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**Cytokinins and phase change in *Pinus radiata*:**  
**Morphological, physiological and molecular studies**

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## 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 (*Pr-cr5*) was cloned using a modified mRNA differential display technique. Northern analyses showed that cytokinin promoted and maintained the expression of *Pr-cr5* 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 *Pr-cr5* gene in the regulation of phase change is discussed.

A cDNA sequence (*Pr-cab*) 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*.

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## Table of Contents

<b>Abstract</b>	<b>ii</b>
<b>Acknowledgements</b>	<b>iv</b>
<b>Table of contents</b>	<b>vi</b>
<b>List of figures</b>	<b>xi</b>
<b>List of tables</b>	<b>xv</b>
<b>List of abbreviations</b>	<b>xvi</b>
<b>Chapter 1 Introduction</b>	<b>1</b>
1.1 Phase change	2
1.1.1 Morphological, physiological, biochemical and molecular features	2
1.1.2. Molecular genetic studies of phase change	7
1.1.3. Phase change and plant hormones	11
1.1.4 Rejuvenation	17
1.1.5 Models of phase change	19
1.2. Cytokinins	23
1.2.1 Cytokinin structures	24
1.2.2. Cytokinin biosynthesis	24
1.2.3 Cytokinin metabolism	29
1.2.3.1. Interconversions of cytokinin free bases, ribosides and nucleotides:	29
1.2.3.2 Reduction of the double bond on the side chain:	30
1.2.3.3 Conjugation:	31
1.2.3.4 Hydrolysis:	32
1.2.3.5 Oxidation:	32
1.2.4 Functions, activity and modes of action of cytokinins	33
<b>Chapter 2 Extraction, purification, isolation and identification of endogenous cytokinins in the buds of <i>Pinus radiata</i></b>	<b>37</b>
2.1 Introduction	37
2.2 Materials and Methods	41
2.2.1 Plant materials	43
2.2.2 Extraction of cytokinins	43
2.2.2.1 Equipment and chemicals:	43
2.2.2.2 Extraction of endogenous cytokinins:	44
2.2.3 Purification and separation of cytokinins	44
2.2.3.1 Polyvinylpyrrolidone column chromatography:	44
2.2.3.2 PVPP-DE52-C <sub>18</sub> column complex chromatography:	45
2.2.3.2a Small PVPP column packing and preconditioning:	45
2.2.3.2b DE52 column packing and preconditioning:	45
2.2.3.2c C <sub>18</sub> column packing and pre-conditioning:	46
2.2.3.2d Linkage of PVPP, DE52 and C <sub>18</sub> columns:	46
2.2.3.2e Sample application, elution and cytokinin recovery:	46

2.2.3.3 High performance liquid chromatography:	46
2.2.3.3a Normal phase HPLC:	47
2.2.3.3b Reverse phase HPLC:	47
2.2.3.4 Purification and separation of cytokinin glucosides by immunoaffinity spin-columns:	48
2.2.3.4a Preparation of anti-cytokinin antibodies:	48
2.2.3.4b Purification and separation of cytokinin glucosides:	49
2.2.4 Enzyme treatments	51
2.2.4.1 Alkaline phosphatase treatment:	51
2.2.4.2 $\beta$ -glucosidase treatment:	51
2.2.5 Detection and identification of cytokinins	51
2.2.5.1 Radioimmunoassay (RIA):	51
2.2.5.2 Electrospray mass spectrometry:	52
2.3 Results	54
2.3.1 PVPP column analysis	54
2.3.2 Separation of nucleotides from the other forms of cytokinin using DE52	54
2.3.3 Recoveries of cytokinins after passage through the PVPP-DE52-C <sub>18</sub> column series	56
2.3.4 HPLC separation of cytokinins	56
2.3.4.1 Bulk separation of cytokinins:	57
2.3.4.2 Separation of individual cytokinins:	58
2.3.5 Radioimmunoassay	58
2.3.6 Mass spectra of standard cytokinins	61
2.3.7 Endogenous cytokinins in the buds of <i>P. radiata</i>	66
2.3.7.1 Cytokinins detected by RIAs:	66
2.3.7.2 Evidence of novel cytokinin glucosides by HPLC analyses and enzyme treatments:	69
2.3.7.3 Confirmation of novel cytokinin glucosides by mass spectrometry:	69
2.3.7.4 No evidence of traditional O-glucosides:	75
2.4 Discussion	75
2.4.1 Methodology of cytokinin analysis	75
2.4.2 Endogenous cytokinins in the buds of <i>P. radiata</i>	83
<b>Chapter 3 Cytokinins and phase change in <i>Pinus radiata</i></b>	<b>87</b>
3.1 Introduction	87
3.2 Materials and methods	87
3.2.1 Cytokinin analyses of field-grown buds	87
3.2.2 Tissue culture material for cytokinin analyses	88
3.2.3. Bud material for morphological studies	89
3.2.4. Light microscopy	89
3.2.5. Cytokinin analyses	90
3.3 Results	91
3.3.1 Changes in bud morphology during phase change in field-grown trees	91
3.3.2. Changes in bud morphology during cytokinin-induced "rejuvenation" <i>in vitro</i>	93



3.3.3 Quantitative analyses of cytokinins in field-grown mature and juvenile buds	98
3.3.3.1. Cytokinin free bases and ribosides:	98
3.3.3.2. Cytokinin nucleotides:	100
3.3.3.3. Glucosylated cytokinins:	100
3.3.4 Metabolism of BA during cytokinin-induced "rejuvenation" <i>in vitro</i>	102
3.3.4.1. Identification of BA metabolites:	102
3.3.4.2 Alterations in the concentrations of BA metabolites during "rejuvenation"	112
3.3.5 Effect of BA treatment on endogenous cytokinin content	115
3.3.6 Changes in endogenous cytokinins in the buds on cytokinin-free medium	116
3.4. Discussion	118
3.4.1. Heteroblasty and phase change in <i>P. radiata</i>	118
3.4.2. Endogenous cytokinin metabolism and phase change	122
3.4.3 Cytokinin-induced "rejuvenation" <i>in vitro</i> in <i>P. radiata</i>	126
3.4.4. Metabolism of BA during cytokinin-induced "rejuvenation" <i>in vitro</i>	127
<b>Chapter 4 Identification of cytokinin-responsive genes using mRNA differential display and characterisation of gene expression</b>	<b>132</b>
4.1 Introduction	132
4.2 Materials and Methods	132
4.2.1 Plant materials	132
4.2.1.1 Tissue culture:	133
4.2.1.2 Tissue cultured materials for RNA and DNA isolation:	133
4.2.2 RNA extraction	134
4.2.3 Genomic DNA extraction	136
4.2.4 Determination of DNA and RNA concentration and quality by spectrophotometry	136
4.2.5 Differential display of mRNA	137
4.2.5.1 The first strand cDNA synthesis:	137
4.2.5.2 Differential display PCR:	138
4.2.5.3 cDNA display electrophoresis:	138
4.2.6 Cloning of selected cDNA sequences	139
4.2.6.1 cDNA preparation:	139
4.2.6.2 Ligation:	139
4.2.6.3 Plasmid transformation of <i>E. coli</i> :	140
4.2.7. Plasmid extraction and purification	140
4.2.8. Confirmation of cDNA inserts	141
4.2.9. Grouping of the cloned cDNA sequences	141
4.2.10. Sequencing of the cloned cDNA	142
4.2.10.1 Plasmid DNA preparation:	142
4.2.10.2 Automated sequencing:	143
4.2.10.3 Manual sequencing:	143
4.2.11 Northern analyses	144
4.2.11.1 Fractionation of total RNA by electrophoresis:	144
4.2.11.2 Gel blotting:	144
4.2.11.3 Preparation of [ $\alpha$ - $^{32}$ P]dCTP-labelled probe:	145
4.2.11.4 Prehybridation and hybridisation:	145

4.2.11.5 Stripping hybridised $^{32}\text{P}$ -labelled probes off Northern blot membranes	146
4.2.12 Southern analyses	147
4.2.12.1 Genomic DNA digestion by restriction enzymes and electrophoresis:	147
4.2.12.2 Stripping hybridised $^{32}\text{P}$ -labelled probes off Southern blot membranes	147
4.2.13 Sequence data analysis:	147
4.3 Results	148
4.3.1 Quality and yields of total RNA preparation	148
4.3.2 mRNA differential display	150
4.3.3 Cloning of <i>Prcr5</i> and <i>Prcab</i> bands	154
4.3.3.1. cDNA extraction from display agarose gel:	154
4.3.3.2. Cloning of the cDNA:	154
4.3.4 cDNA sequence grouping of single size band	154
4.3.5 Characterisation of <i>Prcr5</i> cDNA and <i>Prcab</i> cDNA	158
4.3.5.1. Nucleotide sequence of <i>Prcr5</i> cDNA:	158
4.3.5.2: Amino acid sequence of the deduced polypeptide from the cDNA:	158
4.3.5.3 Relationship of <i>Prcr5</i> to other gene sequences:	162
4.3.5.4. Comparison of 3' untranslated region of <i>Prcr5</i> with other sequences:	163
4.3.6 Characterisation of <i>Prcab</i> gene cDNA	163
4.3.6.1 Characterisation of the sequence:	163
4.3.6.2 Relationship to other genes:	167
4.3.7 Genomic analysis of the cloned <i>Prcr5</i> and <i>Prcab</i> genes	167
4.3.7.1. DNA preparations:	167
4.3.7.2. Southern analyses:	167
4.3.8 Northern analyses of gene transcription	172
4.3.8.1 Exogenous cytokinin treatment promotes and maintains the transcription of the <i>Prcr5</i> gene:	172
4.3.8.2. <i>Prcr5</i> gene transcription responds to cytokinin in a dose-dependent manner:	175
4.3.8.3. High level gene transcription of <i>Prcr5</i> lasts longer in originally more juvenile buds after removal of cytokinin treatment:	175
4.3.8.4. <i>Prcr5</i> mRNA does not accumulate in fully developed leaves:	175
4.3.8.5. <i>Prcab</i> gene expression occurs without the requirements of light and exogenous cytokinin treatments:	175
4.3.8.6. No significant difference in the expression of the <i>Prcab</i> gene exists between the primary and secondary needles of different maturation states:	179
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:	179
4.4 Discussion	183
4.4.1. Nucleic acid isolation	183
4.4.1.1 RNA extraction:	183
4.4.1.2 DNA extraction:	185
4.4.1.3 Simultaneous isolation of total RNA and genomic DNA:	186
4.4.2 Strategy for isolating differentially expressed gene cDNAs	186
4.4.3 <i>Prcr5</i> gene	188
4.4.3.1 <i>Prcr5</i> gene may code a protein relating to RNases:	188
4.4.3.2 The expression of <i>Prcr5</i> gene is regulated by cytokinins:	189

4.4.3.3. Possible function of <i>Prcr5</i> gene in the resetting of needle development in the buds of <i>P. radiata</i> during culture:	192
4.4.4 <i>Prcab</i> gene	194
4.4.4.1 The cloned sequences are chlorophyll a/b binding protein cDNA:	194
4.4.4.2 <i>Prcab</i> gene can be expressed in the cultured buds of <i>P. radiata</i> in the complete dark without requirement of exogenous cytokinin:	194
4.4.4.3 <i>Prcab</i> gene is not differentially expressed between mature and juvenile tissues:	196
4.4.5 Summary	198
<b>Chapter 5 Final discussion and conclusions</b>	<b>200</b>
<b>References</b>	<b>206</b>
<b>Appendices</b>	
Appendix A - Cross reactivity of antibodies with cytokinins	235
Appendix B - Tissue culture medium	235

## List of Figures

Figure 1.1	Kester's and Poethig's models of phase change.	21
Figure 1.2	Alternate models of phase change	22
Figure 1.3	Cytokinin structures - modifications on the purine ring	25
Figure 1.4	Cytokinin structures - modification on the side chain.	26
Figure 2.1	Procedures used for extraction, purification, separation and quantification of different individual cytokinins in <i>Pinus radiata</i> buds.	42
Figure 2.2	The procedures for purification and separation of novel cytokinin glucosides from traditional <i>O</i> -glucosides.	49
Figure 2.3	Elution profiles of cytokinins through PVPP column.	55
Figure 2.4	Separation profiles of cytokinins through DE52 columns.	57
Figure 2.5	Normal phase HPLC separation of cytokinin standards.	59
Figure 2.6	Reverse phase HPLC separation of cytokinin standards.	60
Figure 2.7	Examples of radioimmunoassay standard curves for antibodies employed in RIAs in this thesis.	62
Figure 2.8	MS/MS spectrum of isopentenyladenosine.	64
Figure 2.9	MS/MS spectrum of zeatin riboside- <i>O</i> -glucoside.	65
Figure 2.10	MS/MS spectrum of dihydrozeatin riboside- <i>O</i> -glucoside.	67
Figure 2.11	Cytokinins detected in 8-year-old mature buds of <i>Pinus radiata</i> using RIAs.	68
Figure 2.12	HPLC spectra of the immunoaffinity-purified novel cytokinin glucosides before (solid line) and after (broken line) $\beta$ -glucosidase treatment.	70
Figure 2.13	RIA profiles of individual 0.5 min fractions of the HPLC eluate of the immunoaffinity-purified novel cytokinin glucosides before (dark bars) and after (light bars) $\beta$ -glucosidase treatment.	71
Figure 2.14	MS/MS spectrum of novel cytokinin ZRx.	73
Figure 2.15	MS/MS spectrum of novel cytokinin DZRx.	74
Figure 2.16	MS/MS spectrum of novel cytokinin iPAx.	76
Figure 2.17	Proposed structures of identified novel cytokinins derived from buds of <i>Pinus radiata</i> .	77
Figure 2.18	Mass spectra of the fraction that would contain traditional <i>O</i> -glucosides if any were present.	78

Figure 3.1	Morphology of buds from juvenile and mature phases in <i>P. radiata</i> .	92
Figure 3.2	Changes in morphology of eight-year-old mature buds under different cytokinin treatment regimes.	94
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.	96, 97
Figure 3.4	Concentration of endogenous cytokinin free base and ribosides in the buds from <i>P. radiata</i> trees.	99
Figure 3.5	The concentration of endogenous phosphorylated cytokinins in the buds from <i>P. radiata</i> trees.	101
Figure 3.6	Logit transformation curves of the cross reactivity of BA and its derivatives to clone 12 and clone 16.	105
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.	106
Figure 3.8	Electrospray MS spectrum of 6-benzylaminopurine (A), 6-benzylaminopurine riboside (B) and electrospray MS/MS spectrum of 6-benzylaminopurine glucoside (C).	107
Figure 3.9	The quantitative HPLC-RIAs profiles of BA glucosides extracted from bud fragments cultured on BA-containing medium.	109
Figure 3.10	Electrospray MS/MS spectrum of 6-benzylaminopurine-9-ribosyl-glucoside (BAR-G).	110
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 <i>Pinus radiata</i> .	111
Figure 3.12	The qualitative HPLC-RIAs profiles of phosphorylated BA metabolites extracted from the bud fragments cultured under different BA regimes.	113
Figure 3.13	Comparisons of individual metabolites of BA extracted from the bud fragments under different exogenous BA regimes in culture.	114
Figure 3.14	Endogenous cytokinins extracted from eight-year-old mature buds (M8) and the M8 bud fragments cultured under different BA regimes.	117

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.	119
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 or on cytokinin-free medium for 50 days.	120
Figure 4.1	UV-light absorbance spectrum of purified total RNA from the buds of <i>P. radiata</i> .	149
Figure 4.2	Electrophoresis of total RNA on a 1.2% denaturing agarose gel.	151
Figure 4.3	The differential display profiles of cDNAs amplified using (T <sub>12</sub> VG) as anchored primer, 5' CGGCARGTNACNTT3' as random primer and the first strand cDNA as template.	152
Figure 4.4	The differential display patterns of cDNAs multiplied using (T <sub>12</sub> VC) as anchored primer, 5' CGGCARGTNACNTT3' as random primer and the first strand cDNA as template.	153
Figure 4.5	Estimation of sample plasmid DNA mass by comparing fluorescence of sample and mass ladder standards.	155
Figure 4.6	Confirmation of the presence of <i>Prcr</i> cDNA inserts in TA-cloning pCRII vectors derived from transformed <i>E. coli</i> colonies.	156
Figure 4.7	Confirmation of the presence of <i>Prcab</i> cDNA inserts in TA-cloning pCRII vectors derived from transformed <i>E. coli</i> colonies.	157
Figure 4.8	cDNA sequence grouping by restriction enzyme digestion fingerprinting.	159
Figure 4.9	Sequence of cloned <i>Prcr5</i> cDNA and deduced amino acid sequence of the protein PrCR5.	160
Figure 4.10	Hydropathy plot of the deduced amino acid sequence of PrCR5.	161
Figure 4.11	Common patterns of conserved amino acids between the deduced PrCR5 protein sequence and other PR-10 protein sequences.	164
Figure 4.12	Dendogram displaying the relatedness of the deduced PrCR5 protein sequence to other related proteins.	165

Figure 4.13	Nucleotide sequence of cloned <i>Prcab</i> cDNA and the deduced amino acid sequence of the protein PrCAB	166
Figure 4.14	Comparison of conserved amino acids between deduced PrCAB and other chlorophyll <i>a/b</i> binding proteins of conifers.	168
Figure 4.15	Restriction enzyme digestion of genomic DNA of <i>P. radiata</i> .	169
Figure 4.16	Southern analysis of <i>Prchr5</i> gene arrangement in genomic DNA in <i>P. radiata</i> .	170
Figure 4.17	Southern analysis of <i>Prcab</i> gene arrangement in genomic DNA in <i>P. radiata</i> .	171
Figure 4.18	Southern analysis of 28 S rRNA gene arrangement in genomic DNA in <i>P. radiata</i> .	173
Figure 4.19	Northern analysis of <i>Prchr5</i> gene expression in the mature <i>P. radiata</i> buds cultured over a period of 7 days in the presence or absence of exogenous cytokinin.	174
Figure 4.20	Northern analysis of the effect of exogenous cytokinin on <i>Prchr5</i> gene expression.	176
Figure 4.21	Northern analysis of the effect of exogenous cytokinin dose on the <i>Prchr5</i> gene expression.	177
Figure 4.22	Northern analysis of <i>Prchr5</i> and <i>Prcab</i> gene expression in primary and secondary needles from <i>Pinus radiata</i> trees of different maturation states.	178
Figure 4.23	Northern analysis of <i>Prcab</i> gene expression in the mature <i>P. radiata</i> buds cultured over a period of 7 days in the presence (5.0 mg/L) or absence of exogenous cytokinin.	180
Figure 4.24	Northern analysis of the effect of exogenous cytokinin dose on the <i>Prcab</i> gene expression.	181
Figure 4.25	Northern analysis of <i>Prcab</i> gene expression in different buds of <i>P. radiata</i> under different cytokinin regimes.	182

## List of Tables

Table 1.1	Morphological and physiological features that distinguish the juvenile and mature phases of a woody species - English ivy ( <i>Hedera helix</i> L.) and a herbaceous species - maize ( <i>Zea mays</i> ) (Poethig, 1990; Hackett et al., 1995)	4
Table 2.1.	The performance of the entire model PVPP-DE52-C <sub>18</sub> column complex.	58
Table 2.2	Diagnostic positive ions in MS/MS spectra of standard cytokinins.	63
Table 3.1	The concentrations of glucosylated cytokinins in the different types of buds.	102
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.	104
Table 4.1	The quality and the quantity of total RNA obtained using modified RNA extraction procedures.	148
Table 4.2	Similarities and identities between the deduced polypeptide sequence of <i>Pr-cr5</i> and the other protein sequences	162
Table 4.3.	Chlorophyll contents in different types of needles of <i>P. radiata</i> .	183



## List of Abbreviations

AMP	adenosine-5'-monophosphate
<i>amp1</i>	<i>altered meristem programming 1</i> mutation
amu	atomic mass units
<i>AP1</i>	<i>APETALA1</i> 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-(glucopyranosyl-1,3-ribosyl)-phosphate
BSA	bovine serum albumin
BV	column volume
<i>cab</i>	chlorophyll <i>a/b</i> binding protein gene
<i>Cg</i>	<i>Corngrass</i> gene
CHS	chalcone synthase
CK	cytokinin
cpm	counts per minute
CPPU	N-(2-chloro-4-pyridyl)-N'-phenylurea
CTAB	cetyltrimethylammonium bromide
<i>cyr1</i>	cytokinin-resistant mutant
<i>cZ</i>	<i>cis</i> -zeatin
<i>cZR</i>	<i>cis</i> -zeatin riboside
$\Delta^2$ -iPP	$\Delta^2$ -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- <i>O</i> -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- <i>O</i> -glucoside
EDTA	ethylenedinitrilotetraacetic acid
ELISA	enzyme-linked immunosorbant assay
<i>EMF1</i>	<i>EMBRYONIC FLOWERING</i> gene 1
<i>EMF2</i>	<i>EMBRYONIC FLOWERING</i> gene 2
ES MS/MS	electrospray tandem mass spectrometry
FAA	formalin-acetic acid-alcohol fixative

FPF1	flowering promoting factor 1
FW	fresh weight
GA	gibberellin
GA <sub>3</sub>	gibberellic acid
GC-MS	gas chromatography mass spectrometry
<i>GL15</i>	<i>GLOSSY15</i> 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)
<i>ipt</i>	isopentenyl transferase gene
J4	juvenile-looking buds from 4-year-old trees
LC-MS	liquid chromatography-linked mass spectrometry
<i>lec</i>	<i>leafy cotyledon</i> mutation
<i>LFY</i>	<i>LEAFY</i> gene
LP5	LP medium containing 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- <i>O</i> -glucoside
mTR	meta-topolin riboside
mTR5'P	meta-topolin riboside-5'-monophosphate
mTROG	meta-topolin riboside- <i>O</i> -glucoside
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PR-10	pathogenesis-related protein 10
<i>Prcab</i>	<i>Pinus radiata</i> chlorophyll a/b binding protein gene
<i>Prcr5</i>	<i>Pinus radiata</i> cytokinin-responsive gene
<i>psd</i>	<i>paused</i> 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
<i>Tp</i>	<i>Teopod</i> gene
Tris	tris(hydroxymethyl)aminomethane
<i>tZ</i>	<i>trans</i> -zeatin
<i>tZR</i>	<i>trans</i> -zeatin riboside
UTR	untranslated region
UV	ultraviolet
v/v	volume/volume
<i>vp8</i>	<i>viviparous8</i> mutation
<i>xtc1</i>	<i>extra cotyledon 1</i> mutation
<i>xtc2</i>	<i>extra cotyledon 2</i> 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- <i>O</i> -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- <i>O</i> -glucoside
ZRTA	zeatin riboside trialcohol