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Phytohormone profiles during male and female cone initiation and early differentiation in long-shoot buds of lodgepole pine

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Running title: Cone gender and endogenous hormones in lodgepole pine

Abstract

In lodgepole pine (*Pinus contorta* Dougl. ex Loud. var. *latifolia* Engelm), cone initiation and gender differentiation are site-specific in long-shoot buds with female cones in the distal portion and male cones in the proximal portion. By using high performance liquid chromatography-electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) in multiple reaction monitoring (MRM) mode, cytokinins, indole-3-acetic acid (IAA), gibberellins (GAs), abscisic acid (ABA) and their selected metabolites, were investigated in developing long-shoot buds from multiple genotypes. Spatially, higher concentrations of *trans*-zeatin riboside (*t*-ZR) and dihydrozeatin (dhZR) existed in the distal parts of long-shoot buds, whereas concentrations of isopentenyl adenosine (iPA), IAA, GA_{24}, ABA, ABA glucose ester (ABA-GE) and phaseic acid (PA) were higher in the proximal parts in all investigated genotypes. In long-shoot buds of genotypes with a history of high female cone yield, concentrations of *t*-ZR and the ratio of zeatin-type to isopentenyl-type cytokinins were higher in the entire buds, whereas dhZR or IAA was higher either in the distal or in the proximal part respectively. In low female cone yielding genotypes, concentrations of *c*-ZR, iPA, ABA-GE and PA were higher in both of the parts, whereas ABA was higher mainly in the distal part with higher GA_{24} in the proximal part**.** Temporally, concentrations of several hormone-related compounds showed obvious changes in late June and late July, prior to male and female cone bud differentiation. This study reveals that the local hormonal status in a long-shoot bud at specific developmental stages may play an important role in gender determination and cone yield.

Abbreviations: HPLC-ESI-MS/MS, high performance liquid chromatography-electrospray ionization tandem mass spectrometry; MRM, multiple-reaction monitoring; GA, gibberellin; ABA, abscisic acid; PA, phaseic acid; DPA, dihydrophaseic acid; 7'-OH ABA, 7'-hydroxy ABA; *neo*PA, *neo*phaseic acid; ABA-GE, abscisic acid glucose ester; IAA, indole-3-acetic acid; IAA-Asp, indole-3-acetic acid aspartate; IAA-Glu, indole-3-acetic acid glutamate; *t*-Z, *trans*-zeatin; *t*-ZR, *trans*-zeatin riboside; *c*-ZR, *cis*-zeatin riboside; *t*-Z-*O*-Glu, *trans*-zeatin-*O*-glucoside; dhZ, dihydrozeatin; dhZR, dihydrozeatin riboside; 2iP, isopentenyl adenine; iPA, isopentenyl adenosine.

Introduction

Cone bud initiation and gender differentiation is site-specific in *Pinus* species. Female cone buds only develop at the distal region of the axis in a long-shoot bud whereas male cone buds form at the proximal region along the axis (Ross and Pharis 1987; O'Reilly and Owens 1987; 1988). The mechanism controlling such site-restricted gender determination remains unclear. In addition, cone bud initiation and differentiation also varies by gender: male cones differentiate earlier than females (O'Reilly and Owens 1987; 1988).

Lodgepole pine (*Pinus contorta* Dougl. ex Loud. var. *latifolia* Engelm) is an important forest species in North America. Currently, seed demand for this species has increased due to the outbreak of mountain pine beetle in British Columbia. To produce more elite seeds in forest seed orchards there is interest in increasing both female cone yield and seed yield.

Plant hormones play an important role in the floral initiation (Bernier et al. 1993; King et al. 2006) and development (Li et al. 2010). In *Arabidopsis thaliana*, increase in endogenous cytokinins enhanced meristematic activity, floral organ development, both of which subsequently had a positive influence on seed yield (Bartrina et al. 2011). Exogenously applied plant growth regulators (PGRs) increased cone yield by altering cone bud differentiation within long-shoot buds, resulting in more female buds (Wakushima 2004; Kong et al. 2011). Induction treatments to stimulate female cone formation have been routinely applied to coniferous trees to increase seed yield. Induction of cones by PGRs depends on multiple factors, such as the quality and quantity of PGR, timing of application, tree genotype, as well as its physiological

condition (Bonnet-Masimbert and Zaerr 1987). Genotypes may vary characteristically in the proportions of male and female buds. Given that the different types of buds are spatially segregated, hormone analysis of these different regions may provide important background information in the eventual study of the physiological and molecular regulation of gender determination. Hormone analysis will also provide practical information for the further development of induction treatments to assist orchard managers in boosting cone yield.

High performance liquid chromatography-electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) in multiple-reaction monitoring (MRM) mode has been applied in analyses of endogenous phytohormones and metabolites in a number of coniferous species and different plant materials, such as seed (Feurtado et al. 2004; 2007; Chiwocha et al. 2007) and long shoots (Kong et al. 2008; 2009). The major advantage of this approach is that multiple compounds can be analyzed in a same plant sample.

The objective of this study was to investigate endogenous phytohormones and metabolites in distal or proximal parts of long-shoot buds during cone bud initiation and differentiation in lodgepole pine. Two groups of genotypes differing in female cone productivity were used in this study. Multiple phytohormone-related compounds, including gibberellins (GAs), cytokinins, auxin, abscisic acid (ABA) as well as their selected metabolites, were investigated using HPLC-ESI-MS/MS in MRM mode.

Materials and Methods

Plant material and experimental design

Plant samples were collected in a clonal seed orchard of Vernon Seed Orchard Company (50°13′N, 119°19′W), which is located at Vernon, British Columbia. Based on cone yield data in previous years, six genotypes were selected and divided into two groups. The first group included three ramets from three genotypes of high female cone yield. Equally, the second group included three ramets from three genotypes of low female cone yield (Table 1). The difference in average female cone yield per tree was 4-fold between the high and the low yield groups. One sample was collected from each ramet at each time point. Data was subject to one-way analysis of variance (ANOVA) using MINITAB software (MINITAB Inc., State College, PA, USA). Significance of means was analyzed by the Tukey test. Overall, levels of significance were set to $P < 0.05$.

Sample collection, processing and storage

Samples of long-shoot buds were collected during cone initiation and differentiation at regular intervals of two weeks between the last week of June and the first week of August. A final sample was added in the middle of September, as trees entered winter dormancy. The number of long-shoot buds for one sample ranged from as many as twenty buds at the early season to as few as ten in the late season. The reason for this difference in collection samples was related to mass – early in the season buds were much smaller and collecting sufficient sample for analysis required more buds than later in the same season when buds were much larger.

After collection, long-shoot buds were divided unequally into a distal and a proximal portion as follows: the upper 2/5 were separated from the lower 3/5 of the bud, because the lower, proximal portion, which is also the site where male buds form, occupies more than half of the long-shoot bud (Fig. 1). These parts were then wrapped in tin foil and frozen in liquid nitrogen and kept frozen. Subsequently, the samples were lyophilized in a freeze-drier for 48 h. Dry samples were sealed in plastic bags and stored in a freezer.

Analysis of hormones and their metabolites

Chemicals

Bulk amounts of the pure hormones, used to create calibration curves and quality controls (QCs), were obtained as follows: dihydrophaseic acid (DPA), abscisic acid glucose ester (ABA-GE), phaseic acid (PA), 7'-hydroxy ABA (7'-OH ABA), *neo*-phaseic acid (*neo*PA), and indole-3-acetic acid glutamate (IAA-Glu) from the Plant Biotechnology Institute of the National Research Council of Canada (PBI-NRC Saskatoon, SK, Canada); ABA, indole-3-acetic acid aspartate (IAA-Asp), IAA, *trans*-zeatin (*t*-Z), *trans*-zeatin riboside (*t*-ZR), isopentenyl adenosine (iPA), and isopentenyl adenine (2iP) were purchased from Sigma-Aldrich (Oakville, ON, Canada); dihydrozeatin (dhZ), dihydrozeatin riboside (dhZR), and *trans*-zeatin-*O*-glucoside (*t*-Z-*O*-Glu) were purchased from Olchemim Ltd. (Olomouc, Czech Republic). Gibberellins (GAs), *i. e.* GA_1, GA_3, GA_4 GA_7, GA_8, GA_9, GA_{19}, GA_{20}, GA_{24} GA_{29}, and GA_{44}, were obtained from Prof. Lewis Mander (Australian National University, Canberra, Australia). Bulk amounts of the deuterated forms of the hormones, used as internal standards, were obtained as follows: d_3-DPA, d_5-ABA-GE, d_3-PA, 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, SK, Canada); d_5-IAA from Cambridge Isotope Laboratories (Andover, MA, USA); d_3-dhZ, d_3-dhZR, d_5-*t*-Z-*O*-Glu, d_6-iPA, and d_6-2iP from Olchemim Ltd. (Olomouc, Czech Republic); d_2-GAs were purchased from Prof. Lewis Mander (Australian National University, Canberra, Australia). Bulk amounts of the deuterated forms of selected hormones, used as recovery standards, were obtained as follows: d_6-ABA and d_2-ABA-GE from PBI-NRC (Saskatoon, SK, Canada).

Extraction, purification and quantification by HPLC-ESI-MS/MS

Extraction and purification steps were carried out according to previous method (Kong et al. 2008). The procedure used for quantification of phytohormones and metabolites, including auxins (IAA, IAA-Asp and IAA-Glu), ABA and metabolites (ABA, PA, DPA, 7'-OH ABA, *neo*PA and ABA-GE), GAs (GA_1, GA_3, GA_4 GA_7, GA_8, GA_9, GA_{19}, GA_{20}, GA_{24} GA_{29} and GA_{44}) and cytokinins (2iP, iPA, *t*-Z, *t*-ZR, dhZ, dhZR, *t*-Z-*O*-Glu), is as described in Chiwocha et al. (2003; 2005) with modifications. Samples were injected onto a Genesis C18 HPLC column (100 × 2.1 mm, 4 µm, Chromatographic Specialties, Brockville, ON, Canada) and separated by a gradient elution of water against an increasing percentage of acetonitrile and methanol, and containing 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). QC samples, internal standard blanks, and solvent blanks were also prepared and analyzed along with each batch of tissue samples.

Results

Cytokinins

Concentration levels of *t*-ZR were similar in both distal and proximal parts sampled on June 24. In a mater independent of cone production capability, t-ZR concentrations decreased, up to 2-fold, in the proximal part, whereas it showed little changes in the distal part (Fig. 2 A & B). The overall patterns of t-ZR changes were significantly different between distal parts and proximal parts in either the good cone producers (F=15.31, *P*=0.001) or the poor cone producers (F= 8.77, *P*=0.006). In distal parts, *t*-ZR concentrations were slightly higher in good cone producers than in poor ones. However, the difference between the overall patterns was not significant (F = 0.52, *P*=0.475). No difference (F = 0.41, *P*=0.527) was found in proximal parts between the good and the poor cone producers except in the samples of June 24 when higher *t*-ZR concentration existed in poor cone producers (Fig. 2 C).

On June 24, *c*-ZR concentrations were similar in the two bud parts of good cone producers (Fig. 3 A), whereas they were higher in proximal parts in the poor cone producers (Fig. 3 B). Thereafter, *c*-ZR concentrations declined at different rates in both of the bud parts resulting in higher levels, up to 2-fold, in distal parts than those in proximal parts in all genotypes (Fig. 3 A & B). Differences of the overall pattern were spatially significant in either the good cone producers (F= 9.00, *P*=0.006) or the poor cone producers (F= 6.89, *P*=0.014). In both distal and proximal parts, *c*-ZR concentrations were slightly higher in genotypes of low cone yield. However, no overall significant differences were found related to cone productivity in both distal parts

(F=2.75, *P*=0.109) and proximal parts (F=1.89, *P*=0.180) except for two time points in the later case (Fig. 3 C).

Concentrations of dhZR were higher in distal parts than those in proximal parts in all genotypes in spite of cone production capability (Fig. 4A & B). Differences of the overall pattern were spatially significant in good cone producers (F= 15.31, *P*=0.001) or in poor cone producers (F= 5.50, *P*=0.026). No significant differences were related to cone productivity in either distal parts (F = 0.27, *P*=0.610) or proximal parts (F= 0.19, *P*=0.666).

Initially, iPA concentrations were higher in proximal parts than those in distal parts at June 24 in all genotypes (Fig. 5 A & B). Thereafter, no difference existed in iPA concentrations until Sep 16, when iPA concentration was higher in distal parts of good cone producers (Fig. 5A). The overall patterns were not different significantly (F= 0.81, *P*=0.375 for good cone producers; F= 0.13, *P*=0.725 for poor cone producers). Compared to good cone producers, although average iPA concentrations were slightly higher in proximal parts of low cone yielding genotypes, no significant difference was found relating to cone productivity (F=2.90, *P*=0.099 for distal parts; F=0.02, *P*=0.881 for proximal parts).

The ratios of zeatin (Z)-type cytokinins, including *t*-ZR, *c*-ZR, dhZR, to isopentenyl (iP)-type cytokinins, including iPA and 2iP, were at similar levels in both distal and proximal parts of long-shoot buds in good cone producers (Fig. 6 A). In poor cone producers, this ratio was similar in both parts of the bud on June 24. It then became higher in proximal parts before it rose higher in distal parts at the end of July. Thereafter, this ratio remained higher in distal parts (Fig. 6 B). When compared with

poor cone producers, this ratio was much higher in distal parts of the good cone producers at July 22 prior to female cone differentiation (Fig. 6 C). A similar pattern of the ratio changes was also found in proximal parts (Fig. 6 D).

Auxin

Concentrations of IAA were higher, up to 2.6-fold, in proximal parts than those in distal parts in all genotypes between June and August (Fig. 7 A & B). Differences of the overall pattern were spatially significant in long-shoot buds of both the good cone producers (F=10.90, *P*=0.003) and the poor cone producers (F=8.77, *P*=0.006). No significant differences were found related to cone productivity in distal parts (F=0.05, *P*=0.825) and proximal parts (F=1.63, *P*= 0.213), although in proximal parts, the average IAA concentrations were slightly higher in genotypes with higher cone yield. Concentrations of both IAA-Asp and IAA-Glu were below quantifiable levels in all of the samples.

Gibberellins

Of 11 GAs investigated, GA_{24} was the only one that could be detected and quantified consistently. There was no difference between GA_{24} concentrations in distal and proximal parts of good cone producers except for those in June 24 samples (Fig. 8), whereas GA_{24} concentrations were higher in proximal parts during July and August. Higher concentrations of GA_{24} were found in proximal parts of poor cone producers when compared to those of good cone producers. Differences of the overall pattern were spatially significant in the poor cone producers (F = 4.86, *P* =0.036) but not in the good cone producers (F= 0.06, *P*= 0.806). Significant differences were found related to cone productivity only in proximal parts (F= 5.67, *P*=0.024), but not in distal parts (F=

0.02, P=0.891). GA_1, GA_4 and GA_7 were quantifiable in only a few samples at low concentrations (data not shown). Other GAs, *i.e.* GA_3, GA_8, GA_9, GA_{19}, GA_{20}, GA_{29}, and GA_{44} were either undetectable or below quantifiable levels.

Abscisic acid

Concentrations of ABA were higher, up to 1.7-fold, in proximal parts than those in distal parts in all genotypes between June 24 and August 5 (Fig. 9A & B). The overall patterns of ABA were not significantly different between distal parts and proximal parts, *i.e.* F=1.13, *P*=0.297 for good producers; F= 0.32, *P*= 0.576 for poor cone producers. Also, there were no significant differences between good and poor cone producers (F=0.91, *P*=0.348 for distal parts; F=0.06, *P*=0.808 for proximal parts).

In all genotypes, concentrations of ABA-GE were up to 5-fold higher in proximal parts than in distal parts at most sampling points (Fig. 10 A & B). Compared with good cone producers, concentrations of ABA-GE were higher in poor cone producers in both distal (Fig.10 C) and proximal parts (Fig.10 D). The overall patterns of ABA-GE were significantly different between the two parts, *i.e.* F = 8.02, *P*= 0.008 for good cone producers and F = 5.39, *P*= 0.028 for poor producers. However, the difference was not related to cone productivity. When comparisons were made between the good cone producers and the poor producers, significant differences were found in neither distal parts (F=2.43, *P*=0.131) nor proximal parts (F=1.12, *P*=0.300).

Concentrations of PA were generally higher in proximal parts than in distal parts (Fig. 11 A & B) in all genotypes. This spatial difference was larger in genotypes of low female cone yield (Fig. 11 B). Compared with the good cone yielder, PA concentrations were higher in genotypes of low cone yield in both distal and proximal parts after June

24 (Fig. 11 C & D). Differences of the overall PA pattern were spatially significant in the poor cone producers (F= 5.05, *P*=0.033) but not in the good cone producers (F= 0.40, *P*=0.530). Significant differences were found related to cone productivity only in proximal parts (F=6.86, *P*=0.014), but not in distal parts (F=1.35, *P*=0.255). Dihydrophaseic acid was quantifiable in a few samples (data not shown), whereas concentrations of both 7'-OH ABA and *neo*PA were under quantifiable levels.

Discussion

On to the well-documented distribution of male and female cone buds in lodgepole pine long-shoots (O'Reilly and Owens 1987, 1988) we can now add spatial and temporal distributions of certain classes of plant growth regulators. Although it is tempting to correlate these patterns, a more conservative interpretation would be to use the hormone information in future experiments involving gene expression or applied treatments to increase cone yields.

Spatial differences were observed in several hormones and their metabolites. Higher concentrations of t-ZR, c-ZR and dhZR were found consistently in distal (female) parts of long-shoot buds. Higher concentrations of IAA, ABA, AGA-GE and PA were found in the proximal (male) parts. Temporal differences in several hormones and their metabolites were also noted. Two periods in particular warrant closer attention. Between June 24 and July 8 concentrations of t-ZR, c-ZR, iPA and GA_{24} showed substantial changes, especially in the proximal (male) parts. This period corresponds to a time point prior to male cone differentiation (von Aderkas et al. 2007). The second time point was between July 22 and August 5 when changes in concentrations of dhZR,

ABA, ABA-GE and the ratio of Z-type to iP-type cytokinins were noted. These changes occurred in both distal and proximal portions. Female cone differentiation is after male cone differentiation, which means at this point both types of cones are differentiating along the long-shoot axis. These two points (June 24-July 8, and July 22-Aug 8) may represent points at which exogenous application of plant growth regulating substances (e.g. commercial gibberellin mixtures used in seed orchards) may have most effect. A cautious interpretation is that half the periods that we sampled show concentration changes, whereas the other did not, which is hardly grounds for recommending that endogenous hormone changes should be measured to establish optimal application periods. Knowing when differentiation occurs should be sufficient. Since this is already known at the morphological level (O'Reilly and Owens, 1986; von Aderkas et al. 2007), as well as on the seed orchard management level (Almqvist 2003; Ross and Pharis 1987), we can only conclude that the dynamics of endogenous hormone levels appear to correlate with known induction periods.

One group of hormones and related metabolites that deserve further exploration in pine cone bud initiation are cytokinins. In conifers, cytokinins participate in regulation of bud differentiation and development (Bollmark et al. 1995; Chen et al. 1996; Zhang et al. 2001; 2003). Exogenously applied benzyl adenine purine (BAP), a commercially synthesized and stable cytokinin, induced female cone buds from male bud sites in Japanese red pine and Japanese black pine (Wakushima 2004). This suggests that cytokinin metabolism may provide targets for cone induction treatments. In addition to BAP, other commercial cytokinins that could be tried include thidiazuron (TDZ), iP and iPA.

At present, the biological significance of the ratio between Z-type and iP-type cytokinins is unclear. Since Z-type cytokinins are derived from iP-type compounds and not vice versa (Kakimoto 2003; Sakakibara 2006), the low ratio of Z- to iP- cytokinins indicates lower activity of cytokinin synthesis. It has been suggested that Z/iP is a useful index of ageing and vigour in radiata (Valdés and et al. 2002; 2003) and Scots pine (Valdés et al. 2007). In Douglas-fir, concentrations of Z-type cytokinins were relatively higher in female and vegetative buds, whereas iP-type cytokinins were higher in male buds (Morris et al. 1990). In our study, a higher ratio of Z- to iP-type cytokinins occurred during bud primordia formation and bud differentiation. Recent evidence indicates that different cytokinin receptors may have different affinities for either Z- or iP-type cytokinins (Spíchal et al. 2004; Romanov et al. 2006) since xylem sap mainly contains Z-type cytokinins, while phloem sap mainly contains iP-type cytokinins (Corbesier et al. 2003), such compartmentalization might function in regulating cytokinin signaling (Hirose et al. 2008). Cell fate and organ formation may also be associated with local concentrations of Z- and iP-type cytokinins (Frugis et al. 2001).

Concentrations of IAA were higher in proximal parts during early summer bud growth. Involvement of IAA in regulation of cambial activity in conifer species has been reported (Sundberg et al. 1991; Uggla et al. 2001). Although it is unknown if cambial growth is occurring in long-shoot buds, IAA may be involved in male cone development since higher IAA concentrations were consistently observed in the proximal parts during male cone development. For cone induction, auxins are usually applied together with GAs in order to enhance GA effects (Pharis et al. 1980). When applied alone, auxin stimulated male cone formation (Sheng and Wang 1990). Usually auxin is regarded

synthesized at the apical location and transported downwards. In this case, IAA concentrations in distal parts should be higher than those in proximal parts. Recently, Rasmussen et al (2009) provided new evidence supporting local hormone synthesis in plants. Concentrations of IAA in proximal part were higher in the good female cone producers than in the poor ones. Whether the spatial gradient of IAA concentrations between these two parts could affect gender determination needs further study. Another possible interpretation is that higher IAA concentrations in the lower part of good genotype may contribute to vigorous long shoot growth, resulting in high female cone yield. In our previous studies, higher IAA concentrations existed in developing long shoots of good cone producers in Douglas-fir (Kong et al. 2009).

Gibberellins have long played a key role in exogenously applied cone induction treatments (Pharis et al. 1980). MS methods that have focused strictly on gibberellins in conifers have identified GA_1, GA_3, GA_4, GA_7, GA_9, GA_{12}, an GA_{20} (Odén et al. 1987; 1994; Moritz et al. 1990; Doumas et al. 1992; Wang et al. 1995; 1996; Kong et al. 1997; Fernández et al. 2003). Physiologically active gibberellin concentrations are orders of magnitude lower than many other hormone classes and provide a particular challenge to the mass spectrometrist. This is compounded in these bud samples of lodgepole pine, which are particularly rich in interfering secondary metabolites. We had difficulties in detecting gibberellins using our multiple ion approach. We would suggest that this work could be better accomplished with an approach focused directly on these lower abundance molecules. We report high concentrations of GA_{24} for the first time in lodgepole pine long-shoot buds, which is also a first report for conifers. GA_{24} is a precursor of GA_4 via GA_9 during GA biosynthesis (Takahashi et al. 1986). GA_4 is a less

polar molecule that is known to play an important role in regulating cone formation (Pharis and King 1985). GA_4 could enhance female cone formation and also stimulate male cone formation (Hare 1984). Endogenous GA_4 concentrations were much higher in male cone buds than vegetative buds in radiata pine (Fernández et al. 2003). In this study, GA_{24} concentrations were always higher in proximal parts of long-shoot buds where male cone buds develop.

Concentrations of ABA have never before been measured in long-shoot buds of lodgepole pine. We found that they were consistently lower in distal regions of the long-shoot during its development and differentiation. Furthermore, ABA was lower in good cone producers than in poor cone producers. ABA metabolites ABA-GE and PA were also higher in the distal portions of these poor-yield genotypes. Though hardly conclusive, this is supported by other evidence of high concentrations of ABA inhibition of female cone formation in female-sterile Chinese red pine (Bao and Zheng 2005).

Interactions among various plant hormones could result in complex mechanisms regulating physiological processes (Weiss and Ori 2007). Cytokinins and auxin can stimulate cell division and cell growth while ABA may inhibit such activities. Bao and Zheng (2005) reported that high concentrations of ABA and low concentrations of IAA and ZR led to female gametophyte abortion in the female-sterile genotype in *Pinus tabulaeformis*. Higher cytokinin and lower ABA concentrations in the distal parts of long-shoot buds in lodgepole pine may favor female cone initiation and differentiation.

In this paper we have shown that temporal and spatial differences exist within a long-shoot bud in a number of classes of phytohormones. Although there are indications that some of these changes, particularly in cytokinin and ABA distributions, may

correlate well with known gender differences within long-shoot buds, further research is required. The MRM approach allows us to create profiles of endogenous hormones during development and differentiation of the long-shoot bud, which in turn provides a basis upon which to devise further experiments to establish the basis of cone gender determination in future.

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Table 1. Female cone yield of six different lodgepole pine genotypes. These genotypes were divided into the high yield and low yield groups on the basis of averaged female cone production per ramet from 2005 to 2007. Significant differences at $P < 0.05$ are indicated by different letters within a same row. Mean ± SE, n = 9.

High		**Low**	
Genotype	♀ cone yield / tree	Genotype	♀ cone yield / tree
472	128.7, 114.6, 72.1	224	27.5, 25.3, 21.8
1779	105.2, 116.5, 105.2	423	21.3, 25.0, 20.5
502	113.2, 107.5, 85.2	402	23.5, 26.2, 19.8
	105.4 ± 5.7 (a)		**23.4 ± 0.9 (b)**

Figures

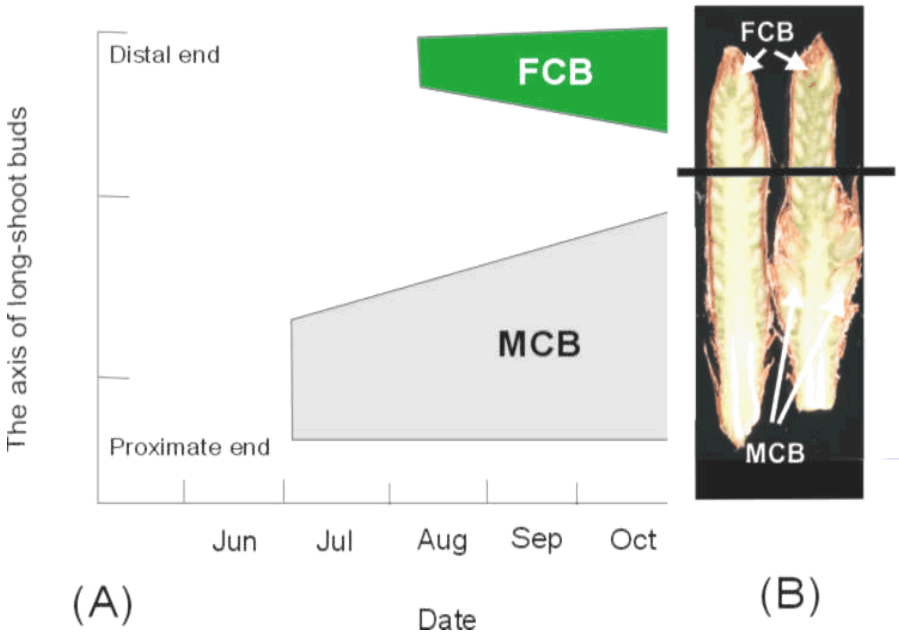

Figure 1. Development of cone buds in a long-shoot bud of lodgepole pine. (A) A graph showing a temporal and spatial relationship for both male cone and female cone bud development. (B) A photo showing two longitudinally-cut long-shoot buds. The horizontal line indicated the cut for separating distal and proximal parts of long-shoot buds for hormone analysis. Male cone bud (MCB), female cone bud (FCB).

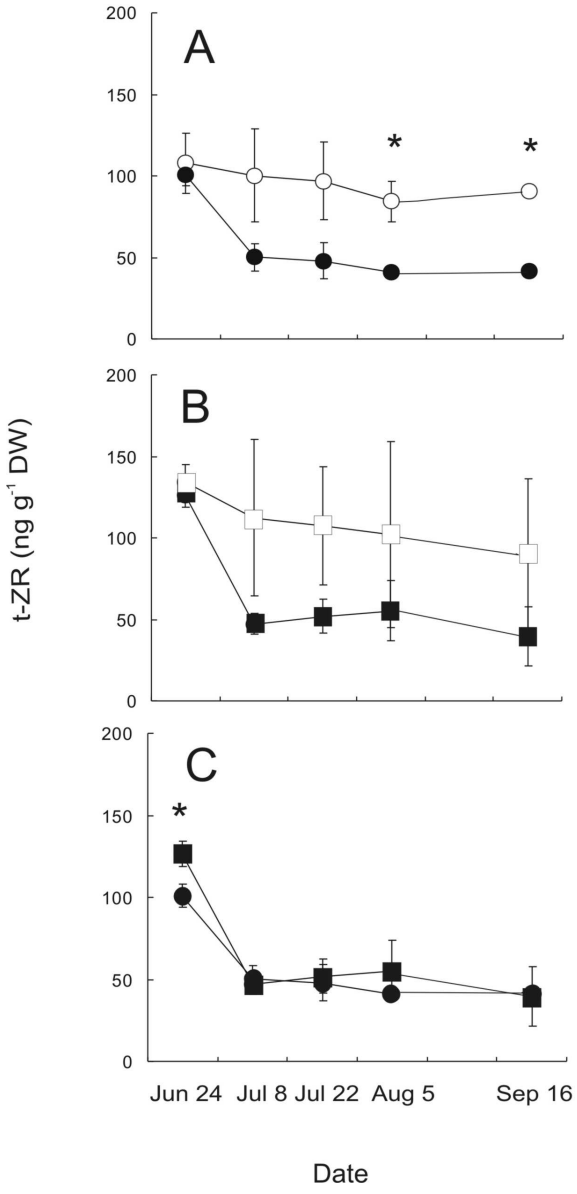

Figure 2. Changes in concentrations of endogenous t-ZR in lodgepole pine long-shoot buds from summer to the fall. (A) The two parts of good female cone producer; (B) the two parts of poor female cone producer; (C) proximal parts of good and poor female cone producer. Circle - good female cone producer; Square - poor female cone producer; Open - distal parts of long-shoot buds; Solid - proximal parts of long-shoot

buds. Mean ± SE, n=3 genotypes. Asterisk (*) indicates significant difference (P < 0.05) between two different samples at each individual time point.

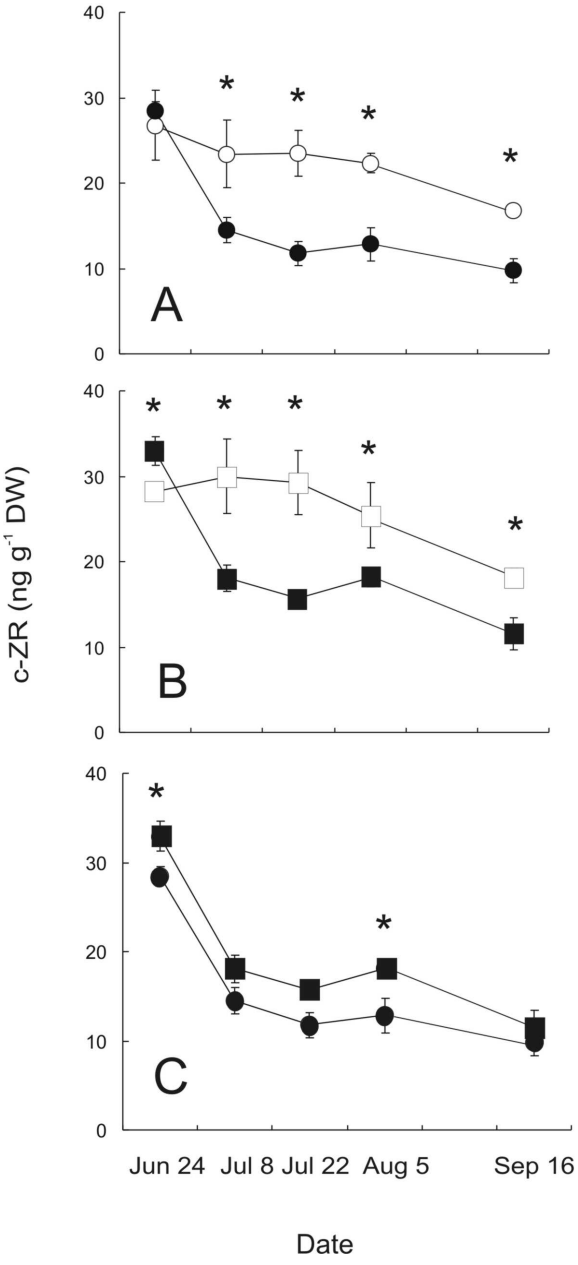

Figure 3 Changes in concentrations of endogenous c-ZR in lodgepole pine long-shoot buds from summer to the fall. (A) The two parts of good female cone producer; (B) the two parts of poor female cone producer; (C) proximal parts of good and poor female

cone producer. Circle - good female cone producer; Square - poor female cone producer; Open - distal parts of long-shoot buds; Solid – proximal parts of long-shoot buds. Mean ± SE, n=3 genotypes. Asterisk (*) indicates significant difference (P < 0.05) between two different samples at each individual time point.

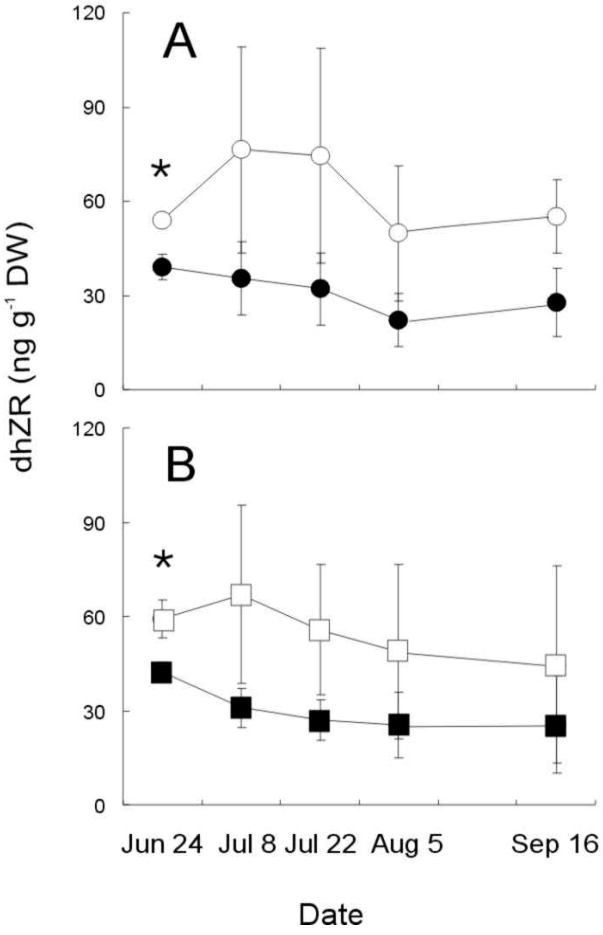

Figure 4 Changes in concentrations of endogenous dhZR in lodgepole pine long-shoot buds from summer to the fall. (A) The two parts of good female cone producer; (B) the two parts of poor female cone producer. Circle - good female cone producer; Square - poor female cone producer; Open - distal parts of long-shoot buds; Solid – proximal

parts of long-shoot buds. Mean ± SE, n=3 genotypes. Asterisk (*) indicates significant difference ($P < 0.05$) between two different samples at each individual time point.

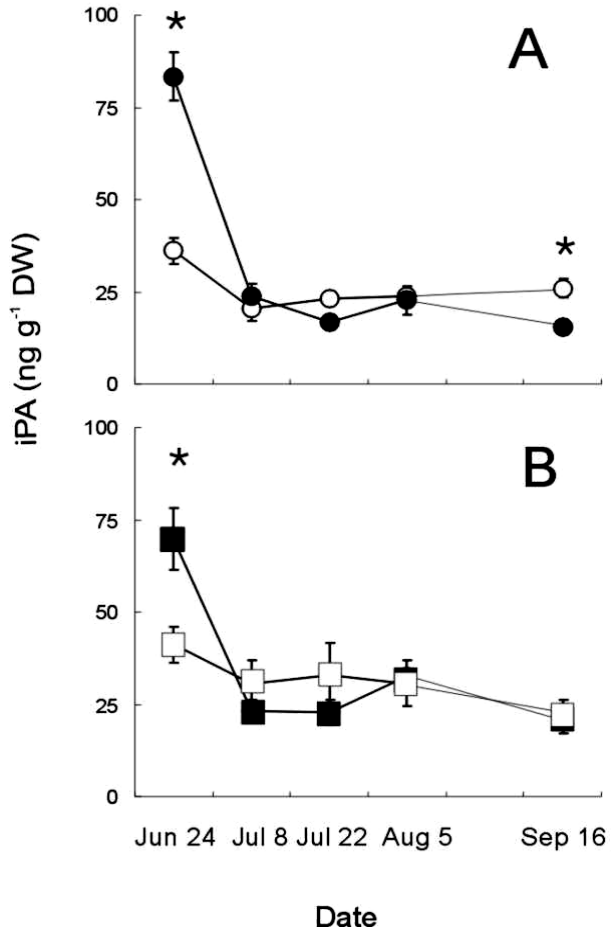

Figure 5 Changes in concentrations of endogenous iPA in lodgepole pine long-shoot buds from summer to the fall. (A) The two parts of good female cone producer; (B) the two parts of poor female cone producer. Circle - good female cone producer; Square - poor female cone producer; Open - distal parts of long-shoot buds; Solid – proximal parts of long-shoot buds. Mean ± SE, n=3 genotypes. Asterisk (*) indicates significant difference ($P < 0.05$) between two different samples at each individual time point.

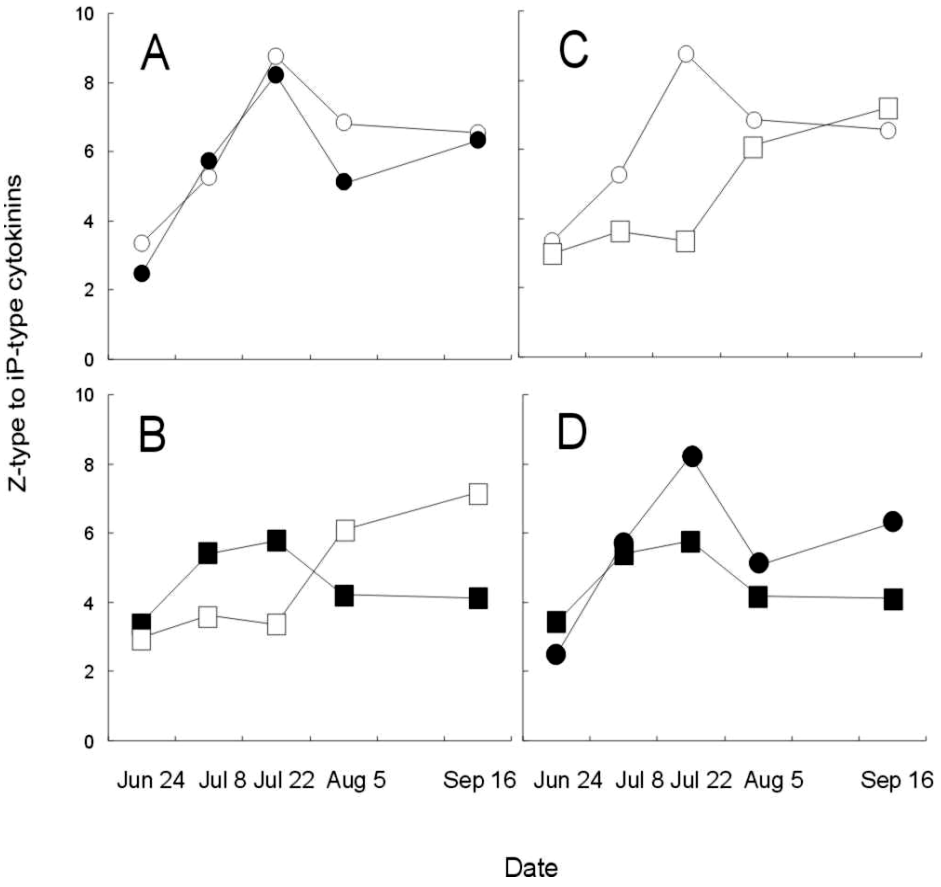

Figure 6 Changes in the ratio of Z-type to iP-type cytokinins in lodgepole pine long-shoot buds from summer to the fall. (A) The two parts of good female cone producer; (B) the two parts of poor female cone producer; (C) distal parts of good and poor female cone producer; (D) proximal parts of good and poor female cone producer. Circle - good female cone producer; Square - poor female cone producer; Open - distal parts of long-shoot buds; Solid - proximal parts of long-shoot buds. Mean ± SE, n=3 genotypes.

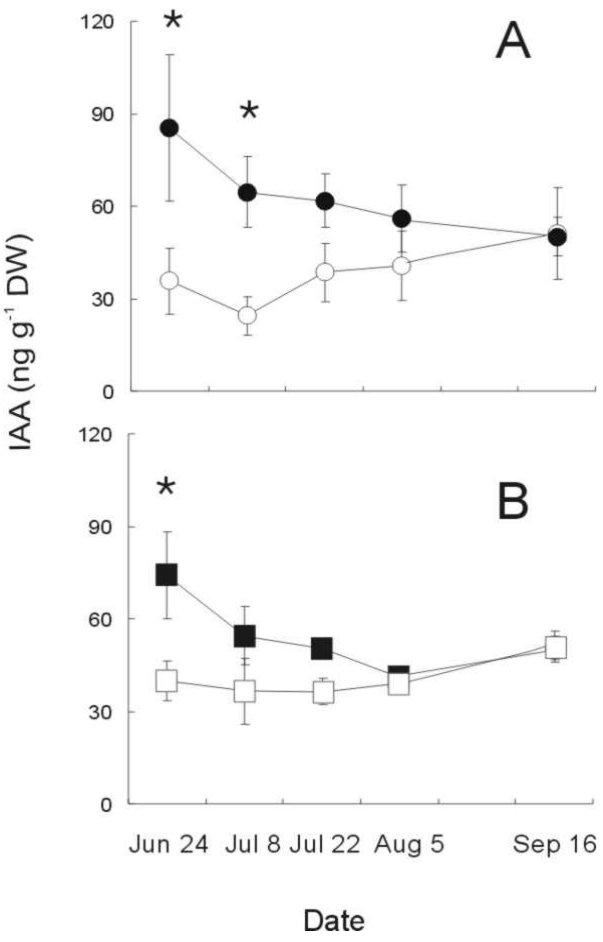

Figure 7 Changes in concentrations of endogenous IAA in lodgepole pine long-shoot buds from summer to the fall. (A) The two parts of good female cone producer; (B) the two parts of poor female cone producer. Circle - good female cone producer; Square - poor female cone producer; Open - distal parts of long-shoot buds; Solid – proximal parts of long-shoot buds. Mean ± SE, n=3 genotypes. Asterisk (*) indicates significant difference ($P < 0.05$) between two different samples at each individual time point.

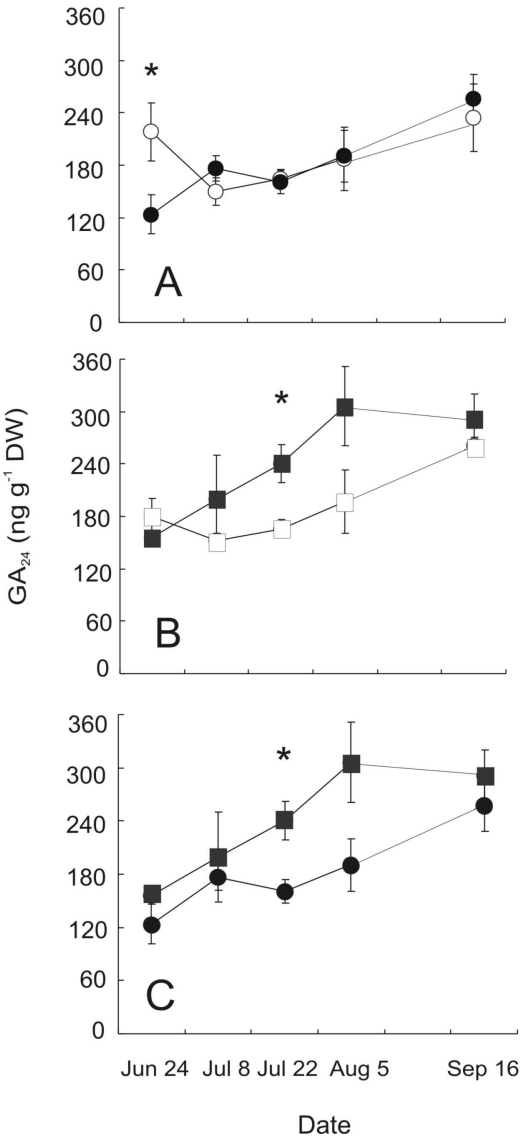

Figure 8. Changes in concentrations of endogenous GA_{24} in lodgepole pine long-shoot buds from summer to the fall. (A) The two parts of good female cone producer; (B) the two parts of poor female cone producer; (C) proximal parts of good and poor female cone producer. Circle - good female cone producer; Square - poor female cone producer; Open - distal parts of long-shoot buds; Solid – proximal parts of long-shoot buds. Mean ± SE, n=3 genotypes. Asterisk (*) indicates significant difference ($P < 0.05$) between two different samples at each individual time point.

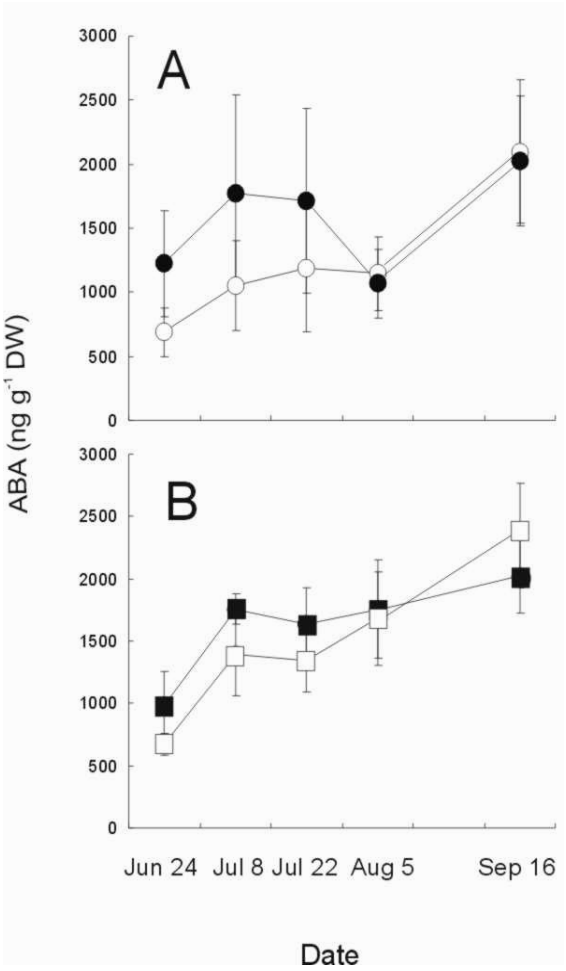

Figure 9 Changes in concentrations of endogenous ABA in lodgepole pine long-shoot buds from summer to the fall. (A) The two parts of good female cone producer; (B) the two parts of poor female cone producer. Circle - good female cone producer; Square - poor female cone producer; Open - distal parts of long-shoot buds; Solid – proximal parts of long-shoot buds. Mean ± SE, n=3 genotypes.

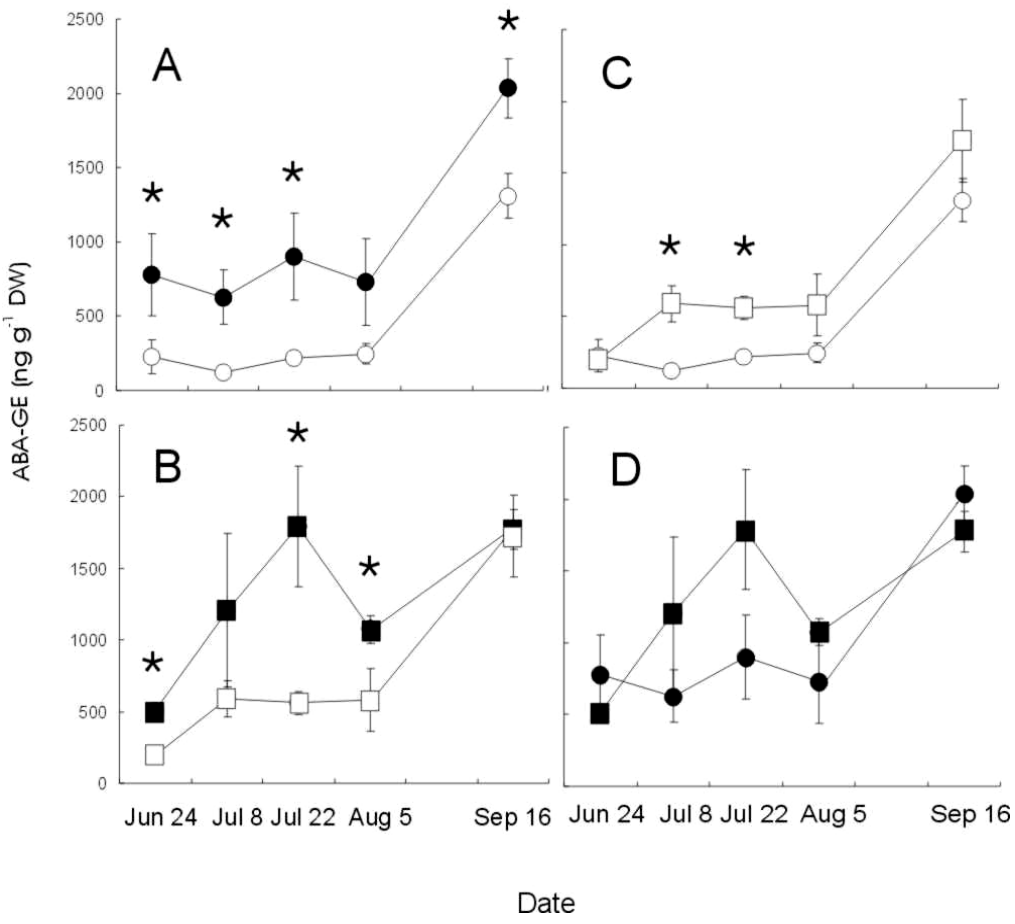

Figure 10 Changes in concentrations of endogenous ABA-GE in lodgepole pine long-shoot buds from summer to the fall. (A) The two parts of good female cone producer; (B) the two parts of poor female cone producer; (C) distal parts of good and poor female cone producer; (D) proximal parts of good and poor female cone producer. Circle - good female cone producer; Square - poor female cone producer; Open - distal parts of long-shoot buds; Solid – proximal parts of long-shoot buds. Mean ± SE, n=3 genotypes. Asterisk (*) indicates significant difference ($P < 0.05$) between two different samples at each individual time point.

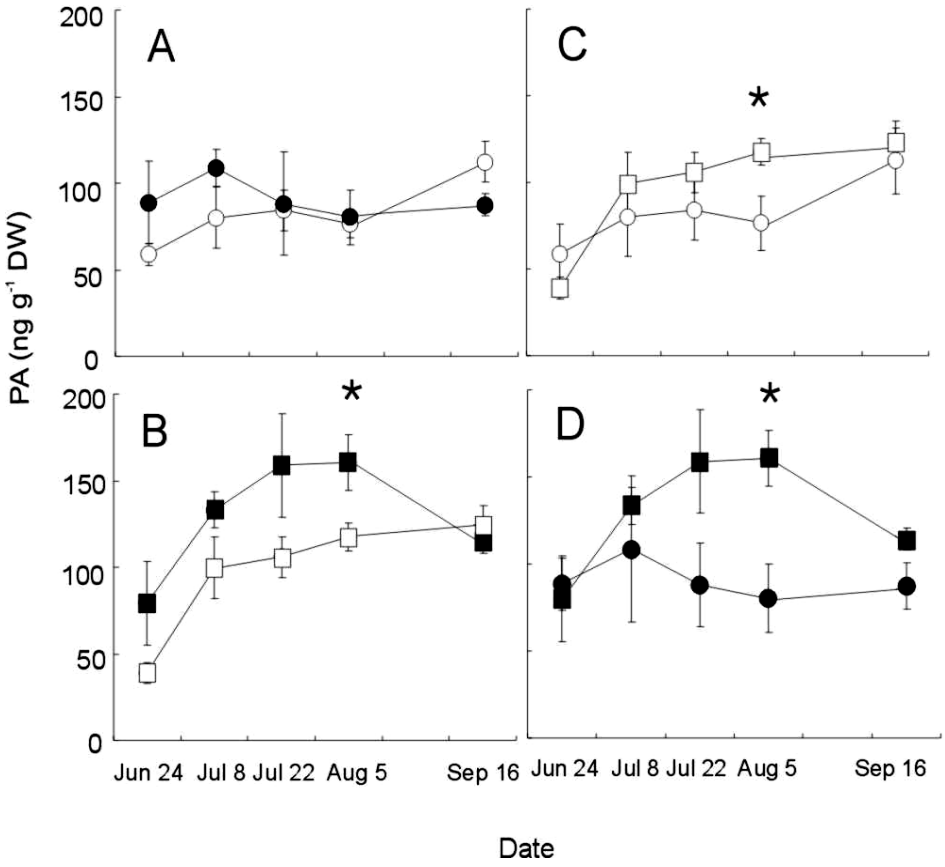

Figure 11 Changes in concentrations of endogenous PA in lodgepole pine long-shoot buds from summer to the fall. (A) The two parts of good female cone producer; (B) the two parts of poor female cone producer; (C) distal parts of good and poor female cone producer; (D) proximal parts of good and poor female cone producer. Circle - good female cone producer; Square - poor female cone producer; Open - distal parts of long-shoot buds; Solid – proximal parts of long-shoot buds. Mean ± SE, n=3 genotypes. Asterisk (*) indicates significant difference ($P < 0.05$) between two different samples at each individual time point.