

***In vivo* function of *Pgβglu-1* in the release of acetophenones in white spruce**

Melissa H. Mageroy^{1,2}, Denis Lachance³, Sharon Jancsik¹, Geneviève Parent^{4,5}, Armand Séguin³, John Mackay^{4,5} and Joerg Bohlmann^{1,6,7}

¹Michael Smith Laboratories, University of British Columbia, Vancouver, BC V6T 1Z4, Canada,

²Norwegian Institute of Bioeconomy Research, PO Box 115, N-1431 Ås, Norway

³Natural Resources Canada, Laurentian Forestry Centre, Québec, QC G1V 4C7, Canada,

⁴Department of Wood and Forest Sciences, Université Laval, Québec, QC G1V 0A6, Canada,

⁵Department of Plant Sciences, University of Oxford, South Parks Road, Oxford, OX1 3RB, UK

⁶Department of Forest and Conservation Sciences, University of British Columbia, Vancouver, BC V6T 1Z4, Canada

⁷Department of Botany, University of British Columbia, Vancouver, BC V6T 1Z4, Canada

Corresponding author:

Joerg Bohlmann

Email address: bohlmann@mssl.ubc.ca

1 **ABSTRACT**

2 Eastern spruce budworm (*Choristoneura fumiferiana* Clemens) (ESBW) is a major forest pest
3 which feeds on young shoots of white spruce (*Picea glauca*) and can cause landscape level
4 economic and ecological losses. Release of acetophenone metabolites, piceol and pungenol, from
5 their corresponding glycosides, picein and pungenin, can confer natural resistance of spruce to
6 ESBW. A beta-glucosidase gene, *Pgβglu-1*, was recently discovered and the encoded enzyme
7 was characterized *in vitro* to function in the release of the defensive acetophenone aglycons.
8 Here we describe overexpression of *Pgβglu-1* in a white spruce genotype whose metabolome
9 contains the glucosylated acetophenones, but no detectable amounts of the aglycons. Transgenic
10 overexpression of *Pgβglu-1* resulted in release of the acetophenone aglycons *in planta*. This
11 work provides *in vivo* evidence for the function of *Pgβglu-1*.

12
13 **INTRODUCTION**

14 Eastern spruce budworm (*Choristoneura fumiferiana* Clemens) (ESBW) is considered the most
15 detrimental pest of spruce and fir forests in eastern North America. While populations of ESBW
16 usually persist at endemic levels, outbreaks can last for years incurring landscape level
17 ecological changes and major economic losses (Chang et al. 2015; MacLean 2016). With climate
18 change, outbreaks of ESBW are predicted to increase in frequency and severity (Hennigar et al.
19 2013). Current forest management practices to control ESBW outbreak include costly aerial
20 spraying of *Bacillus thuringiensis* (Bt) and the insect growth regulator Mimic® (NRCAN 2016).
21 The Bt *cryIAb* gene has been successfully overexpressed in white spruce and shown to be
22 effective against ESBW (Lachance et al. 2007), however commercial deployment of transgenic
23 trees is not permitted in Canada.

24
25 Recently, natural resistance to ESBW was discovered in white spruce (*Picea glauca*). Resistant
26 genotypes accumulated the acetophenone aglycons piceol and pungenol as well as the
27 corresponding glucosides picein and pungenin. Non-resistant genotypes only accumulated the
28 acetophenone glucosides (Delvas et al. 2011). Parent et al. (2017) showed that the aglycons
29 piceol and pungenol are the active defense compounds that contribute to resistance. We also
30 showed that gene expression of *Pgβglu-1* was positively correlated with resistance, and in *in*

31 *in vitro* assays the encoded PgβGLU-1 enzyme cleaved the acetophenone glucosides, picein and
32 pungenin, producing the biologically active aglycons (Mageroy et al. 2015). However, function
33 of PgβGLU-1 has not yet been proven *in planta* and remained a critical but elusive part of the
34 proof of function.

35

36 Here we report the successful overexpression of *Pgβglu-1* in a white spruce genotype Pg653.
37 While wildtype Pg653 plants do not accumulate detectable amounts of the acetophenone
38 aglycons, overexpression of *Pgβglu-1* resulted in the *in planta* formation of piceol and pungenol.

39

40 **METHODS**

41 **Vector construction, *Agrobacterium* transformation and plant regeneration of *Pgβglu-1*** 42 **overexpression white spruce**

43 The full-length cDNA of *Pgβglu-1* (GenBank KJ780719) or a modified green fluorescence
44 protein (*gfp*) (Cambia) coding sequence were first cloned using the Gateway System (Invitrogen)
45 into vector pMJM, containing the maize (*Zea mays*) ubiquitin promoter and the 35S terminator
46 (Levéé et al. 2009), then digested with *SbfI* and sub-cloned into the binary vector
47 pCAMBIA2300 (Supplemental Fig. 1; Supplemental Fig. 2). The resulting constructs were
48 transformed into *Agrobacterium tumefaciens* strain C58 pMP90 (Hellens et al. 2000).
49 *Agrobacterium* transformation of white spruce somatic embryonal masses (line Pg653) and
50 subsequent selection and growth of transformants was performed as described by Klimaszewska
51 et al. (2001). Kanamycin resistance was used as the selection marker. Somatic embryo
52 maturation, germination, acclimatization and transfer of somatic seedlings to soil were
53 performed according to Klimaszewska et al. (2004).

54

55 **RT-qPCR of embryogenic tissue and somatic seedlings**

56 Quantitative reverse transcription PCR (RT-qPCR) was used to confirm transformation and to
57 measure transcript abundance in both embryogenic tissue and somatic seedlings of select
58 *Pgβglu-1* and *gfp* transformed lines. RNA was isolated from up to 100 mg fresh weight of
59 embryogenic tissue or from the pooled epicotyls of two 2-months old somatic seedlings using the
60 RNeasy Plant mini kit (Qiagen) with on-column RNase-Free DNase (Qiagen) treatment. Primers
61 were designed as previously described (Foster et al. 2015) with primers PgβGLU1-f—5'-

62 GCCATAAGGGAGGGAGCAG; Pg β GLU1-r—5'- CTCGCCCACTCAAAGCCGT or GFP-f—
63 5'- GCCCGACAACCACTACCTGA; GFP-r—5'- GCGGTCACGAACTCCAGCAG used to
64 analyze the *g β glu-1* and *gfp* lines respectively. Gene expression analysis was conducted as
65 described by Foster et al. (2015) with the exceptions that PCR thermocycling conditions
66 employed a two-step amplification protocol of 45 cycles with an annealing/elongation step at
67 65°C for 90 seconds and that data was normalized using the two white spruce reference genes
68 YLS8 and EF1 α (Rutledge et al. 2013). Transcript abundance quantification was performed
69 using a Java program based on linear regression of efficiency previously described (Rutledge
70 2011).

71

72 **Plant growth conditions**

73 After growth on germination media for three months, somatic seedlings were planted into cones
74 (Fig. 1b) and maintained in a greenhouse under natural light and growth lights (16 h; 600W
75 HPS). Temperatures were set with a low of 19°C and, within the limitations of a greenhouse that
76 is not fully temperature controlled, to a high of 23.5°C. Plants were allowed to grow for eight
77 months and then placed at 4°C with minimal light for two weeks to induce flushing. Plants were
78 placed on the benchtop at 22°C for one week to transition from the cold and then moved into a
79 growth chamber with 16 h light at 22°C and 8 h dark at 16°C.

80

81 **RT-qPCR of plants grown for 6 months**

82 Total RNA was isolated from needles of plants grown for six months using PureLink® Plant
83 RNA Reagent (ThermoFisher) using approximately 100 mg tissue according to manufacturer's
84 instructions. RNA integrity and concentration was measured using Bioanalyzer 2100 RNA Nano
85 chip assays (Agilent) following the manufacturer's protocol. Equal RNA amounts were used for
86 cDNA synthesis with the iScript Reverse Transcription Supermix (Bio-Rad). qRT-PCR reactions
87 were performed on a Bio-Rad CFX96 Real-time system using the SsoFast kit (Bio-Rad) in
88 triplicate. Relative transcript abundance was calculated using efficiency corrected ΔC_T and $\Delta\Delta C_T$
89 values based on ELF-1 α as the reference gene. Target-specific oligonucleotides were as follows:
90 ELF-1 α -f—5'-CCCTTCCTCACTCCAAGTGCATA; ELF-1 α -r—5'-
91 TCGGCGGTGGCAGAGTTTACATTA; or Pg β GLU1-f—5'-TTGGATCCTCTGAAGGTGT;

92 Pg β GLU1-r—5'-TCCCTCCCTTATGGCTTC. Target specificity was confirmed by sequence
93 verification of representative amplicons.

94

95 **Metabolite analysis**

96 For the time course study of acetophenone glucoside deglycosylation, tissue was ground and left
97 on the bench top for 4 h, 8 h, and 24 h before adding extraction solvent [100% methanol
98 containing 1 mg/ml benzoic acid as the internal standard]. For all other metabolite extractions,
99 100 mg of tissue was placed in a vial and 1 mL of extraction solvent was immediately added.
100 The vial was capped and placed at 4 °C with shaking overnight. The supernatant was removed
101 and placed in a new vial. For liquid chromatography-mass spectrometry (LC-MS) analysis,
102 samples were diluted 1:10 by diluting 100 μ L of supernatant in 900 μ L of 100% methanol. LC-
103 MS analysis was performed using a LC-MSD-Trap-XCT_plus with a SB-C18, 15-cm column
104 (Agilent). An injection volume of 10 μ L was used. Solvent A was water with 0.2% (v/v) formic
105 acid; solvent B was 100% (v/v) acetonitrile with 0.2% (v/v) formic acid. The following gradient
106 was used: increase to 5% solvent B from 0 to 0.5 min; increase to 22% solvent B from 0.5 to 5.0
107 min; increase to 35% solvent B from 5.0 to 10.0 min; increase to 50% solvent B from 10.0 to
108 13.0 min; increase to 95% solvent B from 13.0 to 16.0 min; holding 95% solvent B from 16.0 to
109 17.0 min; decrease to 5% solvent B from 17.0 to 17.1 min. Column flow rate was 0.8 mL min⁻¹.
110 Piceol and picein were identified using the extracted ion 135(-), the parent mass (-1) of piceol.
111 Pungenol and pungenin were identified using the extracted ion 151(-), the parent mass (-1) of
112 pungenol.

113

114 **RESULTS AND DISCUSSION**

115 **Overexpression of SBW defense gene *Pg β glu-1***

116 We overexpressed the cDNA of *Pg β glu-1* in *planta* to validate the function of this gene and its
117 encoded enzyme activity in the release of acetophenone aglycons from the corresponding
118 glucosides in white spruce foliage (Fig. 1). We used the white spruce genotype Pg653 to test the
119 effect of overexpression of *Pg β glu-1* for two reasons: 1) Pg653 is a well-established somatic
120 embryogenic line for white spruce transformations. 2) This line shows a metabolite phenotype
121 that contains the acetophenone glucosides picein and pungenin, which are the proposed *in vivo*
122 substrates for *Pg β glu-1* enzyme activity, but contains minimal detectable amounts of the

123 corresponding aglycons piceol and pungenol. Thus, Pg653 provides a suitable background for *de*
124 *novo* formation of piceol and pungenol in transgenic plants. *Agrobacterium* transformation of
125 the coding region of *Pgβglu-1* driven under maize (*Zea mays*) ubiquitin promoter was used to
126 produce transgenic white spruce lines. A *gfp* reporter gene was overexpressed in white spruce as
127 a control.

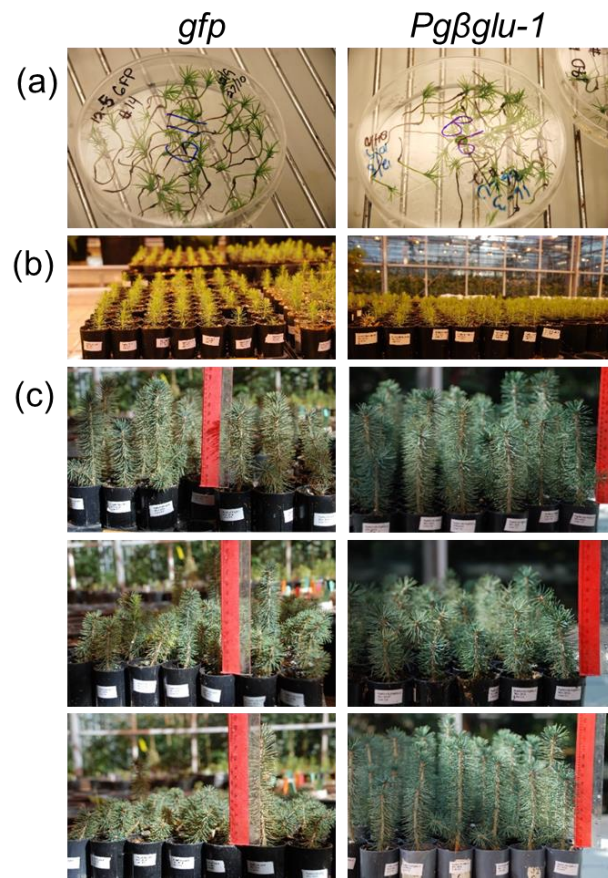


Figure 1. Transgenic white spruce seedlings. The left panels show control white spruce seedlings expressing *gfp*. The right panels show white spruce seedlings overexpressing *Pgβglu-1*. (a) Transgenic white spruce somatic seedlings were grown on selective media for 3 months. (b) Plantlets were then transferred into cones and placed in the greenhouse. (c) After 6 months of growth, on average, the *Pgβglu-1* overexpressing seedlings appeared healthier and taller.

128

129 **Evaluation of possible negative effects of transgene overexpression**

130 Levels of transgene gene expression were evaluated in both embryonal tissue and somatic
131 seedlings using qPCR (Figure 2). Overall transcript levels of the *gfp* transgene were higher in
132 both sample types compared to the *Pgβglu-1* transgene. The lower levels of *Pgβglu-1* transcripts
133 could indicate some phytotoxic effects, as toxic compounds are often glycosylated in plants for

134 self-protection. However, *Pgβglu-1* overexpressing young plants appeared to be healthier than
 135 *gfp* expressing plants (Figure 1c) under greenhouse conditions, including naturally occurring
 136 biotic and abiotic stresses. Although previous studies have shown *gfp* to be non-toxic in plants
 137 (Millwood et al. 2010; Tian et al. 1999), deleterious effects have been noted in mammalian cells
 138 (Liu et al 1999). The observed plant growth difference may be due to physiological adaptations
 139 to cope with effects that may arise from high levels of *gfp* expression (Steward 2001). For
 140 example, under stress conditions, which increases the production of free radicals, plant cells may
 141 not be able to compensate as well for high expression of *gfp* leading to negative growth effects.

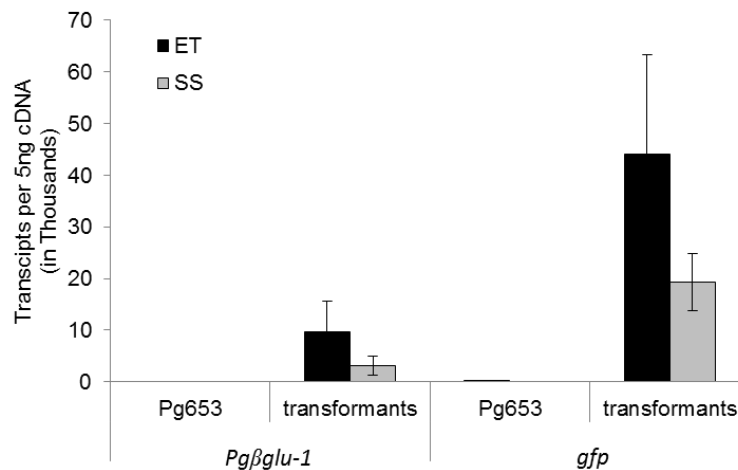


Figure 2. qRT-PCR of transgene expression in embryonic tissue (ET) and somatic seedlings (SS). Transcript abundance was calculated as the number of transcripts in 5 ng of synthesized cDNA. Pg653 represent the untransformed control line. Overall, higher expression of *gfp* was observed in ET and SS than *Pgβglu-1*. Error bars represent standard deviation. N=11

142

143 **Young seedlings overexpressing *Pgβglu-1* do not accumulate acetophenone aglycons**

144 No significant levels of acetophenone aglycons were observed in either *gfp* controls or *Pgβglu-1*
 145 overexpressing transgenic seedlings after the first six months of growing in the greenhouse. To
 146 test if we could observe aglycon production in *Pgβglu-1* overexpressing foliage when tissues
 147 were disrupted, we chose one high, one medium, and one low *Pgβglu-1* expressing line based on
 148 transcript abundance in six-month old seedlings (Figure 3a). Tissue was ground and the disrupted
 149 tissue left at room temperature for up to 24 h before metabolite extraction. In this time course
 150 test, we observed much greater release of the acetophenone aglycons piceol and pungenol in
 151 *Pgβglu-1* overexpressing lines compared to *gfp* controls (Figure 3b). In nature, acetophenone
 152 aglycons are produced in resistant white spruce foliage without tissue disturbance (Mageroy et
 153 al., 2015). The requirement of tissue disruption to produce the aglycons in the *Pgβglu-1*

154 overexpressing lines may indicate that P β GLU-1 protein is prevented from interacting with
 155 glucosides in the young seedlings, perhaps due to differential localization of the enzyme and the
 156 substrate or some reversible inhibition or inactivation of the enzyme. As *Pg β glu-1* was expressed
 157 under a constitutive promoter it is plausible that this expression may be spatially and temporally
 158 amiss or that reversible protein modification rendered it inactive in young seedlings. However,
 159 the ability of *Pg β glu-1* overexpressing lines to produce greater amounts aglycon when tissue was
 160 disturbed provided additional proof for the function of this gene and its encoded protein.

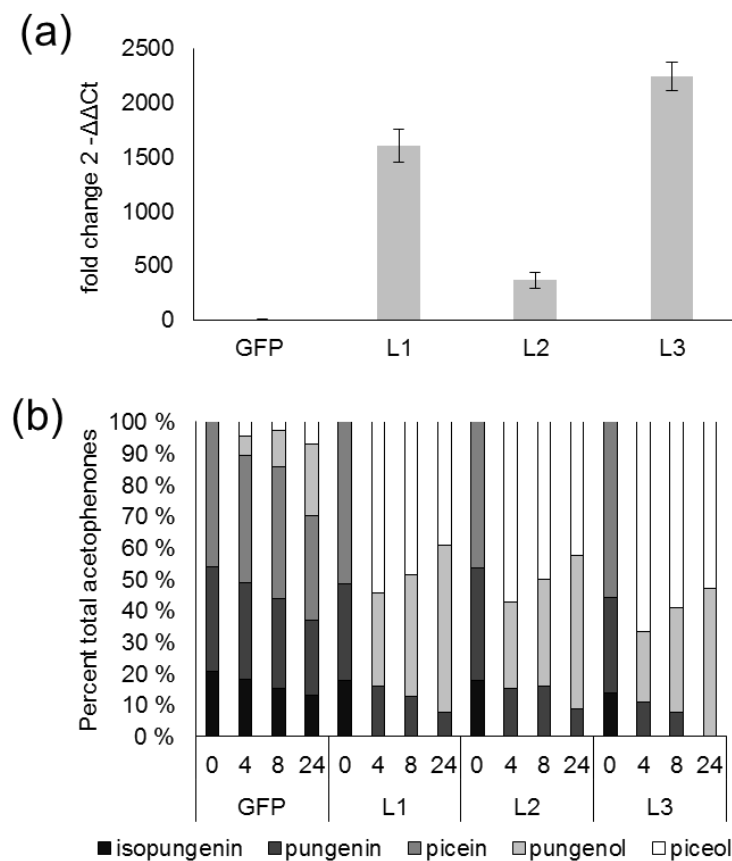


Figure 3. Analysis of transgene expression and β -glucosidase potential in 6-months old transgenic white spruce seedlings. (a) After 6 month of growth in the greenhouse, qRT-PCR was used to determine the fold change in *Pg β glu-1* expression between *gfp* overexpressing trees and three independent line of *Pg β glu-1* overexpressing trees. A low, a medium and a high *Pg β glu-1* expressing lines were chosen for further metabolite comparisons. GFP represents the average of four independent lines. (b) To test if acetophenone glucoside could be released in *Pg β glu-1* overexpressing seedlings, needles were grounds and the disrupted tissue left for 0 h, 4 h, 8 h, and 24 h before extracting metabolites. A much larger proportion of acetophenone aglycons was released in *Pg β glu-1* overexpressing trees compared very small proportion of acetophenone aglycons released in *gfp* overexpressing tissue. Error bars represent standard deviation. N=3

162 **Seedlings overexpressing *Pgβglu-1* accumulate acetophenone aglycons in newly growing**
163 **shoot tissue after induced dormancy**

164 Since white spruce is a perennial tree species, acetophenone production may be influenced by
165 plant development beyond the first growth phase. We tested this possibility by carrying eight-
166 month old seedlings through a simulated complete growth cycle including bud set, winter
167 dormancy, and new bud flush, which involved a cold treatment in the dark and subsequent return
168 to normal light and temperature conditions favorable to active vegetative growth. Bud flush
169 began three weeks after returning trees to normal growth conditions. Following a gap period of
170 no detectable levels of acetophenone glucoside and aglycons in the newly flushing shoots,
171 accumulation of both acetophenone glucosides and the corresponding aglycons was detected at
172 eight weeks after the beginning of new shoot growth (Figure 4a). While both the acetophenone
173 glucosides and aglycons were observed in the *Pgβglu-1* overexpressing seedlings, no substantial
174 quantities of the aglycons were detected in the *gfp* transgenic control seedlings. Under these *in*
175 *planta* conditions, overexpression of *Pgβglu-1* led to higher amounts of the picein-derived
176 aglycon piceol compared to the pungenol aglycon (Figure 4b). Accumulation of piceol also
177 correlated with the difference of *Pgβglu-1* transcript levels in low, medium and high expressing
178 lines (Figure 3a and Figure 4b). These results conclusively confirm *in planta* function of
179 *Pgβglu-1* in the release of acetophenone aglycons and their accumulation in intact plant tissue. In
180 previous work, we reported the *in vitro* kinetic parameters of the PgβGLU-1 enzyme with picein
181 as the substrate, but not for pungenin as this substrate is not a commercially available (Mageroy
182 et al. 2015). The present results suggest that PgβGLU-1 is more active on picein, compared to
183 pungenin, *in planta*.

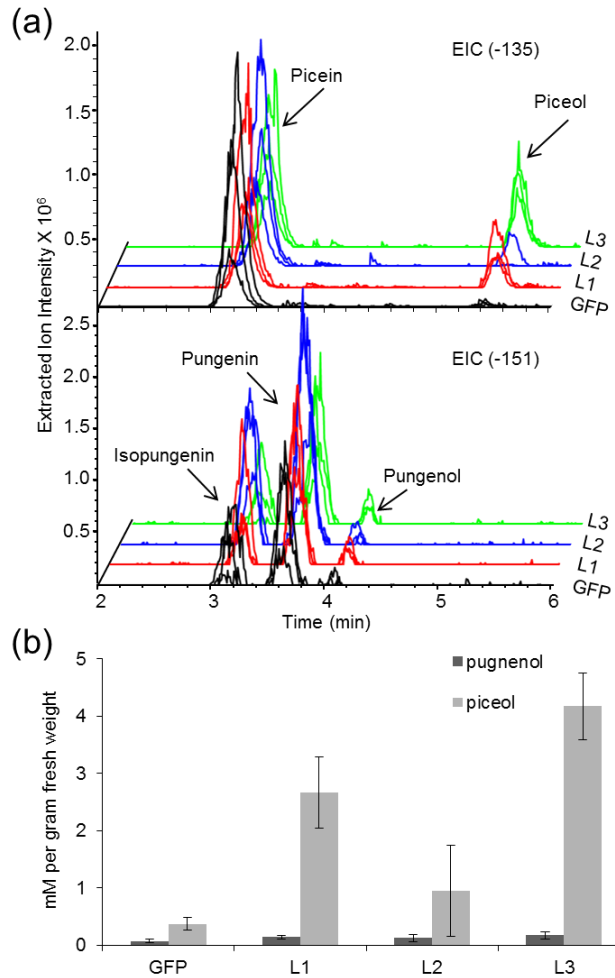


Figure 4. Altered acetophenone glucoside and aglycon profiles in new shoots of white spruce seedlings after bud flush. (a) 8 weeks after the beginning of bud flush, acetophenone aglycons were detected in extraction from intact *Pgβglu-1* overexpressing shoots. The top graph shows the extracted ion chromatogram (EIC) for the parent mass of piceol (-135). The bottom graph shows the EIC for the parent mass of pungenol (-151). (b) Piceol and pungenol were quantified using authentic standards. A higher amount of piceol was released in *Pgβglu-1* overexpressing shoots compared to the amount of pungenol. Error bars represent standard error. N=3

184

185 CONCLUSIONS

186 We showed that overexpressing *Pgβglu-1* in a white spruce genotype that does not naturally
 187 contain acetophenone aglycons leads to the *in planta* formation of the resistance metabolite
 188 piceol, and in disrupted tissues also the additional formation of pungenol. The results validate
 189 previously reported *in vitro* function of *Pgβglu-1* and its encoded PgβGLU-1 enzyme. The
 190 different results obtained with young seedling before bud flush and seedlings that had passed
 191 through bud set and new bud flush point out the need for caution when evaluating phenotypes of

192 young seedling overexpressing a transgene. As we found, the altered metabolite phenotype was
193 not observable in intact tissue until after the first bud flush. Conditions of spruce metabolism that
194 provide the precursors for altered metabolism may vary depending on the developmental stage of
195 seedlings, where precursors for defense metabolism may only become fully accessible after the
196 seedlings have gone through an initial growth phase or a dormancy phase. In future work, effects
197 of the *Pgβglu-1* transgene expression and altered acetophenone profiles in transgenic Pg653 trees
198 will be tested with insect feeding test, which will require production and maturation of a larger
199 number of young trees.

200 ACKNOWLEDGMENTS

201 We thank Lina Madilao for her assistance with LCMS. JB is a UBC Distinguished University
202 Scholar.

203

204 REFERENCES

205 Chang W-Y., Lantz VA., Hennigar CR., MacLean DA. 2012. Economic impacts of forest pests:
206 a case study of spruce budworm outbreaks and control in New Brunswick, Canada.
207 *Canadian Journal of Forest Research* 42:490–505. DOI: 10.1139/x11-190.

208 Delvas N., Bauce É., Labbé C., Ollevier T., Bélanger R. 2011. Phenolic compounds that confer
209 resistance to spruce budworm. *Entomologia Experimentalis et Applicata* 141:35–44. DOI:
210 10.1111/j.1570-7458.2011.01161.x.

211 Foster AJ., Pelletier G., Tanguay P., Seguin A. 2015. Transcriptome Analysis of Poplar during
212 Leaf Spot Infection with *Sphaerulina* spp. *PloS one* 10:e0138162. DOI:
213 10.1371/journal.pone.0138162.

214 Hellens R., Mullineaux P., Klee H. 2000. A guide to *Agrobacterium* binary Ti vectors. *Trends in*
215 *Plant Science* 5:446–451. DOI: 10.1016/S1360-1385(00)01740-4.

216 Hennigar CR., Erdle TA., Gullison JJ., Maclean DA. 2013. Re-examining wood supply in light
217 of future spruce budworm outbreaks: A case study in New Brunswick. *Forestry Chronicle*
218 89:42–53. DOI: 10.5558/tfc2013-010.

219 Hjältén J., Axelsson EP. 2015. GM trees with increased resistance to herbivores: trait efficiency
220 and their potential to promote tree growth. *Frontiers in plant science* 6:279. DOI:
221 10.3389/fpls.2015.00279.

- 222 Klimaszewska K., Lachance D., Pelletier G., Lelu M-AL., Seguin A. 2001. Regeneration of
223 Transgenic *Picea Glauca* , P . Mariana , and P . Abies After Cocultivation of Embryogenic
224 Tissue With *Agrobacterium Tumefaciens*. *In Vitro Cellular & Developmental Biology -*
225 *Plant* 37:748–755. DOI: 10.1079/IVP2001230.
- 226 Klimaszewska K., Rutledge RG., Séguin A., Seguin A. 2004. Genetic transformation of conifers
227 utilizing somatic embryogenesis. In: Peña L ed. *Transgenic Plants: Methods and Protocols*.
228 Totowa, NJ, United States: Humana Press, 151–64.
- 229 Lachance D., Hamel LP., Pelletier F., Valero J., Bernier-Cardou M., Chapman K., van
230 Frankenhuyzen K., Seguin A. 2007. Expression of a *Bacillus thuringiensis* cry1Ab gene in
231 transgenic white spruce and its efficacy against the spruce budworm (*Choristoneura*
232 *fumiferana*). *Tree Genetics & Genomes* 3:153–167. DOI: 10.1007/s11295-006-0072-y.
- 233 Levée V., Major I., Levasseur C., Tremblay L., MacKay J., Séguin A. 2009. Expression profiling
234 and functional analysis of *Populus* WRKY23 reveals a regulatory role in defense. *New*
235 *Phytologist* 184:48–70. DOI: 10.1111/j.1469-8137.2009.02955.x.
- 236 Liu H-S., Jan M-S., Chou C-K., Chen P-H., Ke N-J. 1999. Is Green Fluorescent Protein Toxic to
237 the Living Cells? *Biochemical and Biophysical Research Communications* 260:712–717.
238 DOI: <http://dx.doi.org/10.1006/bbrc.1999.0954>.
- 239 MacLean DA. 2016. Impacts of insect outbreaks on tree mortality, productivity, and stand
240 development. *The Canadian Entomologist* 148:S138–S159. DOI: 10.4039/tce.2015.24.
- 241 Mageroy MH., Parents G., Germanos G., Giguere I., Delvas N., Maaroufi H., Bauce E.,
242 Bohlmann J., Mackay JJ., Parent GE., Germanos G., Giguere I., Delvas N., Maaroufi H.,
243 Bauce E., Bohlmann J., Mackay JJ., Giguere I., Bohlmann J., Mackay JJ. 2015. Expression of
244 the b-glucosidase gene *Pgbglu-1* underpins natural resistance of white spruce against spruce
245 budworm. :68–80. DOI: 10.1111/tpj.12699.
- 246 Millwood RJ., Moon HS., Stewart Jr. CN. 2010. Fluorescent Proteins in Transgenic Plants. In:
247 Geddes CD ed. *Reviews in Fluorescence 2008*. Springer New York, 387–403. DOI:
248 10.1007/978-0-387-88722-7.
- 249 NRCAN. 2016. Spruce budworm. Available at [http://www.nrcan.gc.ca/forests/fire-insects-](http://www.nrcan.gc.ca/forests/fire-insects-disturbances/top-insects/13383)
250 *disturbances/top-insects/13383* (accessed February 24, 2016).
- 251 Parent GJ., Giguère I., Germanos G., Lamara M., Bauce É., MacKay JJ. 2017. Insect herbivory
252 (*Choristoneura fumiferana*, Tortricidea) underlies tree population structure (*Picea glauca*,
253 Pinaceae). *Scientific Reports, Published online: 16 February 2017; |*
254 *doi:10.1038/srep42273* 199:379–398. DOI: 10.1038/SREP42273.
- 255 Rutledge RG., Stewart D. 2010. Assessing the performance capabilities of LRE-based assays for
256 absolute quantitative real-time PCR. *PLoS ONE* 5. DOI: 10.1371/journal.pone.0009731.

- 257 Rutledge RG. 2011. A Java program for LRE-based real-time qPCR that enables large-scale
258 absolute quantification. *PloS one* 6:e17636. DOI: 10.1371/journal.pone.0017636.
- 259 Stewart CNJ. 2001. The utility of green fluorescent protein in transgenic plants. *Plant cell*
260 *reports* 20:376–382.
- 261 Tian LN., Levee V., Mentag R., Charest PJ., Seguin A. 1999. Green fluorescent protein as a tool
262 for monitoring transgene expression in forest tree species. *Tree Physiology* 19:541–546.