presented in the table below. The values were determined by Dr D. Lewis.

Cytokinin	% Cross Reactivity clone 16	clone 12
ZR	100	2.4
Z	34	1.7
DZR	30	-*
DZ	14	-
Z9G	39	-
DZ9G	8	-
Z7G	0	-
ZOG	0	-
ZROG	0	-
DZOG	0	-
DZROG	0	-
iPA	0.9	100
iP	0	93
iP9G	-	131

*- = not tested.

Appendix B - Tissue culture medium

LP medium: Modified LP basic media contained 1800 mg KNO_3, 360 mg $MgSO_4 \cdot 7H_2O$, 400 mg NH_4NO_3, 270 mg KH_2PO_4, 1200 mg $Ca(NO_3)_2.4H_2O$, 27.8 mg $FeSO_4.7H_2O$, 37.3 mg $Na_2EDTA.2H_2O$, 1 mg $MnSO_4.4H_2O$, 6.2 mg H_3BO_3, 8.6 mg $ZnSO_4.7H_2O$, 0.08 mg KI, 0.025 mg $CuSO_4.5H_2O$, 0.25 mg $Na_2MoO_4.2H_2O$, 0.025 mg $CoCl_2.6H_2O$, 0.4 mg Thiamine.HCl, 1000 mg inositol, 30 g sucrose and the pH was adjusted to 5.7. Eight grams of Difco Bacto agar was added in one litre medium to solidify the media.

LP5 medium: basic LP medium plus 5 mg/L BA.

LPCH medium: basic LP medium plus 5 g/L charcoal.

Addendum

1. For all the figures in this thesis, the bars represent standard deviations of the means.
2. 'Liang et al. (1992)' in the text should be replaced by 'Liang and Pardee (1992)'.
3. 'Hackett et al., 1995' on page xv should be replaced by 'Hackett and Murray, 1996'.
4. 'Similarity' should replace the term 'homology' in the text.
5. Last line on page 6 "..., which is involved in upstream DFR in anthocyanin biosynthesis." should read: '..., which is involved in anthocyanin biosynthesis upstream of DFR.'.
6. For Table 3.1, DE represents standard deviation of the mean.