J Plant Growth Regul (2012) 31:478–489
DOI 10.1007/s00344-011-9257-1

Analysis of Phytohormone Profiles during Male and Female Cone Initiation and Early Differentiation in Long-shoot Buds of Lodgepole Pine

Lisheng Kong · Patrick von Aderkas · Irina Zaharia · Suzanne R. Abrams · Tim Lee · Jack Woods

Received: 22 May 2011 / Accepted: 5 December 2011 / Published online: 26 January 2012

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), 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 riboside (dhZR) existed in the distal parts of long-shoot buds, whereas concentrations of isopentenyl adenosine (iPA), IAA, 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 in either the distal or 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. 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.

Keywords Cone gender · Lodgepole pine · Long-shoot bud · Genotype · Phytohormone profile

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Introduction

Cone bud initiation and gender differentiation is site-specific in *Pinus* species. Female cone buds develop only 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 vary by gender: male cones differentiate earlier than female (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 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 and others 1993; King and others 2006) and development (Li and others 2010). In *Arabidopsis thaliana*, increase in endogenous cytokinins enhanced meristematic activity and floral organ development, both of which subsequently had a positive influence on seed yield (Bartrina and others 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 and others 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 the PGR, timing of application, tree genotype, and 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 and others 2004, 2007; Chiwocha and others 2007) and long shoots (Kong and others 2008, 2009). The major advantage of this approach is that multiple compounds in the same plant sample can be analyzed.

The objective of this study was to investigate endogenous phytohormones and metabolites in distal and 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 cytokinins, auxin, and 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 fourfold between the high- and the low-yield group. One sample was collected from each ramet at each time point. Data were 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 bud initiation and differentiation at regular intervals of 2 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. Table 2 shows the relationship between the time point of sampling and the stages of cone bud development. The number of long-shoot buds for one sample ranged from as many as 20 buds at the early season to as few as 10 in the late season. The reason for this difference in collecting samples was related to mass—early

Table 1 Female cone yield of six different lodgepole pine genotypes

High yield		Low yield	
Genotype	♀ cone yield/tree	Genotype	♀ cone yield/tree
472	128.7(a), 114.6(a), 72.1(a)	224	27.5(b), 25.3(b), 21.8(b)
1779	105.2(a), 116.5(a), 105.2(a)	423	21.3(b), 25.0(b), 20.5(b)
502	113.2(a), 107.5(a), 85.2(a)	402	23.59(b), 26.2(b), 19.8(b)
	105.4 (a)		23.4 (b)

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, from left to right. The overall analysis of 3 years of data is given in the bottom row. Significant differences at $P < 0.05$ are indicated by different letters for data generated from the same period of time. Mean, $n = 9$

Table 2 Relationship between the time point of hormone measurements and cone bud developmental stages in a long-shoot bud

24 Jun	8 Jul	22 Jul	5 Aug	16 Sep
Cone buds were undifferentiated in a long-shoot bud.	Differentiation of male cone bud almost completed with well-developed scale primordia.	Differentiation of female cone buds just started with an enlarged apical meristem.	Differentiation of female cone bud continued with small bract primordia at the base area.	Differentiation of female cone bud almost completed with more well-developed bract primordia.

Graphs in the bottom row show, from left to right, an undifferentiated cone bud, a male cone bud, and female cone buds at early developmental stages. Scale bar = 1 mm

in the season buds were much smaller and collecting sufficient samples 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 two-fifths were measured and separated from the lower three-fifths 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 aluminum 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.

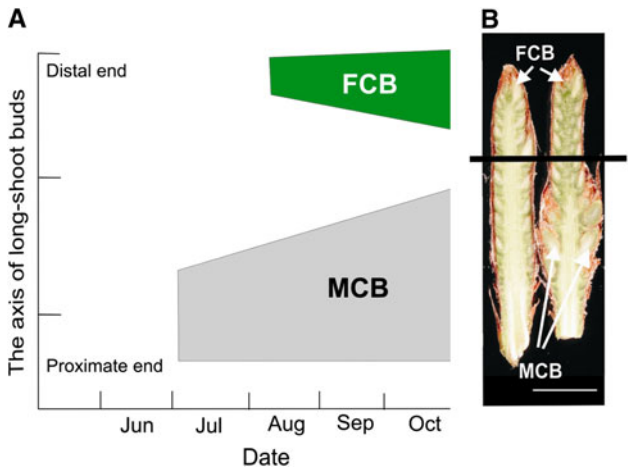

Fig. 1 Development of cone buds in a long-shoot bud of lodgepole pine. **a** Graph showing a temporal and spatial relationship for both male cone and female cone bud development. **b** Photo showing two longitudinally cut long-shoot buds at the stage when differentiation of the female cone bud is almost completed. The horizontal line indicates the cut for separating distal and proximal parts of long-shoot buds for hormone analysis. MCB, male cone bud; FCB, female cone bud. Scale bar = 1 cm

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) were 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), that is, 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 were from PBI-NRC; d_5-IAA was 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 were from Olchemim Ltd.; d_2-GAs were purchased from Prof. Lewis Mander. 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 were from PBI-NRC.

Extraction, Purification, and Quantification by HPLC–ESI–MS/MS

Extraction and purification steps were carried out according to previous methods (Kong and others 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, and *t*-Z-*O*-Glu), is as described in Chiwocha and others (2003, 2005) with modifications. Chromatographically separated *t*-Z, *c*-Z, and dhZ are measured using d_3-dhZ as an internal standard, whereas *t*-ZR, *c*-ZR, and dhZR are measured against d_3-dhZR as an internal standard. 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 and others (2004). QC samples, internal standard blanks, and solvent blanks were also prepared and analyzed along with each batch of tissue samples.

Of the 11 GAs investigated in this study, they were either undetectable or below quantifiable levels due to the presence of interfering compounds in the samples. As a consequence, GAs are not included in the results.

Results

Cytokinins

Concentration levels of *t*-ZR were similar in both distal and proximal parts sampled on June 24. In a manner independent of cone production capability, *t*-ZR concentrations decreased up to twofold in the proximal part, but it showed little changes in the distal part (Fig. 2a, b). The overall patterns of *t*-ZR changes were significantly different between distal parts and proximal parts in both the good cone producers ($F = 15.31$, $P = 0.001$) and 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 concentrations existed in poor cone producers (Fig. 2c).

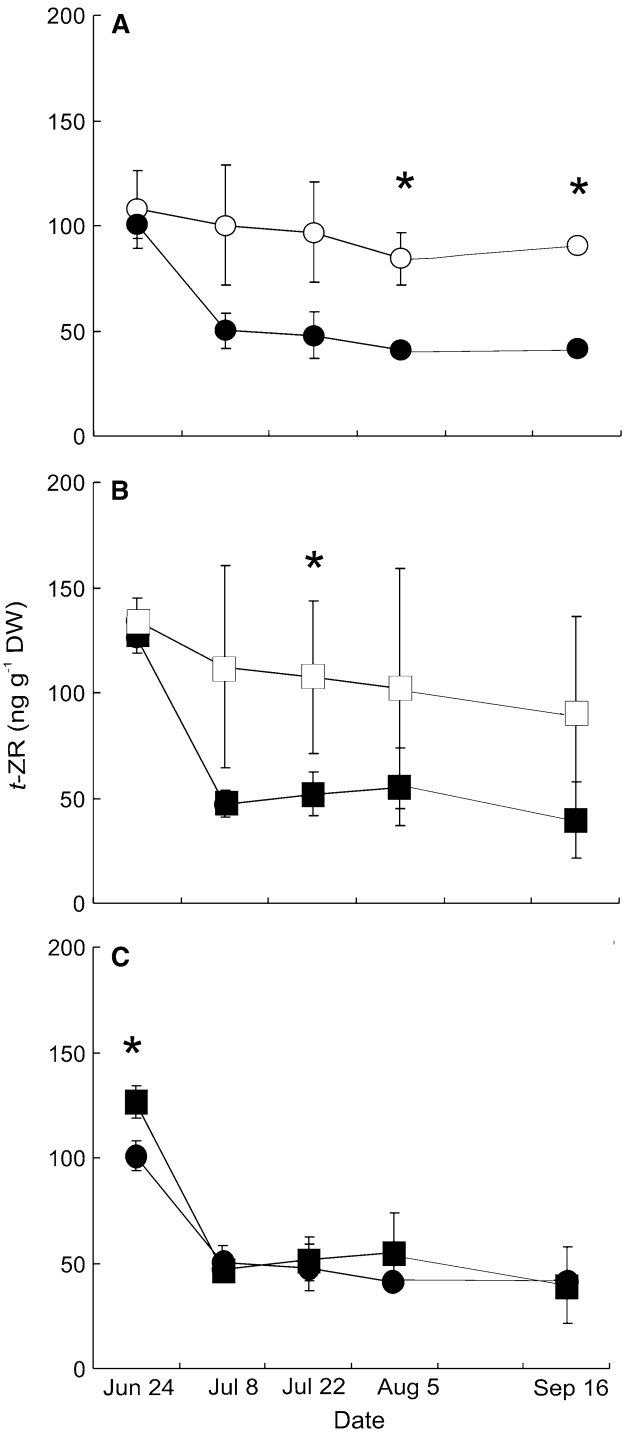

Fig. 2 Changes in concentrations of endogenous *t*-ZR in lodgepole pine long-shoot buds from summer to the fall. **a** The two parts of good female cone producer. **b** The two parts of poor female cone producer. **c** Proximal parts of good and poor female cone producer. Circle, good female cone producer; square, poor female cone producer; open symbol, distal parts of long-shoot buds; solid symbol, 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

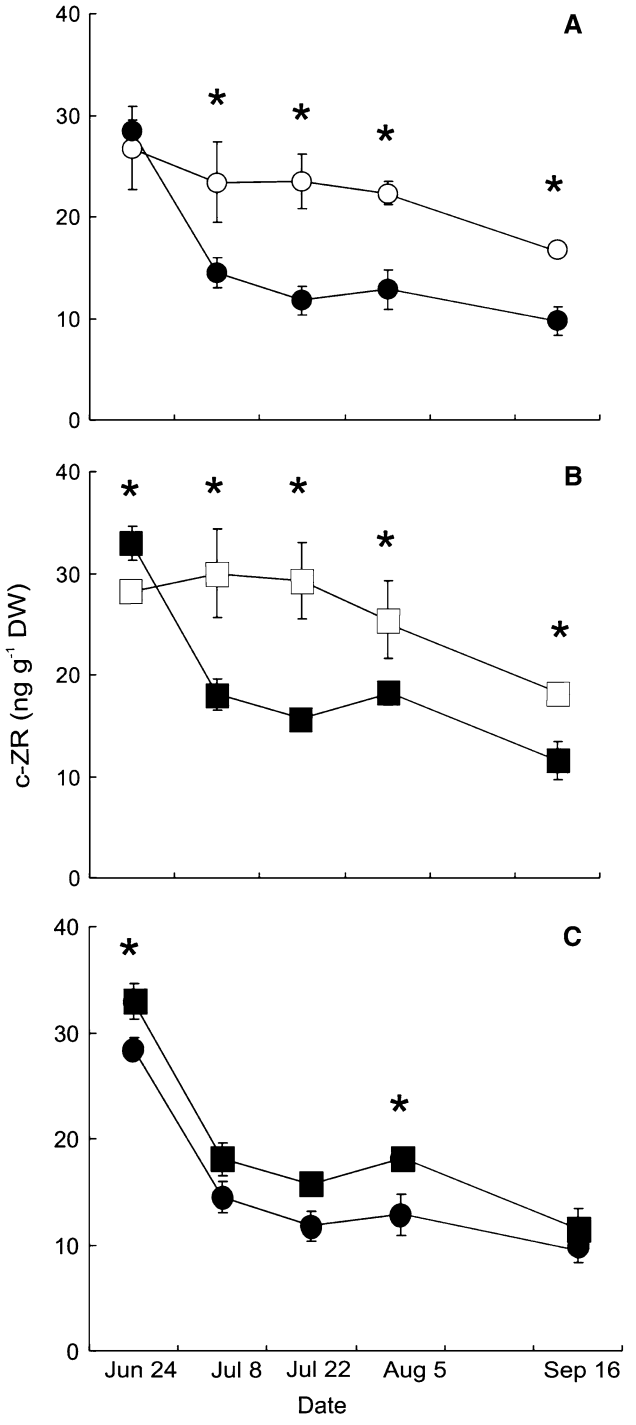

Fig. 3 Changes in concentrations of endogenous *c*-ZR in lodgepole pine long-shoot buds from summer to the fall. **a** The two parts of good female cone producer. **b** the two parts of poor female cone producer. **c** Proximal parts of good and poor female cone producer. Circle, good female cone producer; square, poor female cone producer; open symbols, distal parts of long-shoot buds; solid symbols, 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

On June 24, *c*-ZR concentrations were similar in the two bud parts of good cone producers (Fig. 3a), whereas they were higher in the proximal parts in the poor cone producers (Fig. 3b). Thereafter, *c*-ZR concentrations declined at different rates in both of the bud parts resulting in higher levels, up to twofold, in distal parts than those in proximal parts in all genotypes (Fig. 3a, 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 relating 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 latter case (Fig. 3c).

Concentrations of dhZR were higher in distal parts than in proximal parts in all genotypes in spite of cone production capability (Fig. 4a, c). Differences of the overall pattern were spatially significant in good cone producers ($F = 15.31$, $P = 0.001$) and 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 ($F = 0.19$, $P = 0.666$). Concentrations of dhZ showed little change in all the samples (Fig. 4b, d). Concentrations of *t*-Z were generally below quantifiable levels.

Initially, iPA concentrations were higher in proximal parts than in distal parts at June 24 in all genotypes (Fig. 5a, b). Thereafter, there was no difference in iPA concentrations until September 16, when iPA concentration was higher in distal parts of good cone producers (Fig. 5a). The overall patterns were not significantly different ($F = 0.81$, $P = 0.375$ 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). 2iP could not be detected and quantified consistently.

The ratios of zeatin (Z)-type cytokinins, including *t*-ZR, *c*-ZR, dhZR, and dhZ, to isopentenyl (iP)-type cytokinins, that is, iPA and 2iP, were at similar levels in both distal and proximal parts of long-shoot buds in good cone producers (Fig. 6a). 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. 6b). When compared with poor cone producers, this ratio was much higher in distal parts of the good cone

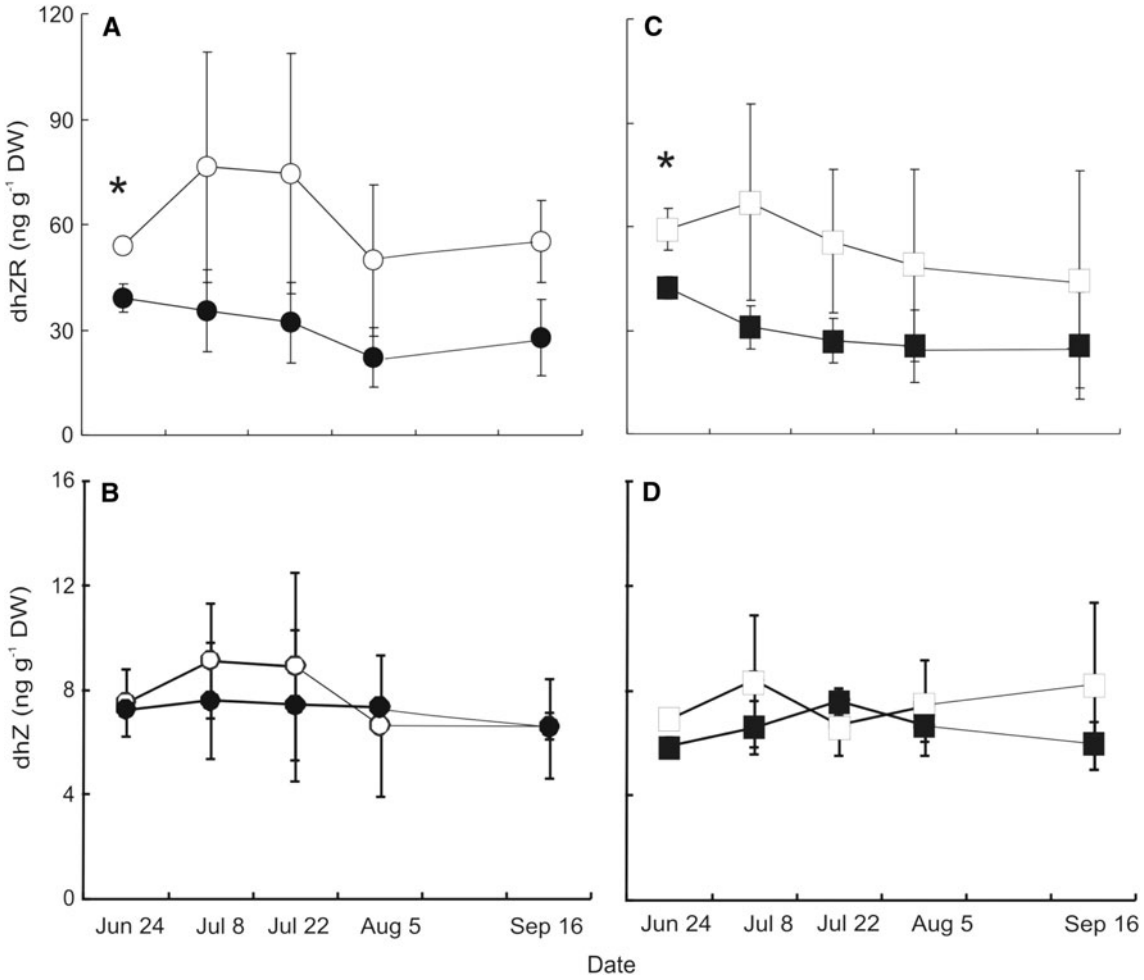

Fig. 4 Changes in concentrations of endogenous dhZR and dhZ in lodgepole pine long-shoot buds from summer to the fall. **a**, **b** The two parts of good female cone producer. **c**, **d** The two parts of poor female cone producer. Circle, good female cone producer; square, poor female cone producer; open symbols, distal parts of long-shoot buds; solid symbols, 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

producers at July 22 prior to female cone differentiation (Fig. 6c). A similar pattern of the ratio changes was also found in proximal parts (Fig. 6d).

Auxin

Concentrations of IAA were up to 2.6-fold higher in proximal parts than in distal parts in all genotypes between June and August (Fig. 7a, 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 relating 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.

Abscisic Acid

Although concentrations of ABA were up to 1.7-fold higher in proximal parts than in distal parts in all genotypes between June 24 and August 5 (Fig. 8a, b), the overall patterns of ABA were not significantly different between distal parts and proximal parts, that is, $F = 1.13$, $P = 0.297$ for good producers and $F = 0.32$, $P = 0.576$ 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 fivefold higher in proximal parts than in distal parts at most

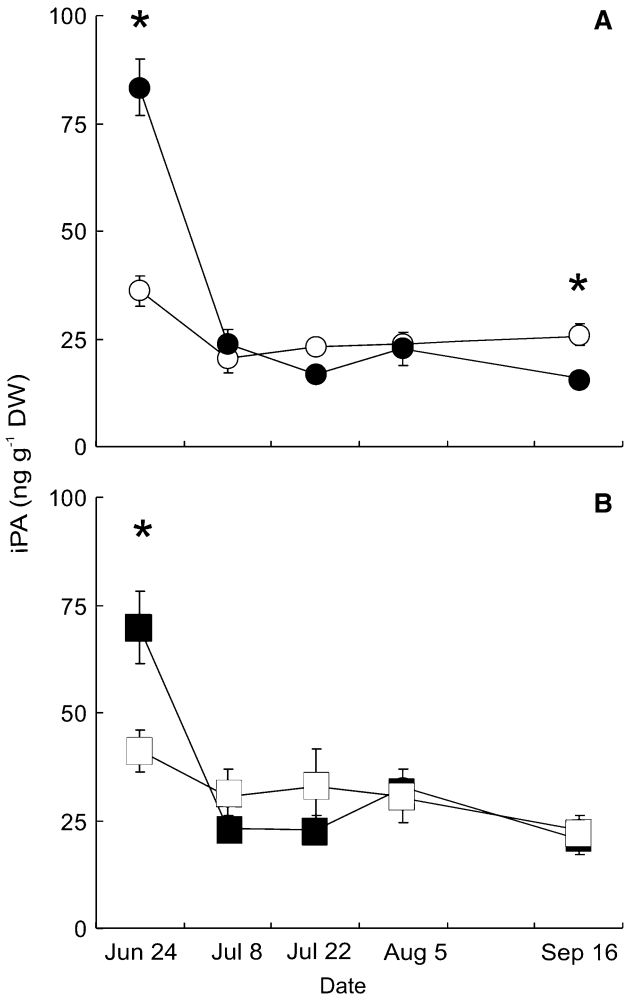

Fig. 5 Changes in concentrations of endogenous iPA in lodgepole pine long-shoot buds from summer to the fall. **a** The two parts of good female cone producer. **b** The two parts of poor female cone producer. Circle, good female cone producer; square, poor female cone producer; open symbols, distal parts of long-shoot buds; solid symbols, 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

sampling points (Fig. 9a, b). Compared with good cone producers, concentrations of ABA-GE were higher in poor cone producers in both distal parts (Fig. 9c) and proximal parts (Fig. 9d). The overall patterns of ABA-GE were significantly different between the two parts, that is, $F = 8.02$, $P = 0.008$ 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, no significant differences were found in either distal parts ($F = 2.43$, $P = 0.131$) or proximal parts ($F = 1.12$, $P = 0.300$).

Concentrations of PA were generally higher in proximal parts than in distal parts (Fig. 10a, b) in all genotypes. This spatial difference was larger in genotypes of low female cone yield (Fig. 10b). 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. 10c, 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 relating 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

Onto 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-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, and iPA showed substantial changes, especially in the proximal (male) parts. This period corresponds to male cone bud differentiation (von Aderkas and others 2007). The second time point was between July 22 and August 5 when changes in concentrations of dhZR, 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 bud differentiation is after male cone bud differentiation, which means at this point female cone buds are differentiating along the long-shoot axis. These two points (June 24–July 8 and July 22–August 8) may represent points at which exogenous application of plant growth-regulating substances (for example, commercial gibberellin mixtures used in seed orchards) may have the most effect when differentiation of male and female cone buds start, respectively (O'Reilly and Owens 1987; Ross and Pharis 1987; Almqvist 2003; von Aderkas and others 2007).

One group of hormones and related metabolites that deserve further exploration in pine cone bud initiation are

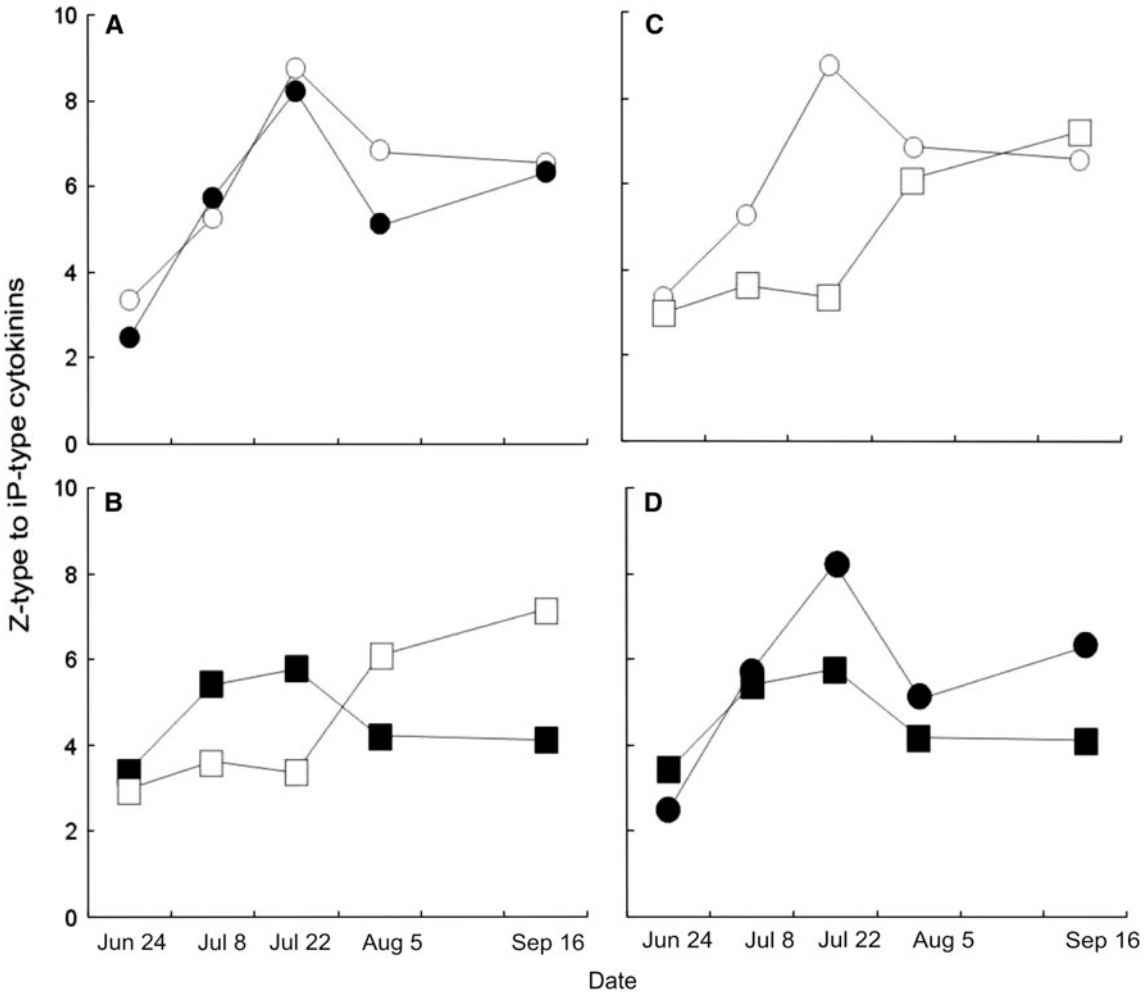

Fig. 6 Changes in the ratio of Z-type to iP-type cytokinins in lodgepole pine long-shoot buds from summer to the fall. **a** The two parts of good female cone producer. **b** The two parts of poor female cone producer. **c** Distal parts of good and poor female cone producer. **d** Proximal parts of good and poor female cone producer. Circle, good female cone producer; square, poor female cone producer; open symbols, distal parts of long-shoot buds; solid symbols, proximal parts of long-shoot buds. Mean ± SE, $n = 3$ genotypes

cytokinins. In conifers, cytokinins participate in regulation of bud differentiation and development (Imbault and others 1988; Bollmark and others 1995; Chen and others 1996; Zhang and others 2001, 2003). Exogenously applied benzylaminopurine (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. Because Z-type cytokinins are derived from iP-type compounds and not vice versa (Kakimoto 2003; Sakakibara 2006), the low ratio of Z-type to iP-type cytokinins indicates lower activity of cytokinin synthesis. It has been suggested that Z/iP is a useful index of aging and vigor in *P. radiata* (Valdés and others 2002, 2003) and Scots pine (Valdés and others 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 and others 1990). In our study, a higher ratio of Z-type 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-type or iP-type cytokinins (Spíchal and others 2004; Romanov and others 2006) because xylem sap contains mainly Z-type cytokinins, whereas phloem sap contains mainly iP-type cytokinins (Corbesier and others 2003); such compartmentalization might function in regulating cytokinin signaling (Hirose and others 2008). Cell

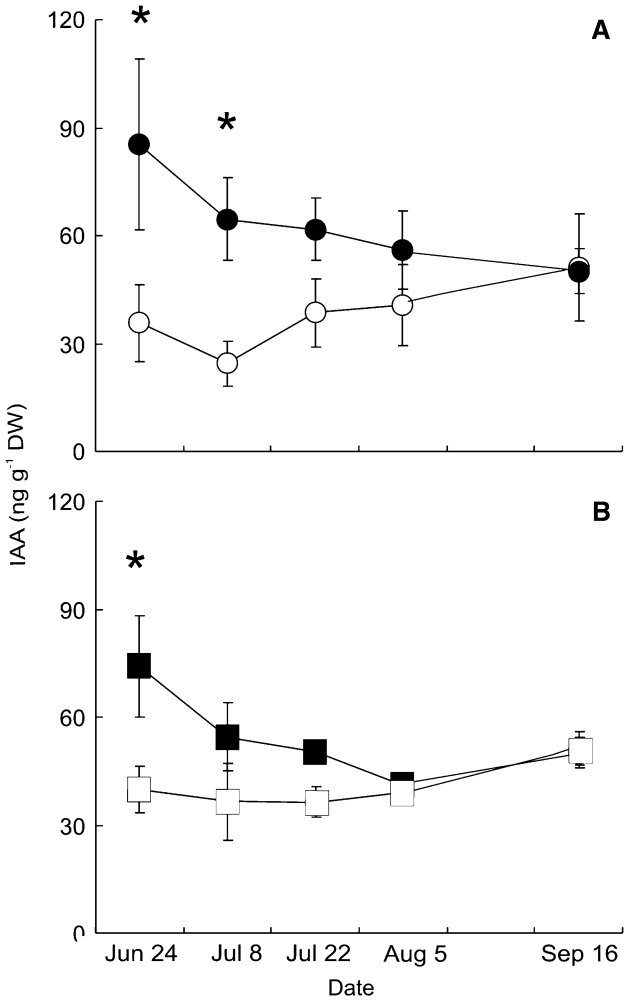

Fig. 7 Changes in concentrations of endogenous IAA in lodgepole pine long-shoot buds from summer to the fall. **a** The two parts of good female cone producer. **b** The two parts of poor female cone producer. Circle, good female cone producer; square, poor female cone producer; open symbols, distal parts of long-shoot buds; solid symbols, 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

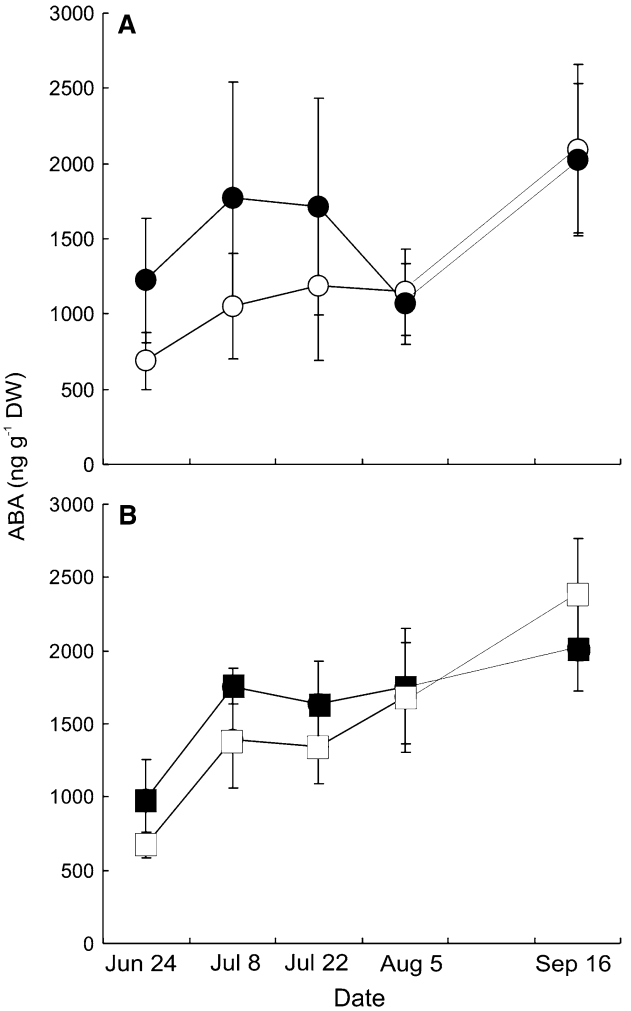

Fig. 8 Changes in concentrations of endogenous ABA in lodgepole pine long-shoot buds from summer to the fall. **a** The two parts of good female cone producer. **b** The two parts of poor female cone producer. Circle, good female cone producer; square, poor female cone producer; open symbols, distal parts of long-shoot buds; solid symbols, proximal parts of long-shoot buds. Mean ± SE, $n = 3$ genotypes

fate and organ formation may also be associated with local concentrations of Z-type and iP-type cytokinins (Frugis and others 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 and others 1991; Uggla and others 2001). Although it is unknown if cambial growth is occurring in long-shoot buds, IAA may be involved in male cone development because higher IAA concentrations were consistently observed in the proximal parts during male cone bud development. For cone induction, auxins are usually applied together with GAs to enhance GA effects (Pharis and others 1980). When applied alone, auxin stimulated male cone formation (Sheng and Wang 1990). Usually, auxin is regarded as being synthesized at the apical location and transported downward. In this case, IAA concentrations in distal parts should be higher than in proximal parts. Recently, local hormone synthesis was reported in coniferous trees (Rasmussen and others 2009) and other plants (Ljung and others 2001). Concentrations of IAA in the proximal part were higher in the good female cone producers than in the poor ones. A possible interpretation is that higher IAA concentrations in the lower parts of good genotypes may contribute to vigorous long-shoot growth, resulting in higher female cone yield. In our previous studies, higher IAA concentrations were found in

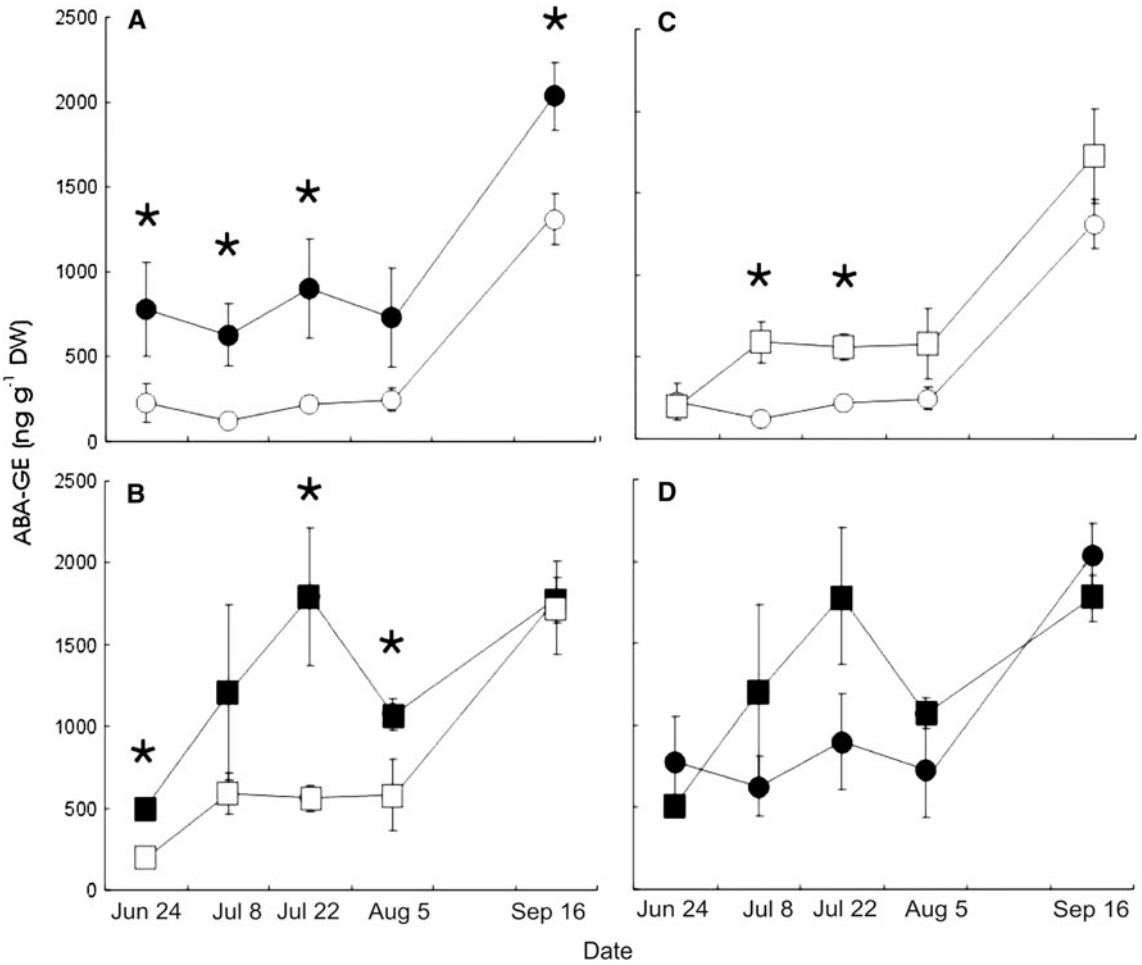

Fig. 9 Changes in concentrations of endogenous ABA-GE in lodgepole pine long-shoot buds from summer to the fall. **a** The two parts of good female cone producer. **b** The two parts of poor female cone producer. **c** Distal parts of good and poor female cone producer. **d** Proximal parts of good and poor female cone producer. Circle, good female cone producer; square, poor female cone producer; open symbols, distal parts of long-shoot buds; solid symbols, 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

developing long shoots of good cone producers in Douglas-fir (Kong and others 2009).

Our study reveals that significant differences exist between different sites within long-shoot buds. This phenomenon may relate to female cone production. We found that ABA metabolites ABA-GE and PA had higher concentrations in the distal portions of poor-yield genotypes than in those of good-yield genotypes. Higher concentrations of ABA metabolites might reflect more active ABA metabolism when ABA level showed no difference. High concentrations of ABA were reported in female-sterile Chinese red pine (Bao and Zheng 2005), whereas ABA-GE is generally regarded as functionally inactive or as a storage form of ABA.

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, whereas 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 bud initiation and differentiation.

In this article we have shown that temporal and spatial differences exist in a number of classes of phytohormones within a long-shoot bud. Some of these changes, particularly in cytokinin and ABA metabolite distributions, correlate well with known gender differences within long-shoot buds. 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 the future.

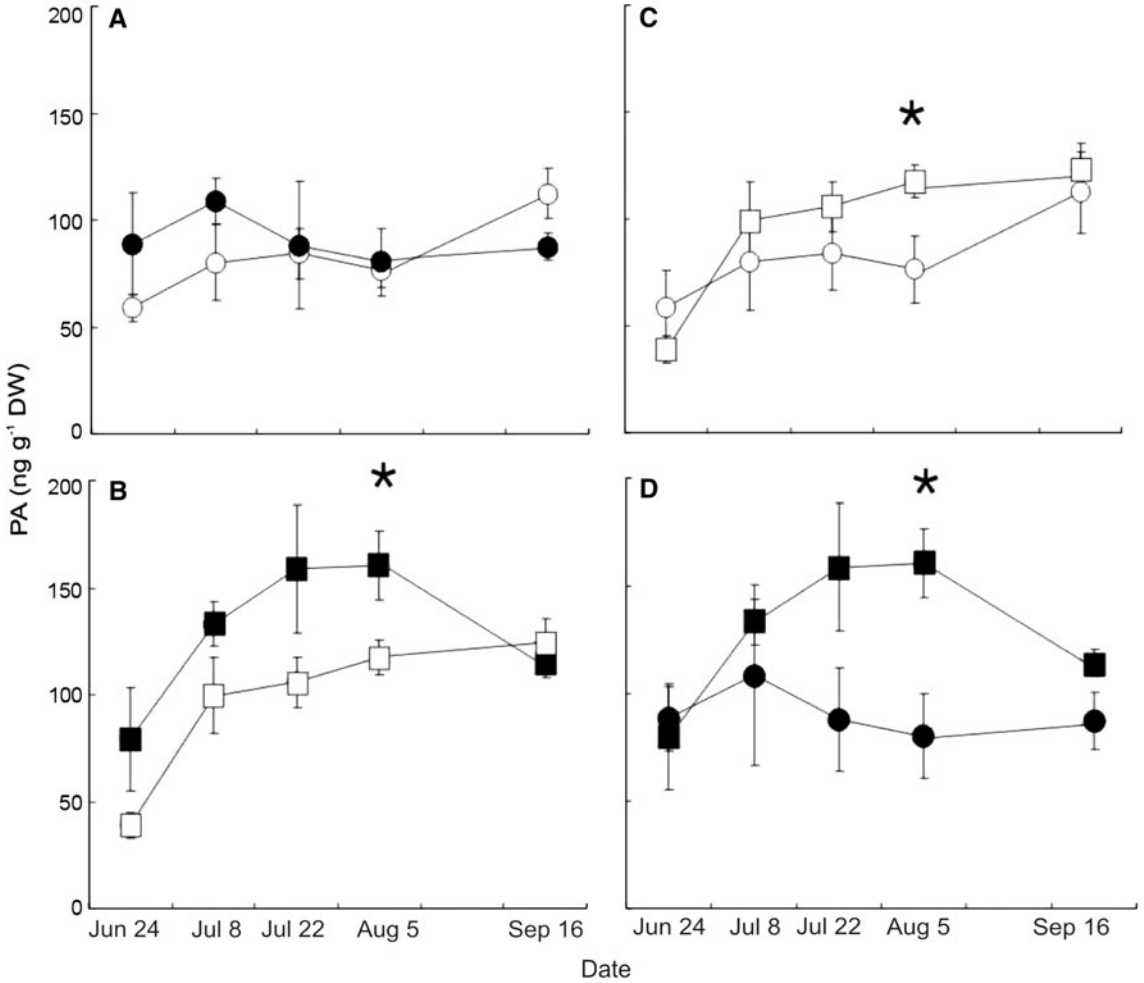

Fig. 10 Changes in concentrations of endogenous PA in lodgepole pine long-shoot buds from summer to the fall. **a** The two parts of good female cone producer. **b** The two parts of poor female cone producer. **c** Distal parts of good and poor female cone producer. **d** Proximal parts of good and poor female cone producer. Circle, good female cone producer; square, poor female cone producer; open symbols, distal parts of long-shoot buds; solid symbols, 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

Acknowledgments The cone induction project was funded through the British Columbia Forest Investment Account, Forest Genetics Conservation and Management Program under the Business Plan of the Forest Genetics Council of British Columbia (FGCBC), and the Natural Sciences and Engineering Research Council of Canada Discovery Program (PvA). We express our appreciation to all members of the project steering committee and Valerie Ashley (Kalamalka Research Station), Tia Wagner and Dan Gaudet (Vernon Seed Orchard Company), Julia Gill (University of Victoria), Dr. Cunxu Zhang (Northwest A & F University, China), and Monika Lafond, Vera Čekić, and Stacey J. Owen (NRC-Plant Biotechnology Institute) for their help in this research.

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