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 (*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*.

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*Now part of the Institute of Molecular Biosciences.

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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