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(Original article)

Comparison of endogenous cytokinins, ABA and metabolites during female cone bud differentiation in low and high cone-producing genotypes of lodgepole pine

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Running title: Endogenous hormones and cone yield in lodgepole pine

Abstract

In lodgepole pine (*Pinus contorta* Dougl. ex Loud. var. *latifolia* Engelm.), cone bud initiation within long-shoot buds varies according to genotype. We chose to study hormone profiles of two genotypes that differed significantly in cone yield. Phytohormone profiles were established by high performance liquid chromatography-electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) in multiple reaction monitoring (MRM) mode with samples collected from genotypes 299 and 233, the typically high and low cone producers. Generally, concentrations of *trans*-zeatin-*O*-glucoside were higher in genotype 299, whereas dihydrozeatin concentrations were higher in genotype 233. Both isopentenyl adenine and isopentenyl adenosine were present at higher concentrations in genotype 233. The ratio of total quantifiable zeatin (Z)-type cytokinins to isopentenyl (iP)-type cytokinins was approximate three-fold higher in genotype 299 during female cone bud differentiation. In genotype 299, ABA concentration was significantly lower than in genotype 233 on the first sampling date, while the phaseic acid concentration was lower consistently throughout the period investigated. Dihydrophaseic acid was present in low concentrations in most samples of genotype 233, but was not quantifiable in genotype 299. Our study reveals that long-shoot buds of the high cone-producing genotype had higher ratios of Z-type cytokinins to iP-type cytokinins than were found in the low cone-producing genotype. High cone-producing buds also contained less ABA, phaseic acid and dihydrophaseic acid during female cone bud differentiation.

Keywords: ABA, cytokinins, female cone yield, genotype, hormone metabolites, lodgepole pine.

***Abbreviations*:** high performance liquid chromatography-electrospray ionization tandem mass spectrometry, HPLC-ESI-MS/MS; multiple reaction monitoring, MRM; gibberellin, GA; abscisic acid, ABA; phaseic acid, PA; dihydrophaseic acid, DPA; 7'-hydroxy ABA, 7'-OH ABA; *neo*phaseic acid, *neo*PA; abscisic acid glucose ester, ABA-GE; *trans*-zeatin, *t*-Z; *trans*-zeatin riboside, *t*-ZR; *trans*-zeatin-*O*-glucoside, *t*-Z-*O*-Glu; dihydrozeatin, dhZ; dihydrozeatin riboside, dhZR; isopentenyl, iP; isopentenyl adenine, 2iP; isopentenyl adenosine, iPA.

Introduction

In pines, both reproductive buds and vegetative buds are initiated along long shoots. In the specific case of female cone bud differentiation in lodgepole pine, initiation is begun in summer and is completed by fall. A further two years are required for these female cones to mature and produce seed (O'Reilly and Owens 1987; 1988). Although many abiotic and biotic factors affect cone productivity, genotypic effects are relatively strong (Longman 1983; Philippe et al. 2006). This is noticeable in seed orchards. Certain genotypes consistently exhibit extremes in productivity. This undermines the purpose of a seed orchard, which is to produce as much seed as possible. Part of this inefficiency lies in the original selection of genotypes. It was not on the basis of seed production, but for fast growth, high wood quality, pest or disease resistance. Consequently, one reason for low seed productivity is low female bud initiation due to genotype.

To overcome genotype-related limitations in cone production, intervention in bud hormone physiology has proven useful. In both angiosperms (Fiehn et al. 2000) and gymnosperms (Kong et al. 2009), different genotypes exhibit unique metabolic and hormone profiles. In Douglas-fir, correlations between female cone yields and endogenous phytohormone levels of shoot buds have been shown (Kong et al. 2009). Studies of endogenous phytohormone levels during cone initiation can provide strategies to overcome cone yield limitations. Most commonly, plant growth regulators (PGRs), such as gibberellins (GAs), are directly applied. Occasionally, cytokinins and even indole-acetic acid have also been used to improve productivity. These plant growth regulators alter concentrations of

78 endogenous phytohormones and boost cone productivity (McMullan 1980; Bonnet-Masimbert
79 and Zaerr 1987; Pilate et al. 1990; Kong et al. 2008).
80 Cytokinins have been shown to play an important role during the reproductive process
81 (Imbault et al. 1988; Morris et al. 1990; Corbesier et al. 2003; Wakushima 2004). Recent
82 studies with *Arabidopsis thaliana* indicate that cytokinin receptors may have different affinities
83 for particular cytokinins, such as zeatin (Z)-type and isopentenyl (iP)-type cytokinins (Spíchal
84 et al. 2004; Romanov et al. 2006). Furthermore, the differential compartmentalization of
85 cytokinins may play a role in long-distance signalling (Corbesier et al. 2003; Hirose et al.
86 2008). In conifers, attention has been paid to the ratio of Z-type to iP-type cytokinins, but this
87 has been mainly in the context of tree ageing or vigour (Valdés et al. 2002; 2003; 2007) and
88 not in reproduction.
89 Abscisic acid (ABA) is involved in the regulation of many physiological processes
90 including the response of plants to environmental stresses (Bravo et al. 1998; Kumar et al.
91 2008). Abscisic acid negatively modulates the effect of other plant hormones, such as GAs
92 (Tompsett 1977) and auxin (Weiss and Ori 2007). This is of interest in conifers, because
93 exogenously applied GA has been proven to be an effective stimulant of cone formation (Ross
94 1983; Pharis and King 1985; Smith and Greenwood 1995; Kong et al. 2008). The main ABA
95 catabolic pathway in many higher plants is ABA oxidation with its end products of phaseic
96 acid (PA) and dihydrophaseic acid (DPA), but in conifers such as Douglas-fir, the dominant
97 product of ABA catabolism in long-shoots is ABA glucose ester (ABA-GE) (Kong et al. 2008;
98 2009).

The purpose of this research was to investigate changes, during female cone bud differentiation, in endogenous cytokinins and ABA as well as some of their metabolites with two genotypes that differed in cone yield. Lodgepole pine (*Pinus contorta* Dougl. ex Loud. var. *latifolia* Engelm.), an important forest species in western North America, was used. Seed improvement programs have been created in this species consequently. Many of these trees are genetically well-characterized. Hormone analyses of samples were completed by high performance liquid chromatography-electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) in multiple reaction monitoring (MRM) mode (Chiwocha et al. 2003; 2005). This method has a number of advantages: multiple compounds can be quantified from the same sample and the need to process separate samples for each class of compounds is eliminated.

Material and Methods

Genotype selection

Genotypes 233 and 299 were selected from an established seed orchard of 15-year-old trees owned by Vernon Seed Orchard Company, located in Vernon, British Columbia (50°13′N, 119°19′W). The selection procedure was based on a ranking according to productivity. Cone yields for the three previous years were pooled for six ramets of each genotype. Significantly different ($P < 0.05$) genotypes were chosen.

Sample collection, processing and storage

Samples were collected five times during female cone bud differentiation between the end of July and mid-October, 2006. Depending on the size of the bud, ten to 20 long-shoot buds were collected from each ramet per sampling time. After collection, the buds were kept frozen at -20 °C for 2 to 3 d before they were lyophilized in a freeze-drier for 48 hrs. The resulting dry samples were sealed in plastic bags and stored at - 20 °C.

Measurement of moisture content

Fresh weight and dry weight of the samples were used to derive moisture content according to the following formula:

$$\text{Moisture content (\%)} = \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Fresh weight}} \times 100$$

Analysis of hormones and their metabolites

Chemicals: Pure hormone standards, used in calibration curve and quality control solutions, were obtained as follows: dihydrophaseic acid (DPA), abscisic acid glucose ester (ABA-GE), phaseic acid (PA), 7'-hydroxy ABA (7'-OH ABA) and *neo*phaseic acid (*neo*PA) from the Plant Biotechnology Institute of the National Research Council of Canada (PBI-NRC, Saskatoon, SK, Canada); IAA, indole-3-acetic acid aspartate (IAA-Asp), indole-3-acetic acid glutamate (IAA-Glu), ABA, *trans*-zeatin (*t*-Z), *trans*-zeatin riboside (*t*-ZR), isopentenyl adenosine (iPA), and isopentenyl adenine (2iP) from Sigma-Aldrich (Oakville, ON, Canada); dihydrozeatin (dhZ),

146 dihydrozeatin riboside (dhZR), and *trans*-zeatin-*O*-glucoside (*t*-Z-*O*-Glu) from Olchemim Ltd.
147 (Olomouc, Czech Republic); GA_1, GA_3, GA_4, and GA_7 from Prof. Lewis Mander (Australian
148 National University, Canberra, Australia). Bulk amounts of the deuterated forms of the
149 hormones, used as internal standards, were obtained as follows: d_3-DPA, d_5-ABA-GE, d_3-PA,
150 d_4-7'-OH ABA, d_3- *neo*PA, d_4-ABA, d_3-IAA-Asp, and d_3-IAA-Glu from PBI-NRC (Saskatoon,
151 SK, Canada); d_5-IAA, d_3-dhZ, d_3-dhZR, d_5-*t*-Z-*O*-Glu, d_6-iPA, and d_6-2iP from Olchemim Ltd.
152 (Olomouc, Czech Republic); d_2-GA_1, and d_2-GA_4 from Prof. Lewis Mander (Australian
153 National University, Canberra, Australia). Bulk amounts of the deuterated forms of selected
154 hormones which were used as recovery standards, namely d_6-ABA and d_2-ABA-GE, were
155 obtained from PBI-NRC. Preparations of ABA and ABA metabolite standards were described
156 by Abrams et al. (2003) and Zaharia et al. (2005).
157
158 *Extraction, purification and quantification by HPLC-ESI-MS/MS:*
159 Extraction and purification steps were carried out as in Kong et al. (2008). The procedure used
160 for quantification of multiple hormones, including abscisic acid and its metabolites, was a
161 modification of Chiwocha et al. (2003; 2005). To the spectrum of 18 hormones originally
162 quantified in these papers, including IAA, IAA-Asp, ABA, 7′-OH ABA, PA, DPA, ABA-GE,
163 GA_1 (*m/z* 347>259), GA_3, GA_4 (*m/z* 331>243), GA_7, *t*-Z, *t*-ZR, dhZ, dhZR, *t*-Z-*O*-Glu, 2iP, and
164 iPA, were added an additional two; namely IAA-Glu (*m/z* 303>146) and *neo*PA (*m/z* 279>205).
165 Samples were injected onto a Genesis C18 HPLC column (100 × 2.1 mm, 4 μm,
166 Chromatographic Specialties, Brockville, ON, Canada) and separated by a gradient elution of

water against an increasing percentage of acetonitrile and methanol plus 0.04% acetic acid. Calibration curves were generated from the MRM signals obtained from standard solutions using the ratio of the chromatographic peak area for each analyte to that of the corresponding internal standard, as described by Ross et al. (2004). Quality control samples, internal standard blanks, and solvent blanks were also prepared and analyzed along with each batch of tissue samples. The concentrations of IAA, IAA-Asp and IAA-Glu were generally below quantifiable limits of <61 ng g^{-1} DW, <58 ng g^{-1} DW, and <58 ng g^{-1} DW, respectively. All GAs analyzed in this study were also below quantifiable levels of <473 ng g^{-1} DW for GA_1, <238 ng g^{-1} DW for GA_3, <116 ng g^{-1} DW for GA_4, and <116 ng g^{-1} DW for GA_7. As a consequence IAA, IAA metabolites and GAs are not included in the results.

Experimental design and statistical analysis

Six ramets were used as replicates for female cone yield evaluation in each genotype, whereas 3 ramets per genotype were used as replicates for hormone analysis. Data were subjected to one-way analysis of variance (ANOVA) using Minitab statistical software (Minitab, State College, Pennsylvania, USA). The variance was analyzed by Tukey's significant difference with the level of significance set to $P < 0.05$. For analysis purposes, concentrations below quantifiable levels were treated as zeros. Significance differences in overall patterns or at each sampling date have been indicated in the text.

188 **Results**

189 *Genotype performance in cone production*

190 Cone yield was significantly different ($P < 0.05$) between the genotypes. The mean value of
191 female cone yield per tree of genotype 299 was 3.5 times higher than that of genotype 233
192 (Table 1).

193

194 *Dry weight and moisture content*

195 The dry weight of long-shoot buds increased as the season advanced (Fig. 1). Overall, no
196 difference in dry weight existed between these two genotypes (F= 1.79, $P = 0.16$). Moisture
197 content of the bud decreased consistently in both of the genotypes over the growth period (Fig.
198 2). No significant difference (F = 0, $P = 0.951$) was found between genotypes 299 and 233.
199 Although the dry weight per bud in genotype 299 was slightly higher than that of genotype 233
200 at weeks 4 and 10, these differences were not significant (F = 4.15, $P = 0.069$ and F = 3.76, $P =$
201 0.081).

202

203 *Cytokinins and metabolites*

204 *Zeatin (Z)-type cytokinins:* The concentration of *t*-ZR was highest at the beginning of August
205 (week 0), reaching 109 ng g^{-1} DW and 90 ng g^{-1} DW in genotypes 299 and 233, respectively
206 (Fig. 3). Concentrations then declined as the season advanced. The average concentration of *t*-
207 ZR in genotype 299 samples was slightly higher than that in genotype 233 until week 6.
208 Concentrations of *t*-ZR in genotype 299 samples then dropped below quantifiable levels. There

209 was no significant difference (F = 0.04, $P = 0.836$) between genotypes 299 and 233 in the
210 overall pattern of *t*-ZR concentration changes. In genotype 233, the concentration of dhZ
211 remained fairly steady until week 4 when it dropped below quantifiable levels (Table 2). In
212 contrast, dhZ levels were below quantifiable levels in all genotype 299 samples at all sampling
213 dates. In genotype 299, the concentration of *t*-Z-*O*-Glu peaked at week 4 before progressively
214 declining. At week 10, its concentration dropped below quantifiable levels (Table 2). Genotype
215 233's *t*-Z-*O*-Glu levels were below quantifiable levels throughout. In both genotypes,
216 concentrations of dhZR and *t*-Z were generally below quantifiable levels.
217
218 *Isopentenyl (iP)-type cytokinins:* A significant difference (F = 10.94, $P = 0.003$) existed in the
219 overall pattern of iPA concentration changes between the two genotypes. The concentration of
220 2iP in genotype 233 peaked at week 2, declined at week 4 and remained unchanged throughout
221 the subsequent sampling dates (Table 2). In genotype 299 samples, 2iP was only quantifiable at
222 week 10. The concentration of iPA in genotype 233 stayed relatively constant throughout the
223 time period studied, varying between 11 and 14 ng g^{-1} DW (Fig. 4). The concentrations of iPA
224 were significantly ($P < 0.05$) lower in the samples of genotype 299 than in genotype 233 at
225 weeks 0, 6 and 10 (Fig. 4). In genotype 299, the highest concentration of iPA (13 ng g^{-1} DW)
226 was observed at week 4.
227
228 *The ratio of Z-type to iP-type cytokinins: S*ignificant difference ($P < 0.05$) existed between
229 these two genotypes in the ratios of Z-type cytokinins (dhZ, *t*-Z-*O*-Glu, *t*-ZR, dhZR) to iP-type

cytokinins (2iP, iPA). This ratio was approximately 3-fold higher in genotype 299 than in genotype 233 during the first four weeks (Fig. 5). It then decreased as the season advanced.

Abscisic acid

Concentrations of ABA were significantly lower in genotype 299 than in genotype 233 only at the first sampling date ($F = 14.49$, $P = 0.019$) (Fig. 6). Afterwards, no significant differences were noted between these genotypes. The overall pattern of a continuous increase in ABA concentration was observed for both genotypes ($F = 2.22$, $P = 0.147$).

ABA metabolites

Concentrations of ABA-GE were initially 343 ng g^{-1} DW and 408 ng g^{-1} DW in long-shoot samples of genotypes 299 and 233, respectively. As with ABA, the concentration of ABA-GE in both genotypes continued to increase as the season progressed (Fig. 7). The overall pattern of ABA-GE concentration change showed little difference ($F = 0.36$, $P = 0.551$) between the two genotypes. Generally, the PA concentration was higher in genotype 233 than in genotype 299 (Fig. 8). The initial concentration of PA was 84 ng g^{-1} DW in genotype 233, and it remained fairly consistent thereafter. Phaseic acid was only quantifiable at low concentrations in genotype 299 during the first four weeks, ranging from 21 to 46 ng g^{-1} DW. The overall pattern of PA concentration changes between these two genotypes was significantly different ($F = 49.03$, $P < 0.001$). Dihydrophaseic acid was only quantifiable in the samples of genotype 233, with concentrations ranging from 9 ng g^{-1} DW to 21 ng g^{-1} DW at all time points except

that of week 6, where the level was below the limit of quantification (data not shown). Dihydrophaseic acid was below quantifiable levels in all genotype 299 samples. Neither 7′-OH ABA nor *neo*PA was quantifiable in either of the genotypes.

Discussion

The ratio of Z-type to iP-type cytokinins in lodgepole pine long-shoot buds differed between genotypes of high and low cone yield. This supports previous studies in which cytokinins were shown to regulate bud differentiation and shoot development in Norway spruce (Bollmark et al. 1995; Chen et al. 1996) and radiata pine (Zhang et al. 2001; 2003). In angiosperms, cytokinin receptors have different affinities for Z-type and iP-type cytokinins (Spíchal et al. 2004; Romanov et al. 2006). Both cell fate and organ formation have been associated with local concentration gradients of Z-type and iP-type cytokinins (Frugis et al. 2001). These hormones may also act as long-distance signals since Z-type cytokinins exist predominantly in xylem sap whereas phloem sap mainly contains iP-type cytokinins (Corbesier et al. 2003; Hirose et al. 2008).

There is some evidence that Z-type cytokinins may favour female cone bud differentiation. The largest differences in the ratio of Z-type to iP-type cytokinins were seen from the middle of summer to early September. In terms of development, this is no longer the bud initiation stage, but corresponds to the early stages of differentiation. These ratio differences disappeared by late in the growing season. In our study, the absolute amounts of Z-type cytokinins were higher than iP-type cytokinins in the higher cone producer. Our results for

lodgepole pine accord with those of Morris et al. (1990) for Douglas-fir, in which concentrations of Z-type cytokinins were also higher than iP-type cytokinins in female cone buds and vegetative buds, but not male cone buds. Z-type cytokinins are derived from iP-type compounds and not vice versa (Kakimoto 2003; Sakakibara 2006). Thus, the lower concentrations of 2iP and iPA in the genotype with better cone production indicates a higher capability for Z-type cytokinin synthesis. *Trans*-zeatin riboside and iPA were the major cytokinins in both of the genotypes, whereas *t*-Z-*O*-Glu was only quantifiable in genotype 299, a good cone producer. These three compounds were also found in Douglas-fir shoots with differentiating cone buds (Kong et al. 2008; 2009).

Abscisic acid metabolism differed between the two genotypes. The ABA oxidation pathway, which leads to DPA and PA, was more active in the low cone-producing genotype. Kong and von Aderkas (2007) reported ABA utilization was genotype-dependent during conifer somatic embryogenesis; genotypes that responded to ABA supplementation during maturation converted more ABA into PA and DPA. Concentrations of ABA in developing buds are also sensitive to physiological intervention. When trees are subjected to cone induction treatments, such as GA injection, ABA concentration decreases (Kong et al. 2008). Abscisic acid metabolism may also vary by the type of bud. In *Pinus tabulaeformis*, Bao and Zheng (2005) found much higher ABA concentrations in female-sterile trees than in fertile trees.

Cytokinins and ABA may play a role in regulating female cone differentiation but, to date, studies are few and evidence is scant and mixed. In Douglas-fir, the lowest ABA

293 concentration was observed when bud primordia began to form (Kong et al. 2008; 2009). In
294 *Pinus tabulaeformis*, high levels of ABA have been correlated with female gametophyte
295 abortion in a female-sterile genotype (Bao and Zheng 2005). In other higher plants, cytokinin
296 levels increased during flowering induction (Lejeune et al. 1994; Corbesier et al. 2003). Higher
297 cytokinin concentrations were found in female gametophytes than in male gametophytes in
298 *Blechnum spicant* (Menéndez et al. 2009).

299 We have shown that a genotype that characteristically had high cone yield also had
300 much higher ratios of Z-type to iP-type cytokinins compared with a low cone yield genotype.
301 Most of the pronounced changes occurred before week 6. This period corresponds to female
302 cone bud differentiation on the basis of our previous structural study of lodgepole pine long-
303 shoot buds (von Aderkas et al. 2007). In addition, the dramatic increases of bud dry weight
304 between weeks 2 and 6 also indicate fast growth during cone bud differentiation and
305 development. Comparison of bud dry weight and moisture content between the high and low
306 yielding genotypes does not indicate a difference in the health of these trees. The buds in both
307 types grew equally well. The difference in cytokinin metabolism should be further investigated,
308 as this pathway may provide opportunities in developing new strategies for cone induction. For
309 example, cone bud gender in pines is known to be developmentally sensitive to exogenously
310 applied cytokinins (Wakushima 2004, Kong et al. 2011). To date, only adenine type cytokinins
311 have been used, e.g. 6-benzylaminopurine, with other more stable phenylurea types (e.g.
312 thidiazuron) untried. Cytokinins would appear to offer some yet unexplored possibilities in
313 female cone induction (Kong et al. 2011).

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Figure legends

Figure 1 Changes in dry weight of long-shoot buds during female cone differentiation in genotype 299 (open circle) and genotype 233 (solid circle). Sample collection started on August 1, Mean ± SE, n > 30.

Figure 2 Changes in moisture content of long-shoot buds during female cone differentiation in genotype 299 (open circle) and genotype 233 (solid circle). Sample collection started on August 1, Mean ± SE, n > 30.

Figure 3 Concentration of *t*-ZR in long-shoot buds during female cone differentiation in genotypes 299 (open circle) and 233 (solid circle). Sample collection started on August 1, Mean ± SE, n=3.

458 **Figure 4** Concentration of iPA in long-shoot buds during female cone differentiation in
459 genotypes 299 (open circle) and 233 (solid circle). Sample collection started on August 1,
460 Mean ± SE, n=3. Asterisk (*) indicates a significant difference ($P < 0.05$) between genotypes
461 at the individual time point.
462 **Figure 5** Ratio of Z-type cytokinins to iP-type cytokinins in long-shoot buds during female
463 cone differentiation in genotypes 299 (open circle) and 233 (solid circle). Sample collection
464 started on August 1, Mean, n=3. Asterisk (*) indicates a significant difference ($P < 0.05$)
465 between genotypes at the individual time point.
466 **Figure 6** Concentration of ABA in long-shoot buds during female cone differentiation in
467 genotypes 299 (open circle) and 233 (solid circle). Sample collection started on August 1,
468 Mean ± SE, n=3. Asterisk (*) indicates a significant difference ($P < 0.05$) between genotypes
469 at the individual time point.
470 **Figure 7** Concentration of ABA-GE in long-shoot buds during female cone differentiation in
471 genotypes 299 (open circle) and 233 (solid circle). Sample collection started on August 1,
472 Mean ± SE, n=3.
473 **Figure 8** Concentration of PA in long-shoot buds during female cone differentiation in
474 genotypes 299 (open circle) and 233 (solid circle). Sample collection started on August 1,
475 Mean ± SE, n=3. Asterisk (*) indicates a significant difference ($P < 0.05$) between genotypes
476 at the individual time point.

Table 1 Female cone production per ramet in lodgepole pine genotypes 299 and 233. Cone yield data was collected by Vernon seed orchard company (VSOC) during a three-year period before sampling. Mean ± SE, n = 6.

Genotype 299		**Genotype 233**	
Ramet	**Cone yield**	**Ramet**	**Cone yield**
BB91	267 ± 22	AA89	83 ± 8
P91	267 ± 44	BB63	38 ± 7
T67	208 ± 22	O93	60 ± 21
U103	300 ± 52	S66	108 ± 33
Y99	217 ± 22	S79	58 ± 8
O100	142 ± 22	V64	47 ± 3
Total	233 ± 17	**Total**	66 ± 8

Table 2 Concentrations (ng g^{-1} DW) of cytokinins in long-shoot buds during female cone differentiation in genotypes 233 and 299. Sample collection started on August 1, Mean ± SE, n=3. NQ stands for not quantifiable.

Week	dhZ		*t*-Z-*O*-Glu		2iP	
	233	**299**	**233**	**299**	**233**	**299**
0	12 ± 2	NQ	NQ	7± 3	15.3 ± 0.1	NQ
2	10± 2	NQ	NQ	7 ±4	18± 2	NQ
4	10± 1	NQ	NQ	10.1± 0.4	14± 2	NQ
6	NQ	NQ	NQ	7 ± 34	14 ±1	NQ
10	NQ	NQ	NQ	NQ	13 ± 1	6 ± 3

Figures

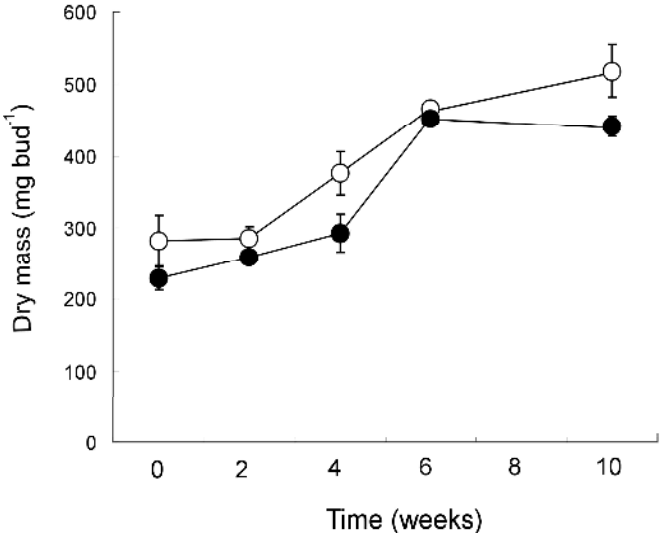
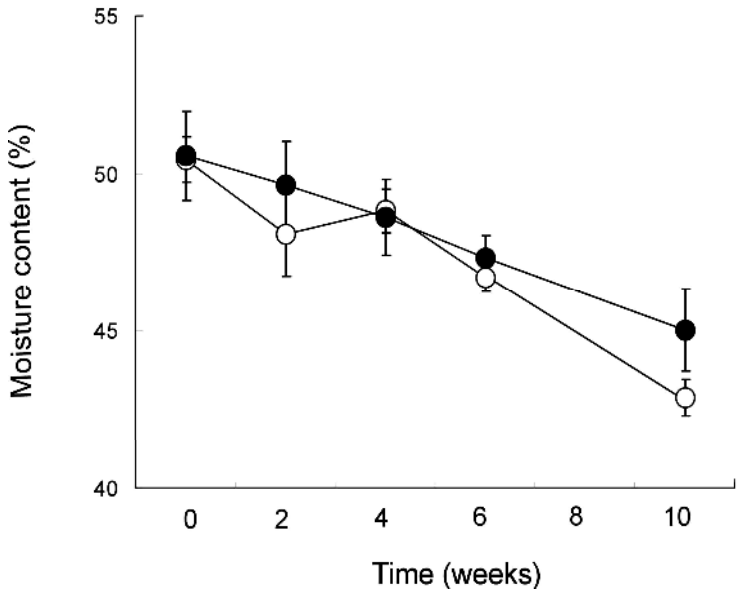


Figure 1



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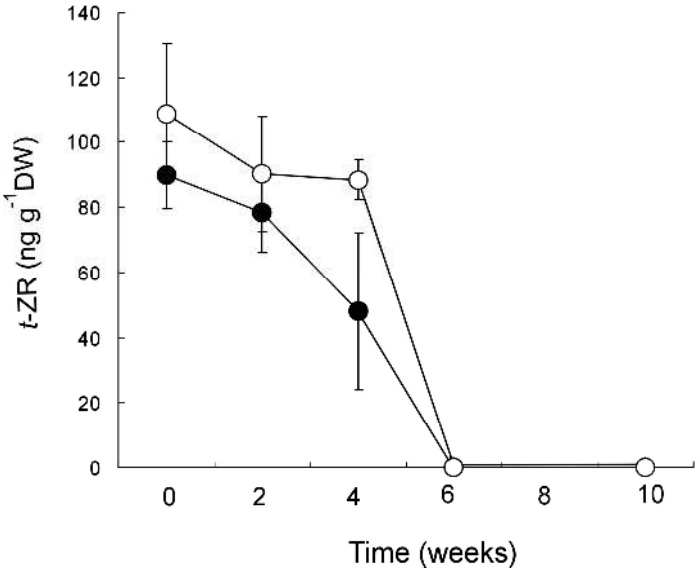

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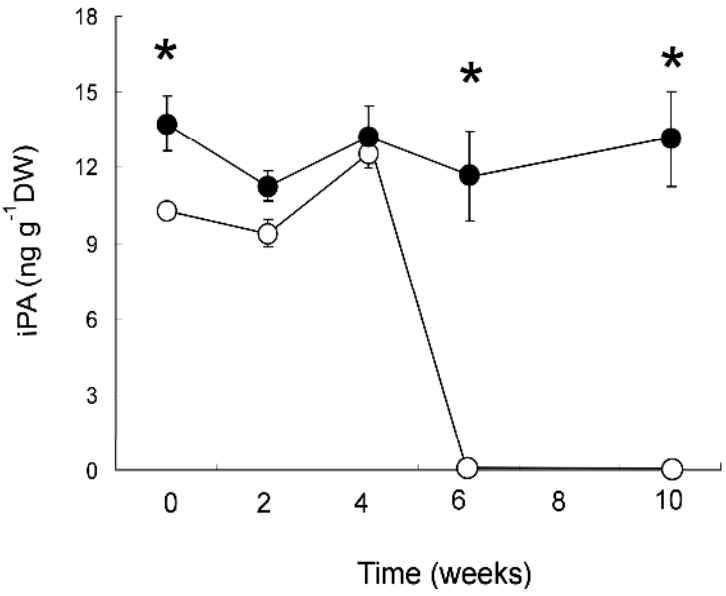

Figure 4

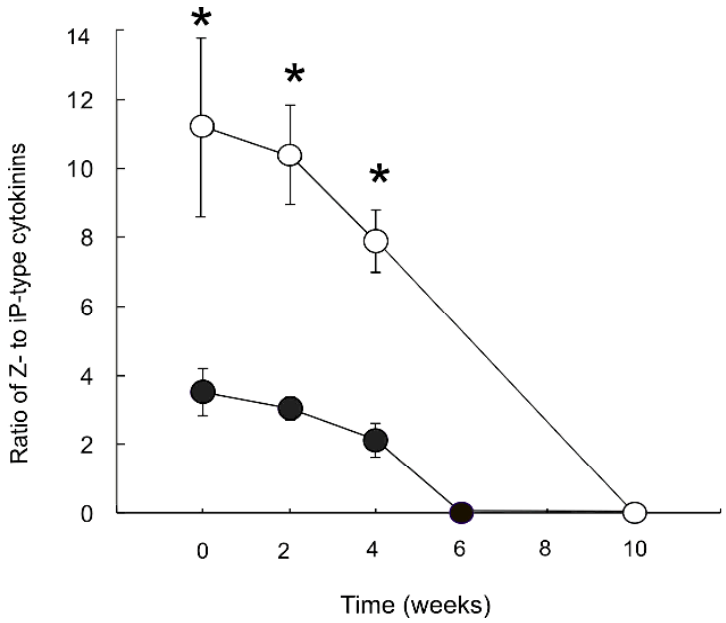

Figure 5

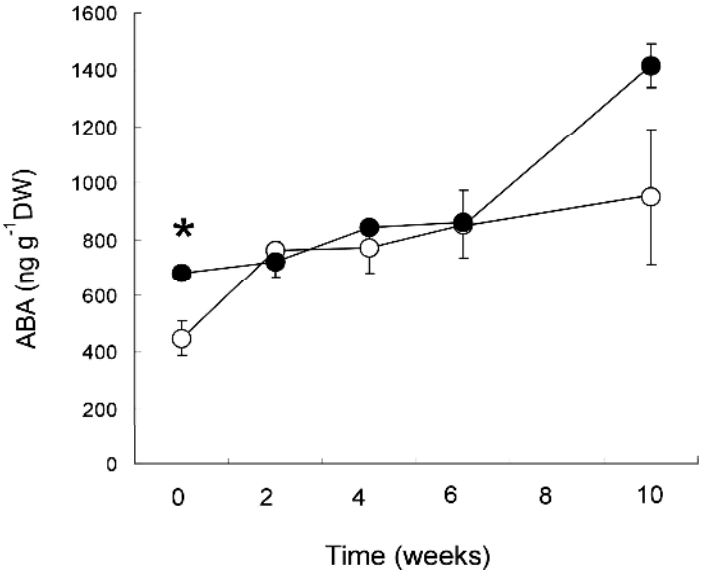

Figure 6

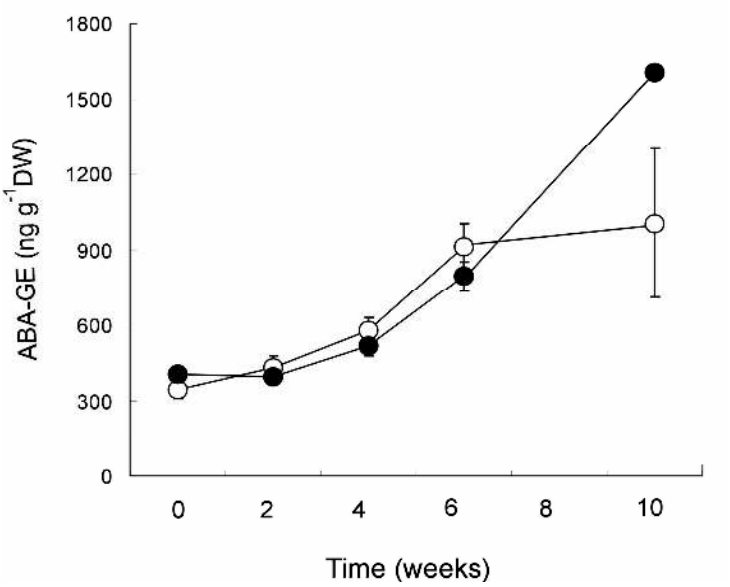

Figure 7

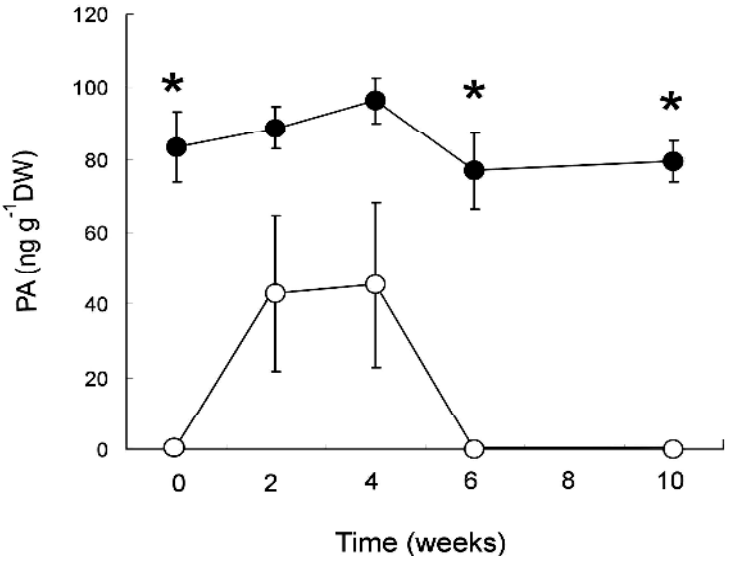

Figure 8