Effects of stem girdling on cone yield and endogenous phytohormones and metabolites in developing long shoots of Douglas-fir (Pseudotsuga menziesii)

Kong, Lisheng; von Aderkas, Patrick; Owen, Stacey J.; Jaquish, Barry; Woods, Jack; Abrams, Suzanne R.

Publisher's version / Version de l'éditeur:
https://doi.org/10.1007/s11056-011-9294-4
New Forests, 43, 4, pp. 491-503, 2011-11-25

Effects of stem girdling on cone yield and endogenous phytohormones and metabolites in developing long shoots of Douglas-fir (*Pseudotsuga menziesii)*

Lisheng Kong (1, 5*), Patrick von Aderkas (1), Stacey J Owen (2),
Barry Jaquish (3), Jack Woods (4), Suzanne R. Abrams (2)

[1] Centre for Forest Biology, University of Victoria, 3800 Finnerty Rd., Victoria, BC, Canada V8W 3N5

[2] Plant Biotechnology Institute, National Research Council of Canada, 110 Gymnasium Place, Saskatoon, SK, Canada S7N 0W9

[3] Kalamalka Research Station, 3401 Reservoir Rd, Vernon BC, Canada V1B 2C7

[4] SelectSeed Company Ltd., 3370 Drinkwater Road, Duncan BC, Canada V9L 5Z2

[5] Warnell School of Forestry and Natural Resources, University of Georgia, Athens, GA 30602 USA

[*] Lisheng Kong (Corresponding author)

Address: Centre for Forest Biology, University of Victoria, 3800 Finnerty Rd, Victoria, BC, Canada V8W 3N5

Tel: 1-(250)-721- 8926

Fax: 1-(250)-721-6611

E-mail: lkong@uvic.ca; lkong@warnell.uga.edu

Key words: *cone yield, Douglas-fir, girdling, genotype, metabolites, phytohormones*

Running title: Girdling, cone yield and endogenous hormones in Douglas-fir

Abstract

Stem-girdling treatments were applied in early spring to stimulate cone formation in two genotypes of interior Douglas-fir (*Pseudotsuga menziesii* var. *glauca* (Beissn.) Franco). After girdling treatments, male cone yield increased significantly in the next growing season. The increase was 14-fold in genotype 9137. In genotype 9550, 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. Multiple phytohormone-related compounds, including gibberellins (GAs), cytokinins, indole-3-acetic acid (IAA), abscisic acid (ABA) and their selected metabolites, were analyzed in developing long shoots after girdling treatments by high performance liquid chromatography-electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) in multiple reaction monitoring mode. Concentrations of GA_4 were slightly higher at week 2 following girdling treatment, whereas at week 8 lower GA_4 concentrations were found in girdled samples. Stem girdling did not affect concentrations of IAA and major cytokinins, such as zeatin riboside and isopentenyl adenosine. Concentrations of ABA differed two-fold between the genotypes. Although girdling treatment did not cause differences in ABA concentrations, it generally resulted in higher concentrations of ABA glucose ester. Concentration increase of 7'-hydroxy ABA by girdling was only found in genotype 9550 at week 8. Girdling caused little change in concentrations of phaseic acid in both genotypes.

57 *Abbreviations*: HPLC-ESI-MS/MS, high performance liquid chromatography-
58 electrospray ionization tandem mass spectrometry; MRM, multiple-reaction monitoring;
59 GA, gibberellic acid; ABA, abscisic acid; PA, phaseic acid; DPA, dihydrophaseic acid;
60 7'-OH ABA, 7'-hydroxy ABA; *neo*PA, *neo*phaseic acid; ABA-GE, abscisic acid glucose
61 ester; IAA, indole-3-acetic acid; IAA-Asp, indole-3-acetic acid aspartate; IAA-Glu,
62 indole-3-acetic acid glutamate; *t*-Z, *trans*-zeatin; *t*-ZR, *trans*-zeatin riboside; *c*-ZR, *cis*-
63 zeatin riboside; *t*-Z-*O*-Glu, *trans*-zeatin-*O*-glucoside; dhZ, dihydrozeatin; dhZR,
64 dihydrozeatin riboside; 2iP, isopentenyl adenine; iPA, isopentenyl adenosine.
65

Introduction

It is a frequent response for many perennial plant species to produce more flowers under stress conditions, such as drought, flooding, or physical damage. Stem girdling or scoring, depending on the amount of bark removal, has been used to enhance flowering and thus higher yield of fruit or seed (Noel 1970; Goren et al. 2004). In some coniferous species, stem girdling increases cone formation (Wheeler et al. 1985; Ross and Bower 1991; Cherry et al. 2007).

Plant hormones regulate tree physiological processes including growth (Savidge and Wareing 1984) and reproduction (Bernier et al. 1993). Abscisic acid (ABA) is a well-known stress hormone. It could increase sink strength (Yang et al. 2003) and function as an endogenous signal (Finkelstein et al. 2002) that adjusts physiological responses to stress (Sauter et al. 2001, 2002; Bray 2002; Himmelbach et al. 2003) by regulating stomatal aperture and the expression of stress-responsive genes (Leung and Giraudat 1998; Finkelstein et al. 2002). Applied gibberellin (GA) can enhance both male and female cone yield in many coniferous species (McMullen 1980; Pharis et al. 1980; Ross 1983; Pharis 1991; Ross and Bower 1991; Kong et al. 2008). More rarely, exogenously applied auxins alone or in combination with GA_{4+7} induce male cones (Pharis et al. 1980; Sheng and Wang 1990), whereas applied cytokinins favor female cone formation (Imbault et al. 1988; Wakushima 2004). Effects of GA_{4+7} on cone bud formation can be enhanced when GA is applied in combination with girdling (Ross and Bower 1991; Cherry et al. 2007).

Stem girdling interrupts phloem transport of carbohydrates (Stock and Silvester 1994; Wang et al. 2006; Urban and Alphonsout 2007), basipetal flow of auxin (Dann et al. 1985) and acropetal flow of cytokinin (Skogerbo 1992; Cutting and Lynne 1993; Havelange et al. 2000). Information about how girdling influences endogenous phytohormone concentrations can possibly be used to improve cone bud induction strategies, such as exogenous application of florigenic PGRs.

The objective of this research project was to investigate effects of stem girdling on cone bud yield and also on concentrations of endogenous phytohormones in the young, elongating long shoots on lateral branches. These are the site for initiation of male and female cone buds. In this research, multiple phytohormones and some selected metabolites were analyzed simultaneously by using high performance liquid chromatography-electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) in multiple-reaction monitoring (MRM) mode. The main advantages of MRM mode are its selectivity, the result of monitoring a specific product ion of the precursor of interest, which reduces interference from matrix components, and its high sensitivity, the result of improving the duty cycle by focusing on only the analytes of interest. Also, no derivitization of the sample is required because volatility of the analytes is not an issue in HPLC, like it is in gas chromatography (GC).

Currently, an MRM method has been applied in analyses of endogenous phytohormones for studies on seed dormancy (Feurtado et al. 2004, 2007), seed parasitism (Chiwocha et al. 2007) and bud development (Kong et al. 2008, 2009, 2011) in coniferous species. In this study, four classes of phytohormones as well as their

selected metabolites were analyzed in two different genotypes of interior Douglas-fir [*Pseudotsuga menziesii* var. *glauca* (Beissn.) Franco].

Materials and Methods

Plant material

Twelve grafted ramets of two genotypes (registration numbers 9550 and 9137) were used for girdling treatment and control in a clonal seed orchard belonging to Pacific Regeneration Technologies Inc. (PRT) in Armstrong, British Columbia (50°26′30″N, 119°11′00″W). Both genotypes are high breeding value parents for volume growth in the Nelson Seed Planning Zone of the B.C. interior Douglas-fir tree breeding program. These 14-year-old ramets had an average stem diameter of 86 ± 18 mm.

Girdling treatment and experimental design

Stem girdling (Figure 1A-B) was applied in May 15, 2007 after bud flushing and before cone differentiation. Two cuts, three inches apart, were made. Each individual was girdled according to standard nursery practice at PRT, meaning through to the xylem, penetrating bark, phloem and cambium. Each cut covered approximately 90% of the stem circumference. To help wound recovery, cuts were covered with cheesecloth following girdling treatment (Figure 1C-D).

Half of the ramets of each genotype were girdled. The others served as controls. To avoid any influence of destructive sampling on cone yield, half of the ramets in each treatment (n=3) were sampled for cone yield, but not for hormone analysis. Data was subject to one-way analysis of variance (ANOVA) using MINITAB

131 software (MINITAB Inc., State College, PA, USA). Significance of means was
132 analyzed by the Tukey test. Overall, levels of significance were set to $P < 0.05$.
133
134 Sample collection, processing and storage
135 Cone yield was assessed in spring of 2008, the year following girdling treatment.
136 Cone production was evaluated from three ramets of each genotype. Samples of long
137 shoots were collected from mid-crown at regular intervals of two or four weeks starting
138 from girdling treatment. Each sample included ten long shoots in the early growing
139 season and a minimum of three in the late season. After collection, needles were
140 removed from the stems of long shoots. Stem samples were wrapped in tin foil,
141 labelled and kept frozen in a - 20 °C freezer for 2 to 3 d. Subsequently, the samples
142 were lyophilized in a freeze-drier for 48 h after the vacuum was stabilized. Dry
143 samples were sealed in plastic bags and stored at - 20 °C.
144
145 Analysis of hormones and their metabolites
146 The analyzed compounds included ABA, 7'-hydroxy ABA (7'-OH ABA), ABA glucose
147 ester (ABA-GE), dihydrophaseic acid (DPA), phaseic acid (PA), *neo*phaseic acid
148 (*neo*PA), trans-zeatin (*t*-Z), *trans*-zeatin riboside (*t*-ZR), *cis*-zeatin riboside (*c*-ZR),
149 dihydrozeatin (dhZ), dihydrozeatin riboside (dhZR), *trans*-zeatin-*O*-glucoside (*t*-Z-*O*-
150 Glu), isopentenyl adenosine (iPA), isopentenyl adenine (2iP), IAA, IAA glutamate
151 (IAA-Glu), IAA aspartate (IAA-Asp), and two gibberellins, GA_4 and GA_7. Compounds

both pure and deuterated, as well as extraction and purification steps were as outlined in Kong et al. (2008). The procedure used for quantification by high performance liquid chromatography-electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) 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 (Ross et al. 2004). QC samples, internal standard blanks, and solvent blanks were also prepared and analyzed along with each batch of tissue samples.

Results

Effects of girdling treatment on cone formation

Girdling treatment significantly increased male cone yield (Table 1). The increase was 14-fold in genotype 9137. In genotype 9550, more than 8,700 male cones were induced by girdling treatment 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 (Table 1).

172 Gibberellins

173 For both genotypes, concentrations of GA_4 were higher in the treated trees than in the
174 controls two weeks after girdling (Table 2). Thereafter, controls were generally higher.
175 GA concentrations were highest four weeks after the beginning of the experiment.
176 Statistically, no significant difference ($P < 0.05$) was found in concentrations of GA_4 or
177 GA_7 between the control and the girdled samples.

178 Cytokinins

179 A few zeatin-type (Z-type) cytokinins were identified and quantified in samples of
180 genotype 9550 (Table 3 and Figure 2). Among Z-type cytokinins, the predominant
181 one is *t*-ZR. In both genotypes, concentrations of *t*-ZR did not significantly change
182 following girdling treatment (Figure 2). Except for a lower concentration of *t*-Z-*O*-Glu
183 in samples of the girdled trees at week 2, no difference caused by girdling was found
184 in other Z-type cytokinins (Table 3). Concentrations of Z and dhZ were below
185 quantification limits. Although 2iP was quantifiable in some samples, its concentration
186 was very low (data not shown), while iPA was quantified in all samples (Figure 2).
187 Again, there was no difference due to treatment.

188 Auxin and metabolites

189 Concentrations of IAA declined after two weeks (Figure 2). By week 8, it had dropped
190 below quantifiable levels in most samples. There was no significant difference in
191 concentrations of IAA in samples of treated and untreated trees. Concentrations of
192 IAA catabolites IAA-Asp and IAA-Glu were generally below detectable levels (results
193 not shown).

194 Abscisic acid and metabolites
195
196 ABA concentration was two-fold higher in genotype 9550 than 9137 (Figure 3). ABA
197 concentration declined as the season advanced. No significant differences between
198 treatments were found. Concentration changes in 7'-OH ABA were similar in both
199 genotypes (Figure 3) although the general level of 7'-OH ABA in genotype 9550 was
200 higher than that in genotype 9137. Except for a higher 7'-OH ABA concentration in
201 the sample of genotype 9550 girdled trees at week 8 after girdling treatment, there
202 was no significant difference between the girdled samples and the controls.
203 Concentrations of PA declined continuously as the season advanced (Figure 3).
204 Girdling treatment in either genotype caused no significant difference in PA
205 concentration. Concentrations of DPA were very low and quantifiable only in few
206 samples (data not shown). *Neo*PA was not quantifiable in any of the samples. ABA-
207 GE increased in the first two weeks and remained during the rest of sampling period
208 (Figure 3). The mean concentrations of ABA-GE appear to be higher in most of the
209 girdled samples with significant difference ($P < 0.05$) at week 4 in genotype 9137. The
210 overall patterns of ABA-GE change after girdling treatment were significantly different
211 between the girdled samples and the control (P=0.046, F=4.69 in genotype 9550;
212 P=0.04, F=4.98 in genotype 9137). For an overall pattern of both genotypes, ABA-GE
213 concentration was 55% higher in the girdled samples than the control (P=0.009,
214 F=7.57, n= 18).

Discussion

Girdling induced much higher yields of male cone in both genotypes. Girdling also induced female cones from zero to small numbers. These results indicate that our girdling treatment was effective and in keeping with previously published reports in which girdling enhanced cone formation in several conifers (Ebell 1971; Bonnet-Masimbert 1982; Wheeler et al. 1985; Ross and Bower 1989). Cone yield can be inconsistent following girdling, as has been recorded for Norway spruce (Bonnet-Masimbert 1987). For Douglas-fir the most important factor influencing cone induction is the date of girdling. The optimal date varies by year, but corresponds to the period in which vegetative buds begin to swell (Ross and Bower 1989). This is similar to larch in which girdling is optimal during long shoot bud elongation (Melchior 1960). Our result of lower female cone yield compared to male cone yield is also similar to cone yields from girdling experiments in a number of French seed orchards (Philippe et al. 2006). In British Columbia, girdling of Douglas-fir trees has been similarly inconsistent, often leading to male-only cone crops (Woods 1989).

Differences in ABA levels between the two genotypes were relatively consistent, with 9550 having higher ABA concentrations than 9137. Genotypic differences were also found in our previous study (Kong et al. 2009). Although concentrations of ABA, a stress hormone (Kempa et al. 2008), were not influenced by girdling treatment, ABA metabolites , ABA-GE and 7'-OH ABA were affected by girdling. This is the first report on a general ABA-GE increase by girdling treatment. In other studies, Stem-girdling treatment resulted in accumulation of soluble sugar and starch in a girdled tree above

its girdling zone (Dann et al. 1985; Li et al. 2003). Soluble sugar, especially glucose, might favour ABA-GE synthesis at higher concentrations after girdling treatment. ABA-GE is a catabolite of ABA located at the end of one of the major ABA metabolic pathways (Nambara and Marion-Poll 2005). ABA-GE is regarded as physiologically inactive.

No significant changes were found in the concentrations of major cytokinins after girdling. Cytokinins are mainly synthesized in the root system and transported to the tree crown through the xylem (Baker 2000). It has been suggested that stem girdling may block phloem transportation of synthesized nutrients from the crown to the root, which leads to root starvation and lower cytokinin levels (Cutting and Lyne 1993). On the other hand, more recent evidence has been found to support local cytokinin synthesis, such as in crown, in conifer trees (Rasmussen et al. 2009). It was suggested that the ratio of sucrose to cytokinins might play an important role during flowering in *Sinapis alba* (Havelange et al. 2000). This ratio could be affected by changes in sugar concentrations without changes in cytokinin levels. This hypothesis might explain girdling effects on cone bud formation since stem girdling enhances sugar accumulation in tree crowns (Dann et al. 1985; Li et al. 2003, Murakami et al. 2008) although more evidence is needed for coniferous species.

In this study, the girdling treatment enhanced male cone formation without concentration changes in endogenous IAA. Kong et al. (2008) found that stem-injected GA_{4+7} increased both female cone yield and endogenous auxin concentrations in Douglas-fir long shoots at concentrations of either 40 or 400 mg

259 GA_{4+7} per tree. However, male cone formation was enhanced only when the higher
260 amount of GA was injected. The higher GA_{4+7} treatments might generate some
261 unknown stress-like effect in addition to IAA increase, resulting in better male cone
262 formation.

263 Concentration changes in phytohormones and metabolites in this study
264 indicated that the metabolic pathways of GA showed little response to stem girdling.
265 In previous reports (Kamienska and Reid 1978; Cutting and Lyne 1993), girdling
266 treatment affected endogenous GA concentrations. In our study, only GA_4
267 concentrations differed between treatments: girdling caused a drop of 1/3 to 2/3 in
268 both genotypes. Exogenously applied GAs stimulate female cone formation, and this
269 effect is further enhanced when GA is applied in combination with a girdling treatment
270 (Philipson 1985; Ross and Bower 1991; Cherry et al. 2007). GA regulation of
271 physiological processes may also involve other phytohormones (Weiss and Ori 2007)
272 and/or gene expressions triggered by girdling treatment (Li et al. 2003).

273 During girdling treatment, few differences in our currently invested nonvolatile
274 phytohormones could lead to more attention to ethylene, a volatile phytohormone.
275 Ethylene could be induced by stress and physical injury (Murayama et al. 2006;
276 Achard et al. 2007) and be able to induce flowering in a number of angiosperms
277 (reviewed by Lin et al. 2009). In the present, little information is available about the
278 role of ethylene during cone initiation and differentiation in coniferous species.
279 Application of ethylene precursors or ethylene releasing compounds, alone or in
280 combination with other PGRs, merits further investigation.

Notable increases in male cone yield by girdling treatment and our analysis indicates that male cone yield might not be regulated directly by concentration changes in endogenous ABA, IAA and cytokinins. In future experiments, more sampling points following the treatment could benefit in finding transient changes, if any, in concentrations of analytes. Since flowering process could be controlled by multiple factors and the physiological signals that induce flowering are complex (Pharis 1991, Bernier et al. 1993, Achard et al. 2006), the relationship between the affected phytohormones and/or their metabolites and enhanced cone yield by girdling treatment needs further study.

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). We express our appreciation to all members of the project steering committee and Valerie Ashley (Kalamalka Research Station), Hilary Graham, Kris King and Laura Whitney (Pacific Regeneration Technologies Inc.), Julia Gill (University of Victoria), Dr. Cunxu Zhang (Northwest A & F University, China), Monika Lafond, Vera Čekić and Dr. Irina Zaharia (NRC-Plant Biotechnology Institute) for their help in this research.

References

Achard P, Baghour M, Chapple A, Hedden P, Van Der Straeten D, Genschik P, Moritz T Harberd NP (2007) The plant stress hormone ethylene controls floral transition

via DELLA-dependent regulation of floral meristem-identity genes. Proc Natl Acad Sci USA 104:6484–6489

Achard P, Cheng H, De Grauwe L, Decat J, Schoutteten H, Moritz T, Van Der Straeten D, Peng J, Harberd NP (2006) Integration of plant responses to environmentally activated phytohormonal signals. Science 311:91–94

Baker D (2000) Vascular transport of auxins and cytokinins in *Ricinus*. Plant Grow Regul 32:157-160

Bernier G, Havelange A, Houssa C, Petitjean A, Lejeune P (1993) Physiological signals that induce flowering. Plant Cell 5:1147-1155

Bonnet-Masimbert M (1982) Effect of growth regulators, girdling, and mulching on flowering of young European and Japanese larches under field conditions. Can J For Res 12:270-279

Bonnet-Masimbert M (1987) Flower induction in conifers: A review of available techniques. For Ecol Manage 19:135 -146

Bray EA (2002) Abscisic acid regulation of gene expression during water-deficit stress in the era of the *Arabidopsis* genome. Plant Cell Environ 25:153-161

Cherry ML, Anekonda TS, Albrecht MJ, Howe GT (2007) Flower stimulation in young miniaturized seed orchards of Douglas-fir (*Pseudotsuga menziesii*). Can J For Res 37:1-10

Chiwocha SDS, Abrams SR, Ambrose SJ, Cutler AJ, Loewen M, Ross ARS, Kermode AR (2003) A method for profiling classes of plant hormones and their metabolites using liquid chromatography-electrospray ionization tandem mass

spectrometry: analysis of hormone regulation of thermodormancy of lettuce (*Lactuca sativa* L.) seeds. Plant J 35:405-417

Chiwocha SDS, Cutler AJ, Abrams SR, Ambrose SJ, Yang J, Ross ARS, Kermode AR (2005) The *etr1-2* mutation in *Arabidopsis thaliana* affects the abscisic acid, auxin, cytokinin and gibberellin metabolic pathways during maintenance of seed dormancy, moist-chilling and germination. Plant J 42:35-48

Chiwocha S, Rouault G, Abrams S, von Aderkas P (2007) Parasitism of seed of Douglas-fir (*Pseudotsuga menziesii*) by the seed chalcid, *Megastigmus spermotrophus*, and its influence on seed hormone physiology. Sex Plant Reprod 20:19-25

Cutting JGM, Lyne MC (1993) Girdling and the reduction in shoot xylem sap concentrations of cytokinins and gibberellins in peach. J Horticul Sci 68:619–626

Dann IR, Jerie PH, Chalmers DJ (1985) Short-term changes in cambial growth and endogenous IAA concentrations in relation to phloem girdling of peach, *Prunus persica* (L.) Batch. Aust J Plant Physiol 12:392-402

Ebell LF (1971) Girdling: its effect on carbohydrate status and on reproductive bud and cone development of Douglas fir. Can J Bot 49:453-466

Feurtado JA, Ambrose SJ, Cutler AJ, Ross ARS, Abrams SR, Kermode AR (2004) Dormancy termination of western white pine (*Pinus monticola* Dougl. Ex D. Don) seeds is associated with changes in abscisic acid metabolism. Planta 218:630–639

345 Feurtado JA, Yang J, Ambrose SJ, Cutler A, Abrams S, Kermode AR (2007)
346 Disrupting abscisic acid homeostasis in western white pine (*Pinus monticola*)
347 seeds induces dormancy termination and changes in abscisic acid catabolites. J
348 Plant Growth Regul 26:46-54
349 Finkelstein RR, Gampala SSL, Rock CD (2002) Abscisic acid signaling in seeds and
350 seedlings. Plant Cell 14 (Suppl):S15-45
351 Goren R, Huberman M, Goldschmidt EE (2004) Girdling: physiological and
352 horticultural aspects. Horticul Rev 30:1-36
353 Havelange A, Lejeune P, Bernier G (2000) Sucrose/cytokinin interaction in *Sinapis*
354 *alba* at floral induction: a shoot-to-root-to-shoot physiological loop. Physiol Plant
355 109:343–350
356 Himmelbach A, Yang Y, Grill E (2003) Relay and control of abscisic acid signalling.
357 Curr Opin Plant Biol 6:470-479
358 Imbault N, Tardieu I, Joseph C, Zaerr JB, Bonnet-Masimbert M (1988) Possible role of
359 isopentenyladenine and isopentenyladenosine in flowering of *Pseudotsuga*
360 *menziesii*: endogenous variations and exogenous applications. Plant Physiol
361 Biochem 26:289-295
362 Kamienska A, Reid DM (1978) The effect of stem girdling on levels of GA-like
363 substances in sunflower plants. Bot Gaz 139:18-26
364 Kempa S, Krasensky J, Dal Santo S, Kopka J, Jonak C (2008) A central role of
365 abscisic acid in stress-regulated carbohydrate metabolism. PLoS ONE 3(12):
366 e3935

Kong L, Abrams SR, Owen S, Graham H, von Aderkas P (2008) Phytohormones and their metabolites during long shoot development in Douglas-fir following cone induction by gibberellin injection. Tree Physiol 28:1357-1364

Kong L, Abrams SR, Owen S, Van Niejenhuis A, von Aderkas P (2009). Dynamic changes in concentrations of auxin, cytokinin, ABA and selected metabolites in multiple genotypes of Douglas-fir (*Pseudotsuga menziesii*) during a growing season. Tree Physiol 29:183-190

Kong L, von Aderkas P, Owen S, Wagner T, Abrams SR (2011) Comparison of endogenous cytokinins, ABA and metabolites during female cone bud differentiation in low and high cone-producing genotypes of lodgepole pine. Trees 10.1007/s00468-011-0585-3

Leung J, Giraudat J (1998) Abscisic acid signal transduction. Annu Rev Plant Physiol Plant Mol Biol 49:199-222

Li C-Y, Weiss D, Goldschmidt EE (2003) Girdling affects carbohydrate-related gene expression in leaves, bark and roots of alternate-bearing citrus trees. Ann Bot 92:137-143

Lin Z, Zhong S, Grierson D (2009) Recent advances in ethylene research. J Exp Bot 60: 3311-3336

McMullan EE (1980) Effect of applied growth regulators on cone production in Douglas-fir, and relation of endogenous growth regulators to cone production capacity. Can J For Res 10:405-422

388 Melchior GH (1960) Ringelungsversuche zur Steigerung der Blühwilligkeit
389 anjapanischer Lärche (*Larix leptolepis* (Sieb & Zucc) Gord) und an europäischer
390 Lärche (*Larix decidua* Mill). Silvae Genet 9:105-111
391 Murakami PF, Schaberg PG, Shane JB (2008) Stem girdling manipulates leaf sugar
392 concentrations and anthocyanin expression in sugar maple trees during autumn.
393 Tree Physiol 28:1467-1473
394 Murayama H, Sekine D, Yamauchi Y, Gao M, Mitsuhashi W, Toyomasu T (2006)
395 Effect of girdling above the abscission zone of fruit on 'Bartlett' pear ripening on
396 the tree. J Exp Bot 57:3679-3686
397 Nambara E, Marion-Poll A (2005) Abscisic acid biosynthesis and catabolism. Annu
398 Rev Plant Biol 56:165-185
399 Noel ARA (1970) The girdled tree. Bot Rev 36:162-195
400 Pharis RP (1991) Physiology of gibberellins in relation to floral initiation and early floral
401 differentiation. *In* Symp. on 50th Anniversary Meeting on Isolation of Gibberellins.
402 Eds. N. Takahashi, BV. Phinney and J MacMillan. Springer Verlag, Heidelberg,
403 pp.166-178
404 Pharis RP, Ross SD, McMullan EE (1980) Promotion of flowering in the *Pinaceae* by
405 gibberellins. III. Seedlings of Douglas-fir. Physiol Plant 50:119-126
406 Philippe G, Baldet P, Héois B, Ginisty C (2006) Reprouction sexuée des conifèrs et
407 production de semences en vergers à graines. *Imprimé en France*.- JOUVE, 11,
408 bd de Sébastopol, 75001 PARIS.

409 Philipson JJ (1985) The promotion of flowering in large field-grown Sitka spruce by
410 girdling and stem injections of gibberellin $A_{4/7}$. Can J For Res 15:166-170
411 Rasmussen HN, Veierskov B, Hansen-Møller J, Nørbæk R and Nielsen UB (2009)
412 Cytokinin profiles in the conifer tree *Abies nordmanniana*: whole-plant relations in
413 year-round perspective. J Plant Grow Regul 28:154-166
414 Ross SD (1983) Enhancement of shoot elongation in Douglas-fir by gibberellin $A_{4/7}$
415 and its relation to the hormonal promotion of flowering. Can J For Res13:986-994
416 Ross ARS, Ambrose SJ, Cutler AJ, Feurtado JA, Kermode AR, Nelson K, Zhou R,
417 Abrams SR (2004) Determination of endogenous and supplied deuterated
418 abscisic acid in plant tissues by high performance liquid chromatography-
419 electrospray ionization tandem mass spectrometry with multiple reaction
420 monitoring. Anal Biochem 329:324-333
421 Ross SD, Bower RC (1989) Cost-effective promotion of flowering in a Douglas-fir seed
422 orchard by girdling and mulsed stem injection of gibberellin $A_{4/7}$. Silvae
423 Genet 38:189-195
424 Ross SD, Bower RC (1991) Promotion of seed production in Douglas-fir grafts by
425 girdling + gibberellin A4/7 stem injection, and effect of retreatment. New For
426 5:23-34
427 Sauter A, Davies WJ, Hartung W (2001) The long-distance abscisic acid signal in the
428 droughted plant: the fate of the hormone on its way from root to shoot. J Exp Bot
429 52:1991-1997

430 Sauter A, Dietz K-J, Hartung W (2002) A possible stress physiological role of abscisic
431 acid conjugates in root-to-shoot signalling. Plant Cell Environ 25:223-228
432 Savidge RA, Wareing PF (1984) Seasonal cambial activity and xylem development in
433 *Pinus contorta* in relation to endogenous indol 3-ylacetic and (*S*)-abscisic acid
434 levels. Can J Forest Res 14:676-682
435 Sheng C, Wang S (1990) Effect of applied growth regulators and cultural treatments
436 on flowering and shoot growth of *Pinus tabulaeformis*. Can J For Res 20:679-685
437 Skogerbo G (1992) Effects of root pruning and trunk girdling on xylem cytokinins
438 content of apple. Norweg J Agri Sci 6:499-507
439 Stock PA, Silvester WB (1994) Phloem transport of recently-fixed nitrogen in the
440 *Gunnera-Nostoc* symbiosis. New Phytol 126:259-266
441 Urban L, Alphonsout L (2007) Girdling decreases photosynthetic electron fluxes and
442 induces sustained photoprotection in mango leaves. Tree Physiol 27:345–352
443 Wakushima S (2004) Promotion of female strobili flowering and seed production in
444 two Japanese pine species by 6-benzylaminopurine (BAP) paste application in a
445 field seed orchard. J Plant Grow Regul 23:135-145
446 Wang WJ, Zu YG, Wang HM, Li XY, Hirano T, Koike T (2006) Newly-formed
447 photosynthates and the respiration rate of girdled stems of Korean pine (*Pinus*
448 *koraiensis* Sieb. *et* Zucc.). Photosynthetica 44:147-150
449 Weiss D, Ori N (2007) Mechanisms of cross talk between gibberellin and other
450 hormones. Plant Physiol 144:1240-1246

Wheeler NC, Masters CJ, Cade SC, Ross SD, Keeley JW, Hsin LY (1985) Girdling: an effective and practical treatment for enhancing seed yields in Douglas-fir seed orchards. Can J For Res15: 505-510

Woods JH (1989) Stem girdling to increase seed and pollen production of coast Douglas-fir. B.C Ministry of Forests research note No 103

Yang J, Zhang J, Wang Z, Zhu Q (2003) Hormones in the grains in relation to sink strength and postanthesis development of spikelets in rice. Plant Grow Regul 41:85-195

462 **Tables**

463
464
465
466 Table 1. Cone yield per tree (mean ± SE) of two Douglas-fir genotypes 9550 and 9137
467 subjected to girdling. Asterisk (*) indicates significant difference ($P < 0.05$) between
468 the treatment and control, based on three replicates.

469

Treatment	9550		9137	
	♂ cone	♀ cone	♂ cone	♀ cone
Control	0	0	572 ± 143	0
Girdling	8,723 ± 1,690 *	10 ± 9	8,135 ± 1,356 *	6 ± 5

470

Table 2. Effects of stem girdling on concentrations (ng g^{-1} DW) of endogenous gibberellins in long shoots of Douglas-fir in two genotypes. Mean (± standard error) values of three independent replicates (n=3) are shown. NQ stands for not quantifiable.

		9550		9137	
Week	**Treatment**	GA_4	GA_7	GA_4	GA_7
2	Control	6.5 ± 6.5	2.6 ± 2.6	3.3 ± 3.3	NQ
	Girdling	13.9 ± 1.8	NQ	10.5 ± 6.0	NQ
4	Control	63.1± 23.6	8.8 ± 4.9	26.2 ± 15.6	7.0 ± 5.0
	Girdling	21.1 ± 3.8	7.5 ± 2.6	19.4 ± 7.7	6.8 ± 3.4
8	Control	11.6 ± 2.4	2.3 ± 2.3	2.9 ± 2.9	NQ
	Girdling	NQ	NQ	6.3 ± 6.3	NQ

Table 3. Effects of stem girdling on concentrations (ng g^{-1} DW) of endogenous zeatin-type cytokinins in long shoots of Douglas-fir in genotype 9550. Mean (± standard error) values of three independent replicates (n=3) are shown. Asterisk (*) indicates significant difference ($P < 0.05$) compared with the control at each individual time point. NQ stands for not quantifiable.

Week	Treatment	t-Z-*O*-Glu	c-ZR	dh-ZR
2	Control	6.4 ± 0.1	11.1 ± 1.3	NQ
	Girdling	5.5 ± 0.1*	12.0 ± 0.7	1.6 ± 1.6
4	Control	7.9 ± 0.2	19.0 ± 2.7	7.4 ± 0.6
	Girdling	8.6 ± 0.6	16.9 ± 1.7	8.8 ± 1.4
8	Control	21.1 ± 2.0	13.6 ± 1.3	13.6 ± 1.8
	Girdling	17.0 ± 1.3	13.7 ± 1.7	10.7± 2.2

Figure legends

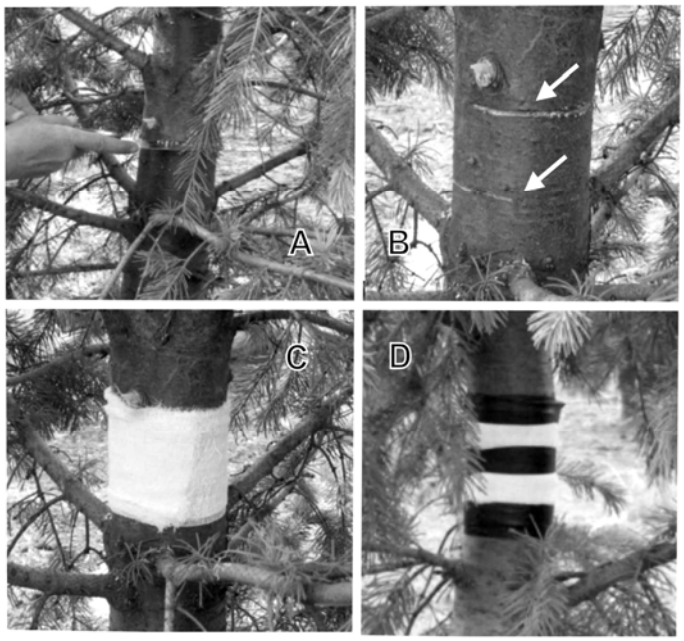
Figure 1. Photos showing the process of stem-girdling treatment in Douglas-fir. Girdling was applied by saw (A) on the stem (B, arrows). Cheesecloth (C) and tape were placed on the girdled area (D) after girdling treatment for protection.

Figure 2. Concentrations of cytokinins and auxin in Douglas-fir long shoots following girdling treatment in May 15, 2007 with genotypes 9550 (left column) and 9137(right column). Girdling treatment (black), control (grey), mean ± SE, n=3. NQ stands for not quantifiable.

Figure 3. Concentrations of ABA and metabolites in Douglas-fir long shoots following girdling treatment in May 15, 2007 with genotypes 9550 (left column) and 9137(right column). Girdling treatment (black), control (grey), mean ± SE, n=3. Asterisk (*) indicates significant difference ($P < 0.05$) compared with the control at each individual time point.

511 **Figures**

512



513

514

515 Figure 1

516

517

518

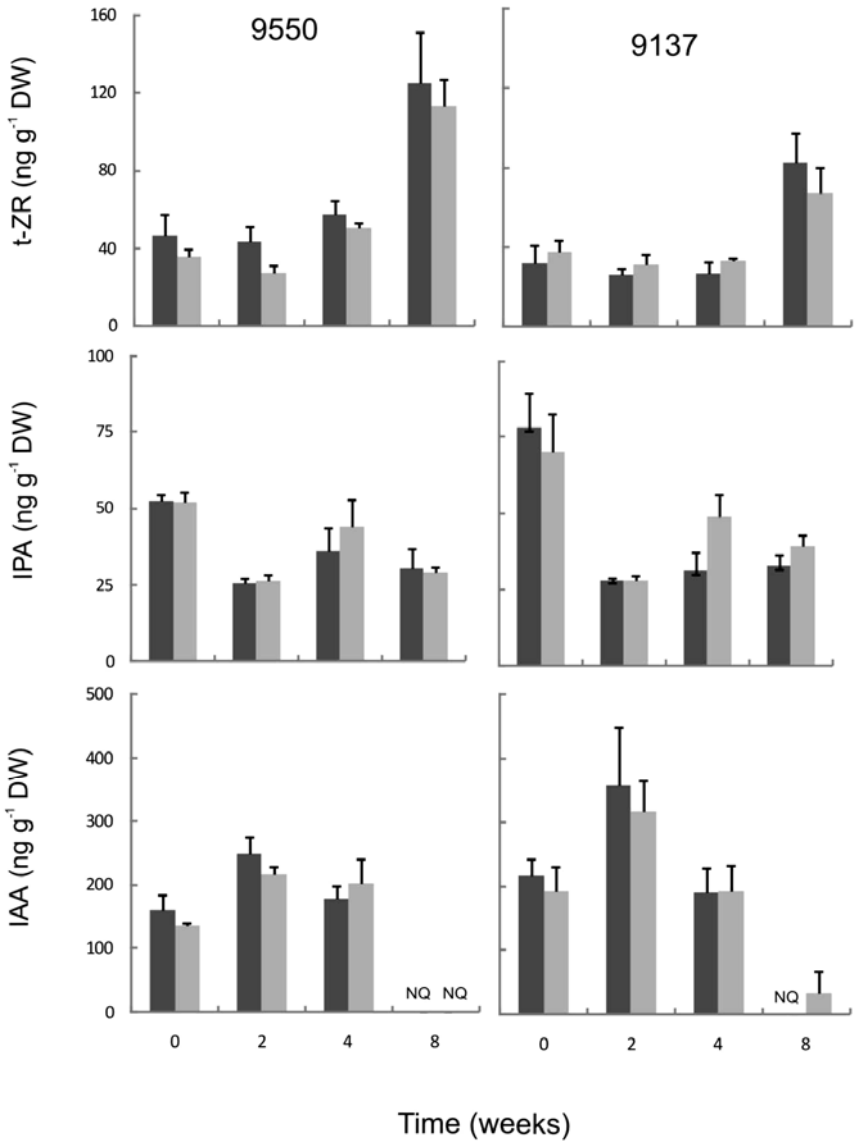
519

520

521

522

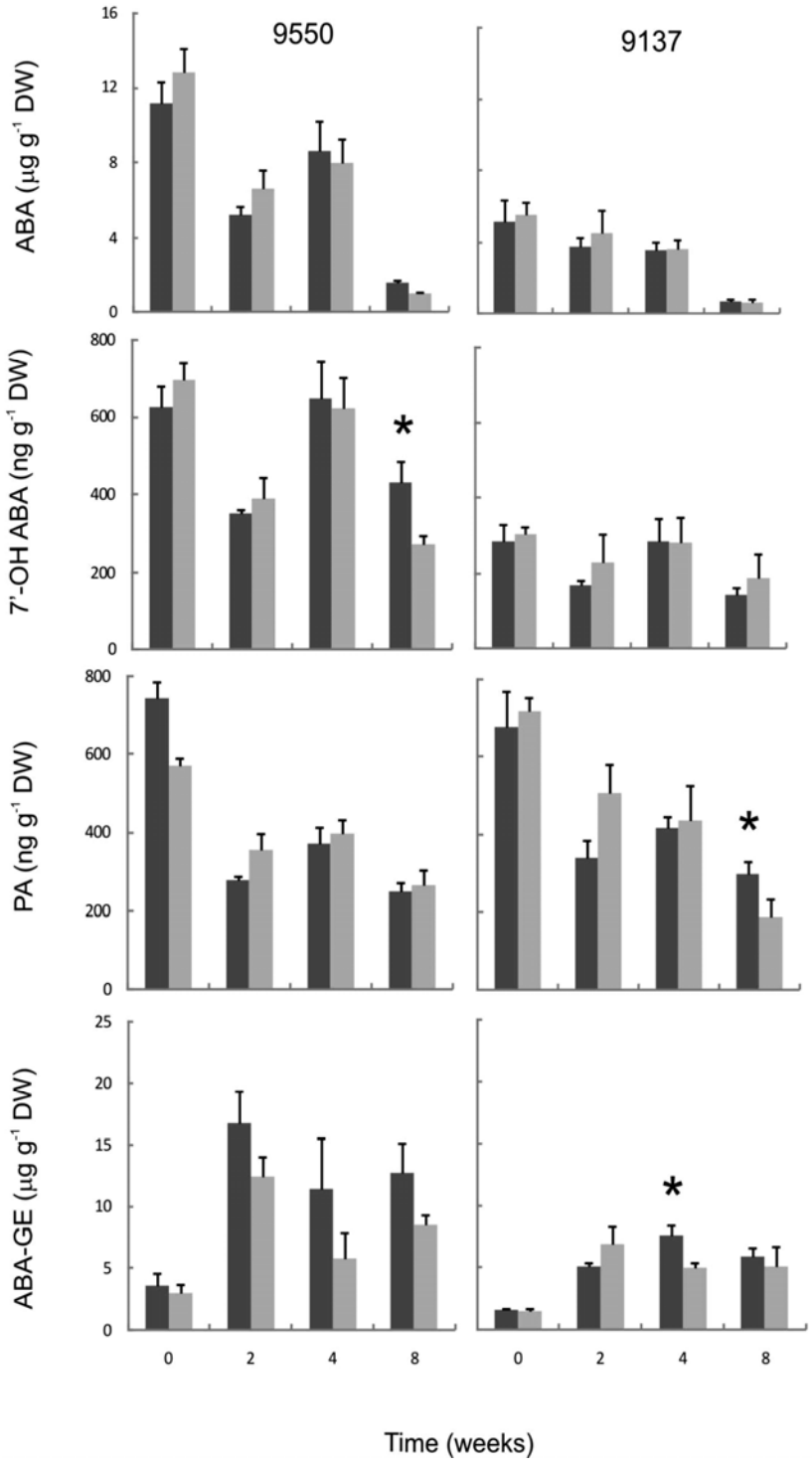
523



524

525 Figure 2

526



527

528 Figure 3