[Internal use only]

Cone induction and hormone metabolomics of lodgepole pine and Douglas-fir

(May 2006 - March 2010)

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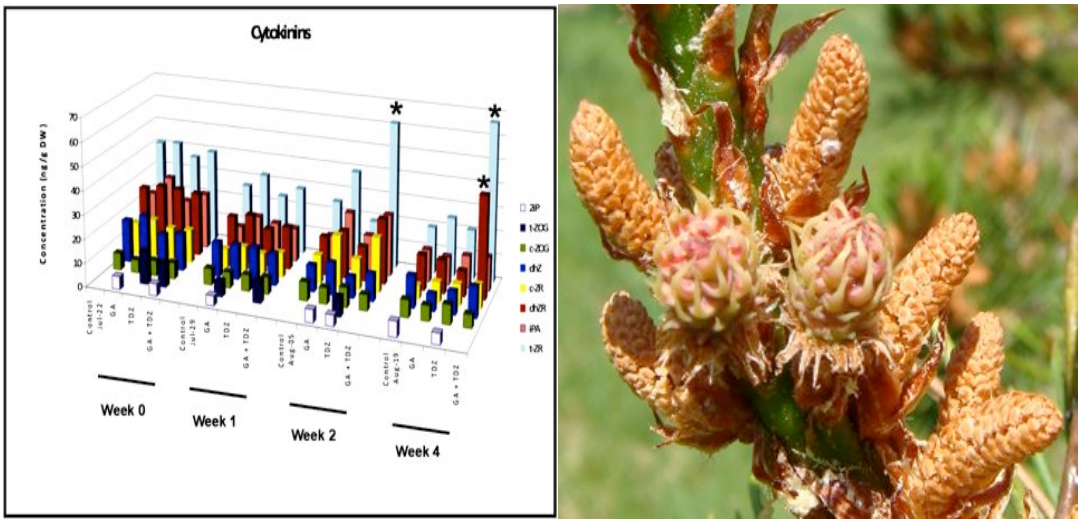

Left: Cytokinin profiles during cone induction; Right: PGR-induced cone gender change in lodgepole pine

Contents

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Summary

A metabolomic approach to cone bud development provides simultaneous profiling of many hormones and their metabolites. The abundant data derived from metabolomics allowed us to separate the physiological effects of physical cone induction treatments from those of chemical cone induction treatments. Although both types of treatment can induce cones only chemical treatments caused large hormonal responses. Cone induction does not appear to be regulated by a single hormone pathway; in fact, cone induction can occur without any accompanying hormonal change. Nevertheless, once metabolomic analysis of phytohormones is used during responses occur, it provides valuable information for method development of cone induction. The data is essential for method development. Cytokinin analysis of pine long-shoot buds provided us with a solid theoretical basis to explore exogenous TDZ treatment, which resulted in many more female cones in lodgepole pine. The encouraging results prove that a research strategy in which metabolomics, a relatively broad bias-free analytical tool, can be combined with field experiments to develop new and successful methods for cone induction.

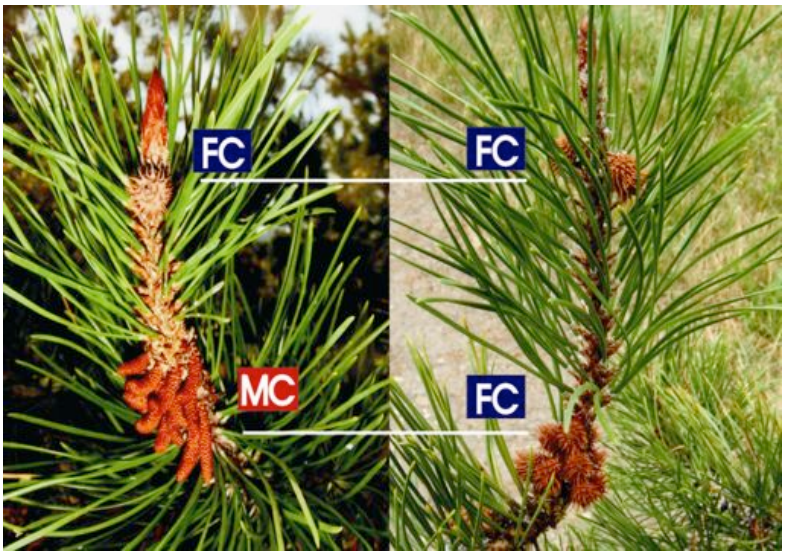

Figure 1. Effects of PGRs on cone gender determination in lodgepole pine. Female cones (FC) were induced at the location where male cones (MC) usually form.

Abbreviations

abscisic acid (ABA)

ABA glucose ester (ABA-GE)

benzyladenine (BA)

cytokinin (CK)

2,4-dichlorophenoxyacetic acid (2,4-D)

dihydrophaseic acid (DPA)

dihydrozeatin riboside (dhZR)

dihydrozeatin (dhZ), electrospray(ES)

electrospray ionisation (ESI)

gibberellin (GA)

7'-hydroxy ABA (7'OH-ABA)

indole-3-acetic acid (IAA)

indole-3-aspartate (IAA-Asp)

indole-3-glutamate (IAA-Glu)

isopentenyladenine (2iP)

isopentynyladenosine (iPA)

mass spectrometry (MS)

multiple reaction monitoring (MRM)

methylglyoxal bis(guanylhydrazone) (MGBG)

naphthylacetic acid (NAA)

*neo*phaseic acid (*neo*PA)

phaseic acid (PA)

plant growth regulator (PGR)

trans-zeatin (*t*-Z)

trans-zeatin-*O*-glucoside (*t*-Z-*O*-Glu)

trans-zeatin riboside (*t*-ZR)

2,3,5-triiodobenzoic acid (TIBA)

thidiazuron (TDZ)

Part 1 Cone induction project

1. Introduction

Conifers are economically and ecologically important forest species. In countries where reforestation efforts use superior genotypes that have been selected and propagated via tree improvement programs, there is high demand for elite seed. Unfortunately, production can vary from one year to the next. A major bottleneck of seed production is the low yield of female cones. Consequently, improving the efficiency of cone induction methods is of great interest to seed-producing orchards.

There are two goals when inducing cone buds. The first one is to induce precocity. Juvenile asexual growth is switched to mature sexual growth, which is useful in accelerating tree-breeding programs. The second is to increase female cone yield from mature trees in order to produce more seeds. Traditional methods for increasing seed yield aim to manipulate physiological conditions of the parent trees, which in turn enhances flowering. Cone yield enhancement is achieved by either physically stressing the trees, altering the tree's nutritional status, or by applying plant growth regulators (PGRs) such as gibberellins (GAs). Physical treatments are either applied to the aboveground parts of trees (shoot training, stem-girdling, scoring) or to the root system (pruning, trickle irrigation, fertigation, confinement). Some treatments are combinations of these methods in order to enhance the effects of each single treatment.

Plant hormones play an important role in generating or maintaining a balance of the factors to initiate flowering process. The history of using exogenously applied PGRs to induce flowers in coniferous species began in the middle of 1950s when GAs were successfully used for inducing cones in Cupressaceae and Taxodiaceae. Thereafter, GAs have been tested and proven effective in cone induction in many coniferous species. Some effort, albeit limited, was also made to use other PGRs alone or in combination for cone enhancement in Pinaceae.

Practical manipulation of sex expression may be possible by using different PGR combinations, but the development of such techniques for operational application requires a better basic understanding of the endogenous hormone levels and regulatory mechanisms involved in certain species. Historically, hormone levels were examined using bioassays, followed by enzyme-linked immunosorbent assays and

radioimmunoassays carried out in the 80s and early 90s. Limitations in sensitivity and specificity of these techniques have been largely overcome with the use of mass spectrometry. In several studies endogenous phytohormones, mainly GAs, have been analyzed in vegetative and reproductive buds of pinaceous conifers. In the last decade an efficient analytical technology has been developed and applied to coniferous species for phytohormone profiling by Chiwocha et al. (2003; 2005) and our papers on conifer applications (Kong et al. 2008; 2009). The original proposal approved by the Cone Induction Steering Committee in 2006 was to conduct a study of cone induction for two species of conifers.

The purpose of the study was to increase orchard seed yield of lodgepole pine (*Pinus contorta*) and Douglas-fir (*Pseudotsuga menziesii*) in British Columbia through the development and refinement of cultural methods for inducing female and male cones without damaging the long-term health and cone-production potential of the trees. This project included investigating profiles of the major hormone groups during cone induction. This would permit design of methods to exploit hormonal and stress-related pathways that regulate aspects of cone induction. It was anticipated that this would lead to new avenues of practical research. Promising methods were tested in existing seed orchards using both novel and existing techniques. Mass spectrometry (MS) was used to examine hormone profiles. MS methods allow identification and resolution of hormones and their related compounds. In addition, multiple reaction monitoring (MRM) permits simultaneous quantification of many hormones and their metabolites. It was anticipated that this work would lead to a much better understanding of hormone effects on bud differentiation, and that ultimately it would provide a basis for designing practical methods useful to seed orchard managers.

The basic research strategy for developing cone induction methods includes three major steps (Figure 1).

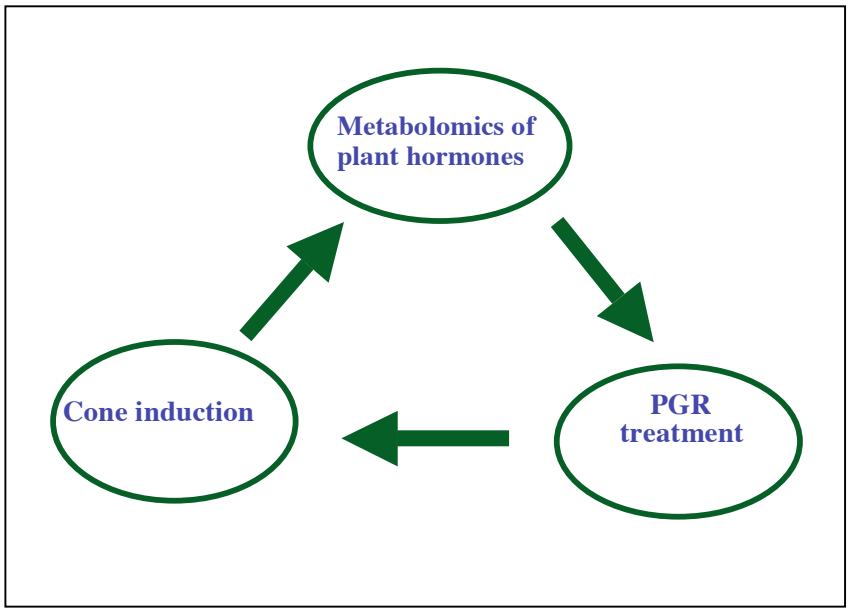

Figure 1. Strategy for method development for cone induction

Step I Apply cone induction treatments by using conventional methods, which include both physical and chemical treatments that have been proven effective for enhancing cone yield.

Step II Study metabolomics of plant hormones. A metabolomic approach is used to profile plant hormones and their metabolites in order to find changes caused by the treatments.

Step III Draw information from metabolomics and apply it to method development for cone induction by PGRs. Method development includes both developing new methods and optimizing the existing methods.

The three steps provide feedback one another allowing new treatments to be designed.

2. Yearly progress

In order to obtain basic information, trees of different untreated genotypes were used to profile plant hormones and their metabolites. Once trends in natural variability were established, treatments were applied. The following year's studies incorporated results of previous treatments. Besides analysis of plant hormones and metabolites, research included applications of either PGR or other chemicals such as phytohormone

inhibitors, methylglyoxal bis(guanylhydrazone) (MGBG) and 2,3,5-triiodobenzoic acid (TIBA) (Table 1). Cone yield data was collected two years after the treatment. In order to evaluate the response of trees to the treatments, estimations of cone yield were also made in the second year after treatments.

Table 1. Experiments from 2006 to 2009

2006-07	2007-08	2008-09	2009-10
Metabolomic studies			
Hormone profiling (Pli)	GA injection (Pli)	GA injection (Pli)	PGR branch Paste (Pli): TDZ, GA and combination
Hormone profiling (DF)	Root pruning-B (Pli)	Analysis of long-shoot buds (Pli)	
Root pruning -A (Pli)	TIBA injection (Pli)	Genotype response to GA injection (DF)	
GA injection (DF)	PGR injections (Pli): GA, BA and combination		
	Stem girdling (DF)		
PGR applications without metabolomic studies			
	GA and BA injections (Pli and DF)	GA and NAA injections (Pli and DF)	PGR bud paste (Pli)
	BA bud paste (Pli and DF)	TIBA, MGBG, TDZ injections (Pli and DF)	PGR branch paste (Pli)
	BA branch paste (Pli and DF)	Bud paste of PGR combinations (Pli)	

3. Current status and further research

This project had a three-year plan initially and was extended one extra year in order to reach the goal (Figure 2). During the period of four years by March 2010, we have had three seasons in which we were able to test induction techniques. We have only had two cone collections.

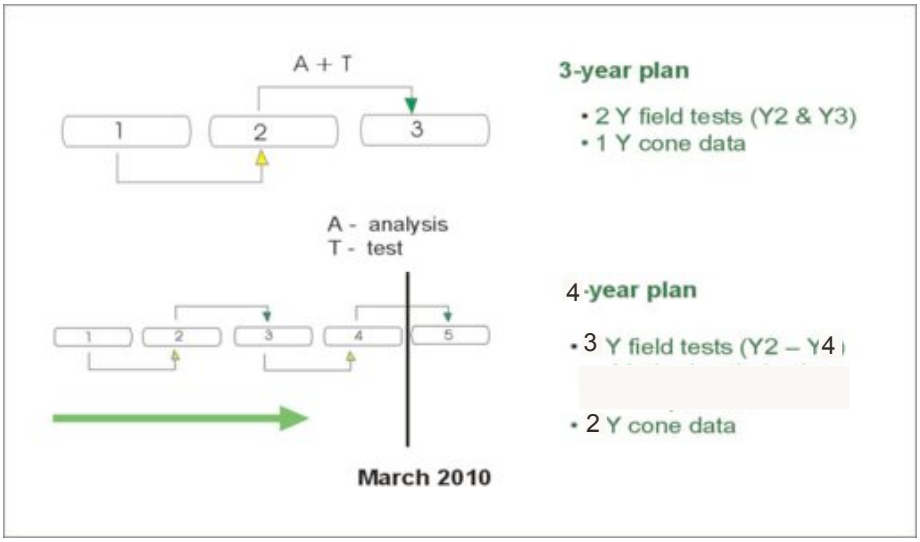

Figure 2. Cone induction plans

Part 2 Lodgepole pine

Lodgepole pine (*Pinus contorta* Dougl. ex Loud.) is an important forest species in western North America. Due to a recent severe outbreak of mountain pine beetle any seed is in great demand, let alone elite seed. Cone yields have been a problem in some of the seed orchards, particularly in the Okanagan region of British Columbia. Some research was carried out approximately 30 years ago on cone induction of this species (Wheeler et al, 1980). High efficiency of cone induction depends on multiple factors including PGR types, concentrations, and timing, method and frequency of application. Responses to PGR treatments appear to be genotype-dependent. Finally, the physiological condition of the trees is important.

Plant materials

Plant materials of lodgepole pine were collected from Vernon Seed Orchard Company, located in Vernon, British Columbia (50°13′N, 119°19′W).

Methods

Hormone profiling

Sample preparation: After collection, needles were removed from the stems of long shoots. Stem samples, or long-shoot buds, were wrapped in tin foil, labeled and kept frozen in a - 20 °C freezer for 2 d. Subsequently, the samples were lyophilized in a freeze-drier for 48 h after the vacuum was stabilized. Dry samples were sealed in plastic bags and stored at - 20 °C.

Metabolomic analysis: Pure hormone standards, used in calibration curve and quality control (QC) 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), dihydrozeatin riboside (dhZR), and *trans*-zeatin-*O*-glucoside (*t*-Z-*O*-Glu) from Olchemim Ltd. (Olomouc, Czech Republic); GA_1, GA_3, GA_4, and GA_7 from Prof. LewisMander (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, 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-GA_1, and d_2-GA_4 from Prof. Lewis Mander (Australian National University, Canberra, Australia). Bulk amounts of the deuterated forms of selected hormones which were used as recovery standards, namely d_6-ABA and d_2-ABA-GE, were obtained from PBI-NRC.

Extraction and purification steps were carried out as in Kong et al. (2008). The procedure used for quantification of multiple hormones was a modification of Chiwocha *et al.* (2003; 2005). 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 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). QC samples, internal standard blanks, and solvent blanks were also

prepared and analyzed along with each batch of tissue samples. Some hormone classes were proved difficult to extract and quantify.

Cone induction treatments

Cone induction methods used in this research include stem-injection of PGRs, bud and branch paste of PGRs, and root pruning. More details can be found in our annual reports.

List of experiments

1. Hormone profiling during long shoot and cone bud development

Experiment: General hormonal profile in relation to genotype and season (Report 2006/2007 pp. 27-30)

Goals: 1) To assess genotype variation of various hormones and their metabolites from nine genotypes of lodgepole pine. 2) To assess seasonal trends in hormone metabolite profiles.

Rationale: By selecting samples at regular time intervals over a one-year period, general seasonal increases and decreases in certain hormones would be apparent.

Results:

Detailed summery can be found in the 2006/07 report. The highlights are the following.

Auxins Endogenous IAA was quantified in most samples. Concentrations of IAA were generally low and no significant change occurred until Jan 24. No IAA catabolites, i.e. low amounts of IAA-Asp and IAA-Glu were detected but could not be quantified.

Cytokinins Endogenous CKs and metabolites were detected and quantified in most samples. *t*-ZR was the dominant one among them. It declined during the period of time from summer to winter. An exception to the common pattern was that of *t*-Z-*O*-Glu, which increased in the autumn (Oct.17). No obvious change was found in iPA concentrations in the apical buds from summer to winter.

Abscisic acid ABA and ABA-GE were detected and quantified in all the samples. Other ABA metabolites were found in only some of the samples. ABA and ABA-GE increased consistently from summer to winter. High amounts of ABA and ABA-GE were found in the samples collected in the winter.

Gibberellins Endogenous GA_1, GA_3, GA_4 and GA_7 were analyzed. Concentrations of endogenous GAs were generally lower than detectable or quantifiable levels.

Conclusions: The predominant cytokinin in the long shoot bud was *t*-ZR, with *t*-Z-*O*-Glu also present in small amounts. The concentration of iPA declined from summer to winter and then peaked in the next spring. Both ABA and ABA-GE exhibited the same pattern of concentration changes: a steady increase from summer to winter, followed by a decline between winter and spring. Concentrations of PA and DPA were lower than quantifiable levels during the summer and fall. They increased up to 135 and 191 ng g^{-1} DW in winter, respectively. During winter and spring, other phytohormones, including IAA, several GAs, were generally lower than quantifiable levels in this study.

Experiment: Dynamics of phytohormones and metabolites in long-shoot buds of multiple genotypes in lodgepole pine (Report 2008/2009 pp. 4-8)

Goals: The objective of this study was to investigate endogenous phytohormones and metabolites in either distal or proximal parts of long-shoot buds during cone bud initiation and differentiation in lodgepole pine.

Rationale: Cone production is genotype-dependent. On a long shoot, female cones are located at the upper part of the shoot while male cones are locating in the lower part. Hormone changes within long shoot buds have never been studied.

Plant materials: Six lodgepole pine genotypes were used in this experiment. These included three good cone producers and three poor ones (Table 2). Samples of long-shoot bud were collected at five time points. Buds were cut into two parts. The top part had one third of the bud length and the rest of the bud was used as the bottom part.

Results: In lodgepole pine, cone initiation and gender differentiation are site-specified within long-shoot buds. Female cones are distal and male cones are proximal (Figure 4). Profiles of cytokinins (Figures 5-9), IAA (Figure 10), GAs (Figure 11), ABA and their selected metabolites (Figures 12-14) showed differences according to location. Higher concentrations of *t*-ZR and dhZR were found in the distal parts, whereas concentrations of iPA, IAA, GA_{24}, ABA, ABA-GE and PA were higher in the proximal parts in all genotypes. Long-shoot buds of high cone producers had higher concentrations of *t*-ZR and higher ratios of zeatin (Z)-type to isopentenyl (iP)-type cytokinins than those of low cone producers. Concentration of dhZR or

IAA was higher either in the distal or in the proximal part respectively. Long-shoot buds of low cone producers had higher concentrations of *c*-ZR, iPA, ABA-GE and PA in both of the parts. Concentration of ABA was higher in the distal part and GA_{24} was higher in the proximal part. Concentrations of several hormone-related compounds changed in both late June and late July, prior to male and female cone bud differentiation.

Conclusions: Concentrations of phytohormones and metabolites were differentially distributed within long-shoot buds. Distal parts contained higher concentrations of several cytokinins and lower concentrations of ABA, auxin and GA_{24}. They also had higher ratios of Z-type cytokinins to iP-type cytokinins. Dynamic changes occurred in early and mid- summer. This research indicates that lower concentrations of ABA and higher concentrations of cytokinins, especially z-type cytokinins, may be related to female cone formation, whereas relatively higher concentrations of GA_{24} and IAA may correlate well with male cone buds.

Table 2. Female cone yield in 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 in three years	Genotype	♀ cone in three years
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)**

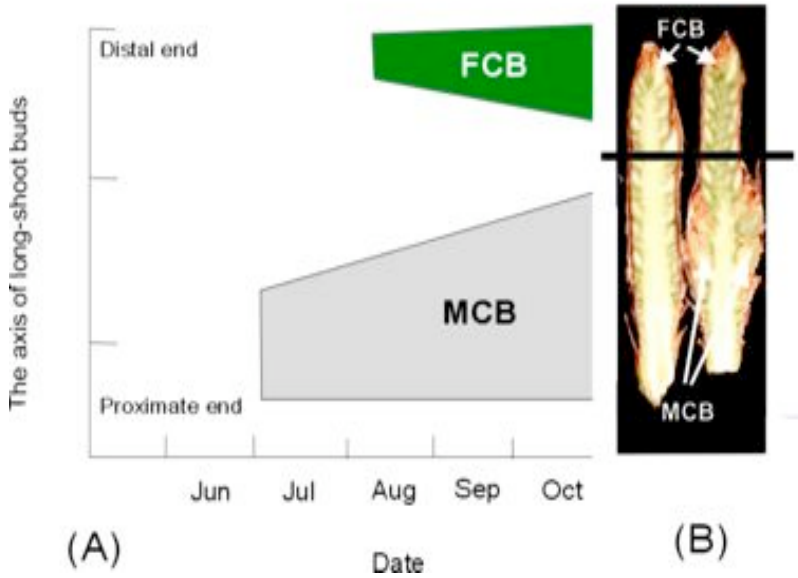

Figure 4. Development of cone buds in a long-shoot bud of lodgepole pine. (A) A graph showing a temporal and spatial relationship for both male cone and female cone bud development. (B) A photo showing two longitudinally-cut long-shoot buds. The horizontal line indicated the location of cut for separating distal and proximal parts of long-shoot buds for hormone analysis. Male cone bud (MCB), female cone bud (FCB).

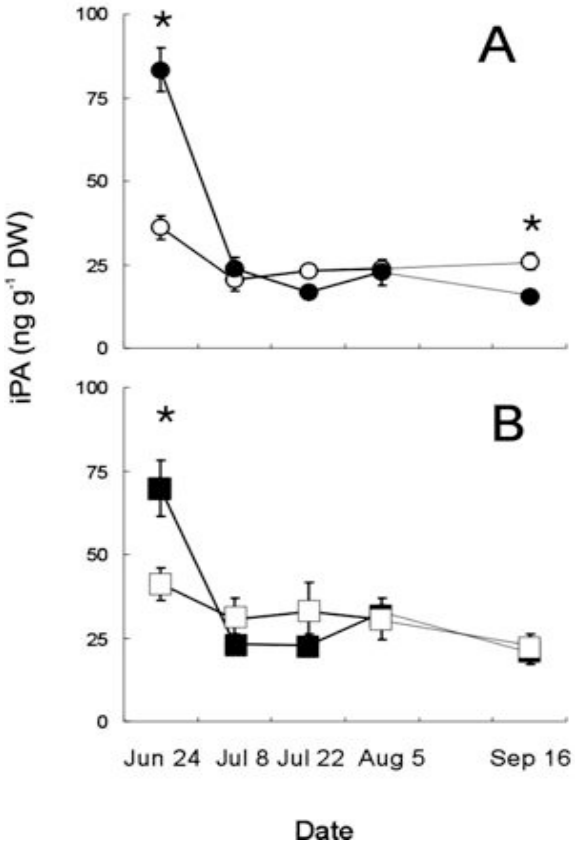

Figure 5. Changes in concentrations of endogenous iPA in lodgepole pine long-shoot buds from summer to the fall. (A) long-shoot buds of good cone producer; (B) long-shoot buds of poor cone producer. Open - distal parts; Solid – proximal parts. Mean ± SE, n=3 genotypes. Asterisk (*) indicates significant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

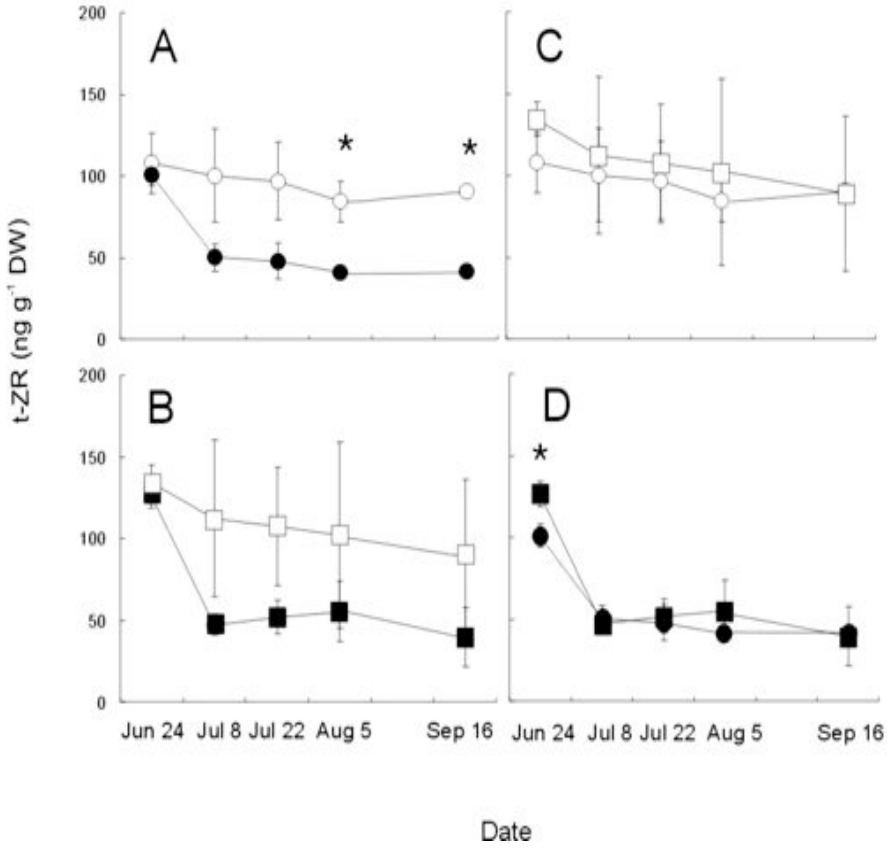

Figure 6. Changes in concentrations of endogenous *t*-ZR in lodgepole pine long-shoot buds from summer to the fall. (A) long-shoot buds of good cone producer; (B) long-shoot buds of poor cone producer; (C) proximal parts of long-shoot buds; (D) distal parts of long-shoot buds. Open circles - distal parts of good female cone producers; Solid circles – proximal parts of good female cone producers, Open square – distal parts of poor female cone producers; Solid square – proximal parts of poor female cone producers. Mean ± SE, n=3 genotypes. Asterisk (*) indicates significant difference ($P < 0.05$) between two different samples at each individual time point.

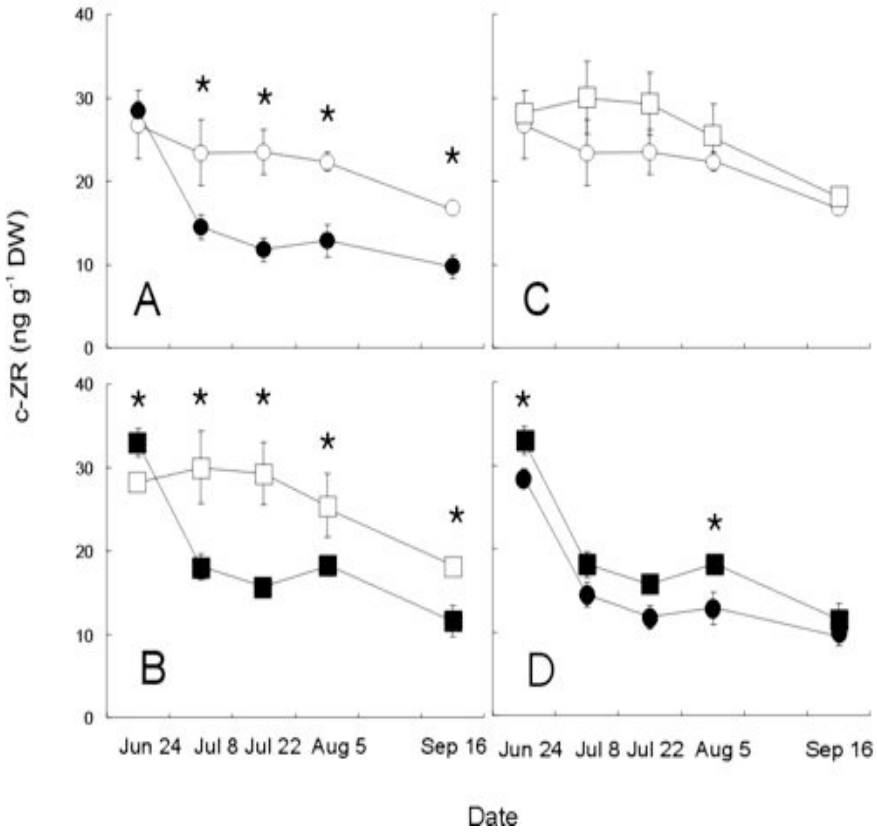

Figure 7. Changes in concentrations of endogenous *c*-ZR in lodgepole pine long-shoot buds from summer to the fall. (A) LSB, good ♀; (B) LSB, poor ♀; (C) proximal LSB; (D) distal LSB. Open circles - distal LSB, good ♀; Solid circles – proximal LSB, good ♀; Open square – distal LSB, poor ♀; Solid square – proximal LSB poor ♀. Mean ± SE, n=3 genotypes. Asterisk (*) indicates significant difference ($P < 0.05$) between two different samples at each individual time point.

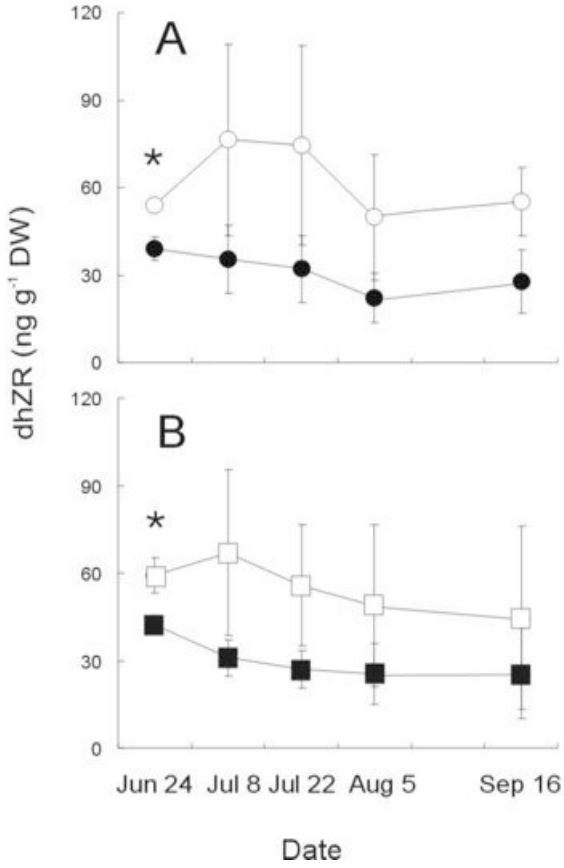

Figure 8. Changes in concentrations of endogenous dhZR in lodgepole pine long-shoot buds from summer to the fall. (A) LSB, good ♀; (B) LSB, poor ♀; Open circles - distal LSB, good ♀; Solid circles – proximal LSB, good ♀; Open square – distal LSB, poor ♀; Solid square – proximal LSB poor ♀. Mean ± SE, n=3 genotypes. Asterisk (*) indicates significant difference ($P < 0.05$) between two different samples at each individual time point.

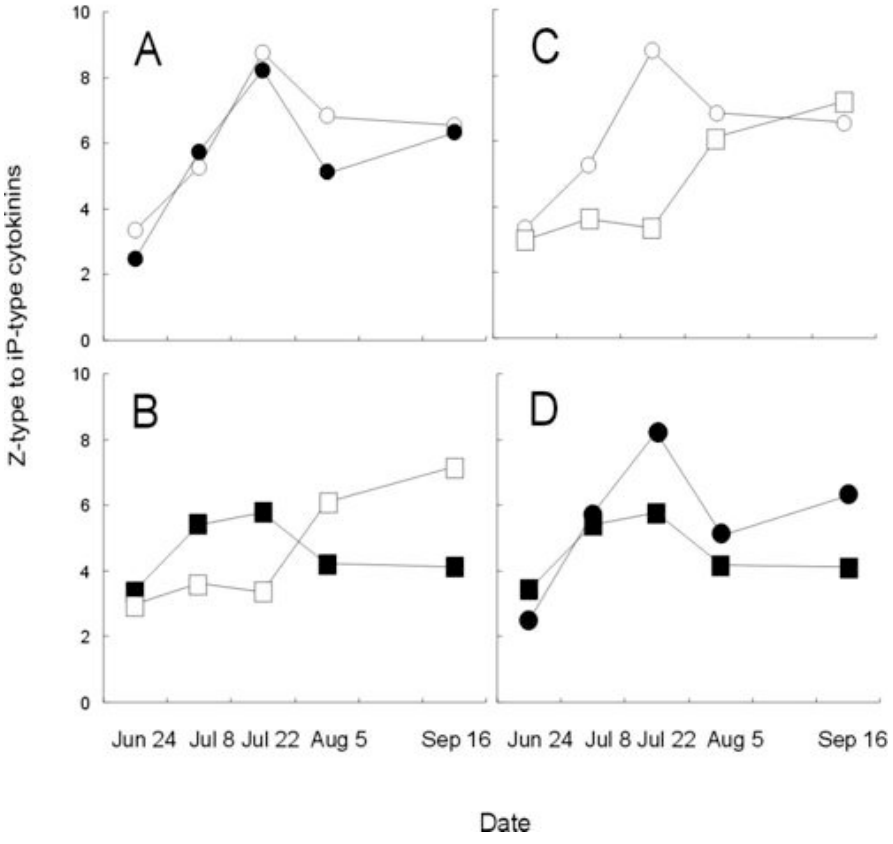

Figure 9. Changes in the ratio of Z-type to iP-type cytokinins in lodgepole pine long-shoot buds from summer to the fall. (A) LSB, good ♀; (B) LSB, poor ♀; (C) proximal LSB; (D) distal LSB. Open circles - distal LSB, good ♀; Solid circles – proximal LSB, good ♀; Open square – distal LSB, poor ♀; Solid square – proximal LSB poor ♀. Mean ± SE, n=3 genotypes.

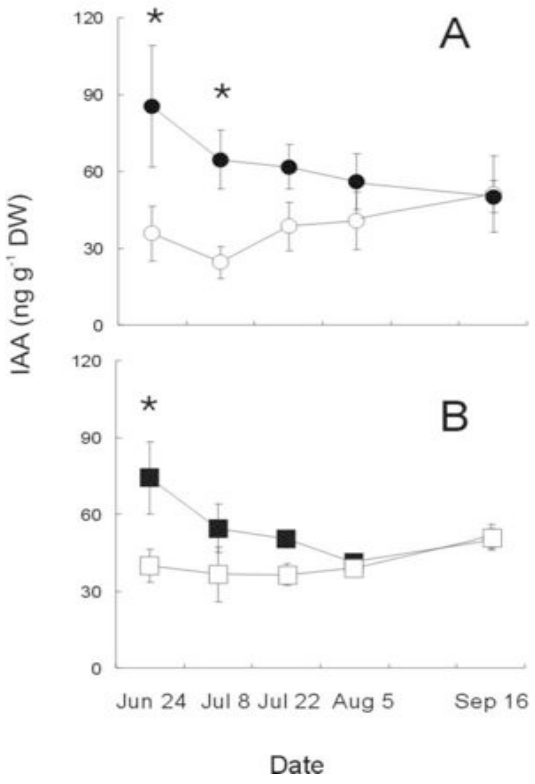

Figure 10. Changes in IAA concentrations in lodgepole pine long-shoot buds from summer to the fall. (A) LSB, good ♀; (B) LSB, poor ♀; Open circles - distal LSB, good ♀; Solid circles – proximal LSB, good ♀; Open square – distal LSB, poor ♀; Solid square – proximal LSB poor ♀. Mean ± SE, n=3 genotypes. Asterisk (*) indicates significant difference ($P < 0.05$) between two different samples at each individual time point.

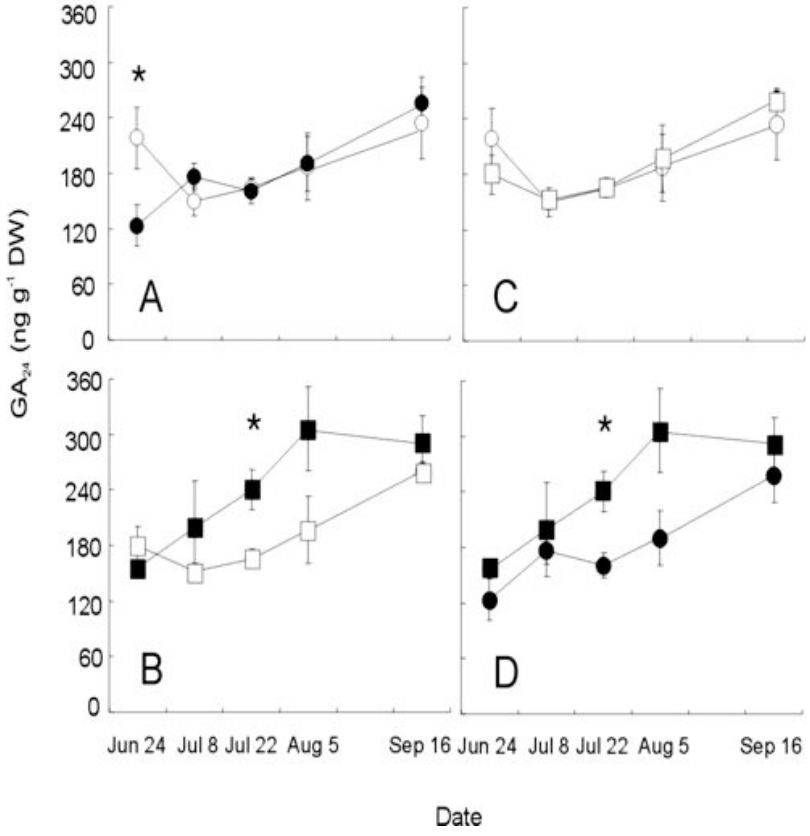

Figure 11. Changes in GA_{24} concentrations in lodgepole pine long-shoot buds from summer to the fall. (A) LSB, good ♀; (B) LSB, poor ♀; (C) proximal LSB; (D) distal LSB. Open circles - distal LSB, good ♀; Solid circles – proximal LSB, good ♀; Open square – distal LSB, poor ♀; Solid square – proximal LSB poor ♀. Mean ± SE, n=3 genotypes. Asterisk (*) indicates significant difference ($P < 0.05$) between two different samples at each individual time point.

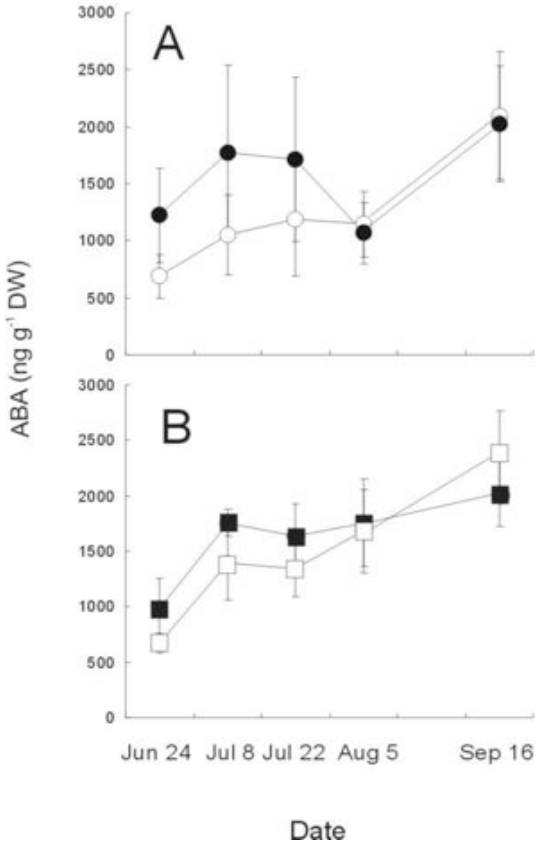

Figure 12. Changes in ABA concentrations in lodgepole pine long-shoot buds from summer to the fall. (A) LSB, good ♀; (B) LSB, poor ♀; Open circles - distal LSB, good ♀; Solid circles – proximal LSB, good ♀; Open square – distal LSB, poor ♀; Solid square – proximal LSB poor ♀. Mean ± SE, n=3 genotypes. Asterisk (*) indicates significant difference ($P < 0.05$) between two different samples at each individual time point.

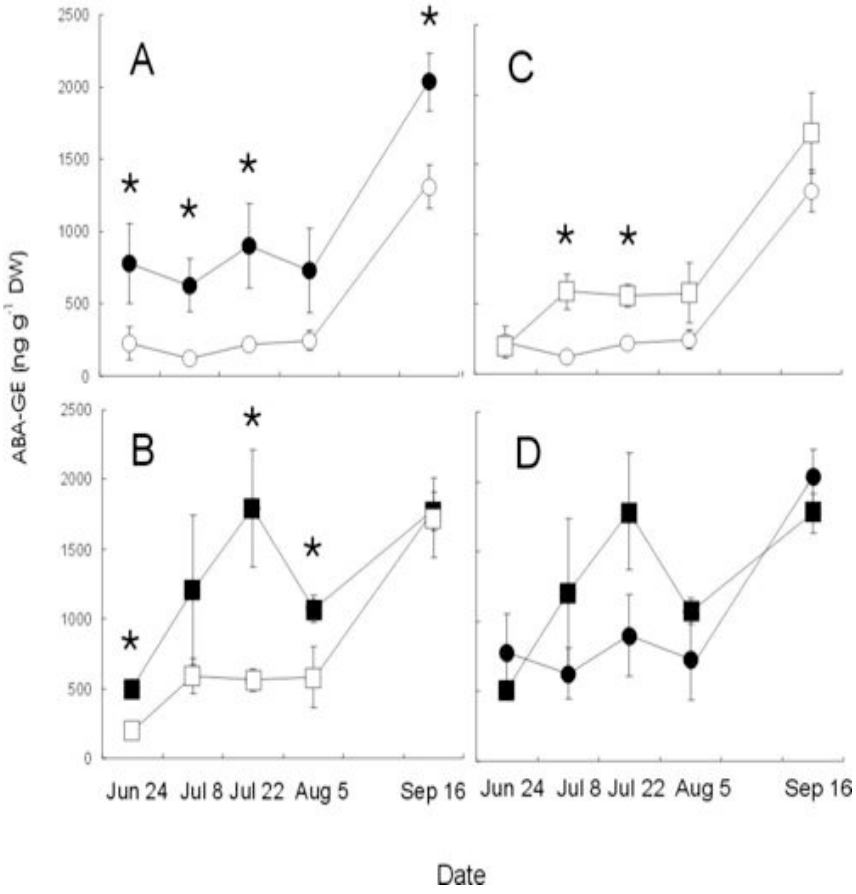

Figure 13. Changes in ABA-GE concentrations in lodgepole pine long-shoot buds from summer to the fall. (A) LSB, good ♀; (B) LSB, poor ♀; (C) proximal LSB; (D) distal LSB. Open circles - distal LSB, good ♀; Solid circles – proximal LSB, good ♀; Open square – distal LSB, poor ♀; Solid square – proximal LSB poor ♀. Mean ± SE, n=3 genotypes. Asterisk (*) indicates significant difference ($P < 0.05$) between two different samples at each individual time point.

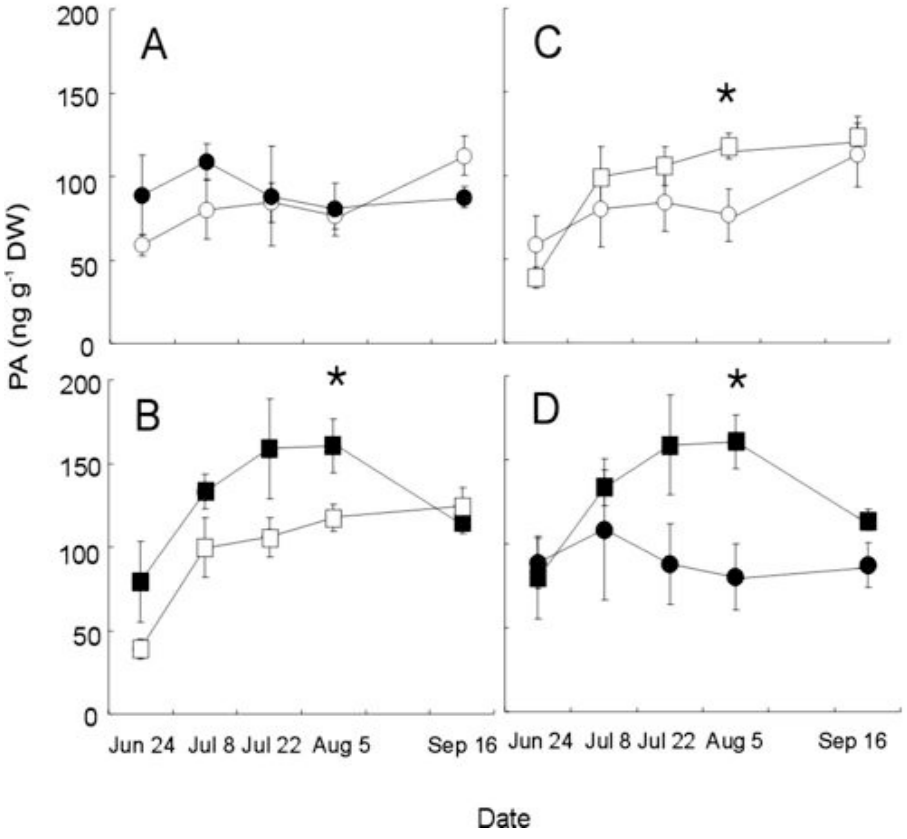

Figure 14. Changes in PA concentrations in lodgepole pine long-shoot buds from summer to the fall. (A) LSB, good ♀; (B) LSB, poor ♀; (C) proximal LSB; (D) distal LSB. Open circles - distal LSB, good ♀; Solid circles – proximal LSB, good ♀; Open square – distal LSB, poor ♀; Solid square – proximal LSB poor ♀. Mean ± SE, n=3 genotypes. Asterisk (*) indicates significant difference ($P < 0.05$) between two different samples at each individual time point.

2. PGR injection

Experiment: GA injections of different amounts (Report 2008/2009, pp. 4-5)

Goals: To investigate responses of lodgepole pine to stem injections with different amounts of GA per tree

Figure 15. Photos showing the process of PGR injection. A. Drilling holes on the stem; B. Injecting PGR; C. Sealing the hole with wax (arrow).

Rationale: Exogenous application of GA is known to influence cone induction in lodgepole pine. How such applications affect endogenous plant hormones and metabolites and how these may be related to cone production needs to be investigated.

Methods: GA was injected into each ramet at different amounts. Samples were collected at five time points. Lodgepole pine ramets (12) from one genotype (1775) were used for cone induction treatments. Treatments were four amounts of GA injection (0, 25, 100, or 200 mg GA per tree). Three ramets were used for each treatment. Injections were made in June 2008. Samples for mass spectrometric analysis were collected at five time points.

Results:

Abscisic acid Injection of GA reduced concentrations of either ABA or ABA-GE. The decrease was also positively correlated to the injected GA amounts. Little effect of GA injection was observed on endogenous PA. We were able to quantify 7'OH-ABA in lodgepole pine samples for the first time. Concentrations of 7'OH-ABA decreased slightly by wk 8.

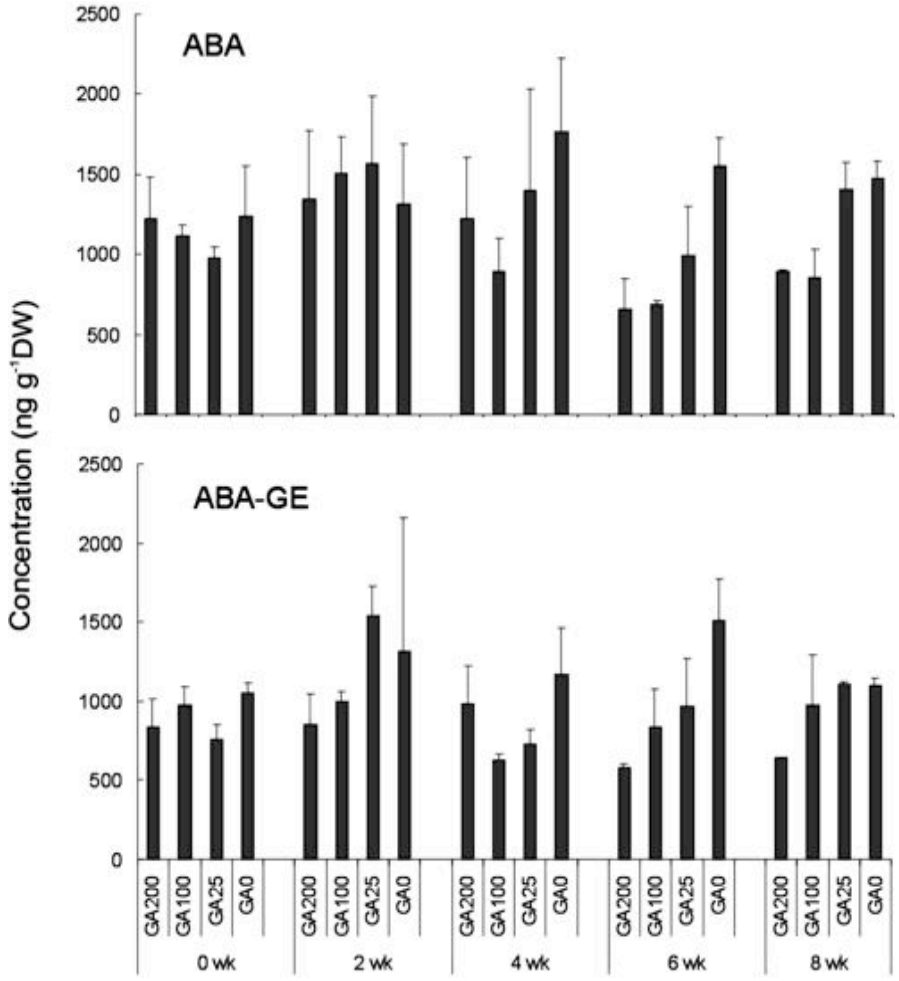

Figure 16. Changes in concentrations of ABA and ABA metabolites in lodgepole pine long-shoot buds after stem injection of GA at 0, 25, 100 or 200 mg per ramet, respectively. Mean ± SE, n=3.

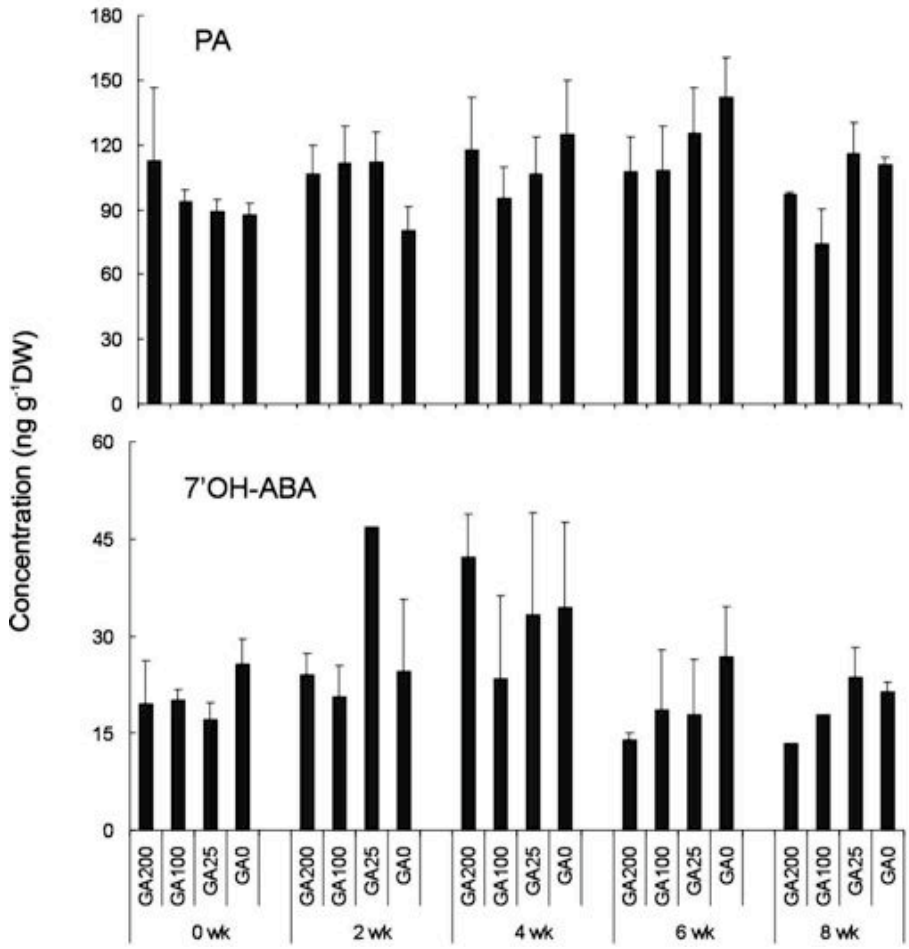

Figure 17. Changes in concentrations of ABA metabolites in lodgepole pine long-shoot buds after stem injection of GA at 0, 25, 100 or 200 mg per ramet, respectively. Mean ± SE, n=3.

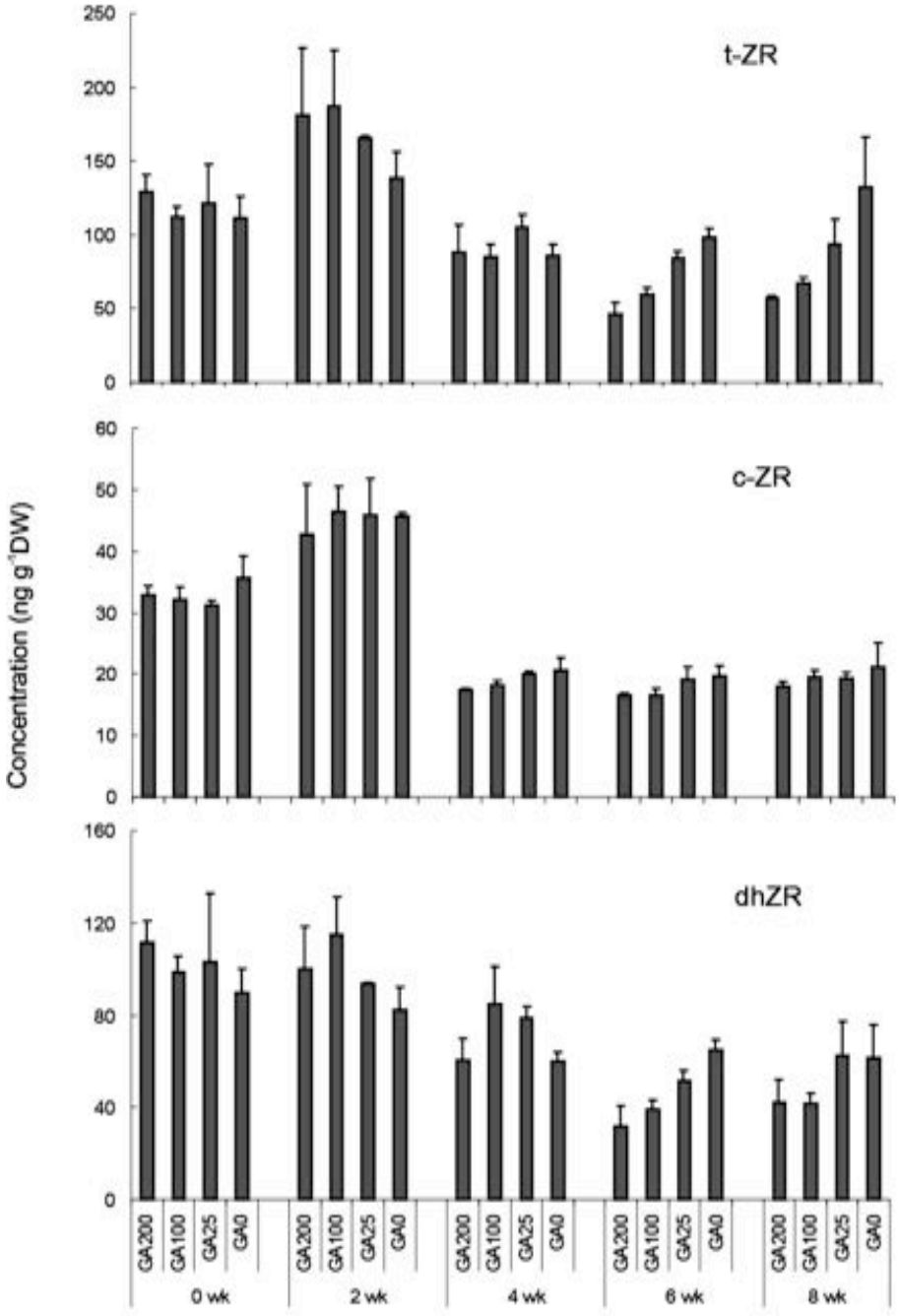

Figure 18. Changes in concentrations of cytokinins in lodgepole pine long-shoot buds before and after stem injection of GA at 0, 25, 100 or 200 mg per ramet, respectively. Mean ± SE, n=3.

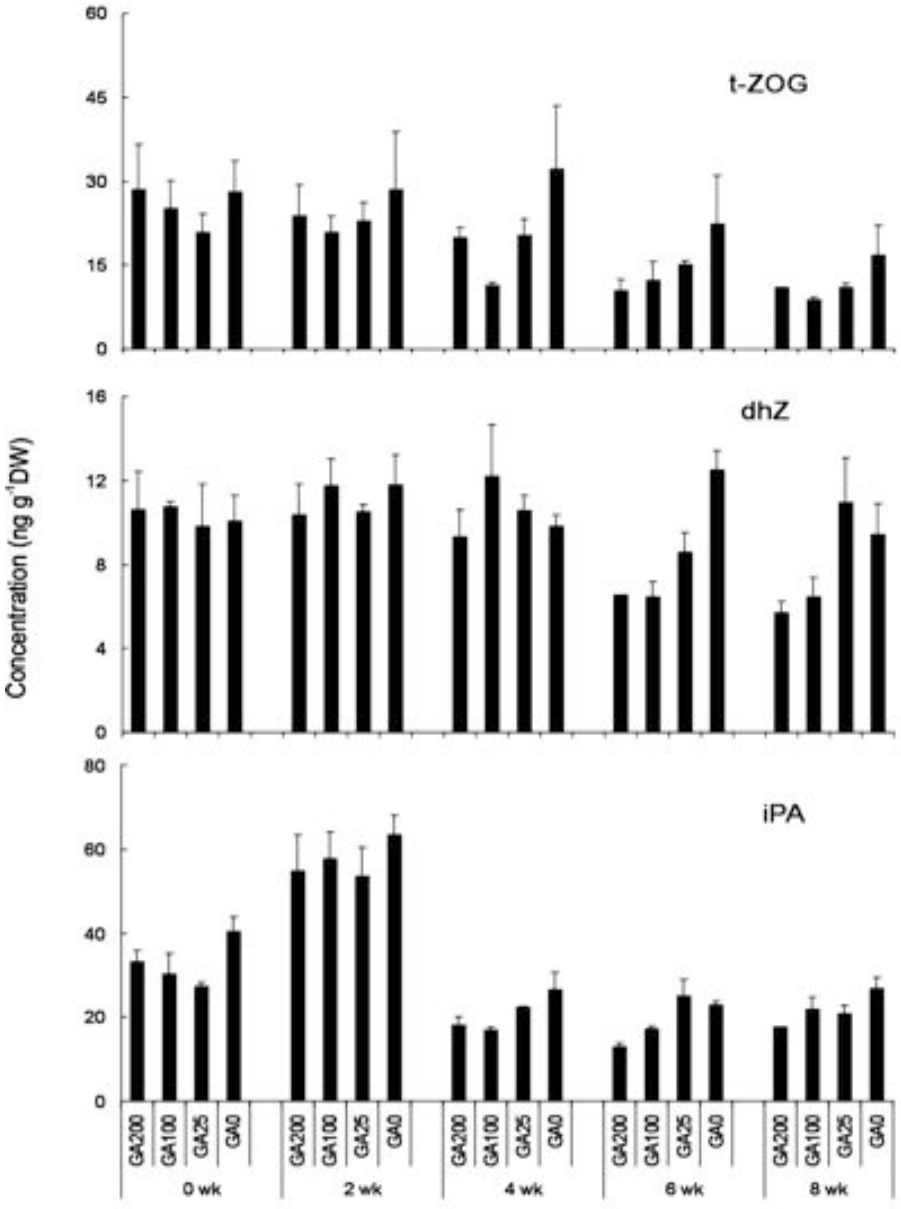

Figure 19. Changes in concentrations of cytokinins in lodgepole pine long-shoot buds before and after stem injection of GA at 0, 25, 100 or 200 mg per ramet, respectively. Mean ± SE, n=3.

Cytokinins Concentrations of *t*-ZR and dhZR decreased with GA injection by wk 6 and wk 8. Little effect was seen in *c*-ZR concentrations. Concentrations of dh-ZR and iPA decreased following GA injection, whereas little change occurred in *t*-Z-*O*-Glu concentrations.

Auxin Endogenous IAA was consistently quantified in lodgepole pine long-shoot buds. GA injection showed little effects on endogenous IAA concentrations.

Gibberellins In this genotype, GAs could last to at least 8 weeks after injection. There was no obvious difference between GA (200 mg) injection and GA (100 mg) injection. However, GA (25 mg) injection showed much lower GA levels in the buds. GA_{24} was quantified consistently in the buds. The average concentrations increased with season advanced. At wk 8, concentrations of GA_{24} were higher in GA-treated samples.

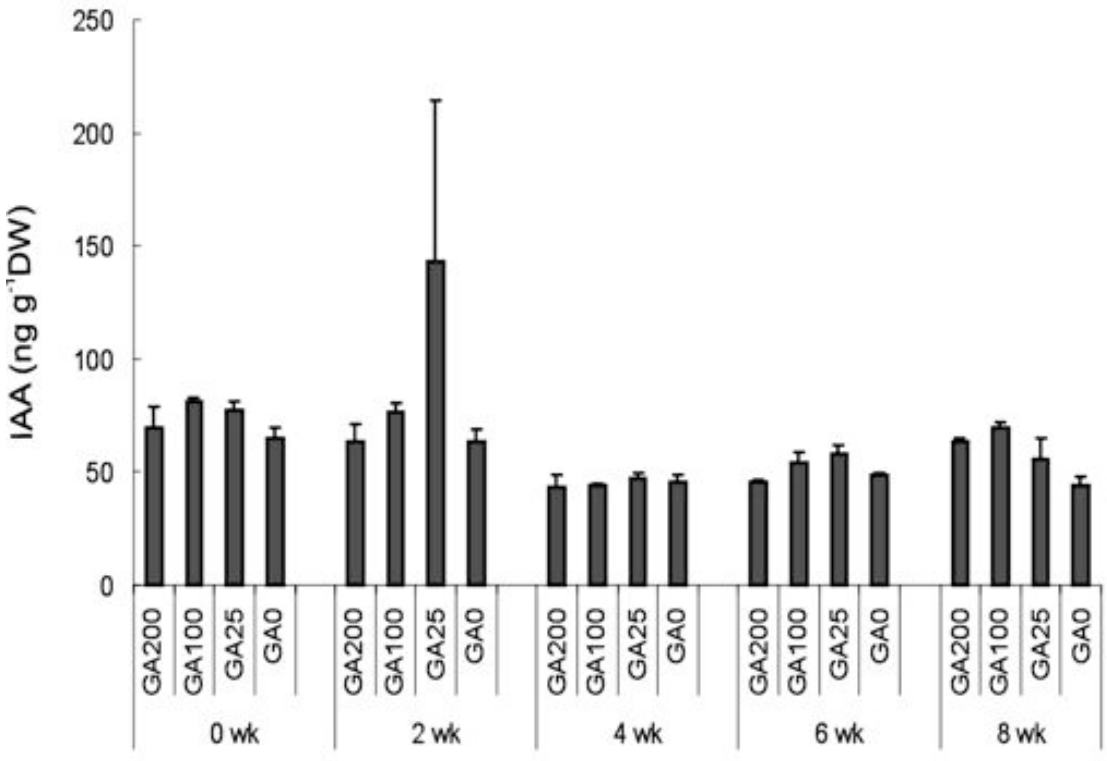

Figure 20. Changes in concentrations of IAA in lodgepole pine long-shoot buds before and after stem injection of GA at 0, 25, 100 or 200 mg per ramet, respectively. Mean ± SE, n=3.

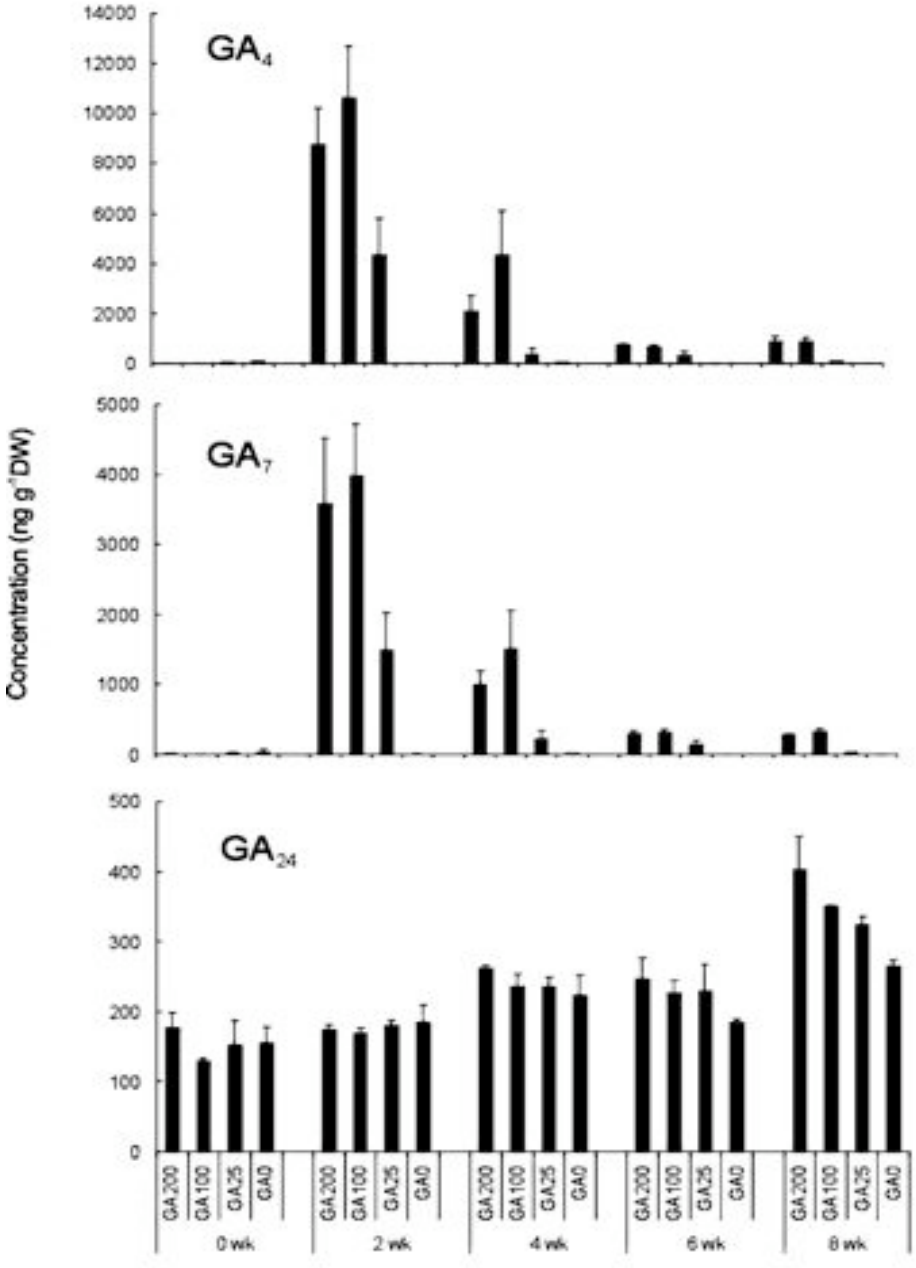

Figure 21. Changes in concentrations of GAs in lodgepole pine long-shoot buds before and after stem injection of GA at 0, 25, 100 or 200 mg per ramet, respectively. Mean ± SE, n=3.

Conclusions: In genotype 1775, high concentrations of GA_4 and GA_7 could last for at least 8 weeks after GA injection. GA_{24} was quantified consistently in the buds. At wk 8, concentrations of GA_{24} were higher in GA-treated samples. GA injection reduced concentrations of ABA and ABA-GE. 7'OH-ABA concentrations decreased

slightly by GA injection at wk 8. Concentrations of *t*-ZR and dhZR decreased with GA injection at wk 6 and wk 8. Similarly, concentrations of dh-ZR and iPA decreased by GA injection, whereas few changes occurred in *t*-Z-*O*-Glu concentrations. GA injection showed little effects on endogenous IAA concentrations.

Experiment: GA and BA injection (Readers can obtain more information from Report 2007/2008 pp. 6-19)

Goals: 1) To assess changes in concentrations of various hormones and their metabolites in developing long-shoot buds following PGR injection in ramets of two lodgepole pine genotypes. 2) To assess cone yield as well as other responses of the trees following PGR injection.

Plant materials and methods: Since a detailed report has been made previously for this experiment, readers can obtain more information from report 2007/2008.

Results:

Gibberellins High concentrations of GAs could be quantified one week after GA injection, alone or in combination with BA, in samples of both genotypes. GAs declined after the first week in genotype 2 samples or after week 3 in genotype 1 samples. In both of the genotypes, GAs were detectable for at least 7 weeks after GA injection. In the control samples without GA injection, GA_4 was undetectable; while GA_7 was quantified at low levels. These results indicated that the increased GAs after the treatments were most likely due to exogenous GA. In the long shoot buds of lodgepole pine, the ratio of GA_4 to GA_7 remained unchanged in genotype 1 and increased a little in genotype 2 until week 7.

Abscisic acid and metabolites Treatments of trees with GA, alone or in combinations with BA, decreased endogenous ABA concentrations in both genotypes 1 and 2 at week 7. In the samples of genotype 2, decrease started at week 5. Little change of ABA concentrations occurred in BA-treated samples. Generally, ABA declined as the season advanced in genotype 1. The trend was not clear in genotype 2. Similarly, lower concentrations of ABA-GE and PA corresponded to treatments with GA, alone or in combination with BA, in both genotypes at week 7 or earlier. DPA was below quantifiable levels in most samples.

Cytokinins and metabolites In both genotypes, the combination of GA and BA increased dhZR and *t*-ZR concentrations at week 7. On the other hand, this

treatment decreased iPA concentration at weeks 5 and 7, resulting in higher ratios of Z-type to iP-type CKS at week 7. Concentrations of other CKs were generally very low or under quantifiable levels. Generally, CK concentrations declined as the season advanced.

Auxins IAA could not be quantified in most of the samples. IAA metabolites, IAA-Glu and IAA-Asp were quantified at low levels. No significant difference was found between any treatments and their controls.

Conclusions: GA injection raised GA levels quickly and noticeably. GA and/or BA treatments brought about decreases in ABA metabolism. Treatments with GA, alone or in combination with BA, altered ratios of Z- to iP- type CKs.

3. Root pruning

Experiment: Root pruning experiment A (Report 2006/2007, pp.31-35)

Goals: To establish hormone profiles before and after pruning in two genotypes.

Plant materials and methods: Read report 2006/2007, pp.31-35.

Results:

Cytokinins Concentrations of endogenous *t*-ZR declined after root pruning in genotype 233 but not in genotype 299. In genotype 233, concentration of *t*-ZR remained lower than that of the controls. Concentrations continuously declined until they were below quantifiable level in the last two samples, i.e. samples collected at Sept 15 and Oct 17.

Abscisic acid A slight increase of endogenous ABA was found in genotype 233 after root pruning. However, genotype 299 showed no such change.

Variation in response to root pruning between genotypes A comparison of endogenous plant hormones has been made between two genotypes, i.e. genotype 299 and genotype 233. Genotype 299 demonstrated high cone productivity consistently (233.3 ± 23 cone/tree) while genotype 233 showed much lower productivity (65.83 ± 11 cone/tree). This number was based on three continuous production years. Seven CKs and CK metabolites were identified and quantified in long shoot buds of genotype 233. Some of these CKs, 2iP and dhZ, were not quantified in genotype 299. Higher ratios of Z-type cytokinins to iP-type cytokinins were found in long-shoot buds of genotype 299 than genotype 233. In samples of Sept 15 and Oct 17, genotype 233 contained higher PA and DPA. No obvious difference was found in ABA and its metabolites between theses two genotypes until Sept 15. On Oct 17,

higher concentrations of ABA and ABAGE were quantified in samples of genotype 233 when compared to genotype 299.

Conclusions: Root pruning did not dramatically influence any pool of phytohormones and their metabolites. No notable change in cone yield was found based on estimation in the second growing season after root pruning. The long-shoot buds of the high cone-producing genotype had higher ratios of Z-type cytokinins to iP-type cytokinins than those of the low cone-producing genotype. High cone-producing buds also contain less ABA, PA and DPA at all or some sampling points.

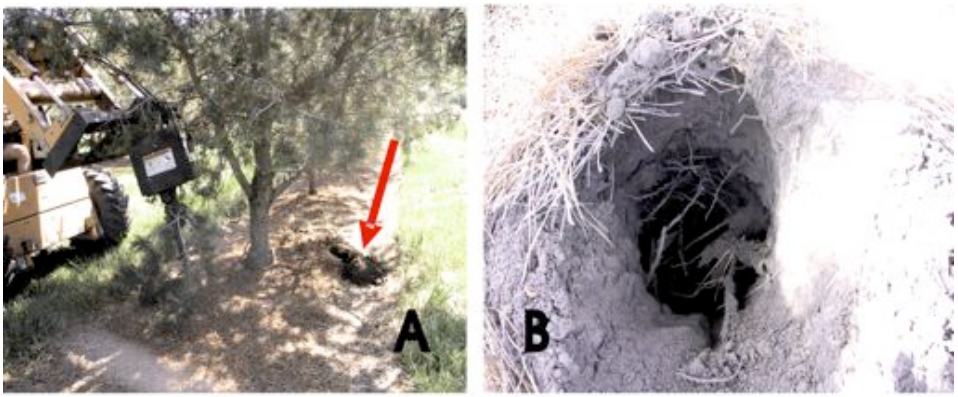

Figure 22. Photos showing the process of root pruning in lodgepole pine. A. drilling holes on the ground near the tree stem; B. A close view of the hole showing injured roots.

Experiment: Root pruning experiment B (Report 2007/2008, pp.19-25)

Goals: To establish hormone profiles before and after root pruning in two genotypes.

Plant materials and methods: Read report 2007/2008, pp.19-25.

Results:

Abscisic acid In both genotypes, no difference was found in concentrations of ABA, ABA-GE and PA between pruning treatment and the control. DPA was only quantified in some of the samples with low concentrations.

Cytokinins Concentration of *t*-Z-*O*-Glu decreased in genotype 1 at Jul 4, 2 weeks after root pruning. No obvious difference was found in concentrations of dhZ, *c*-Z-*O*-Glu, *t*-ZR, *c*-ZR and dhZR between the pruned and the control in both genotypes. In iP-type CKs, no difference was found in concentrations of 2iP and iPA in most

samples except for lower concentrations of 2iP in genotype1 samples at July 17. Concentrations of endogenous *t*-Z were below quantifiable levels.

Auxins IAA metabolites, IAA-Glu and IAA-Asp were quantified at low levels. No difference was found between samples except for the increase of IAA-Asp in genotype 1 samples of the pruned at July 4, two weeks after the treatment. IAA could not be quantified in most of the samples.

Gibberellins GA_4 was quantified in few samples at very low concentrations. GA_7 was only quantified in one sample with a suspect high concentration. It was impossible to make comparison between the treatment and the control samples.

Conclusions: Root pruning did not dramatically influence any pool of phytohormones and their metabolites. No notable change in cone yield was found based on estimation in the second growing season after root pruning.

4. TIBA injection (Report 2007/2008, pp.25-31)

Experiment: TIBA (2,3,5-triiodobenzoic acid) injection

Goals: To establish hormone profiles and cone induction responses after TIBA, an auxin inhibitor, treatment.

Plant materials and methods: Read report 2007/2008, pp.25-31.

Results:

Auxins In samples of both TIBA-L (low amount) and TIBA-H (high amount) samples, concentration of IAA were decreased at week 2 following the first injection. Thereafter, IAA was below quantifiable levels in most samples disregarding the treated ones or the control. IAA catabolites, *i.e.* IAA-Asp and IAA-Glu, were detected in some samples, but unquantifiable in most samples due to their trace amounts.

Cytokinins No difference was found in the concentrations of *t*-ZR, *c*-ZR and iPA in all the samples, while higher concentrations of dhZR than those of the control existed in the samples of week 6 after the first injection resulting in a similar pattern in the total Z-type CKs. Other CKs and metabolites were generally low or unquantifiable.

Abscisic acid At week 6, ABA and ABA-GE were slightly higher in the samples of TIBA-L treatment than the control and lower concentration of PA in the samples of TIBA-H treatment. No difference was found in samples at other time points.

Gibberellins Endogenous GAs were very low or unquantifiable.

Conclusions: Endogenous IAA declined and some ABA metabolites changed in concentrations with TIBA treatments. No notable increase or decrease in cone yield was found with TIBA treatment in most genotypes based on initial estimation.

5. PGR paste treatment

a) Effects of branch paste on hormone profiles

Experiment: Effects of branch paste on hormone profiles and cone gender determination (2009/2010)

Goals: To investigate effects of PGRs on cone gender determination and hormone profiles when GA and TDZ were applied with branch paste

Rationale: Bud paste of GA and TDZ is known to influence cone gender determination in lodgepole pine on our preliminary results. How such paste applications affect endogenous plant hormones and metabolites and how these applications influence cone gender and yield need further investigation.

Methods: GA and TDZ were dissolved and made into paste, respectively and in combination. The method of PGR paste treatment was based on the report by Wakushima (2004). Paste was applied on branches (Figure 23) at July 22, 2009. Long shoot buds were collected for analysis once two weeks. Lodgepole pine ramets from genotype 1822 were used for cone induction. Samples for mass spectrometric analysis were collected at five time points before and after paste applications. Phytohormones and their selected metabolites were analyzed for the lower parts of long-shoot buds. Information about the samples collected for this experiment is provided in Table 3. Cone yield data will be available in late spring of 2010.

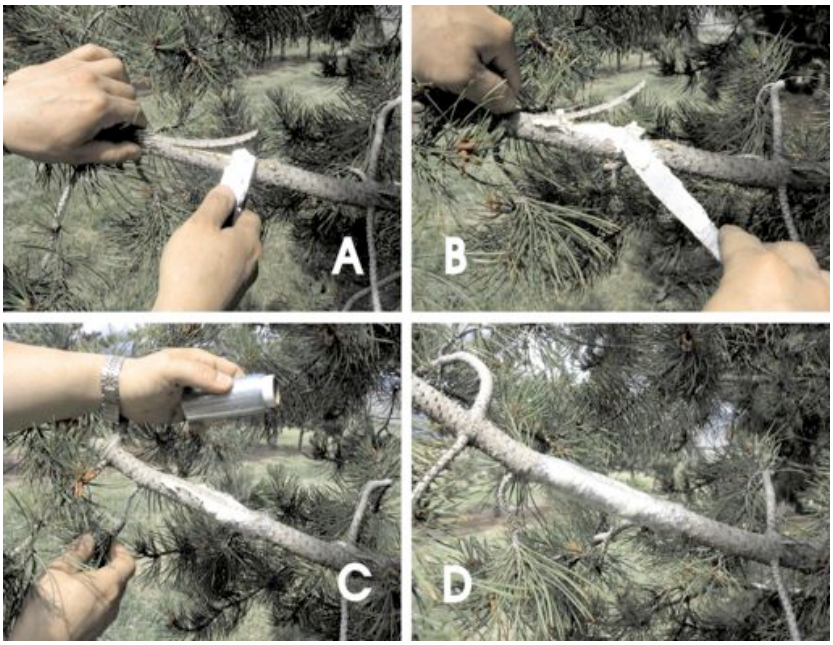

Figure 23. Photos showing the process of paste treatment. A. Making a cut on the branch; B. Applying BA paste; C-D. Wrapping the cutting with plastic wrap.

Table 3. Cone induction treatments by PGR paste in lodgepole pine. Ramets of genotype 1822 were used for branch paste treatments in July 22 2009.

Genotype	Ramet	Treatment
1822	Q17 U34 X25	GA (2mg/ml)
1822	I61 V73 BB68	GA+TDZ (2mg/ml; 0.2 mg/ml)
1822	Y18 AA13 AA34	TDZ (0.2 mg/ml)
1822	Y124 II126 R109	No PGRs

Results:

Gibberellins Concentrations of GA_4 and GA_7 increased dramatically after GA paste treatment (Figure 24). Compared with GA injection, GAs in plant samples with GA paste treatments lasted for a much longer period of time. Concentrations of GA_4

even increased at wk 4 with GA+TDZ paste treatment. With this treatment, GA_1 also increased at wk 4.

Abscisic acid Concentrations of ABA, ABA-GE and 7'OH-ABA decreased after PGR paste (Figure 25). The most effective PGR treatments were GA and GA plus TDZ.

Cytokinins Paste treatments of GA and TDZ remarkably increased concentrations of *t*-ZR and dh-ZR in lodgepole pine long shoot buds (Figure 26).

Auxins IAA concentrations increased at wk2 following the paste treatment of GA or GA plus TDZ (Figure 27). IAA levels were under quantification at week 4 in all the samples.

Conclusions: GA paste raised levels of GA_4 and GA_7 quickly and these high levels could last for 4 weeks without decline when GA plus TDZ paste was applied. GA or GA plus TDZ treatments brought about decreases in ABA metabolism. Treatments with GA, alone or in combination with TDZ, increased concentrations of *t*-ZR and dhZR significantly. In addition, GA plus TDZ increased GA_1 concentrations at week 4 after paste treatments. Data of cone yield will be available in the late spring of 2010.

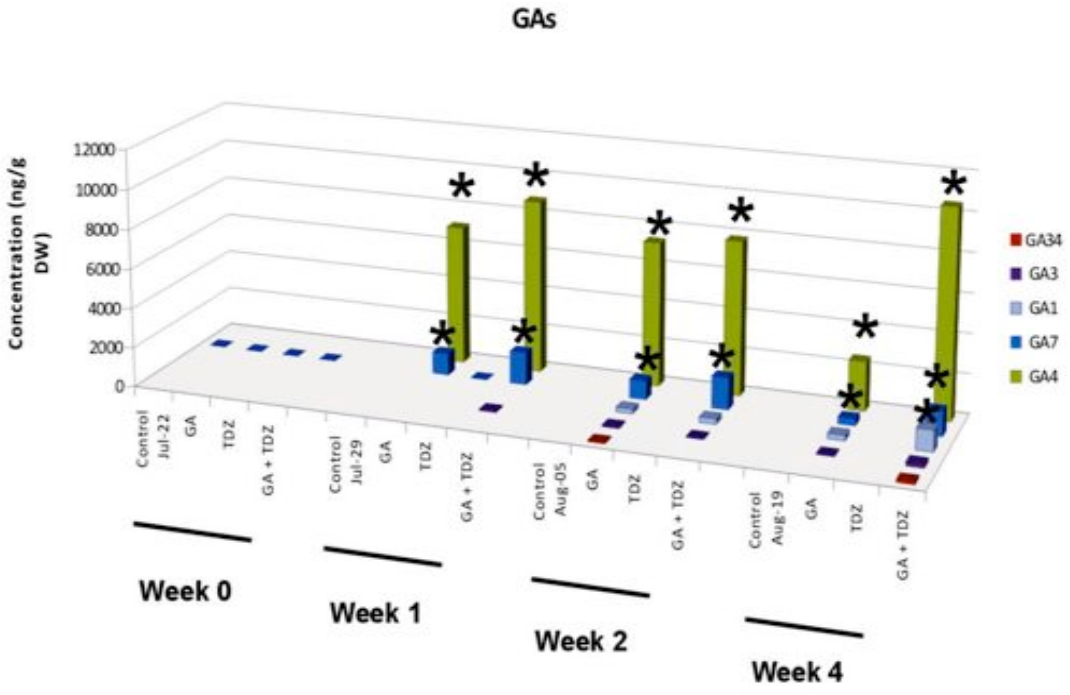

Figure 24. Changes in concentrations of GAs in lodgepole pine long-shoot buds in genotype1802. Asterisk (*) indicates significant difference ($P < 0.05$) between the treatment and control, mean ± SE, n=3.

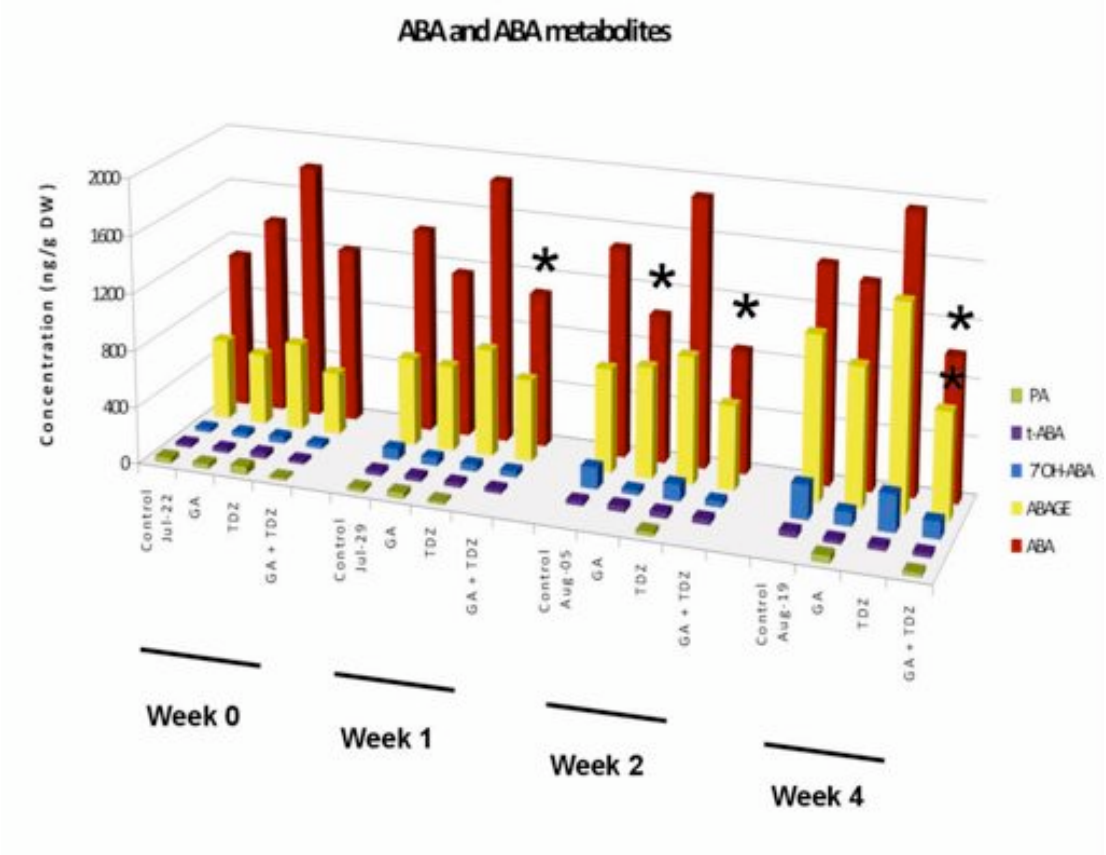

Figure 25. Changes in concentrations of ABA and ABA metabolites in lodgepole pine long-shoot buds in genotype1802. Asterisk (*) indicates significant difference ($P < 0.05$) between the treatment and control, mean ± SE, n=3.

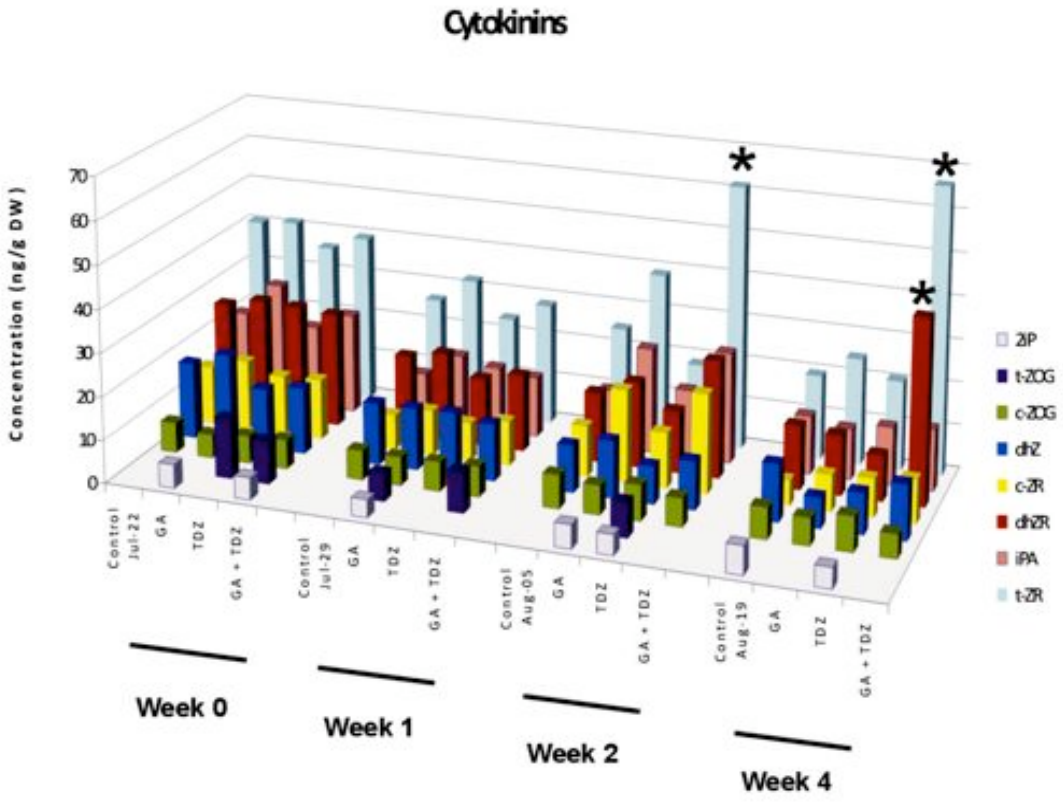

Figure 26. Changes in concentrations of cytokinin and metabolites in lodgepole pine long-shoot buds in genotype1802. Asterisk (*) indicates significant difference ($P < 0.05$) between the treatment and control, mean ± SE, n=3.

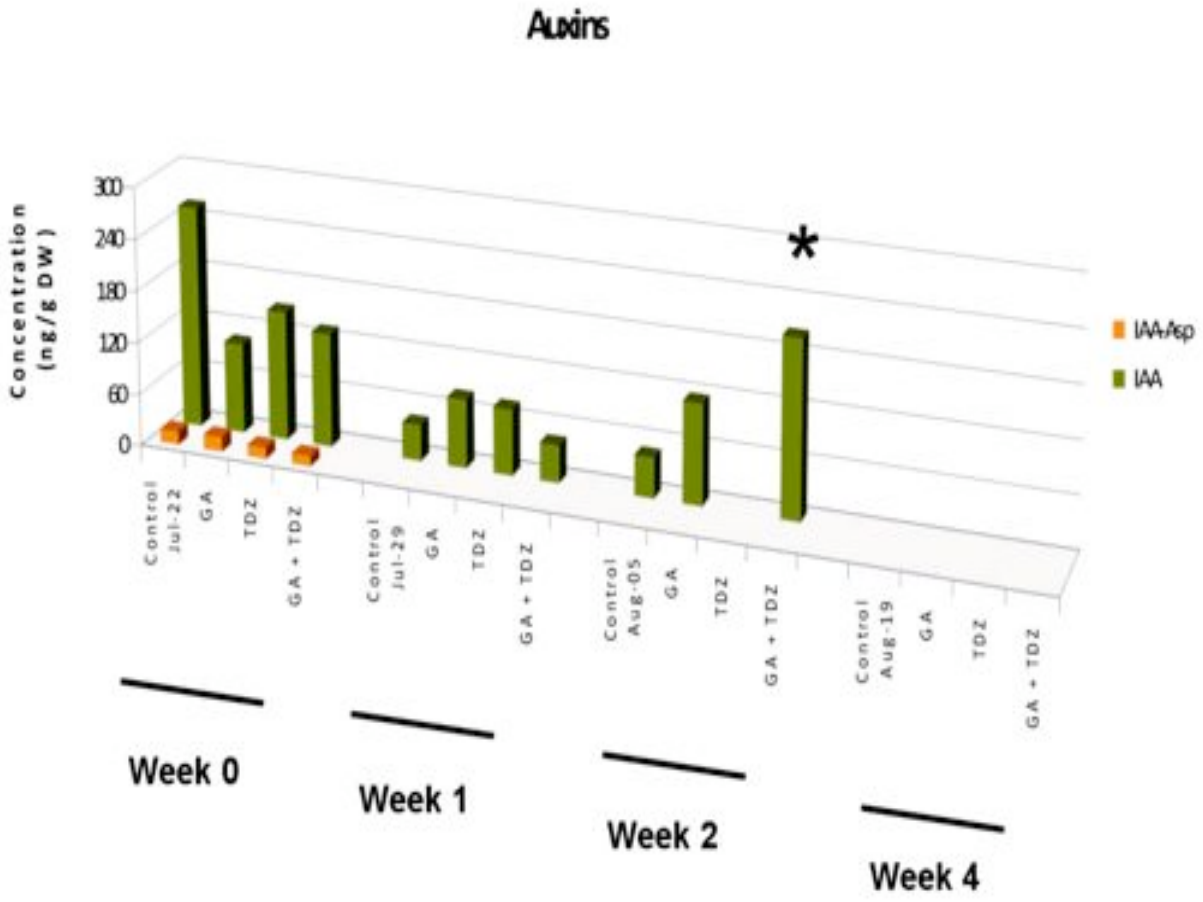

Figure 27. Changes in concentrations of IAA and metabolites in lodgepole pine long-shoot buds in genotype1802. Asterisk (*) indicates significant difference ($P < 0.05$) between the treatment and control, mean ± SE, n=3.

c) Bud paste and cone gender plasticity (2008/2009)

Experiment: Initial trial of bud paste with GA and other chemicals (Report 2008/2009, pp.10-11)

Goals: Initial test of PGR bud paste on cone gender determination in order to find effective treatments.

Method: Bud paste of GA alone or in combination with NAA, TDZ, TIBA or MGBG was applied to lodgepole pine long-shoot buds (Table 4). Ramets from three genotypes (1775, 1822 and 1795) were used in VSOC seed orchard. Three buds on each

branch and two branches per tree were paste-treated. Paste treatments were applied. All paste treatments were completed between June 24 and July 7, 2008.

Results:

1) The treatments of GA plus TDZ or GA only induced female cone formation at the location where male cones usually generate (Figures 28-30).
2) The most sensitive genotype is genotype1822, followed by genotype 1775.
3) On one small branch, up to 14 female cones were observed with the best treatment. Whereas, only one or two female cones usually form on the branch.
4) The better application time point is July 7 in 2008 treatments.

Conclusions: Bud paste with GA plus TDZ or GA only could induce female cones from sub-apical location where male cones usually form.

Table 4. The initial trail for cone induction in lodgepole pine by bud paste.

PGR paste treatment	**1775**	**1822**	**1795**
GA	M8	O4	M10
GA+TIBA	P5	Q17	D53
GA+TDZ	L41	O36	P13
GA+NAA	O27	R54	D31
GA+MGBG	R29	O44	A41

Figure 28. Induction of cone gender changes in lodgepole pine. Paste treatments of PGRs were applied before cone gender determination. One or two female cones (arrows) were induced among male cones.

Figure 29. Induction of cone gender changes in lodgepole pine. Paste treatments of PGRs were applied before cone gender determination. More than ten female cones (FC) were induced at the lower part of the branch where is usually for male cones (MC).

Figure 30. Induction of cone gender changes in lodgepole pine. Paste treatments of PGRs were applied before cone gender determination. Multiple female cones were induced at the lower branch part where is usually for male cones. The female cones are developing into seed cones.

Experiment: Trial of bud paste with TDZ and GA (2009/2010)

Goals: Further investigation of TDZ and GA on cone gender determination

Methods: Bud paste of TDZ and GA, respectively or in combination, was applied to multiple genotypes at different time points from middle July, when the funding was released, to early September in 2009 (Table 5).

Results: Cone induction results will be available in the spring of 2010.

Conclusions: Conclusion will be made on the basis of cone induction data that could be available in the late spring of 2010, depending on funding availability.

Table 5. Bud paste applications in 2009.

Jul-22			**Jul-29**		
1822	Q17	GA	1822	JJ2	GA, GA+TDZ, TDZ
	U34			MM8	
	X25			MM16	
1822	I61	GA+TDZ	1775	MM10	
	V73			JJ21	
	BB68			KK32	
1822	Y18	TDZ	1799	JJ4	
	AA13			PP19	
	AA34			HH30	
1822	Y124	No PGRs	**Aug-05**		
	Y126			F127	GA, GA+TDZ, TDZ
	R109			E126	
1775	C6	GA GA+TDZ		F114	
	B21		**Aug-19**		
	B28		1822	DD126	GA, GA+TDZ, TDZ
1799	C3			II126	
	I27			HH118	
291	D5		**Sep-02**		
250	D12		1822	LL127	GA, GA+TDZ, TDZ
				OO113	
				QQ127	

4. BA treatments

Experiment: Effects of BA on lodgepole pine cone induction (Report 2007/2008, pp.32-36)

Goals: To assess cone yield as well as other responses of the trees following BA treatments (injection and / or paste).

Results and conclusions: BA enhanced lateral bud formation. No obvious increase appears to have been found in female cone yield after BA treatments. No gender change has been observed following BA treatment, either by BA paste treatment or by the combinations with BA injection. This does not, at this point, appear to be an effective method.

Figure 31. Photos showing bud paste treatment and the response of the buds to the treatment. A-B. Showing long-shoot-buds with paste in the same growing season as the treatment. C-D. Showing the treated buds in the spring of the second growing season.

6. Summary and future studies

Summary: Among all cone induction methods tested in this research, the most effective one is bud paste with TDZ plus GA for lodgepole pine. On our preliminary result, this treatment induced cone gender change, from male cone buds to female ones, which remarkably increased the number of female cones (up to seven fold). This method has demonstrated its potential in practice use. Metabolomic profiling revealed that branch paste treatment of TDZ and GA combination increased concentrations of a few cytokinins significantly. Meanwhile this treatment decreased concentrations of ABA and some ABA metabolites. Generally, PGR application, either injection or paste treatments, were able to alter concentrations of various plant hormones and some of their metabolites. However, by now no satisfactory cone yield data was achieved with PGR stem injection in lodgepole pine. Also, no significant change in either hormone profiles or female cone yield was observed with physical treatments such as root pruning.

Further studies: Future research with lodgepole pine may answer the following questions.

A. Why injections of PGR cannot cause cone gender change?

The following factors might need investigation:

- Strength of PGRs when reach the bud
- Duration of PGRs at suitable levels
- Light effects by bud paste
- A ground-ward gradient of applied PGRs caused by bud paste
- Lanolin effects

B. What structural changes occurred during cone induction?

- Bud structural indication for right PGR application timing
- Gene expression: plant hormone and gene control
- Bud differentiation during induction (female cone potential of lateral buds, needle buds, or male buds)

C. Could spray method be developed for industrial application with high cone induction effects?

- Liquid viscosity that affects spray
- Spray equipment
- Foliage vs. buds applications
- Spray frequencies (Single or multiple)
- Sticky ability to forage and capability to stand washing off by rain or shower watering in the orchard.

Part 3 Douglas-fir

Introduction

Cone induction has been better studied in Douglas-fir (*Pseudotsuga menziesii* var. *glauca* (Mirbel) Franco) than in most other conifers. There have been detailed studies of endogenous GAs and other hormones. In our study, Douglas-fir was mainly used as a model system of metabolomic study to develop cone induction method in lodgepole pine.

Plant materials

Plant materials of Douglas-fir were collected from Pacific Regeneration Technologies Inc. in Armstrong, British Columbia (50°26′30″N, 119°11′00″W) or the seed orchard of Western Forest Products Ltd. located in Saanichton, British Columbia (48°35′39″N, 123°24′51″W).

Methods

Hormone profiling Hormone profiling method is similar to that for lodgepole pine.

Cone induction treatments Cone induction methods used in this research project include stem-injection of PGRs, bud and branch paste of PGRs, and stem girdling. More details can be found in our annual reports and publications.

1. Hormone profiling

Experiment: General hormonal profiles (Report 2006/2007, pp.36-39; Kong et al., 2009)

Goals: 1) To assess between genotype variation in the profile of various hormones and their metabolites from nine genotypes of Douglas-fir. 2) To assess general trends in hormone physiological behavior over the course of a year.

Plant materials and methods: Read report 2006/2007, pp.36-39.

Results:

Auxins IAA existed in all the samples, varying in concentration. IAA catabolites (IAA-Asp & IAA-Glu) were generally under detectable levels. Higher concentrations of IAA were quantified at the stages corresponding to the rapid growth of branches.

Cytokinins iPA and *t*-Z-*O*-Glu were found and quantified in all the samples. A marked increase of *t*-ZR and *t*-Z-*O*-Glu was found at stage 5 (Sept 12). The sharp increase of CKs may be responsible for female flower differentiation.

Abscisic acid ABA declined during the growth season with increased ABA-GE. A sharp decrease of ABA-GE with increased ABA occurred in the buds at stage 2.

Gibberellins Endogenous $GA_{1,}$ GA_3 GA_4 and GA_7 were analyzed. Concentrations of all the GAs were too low to be quantified.

Conclusions: Averaged across genotypes, IAA concentration was high at stages 1 and 3. The only pattern that correlated with cone productivity was one that was unique to IAA, in which high concentrations at stages 3 and 4 were found in all genotypes with high female cone productivity. Concentrations of iPA decreased and ZR concentrations increased as buds initiated and differentiated. Z-*O*-Glu was

30 ng g^{-1} DW at stage 1, declining at stages 2 and 3, before increasing at stages 4 and 5. High ABA concentrations were positively correlated with rapid shoot elongation (stages 1 & 2), but as growth slowed and terminated, ABA concnetrations decreased. ABA-GE concnetration altered inversely with changes of ABA concnetrations. Between stages 1 and 2, ABA-GE decreased from 10 to 0.2 µg g^{-1} DW and increased thereafter. 7'-hydroxy ABA was about 2 µg g^{-1} DW at stage 1, declined at stages 2 and 3, and increased at stages 4 and 5; PA concentrations were low at all stages, whereas DPA was detected only at stages 4 and 5.

2. Girdling treatment (Report 2007/2008, pp. 37-49)

Experiment: Effects of girdling treatment on metabolomic files and cone yields

Goals: 1) To assess differences in concentrations of various hormones and their metabolites in the samples of girdling treatment and the control in two genotypes. 2) To assess effects of girdling treatment on cone induction.

Figure 32. Photos showing the process of stem-girdling treatment in Douglas-fir. A-B. Showing double girdling on the stem; C-D. Placing bandage on the girdled area.

Plant materials and methods: Read report 2007/2008, pp. 37-49.

Results:

Cytokinins A few Z-type-CKs were quantified in most of the samples in both genotypes, including *t*-Z-*O*-Glu, *c*-Z-*O*-Glu, *t*-ZR, *c*-ZR and dhZR. However, no difference was observed between girdling treatment and the control. Similarly, no difference was observed concentrations of 2iP and iPA as well as the ratios of Z-type CKs to iP-type CKs between girdling treatment and the control.

Auxins IAA was unquantifiable in the samples of genotype 1 (genotype 9550) at week 6 after girdling treatment. It existed in all the samples, varying in concentration in genotype 2 (genotype 9137) samples. There was no difference in concentrations of IAA in the samples of the girded and the control in both of the genotypes. Concentrations of IAA catabolites (IAA-Asp & IAA-Glu) were generally low or below detectable levels.

Abscisic acid ABA declined during growing season with increased ABA-GE concnetrations. No obvious difference in ABA concentrations was observed in the samples of the girded and the control in both genotypes at all sampling points after girding treatment. Increased concentrations of ABA-GE were found in girdling samples of genotype 1 at weeks 2 to 8. ABA-GE increase also existed in samples of genotype 2 at week 4. Differences caused by girdling treatment in other ABA metabolites included an increase in 7' OH-ABA concentrations in genotype 1samples, but not in genotype 2 at week 8. Concentrations of PA in genotype 2 samples decreased at week 2 and increased at week 8.

Gibberellins Endogenous GA_4 could be quantified in most of the samples. However, no difference was observed in the samples of girding treatment and the control. GA_7 was quantified only in the samples of week 0 and week 4 and no difference was observed in the samples after girdling treatment.

Conclusions: After girdling treatments, male cone yield increased significantly in the next growing season. The increase was 14-fold in genotype 9137 (genotype 2). In genotype 9550 (genotype 1), more than 8,700 male cones were induced from each tree whereas no male cones were found in controls. Female cone yield was zero in controls and low for girdled trees in both genotypes. Although concentrations of GA_4 were slightly higher at week 2 and lower at week 8 in girdled samples following girdling treatment, these changes were not significant ($P>0.05$). Stem girdling did not affect concentrations of IAA and major cytokinins, such as ZR and iPA.

Concentrations of ABA differed two-fold between the genotypes. Girdling treatment did not cause differences in ABA concentrations. Higher concentrations of ABA glucose ester were found in most samples of girdled trees. Concentrations of 7'-OH ABA increased in genotype 9550 at week 8 after girdling, whereas no change was found in genotype 9137. Girdling caused little change in concentrations of PA in both genotypes.

3. PGR injection

a) GA concentrations (Report 2006/2007, Kong et al. 2008)

Experiment: Effects of exogenous gibberellins on metabolomics files during cone induction

Goals: To assess the profile of various hormones and their metabolites from one genotype of Douglas-fir subjected to four different concentrations of gibberellin.

Plant materials and methods: Read report 2006/2007, pp. 40-46.

Results:

Gibberellins Endogenous GAs could be quantified one week after GA injection. GAs become undetectable 5 weeks thereafter in the trees with the least amount of injected GA (4 mg). These results indicated that detected GAs were most likely due to exogenous GA. In Douglas-fir, GA_4 declined rapidly whereas GA_3 increased significantly. GA_7 remained unchanged until week 3. Endogenous concentrations of all GAs began to decline 3 weeks after GA injection. The changes in GA in the course of this study suggest a pathway of GA metabolism from GA_4 to GA_7, changing to GA_3.

Dry weight Exogenous GA application influenced dry weight accumulation in terminal buds. At 4 mg or 40 mg GA, dry weight increased significantly following injection, compared with controls (0 mg) or a higher concentration of 400 mg.

Auxins About two weeks after GA injection, endogenous IAA began to increase. The increase was proportional to concentration of injected exogenous GA, i.e. the highest IAA was found in the trees injected with 400 gm GA. Furthermore, concentrations of endogenous IAA peaked at different time points with different amounts of GA supplied. For example, IAA peaked at week 2 or week 3 in trees injected with either 40 mg or 400 mg GA respectively. Increased dry weight was

found in trees injected with more GAs, *i.e.* 40 mg and 400 mg, 3 weeks after GA injection and later.

Abscisic acid No obvious changes were caused by stem-injected GA in ABA and its metabolites. ABA declined in concentration as the season advanced.

Cytokinins No obvious changes were found in endogenous CKs after GA treatments. Concentrations of CKs were generally very low. In most of the samples only iPA could be quantified.

Conclusions: One week after the injection, concentrations of GAs were elevated in all GA-treated samples. The ratio of GA_4 to GA_7 decreased significantly at week 3. Absolute concentrations of all GAs reduced sharply 3 weeks after GA application. After 5 weeks, GA_1 and GA_4 were below detection limits in all of the samples and GA_7 and GA_3 were only found in the samples treated with 40 or 400 mg of GAs. Endogenous IAA showed an increase two weeks following GA injection. Its concentration peaked either at week 2 or week 3, corresponding to the 40 or 400 mg GA treatment, respectively. Injection of 400 mg of GA brought about an increase in IAA up to 2-fold compared to controls. Seed orchard data revealed that injection of either 40 mg or 400 mg GA enhanced female cone formation whereas male cone formation was only enhanced by 400 mg GA. Slight decreases in concentrations of ABA and iPA were observed after GA application. No significant change was found in ABA metabolites except for a slight decrease in the level of 7'-OH ABA. Concentrations of ABA declined during the growing season while ABA-GE increased correspondingly.

b) Genotype reactions to PGR injection (Report 2008/2009, pp. 1-4)

Experiment: Effects of GA, auxin and genotype on cone induction in Douglas-fir

Purpose: To investigate hormonal responses of different genotypes to PGR induction

Rationale: Responses to cone induction treatments among various genotypes may differ in concentration changes of endogenous phytohormones and/or their metabolites.

Plant materials: Based on previous cone induction data, four genotypes were selected: 9115 (high cone yield), 9137 and 9550 (moderate cone yield) and 8208 (low cone yield).

Methods: PGRs were injected into tree stems in different combinations. Injections were made between the middle of May and the beginning of June in 2008. Samples

for mass spectrometric analysis were collected at five time points from three of the four genotypes (Table 6). The highlighted samples were analyzed at PBI.

Results:

Abscisic acid Concentrations of ABA were higher in both genotypes 8208 and 9115 than that of genotype 9137 at the early spring. The levels became similar in all the three genotype two months later (Figure 33). The treatment of GA+NAA decreased ABA concentrations in genotype 9115. It is not obvious with other genotypes. Concentrations of ABA-GE were much higher in genotype 9115 than other two genotypes (Figure 34). No obvious concentration changes was caused with ABA-GE and PA (Figure 35) by the treatments except for genotype 9115, in which ABA-GE levels were higher than the control at wk 4 and wk 6 (Figure 34). Generally, concentrations of 7'-OH ABA were higher in genotype 9115 than others (Figure 36). Responses of 7'-OH ABA concentrations to the treatments differed among genotypes. Stem-injection of GA plus NAA caused an increase of 7'-OH ABA in genotypes 9137 and 8208, whereas, injection of NAA only increased 7'-OH ABA in genotype 9115 (Figure 36).

Table 6. Douglas-fir samples for metabolomic analysis. PGR injections were completed at PRT seed orchard in spring 2008 before cone differentiation. Samples (3 to 6 long shoot stems each sample) were collected at five time points following PGR injection.

PGR (mg / tree)	8208	9115	9137
0	U26	R3	ZC26
0	J29	X7	ZA1
0	N21	ZV16	E6
GA 60	C8	B1	J26
GA 60	G2	D8	ZK16
GA 60	I24	E13	T28
NAA 60	J37	S22	U35
NAA 60	N34	N28	ZB18
NAA 60	Z29	M21	ZW21
GA 60 + NAA 60	Z37	U38	S17
GA 60 + NAA 60	ZA23	Z36	ZE13
GA 60 + NAA 60	ZF20	V13	ZF22

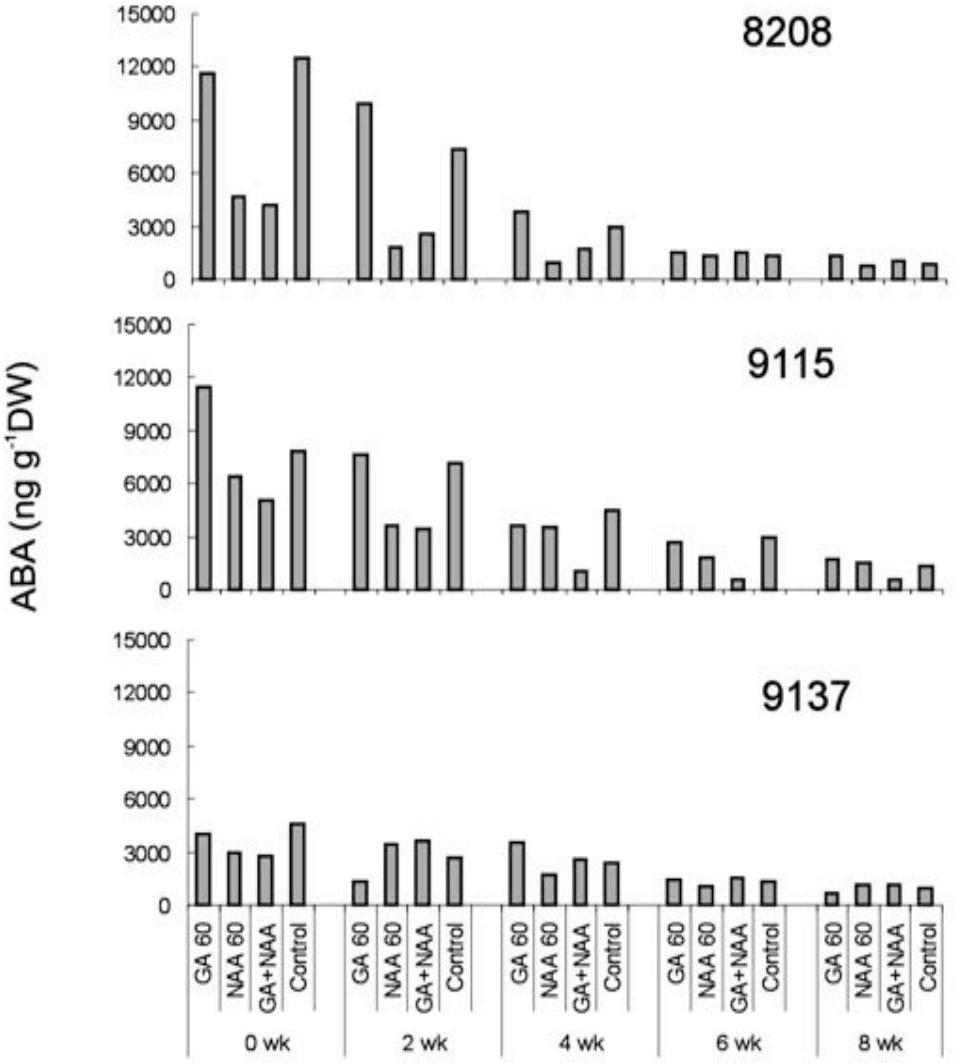

Figure 33. Changes in ABA concentrations in developing long shoots of Douglas-fir before and after PGR injections in three genotypes. Mean of 2 replicates.

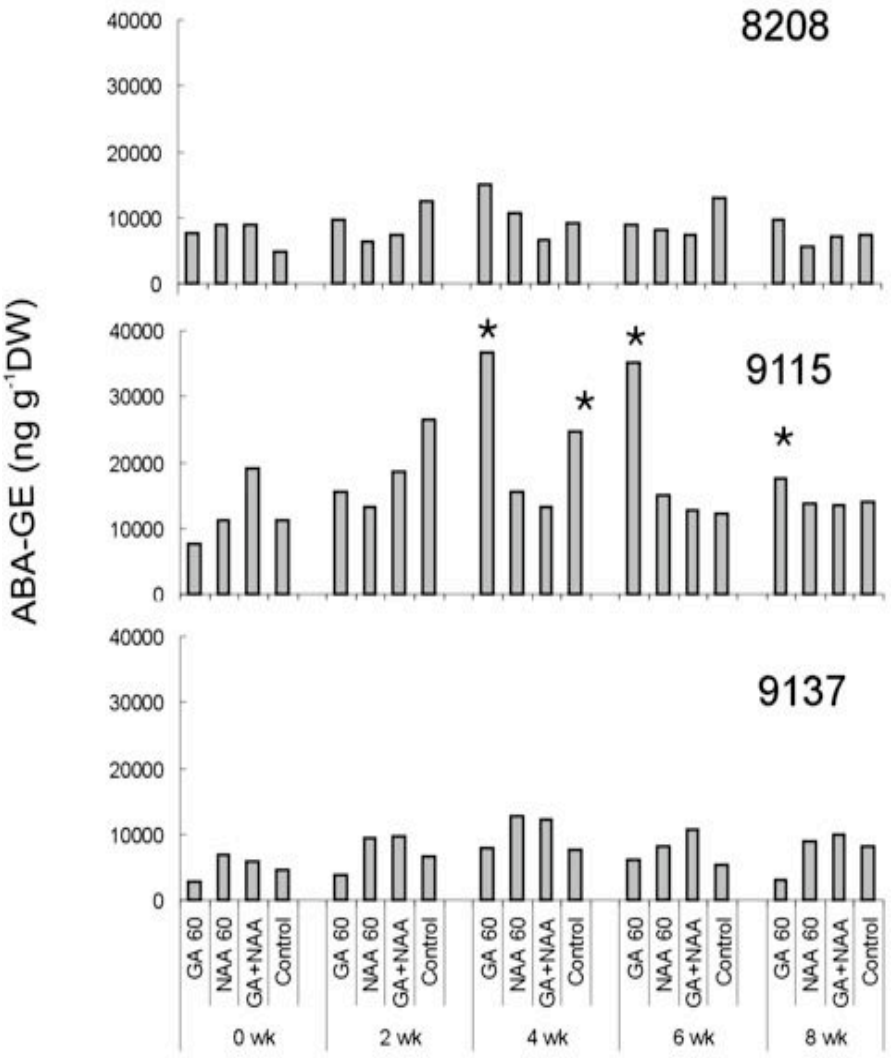

Figure 34. Changes in ABA-GE concentrations in developing long shoots of Douglas-fir before and after PGR injections in three genotypes. Mean of 2 replicates.

* The exact numbers could not be given due to some data was over the highest limitation of quantification and thus the actual quantity should be higher.

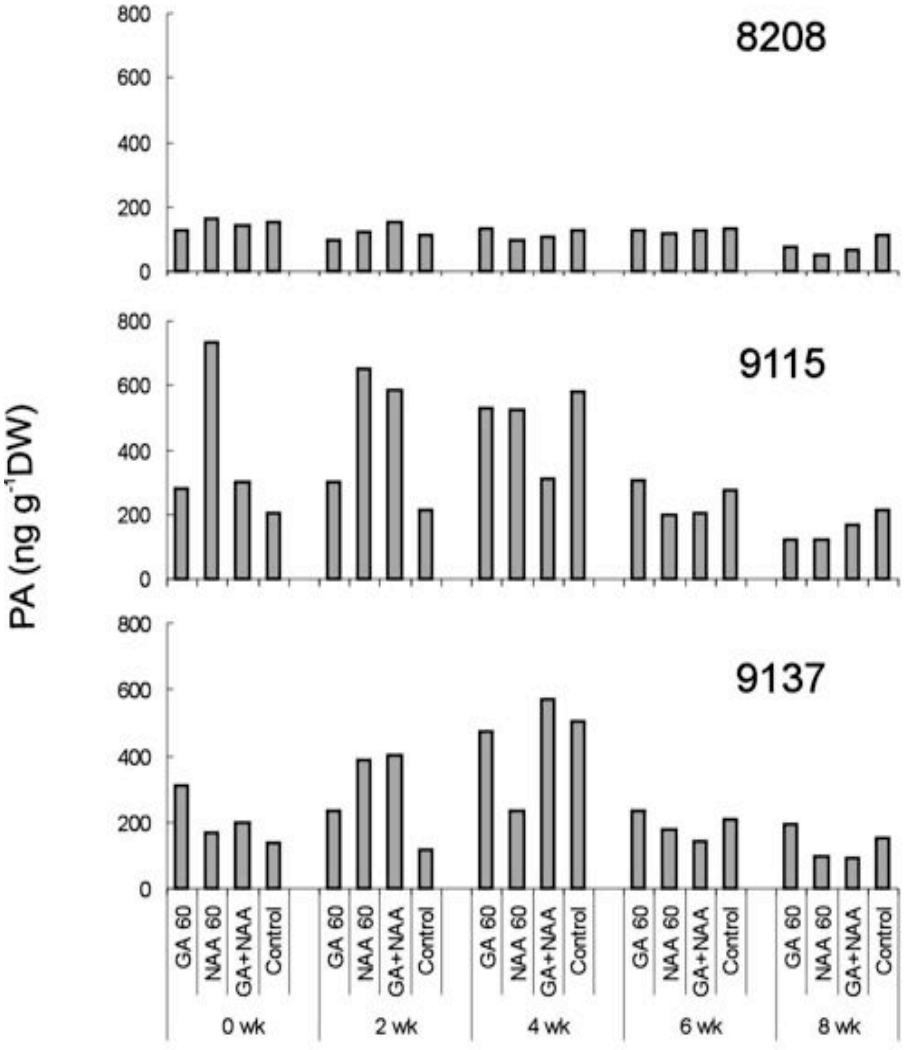

Figure 35. Changes in PA concentrations in developing long shoots of Douglas-fir before and after PGR injections in three genotypes. Mean of 2 replicates.

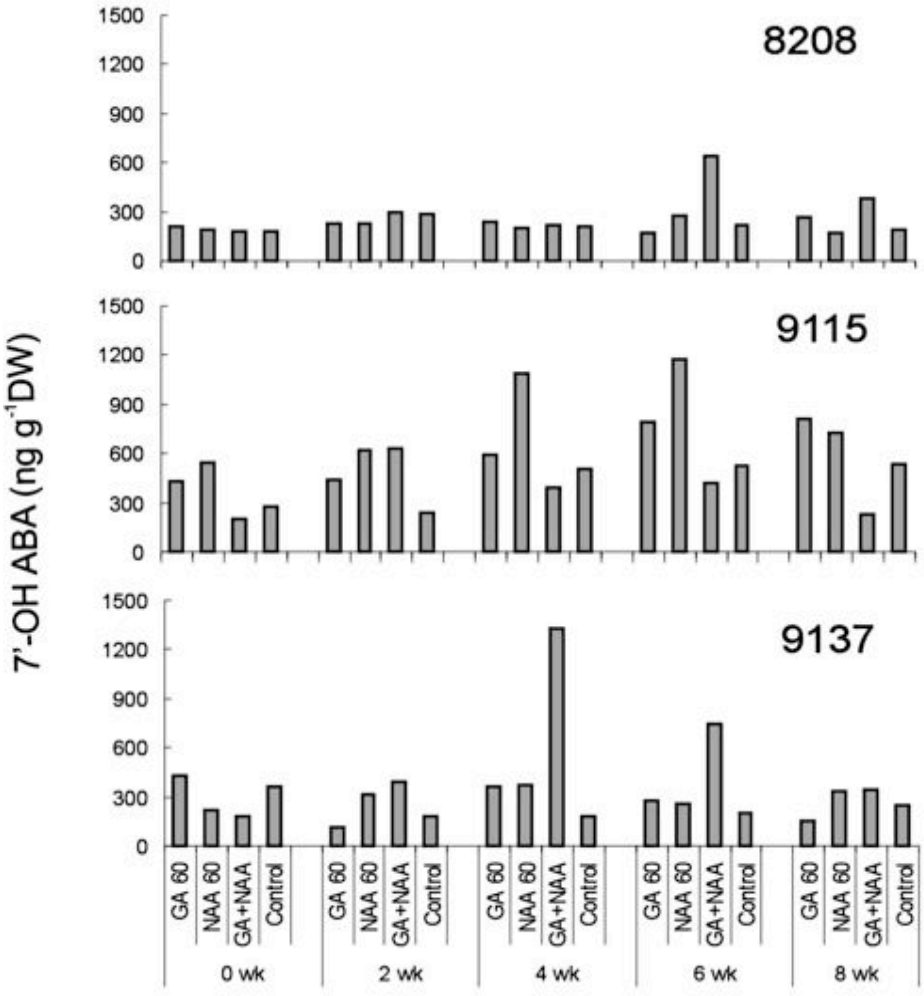

Figure 36. Changes in 7'-OH ABA concentrations in developing long shoots of Douglas-fir before and after PGR injections in three genotypes. Mean of 2 replicates.

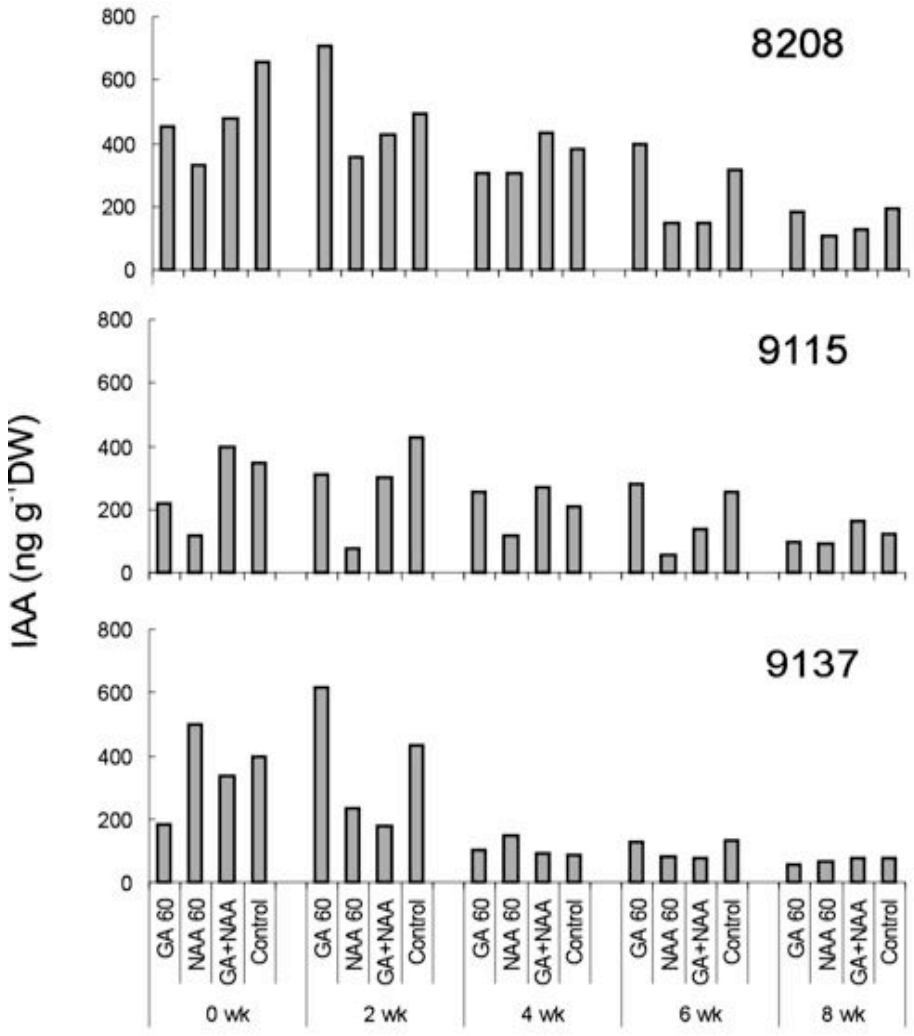

Figure 37. Changes in IAA concentrations in developing long shoots of Douglas-fir before and after PGR injections in three genotypes. Mean of 2 replicates.

Auxin No obvious changes in IAA concentrations was caused by the treatments in any of the genotypes (Figure 37).

Gibberellins Concentrations of GAs decreased quickly in all the genotypes. It could last for about 4 wk. Concentrations of both GA_4 and GA_7 were the lowest in genotype 8208 than other genotypes (Figures 38-39). Concentrations of GAs declined faster with GA plus NAA injection than GA only.

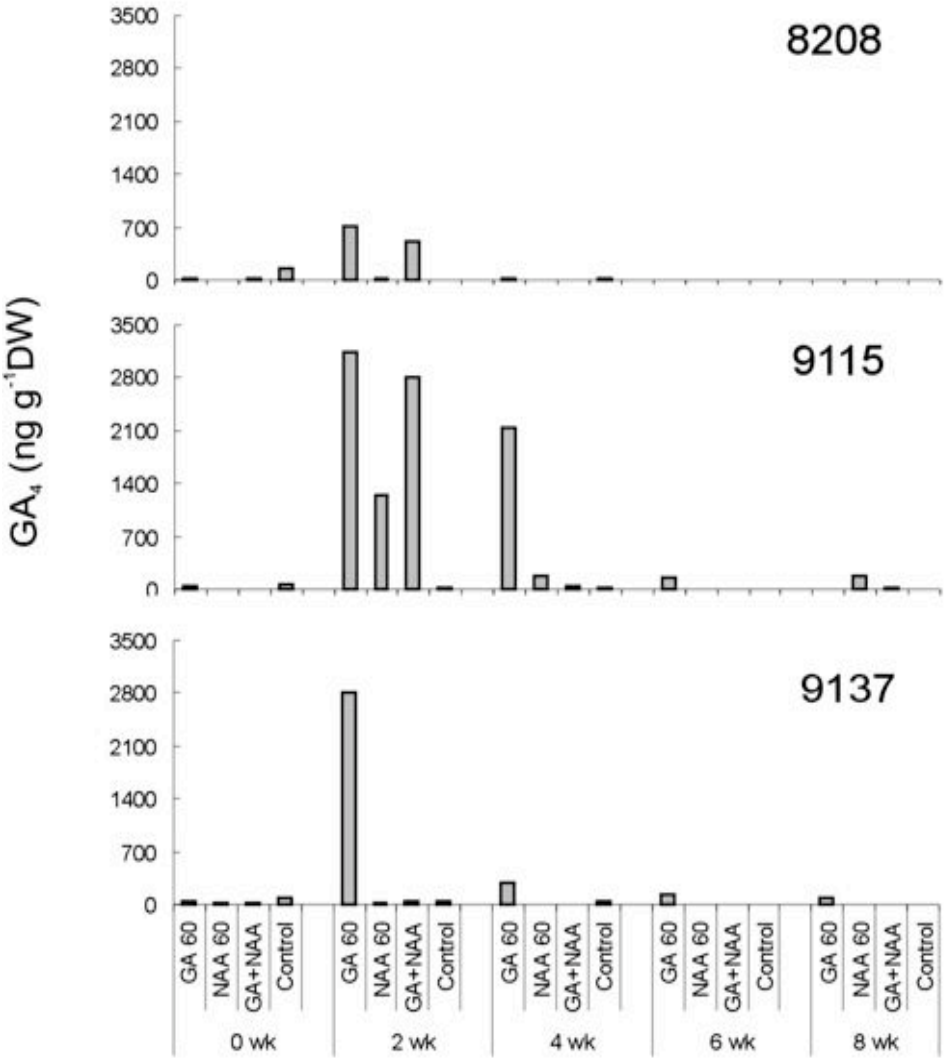

Figure 38. Changes in GA_4 concentrations in developing long shoots of Douglas-fir before and after PGR injections in three genotypes. Mean of 2 replicates.

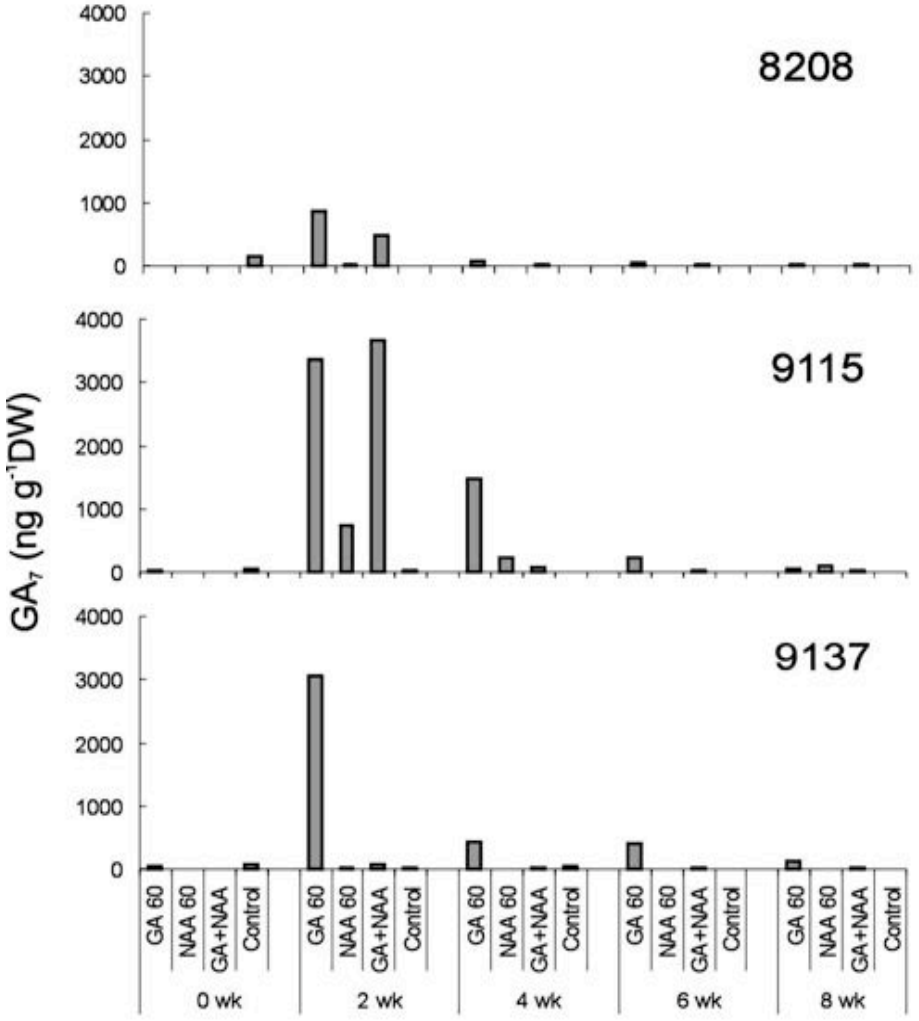

Figure 39. Changes in GA_7 concentrations in developing long shoots of Douglas-fir before and after PGR injections in three genotypes. Mean of 2 replicates.

Cytokinins Concentrations of *t*-ZR increased when season advanced in genotypes 8208 and 9137 (Figure 40). This trend was not clear with genotype 9115. In all the three genotypes, GA+NAA increased *t*-ZR concentrations at either wk 6 or wk 8. Concentrations of iPA were increased by NAA and GA+NAA injections in genotypes 9115 and 9137 at wk 4 (Figure 41).

Conclusions: Plant hormone profiles differ in genotypes. PGR injections altered some hormone profiles and the changes were also different in genotypes. The difference may reflect cone yield capability after induction treatments.

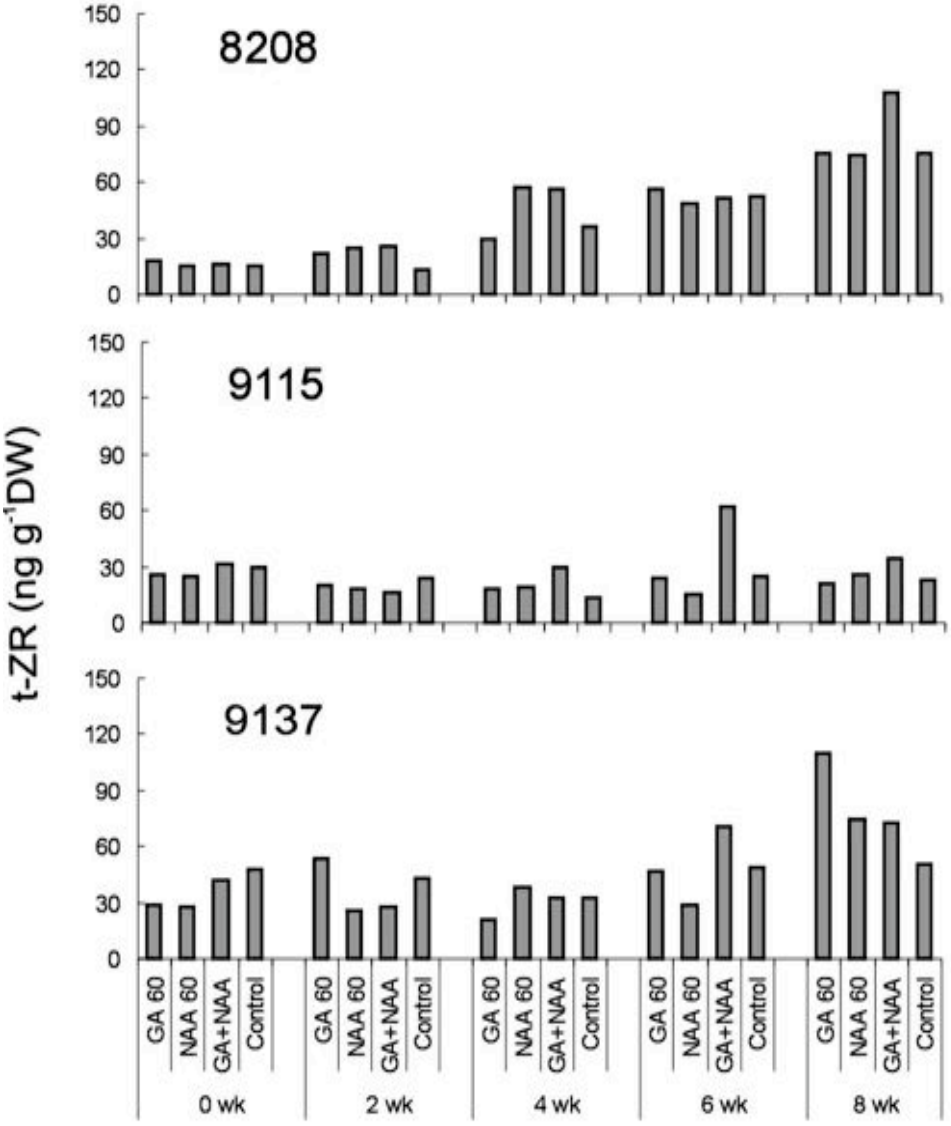

Figure 40. Changes in *t*-ZR concentrations in developing long shoots of Douglas-fir before and after PGR injections in three genotypes. Mean of 2 replicates.

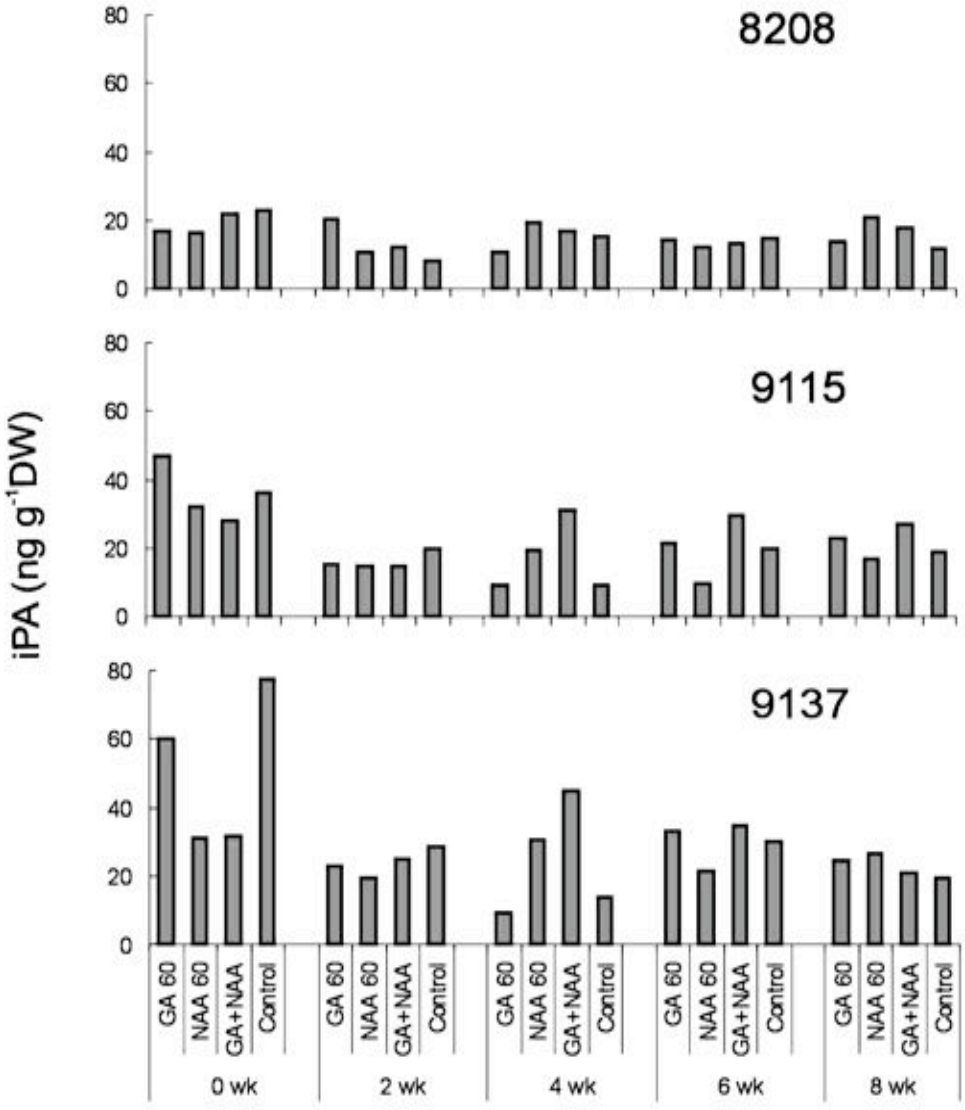

Figure 41. Changes in iPA concentrations in developing long shoots of Douglas-fir before and after PGR injections in three genotypes. Mean of 2 replicates.

b) GA and other chemicals on cone yield without metabolomic analysis (2008/2009)

Experiment: Preliminary trial of stem-Injection of GA and other chemicals in Douglas-fir (Report 2008/2009, pp. 9-10).

Goals: To find stimulators for cone yield enhancement. Further experiments will follow if a positive response is obtained.

Rationale: Cytokinins, polyamines and/or ethylene may affect cone formation

Plant materials: Twenty-one ramets of four Douglas-fir genotypes were used in these tests.

Methods: GA, thidiazuron (TDZ) and/or MGBG (methylglyoxal bis (guanylhydrazone),a polyamine inhibitor that influences polyamine and ethylene synthesis, alone or in combinations were injected into Douglas-fir trees at different quantities. All treatments were carried out between June 3 –25, 2008.

Results: In genotype 8208, female cone yields were increased by a few treatments, such as injections of GA plus other chemicals, *i.e.* NAA, TIBA, or MGBG. Injection of MGBG or TIBA enhanced female cone yields (Figure 42). In genotype 9115 (Figure 43), female cone yield was increased by the injection of GA (60 mg) plus MGBG (100 mg).

Conclusions: Our preliminary results showed that TIBA or MGBG could stimulate cone formation in Douglas-fir. Effects of cone induction was enhanced when these chemicals were applied in combination with GA, which showed potential benefits in cone induction with difficult Douglas-fir genotypes.

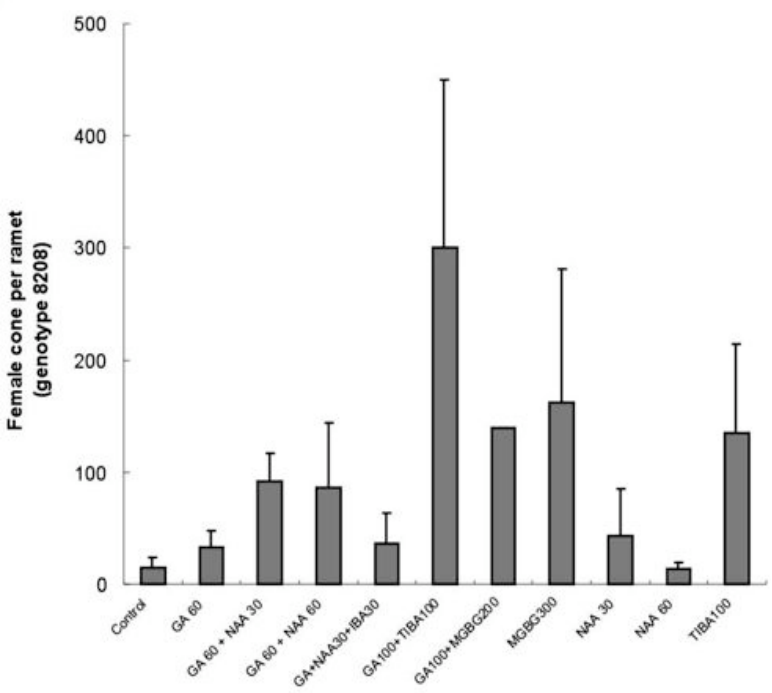

Figure 42. Effects of GA and other chemicals on female cone yield in genotype 8208, Mean ± SE, n= 3.

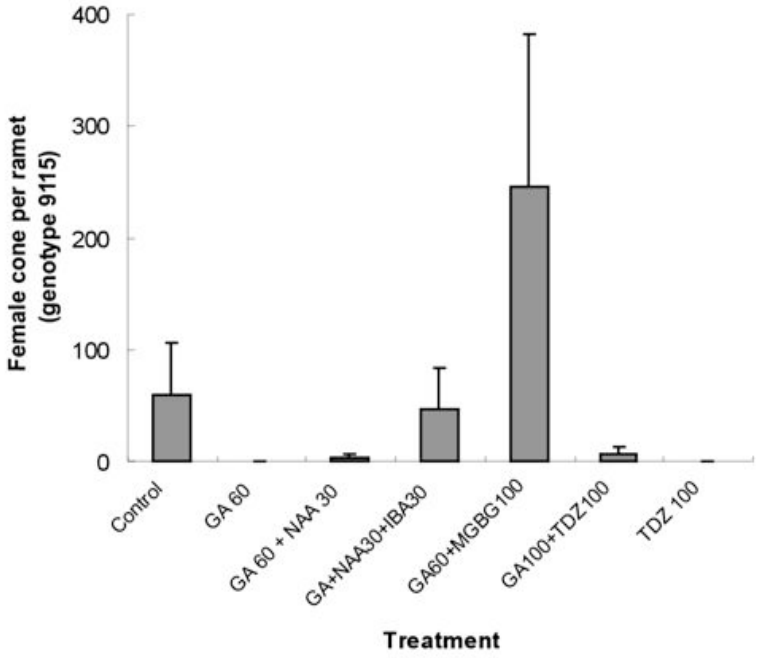

Figure 43. Effects of GA and other chemicals on female cone yield in genotype 9115, Mean ± SE, n= 3.

4. BA treatment (paste and injection) (Report 2007/2008, pp. 50-52.)

Experiment: BA treatment

Goals To assess cone yield as well as other responses of the trees following BA treatments (injection and / or paste) in Douglas-fir.

Results: BA paste or injection had little effect on female cone yield in Douglas-fir. Cone gender was unaffected by either BA injection or BA paste treatment.

Conclusion: Application of BA only had little effect on cone yield in Douglas-fir.

5. Summary

Injection of GAs is an effective method to increase female cone yield in most Douglas-fir genotypes in seed orchards. Meanwhile, cone induction corresponded to increases in GA and IAA concentrations in the developing long shoots where cones formed. Furthermore, our initial results indicate that stem-injections of GA in combination of TIBA or MGBG may solve problems in cone induction with some difficult genotypes that have little response to GA injection.

Part 4 Comparisons of Douglas-fir and lodgepole pine

1. Metabolomic analysis

Four types of major plant hormones as well as some of their metabolites have been quantified in Douglas-fir and lodgepole pine. There were some compounds common to both species, some compounds found only in one or the other species, and patterns of concentration change that differed between species and seasons. In both species, concentrations of ABA were high. Of its catabolites only ABA-GE was high, indicating that ABA glycosylation to ABA-GE was the preferred catabolic pathway. ABA catabolites PA and DPA were always low, and no neoPA was found in either species indicating little activity in either of these catabolic pathways. In both species, the predominant cytokinins were *t*-ZR and iPA. In terms of consistent hormone differences between species, higher IAA concentrations were found in Douglas-fir than in lodgepole pine. Cytokinins dhZ and dhZR were detected only in lodgepole pine. Seasonal differences between these two species were found. A sharp cytokinin increase was seen at the beginning of fall in Douglas-fir but not in lodgepole pine.

Some hormone profiles were similar in both species. Concentration of ABA was high in spring, decreasing during stages of initiation and differentiation in mid-summer. ABA concentrations increased again during the fall, peaking in winter. ABA metabolites PA and DPA also exhibited nearly identical seasonal trends in their concentration changes.

Concentrations of ABA and ABA metabolites such as ABA-GE, PA and DPA were generally higher in Douglas-fir than lodgepole pine (Table 7). A significant inverse correlation was found between ABA-GE and ABA seasonal changes in Douglas-fir but not in lodgepole pine, in which both ABA and ABA-GE concentrations similarly decline over the spring and summer. Concentrations of GAs in conifers are generally low, which poses some problems. GA_4, GA_7, and GA_9 were quantifiable in Douglas-fir long shoots. In comparison, these compounds were below quantifiable levels in lodgepole pine tissue.

Metabolomic hormone analysis showed that endogenous phytohormones other than GAs, such as IAA, could be affected by stem-injection of GAs. Cone induction methods can be either chemical (GAs) or physical (root pruning, stem girdling). Our HPLC-ESI-MS/MS studies show significant differences in these treatments. Physical

treatments do not appear to alter hormone metabolism. In contrast, GA injection of both Douglas-fir and lodgepole pine causes a decline in ABA concentration and alteration in the ratio of Z-type to iP-type cytokinins. In Douglas-fir there is also a pronounced rise in auxin concentration.

Table 7. Plant hormones and their selected metabolites in developing long shoots of Douglas-fir (DF) and lodgepole pine (LP).

	Cytokinins (ng g^{-1} DW)						
	t-Z	**dh-Z**	**t-ZR**	**dhZR**	**t-Z-*O*-Glue**	**iPA**	**2iP**
DF	NQ	NQ	30 to 166	NQ	≤ 30	20 to 95	≤ 12
LP	NQ	≤ 76	≤ 145	≤ 12	≤ 10	≤ 85	≤ 18
	ABA and metabolites (µg g^{-1}DW)						
	ABA	**ABA-GE**	**PA**	**DPA**	**7'-OH ABA**	**neoPA**	
DF	6.7 to 12.5	0.7 to 11	0.2 to 0. 5	≤ 0.05	0.45 to 2.4	NQ	
LP	0.5 to 2.1	0.35 to 2.3	≤ 0.16	≤ 0.02	<0.3	NQ	
	IAA and metabolites (ng g^{-1}DW)						
	IAA	**IAA-glue**	**IAA-Asp**				
DF	11 to 257	NQ	≤ 11				
LP	≤ 85	NQ	NQ				
	GAs (ng g^{-1}DW)*						
	GA_1	**GA_3**	**GA_4**	**GA_7**	**GA_9**	**GA_{12}**	**GA_{24}**
DF	NQ	NQ	≤ 26	≤ 9	NQ	-	-
LP	NQ	NQ	NQ	NQ	NQ	NQ	≤ 320

* GA_{24} was monitored in only one lodgepole pine experiment.

2. Cone yield data collection for treatments in both species

1) PGR injection in Douglas-fir and lodgepole pine

The purity of GA used in 2007 was lower than expected (54% GA_4 and 14% GA_7 in the total weight of GA powder). Effects of GA injection were genotype dependent. Generally, Douglas-fir responds better than lodgepole pine. Injection of BA alone or in combination with GA showed little effect on female cone yield.

2) Stem-girdling treatment in Douglas-fir

Girdling treatment enhanced male cone yield significantly. Although female cone yield was low in both girdled trees and controls, cone production was higher in girdled trees.

3) TIBA, MGBG and GA injection in lodgepole pine and Douglas-fir

TIBA increased lodgepole pine female cone yield in one, a poor cone producer, of the three genotypes (two moderate cone producers and one poor cone producer). TIBA or MGBG injections stimulated cone formation in Douglas-fir. Effects of cone induction were enhanced when these chemicals were applied in combination of GA.

4) BA paste in Douglas-fir and lodgepole pine

Gender was unaffected by BA paste treatment; neither branch paste nor bud paste treatment had any effect. BA treatments resulted in increased lateral bud formation.

5) TDZ and GA paste in lodgepole pine

Cone gender was affected by bud paste treatment. Female cone number could be increased up to 7 fold in the initial test.

6) Root pruning in lodgepole pine

No obvious increase in female cone production occurred following root pruning.

(Preliminary data was collected in the spring of 2008m and 2009. Final cone yield data will be available from seed orchards.)

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Appendix I: Experiments

Lodgepole pine

1) Seasonal hormone profiling (2006-2007)
2) Root pruning A (2006-2007)
1) GA injection (2007-2008)
2) Root pruning B (2007-2008)
3) TIBA injection (2007-2008)
4) Injection of PGR combinations (2007-2008)
5) GA concentrations (2008-2009)
6) Temporo-spatial metabolomics in long shoot buds (2008-2009)
7) PGR paste treatment (2008-2010) -Field test only
8) Cone gender plasticity and PGR paste (2008-2010)

Douglas-fir

9) Seasonal hormone profiling (2006-2007)
10) GA injection (2006-2007)
11) Stem-girdling (2007-2008)
12) Genotype responses (2008-2009)
13) Treatments of PGR combinations (2007-2009) - Field test only
14) Injection of other chemicals (2008-2009) - Field test only

Appendix II: Publications and communications

1. Publications

Kong, L., von Aderkas, P., Abrams, S.R. 2010. A metabolomic plant hormone analysis of cone induction in conifers. In The Flowering Process and its Control in Plants: Gene Expression and Hormone Interaction. M. Yaish and J. Colasanti (eds), Research Signpost, Kerala, India. - In press.

Kong, L., von Aderkas, P., Zaharia, I., Abrams, S.R., Lee, T., Woods, J. Temporospatial dynamics of phytohormones and metabolites in long-shoot buds of lodgepole pine (*Pinus contorta*) during cone bud initiation and gender determination. Manuscript in preparation.

von Aderkas, P., Kong, L., Porter, B., Owen, S.J., Gaudet, D., Abrams, S.R., Barchet, G., Carlson, M., Demdoum, S. & Bonthoux, S. Long shoot development and its seasonal changes in auxin, abscisic acid, cytokinins and metabolites in lodgepole pine. Submitted for publication.

Kong, L., von Aderkas, P. Owen, S., Wagner, T., Abrams, S.R. Phytohormone profiles during female cone differentiation in low and high cone-producing genotypes of lodgepole pine. Submitted for publication.

Kong, L., Abrams, S.R., Owen, S., van Niejenhuis, A. & von Aderkas, P. 2009. Dynamic changes in concentrations of auxin, cytokinin, ABA and metabolites in multiple genotypes of Douglas-fir during a growing season. Tree Physiology 29: 183-190.

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von Aderkas, P., Kong, L., & Micharl Carlson. 2007. One bud, two bud, three bud, four: making lodgepole pine buds count. TICtalk 8: 4-6.

Kong, L. & von Aderkas, P. 2007. Plant growth regulators and cone induction in Pinaceae. Published online by the Forest Genetics Council of British Columbia (http://www.fgcouncil.bc.ca/Pinaceae-cone-ind-lit-rev.pdf).

2. Presentations of conference, meeting or seminar

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