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

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

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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, *Pgßglu-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 *Pgßglu-1* in a white spruce genotype whose metabolome

9 contains the glucosylated acetophenones, but no detectable amounts of the aglycons. Transgenic

10 overexpression of *Pgßglu-1* resulted in release of the acetophenone aglycons *in planta*. This

11 work provides *in vivo* evidence for the function of *Pgßglu-1*.

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 *cry1Ab* 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 *Pgßglu-1* was positively correlated with resistance, and in *in*

31 *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 (Levée et al. 2009), then digested with *Sbf*I 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 **LRE-qPCR of embryogenic tissue and somatic seedlings**

56 Linear regression of efficiency (LRE) qPCR (Rutledge 2011) was used to confirm transformation

57 and to measure and compare absolute transcript abundance levels in both embryogenic tissue and

58 somatic seedlings of 11 selected *Pgβglu-1* and 11 *gfp* transformed lines. RNA was isolated from

59 up to 100 mg fresh weight of embryogenic tissue or from the pooled epicotyls of two 2-months

60 old somatic seedlings using the RNeasy Plant mini kit (Qiagen) with on-column RNase-Free

61 DNase (Qiagen) treatment. Primers were designed as previously described (Foster et al. 2015)

62   with primers PgβGLU1-f—5'- GCCATAAGGGAGGGAGCAG; PgβGLU1-r—5'-

63   CTCGCCCACTCAAAGCCGT or GFP-f—5'- GCCCGACAACCACTACCTGA; GFP-r—5'-

64   GCGGTCACGAACTCCAGCAG used to analyze the *gβglu-1*and *gfp* lines respectively. cDNA

65   synthesis, primer design, and PCR thermocycling conditions were conducted as described by

66   Foster et al. (2015) with the exceptions that a two-step amplification protocol of 45 cycles was

67   used with a 120 second annealing/elongation step at 65°C. Gene expression was normalized

68   using the two white spruce reference genes YLS8 and EF1α (Rutledge et al. 2013). Transcript

69   abundance quantification was performed using a Java program based on linear regression of

70   efficiency previously described (Rutledge 2011).

71

Plant growth conditions

73   After growth on germination media for three months, somatic seedlings were planted into cones

74   (Figure 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

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-1α as the reference gene. Target-specific oligonucleotides were as follows:

90   ELF-1α-f—5'-CCCTTCCTCACTCCAACTGCATA; ELF-1α-r—5'-

91   TCGGCGGTGGCAGAGTTTACATTA; or PgβGLU1-f—5'-TTGGATCCTCTGAAGGTGT;

92   PgβGLU1-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 ℃ 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 μL of supernatant in 900 μL 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 μL 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 *Pgβglu-1***

116   We overexpressed the cDNA of *Pgβglu-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 (Figure 1). We used the white spruce genotype Pg653 to test

119   the effect of overexpression of *Pgβglu-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 Pgβglu-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.
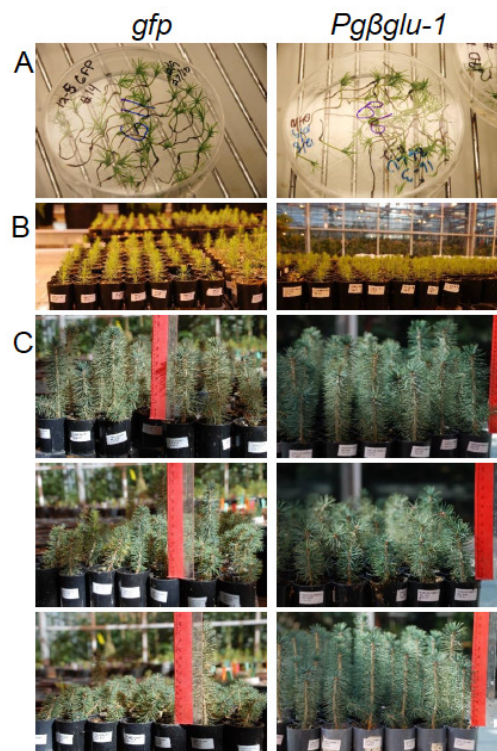
Figure 1. Transgenic white spruce seedlings. A, C, E, G, and I show control white spruce seedlings expressing *gfp*. B, D, F, H, and J show white spruce seedlings overexpressing *Pgβglu-1*. (A-B) Transgenic white spruce somatic seedlings were grown on selective media for three months. (B-C) Plantlets were then transferred into cones and placed in the greenhouse. (D-J) After six 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 increase 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.
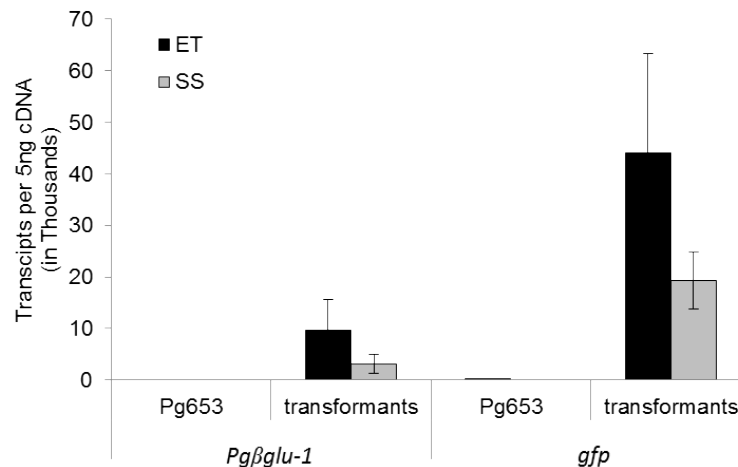
Figure 2. LRE-qPCR quantification of transgene expression in embryonic tissue (ET) and somatic seedlings (SS) of transformed lines. Transcript abundance was calculated as the number of transcripts in 5 ng of synthesized cDNA. Pg653 represent the untransformed control line. Overall, higher expression 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 aceotophenone 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 PgβGLU-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 *Pgβglu-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 *Pgβglu-1* overexpressing lines to produce greater amounts of aglycon when tissue

160 was disturbed provided additional proof for the function of this gene and its encoded protein. It

161 is possible that this increased accumulation of acetophenone aglycons upon tissue distruption

162 may contribute enhanced resistance in plants overexpressing *Pgβglu-1*. This remains to be tested

163 in future work.
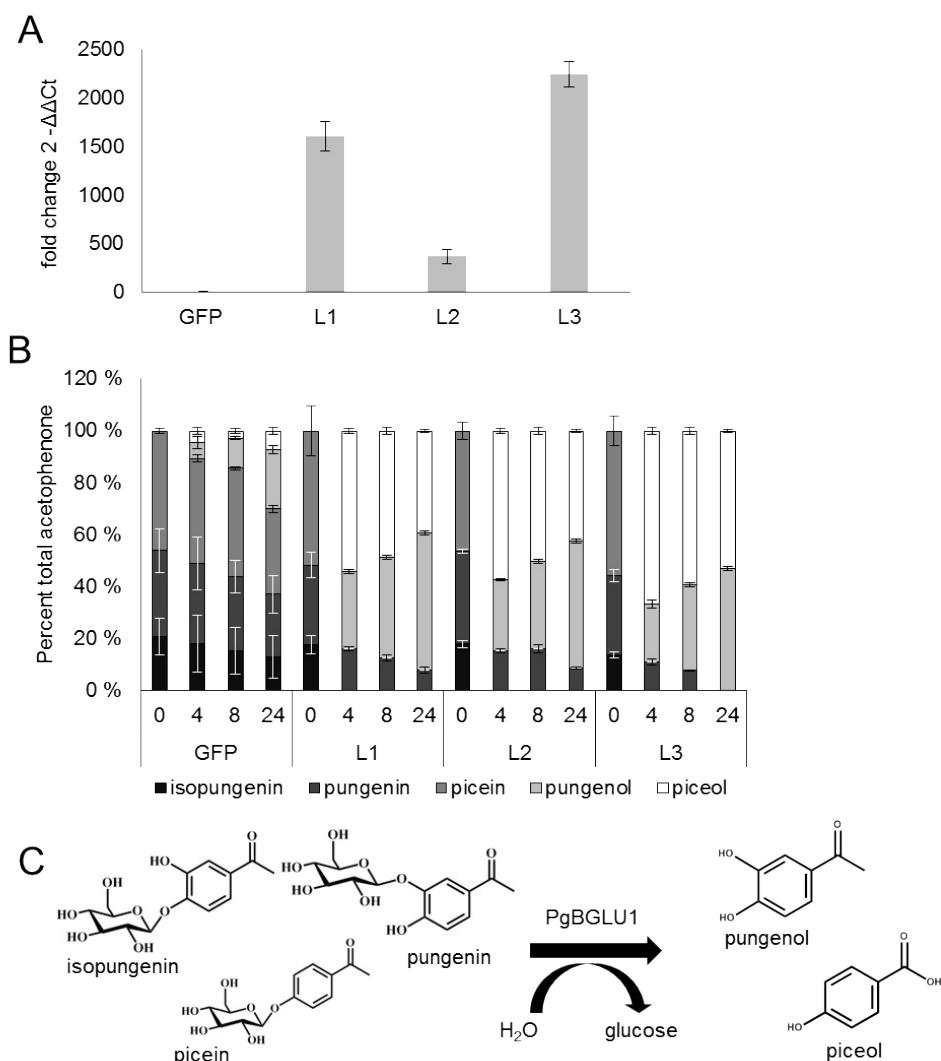
Figure 3. Analysis of transgene expression and β-glucosidase potential in 6-months old transgenic white spruce seedlings. (A) After 6 months of growth in the greenhouse, qRT-PCR

was used to determine the fold change in *Pgβglu-1* expression between *gfp* expressing plants and three independent lines of *Pgβglu-1* overexpressing plants. Representative low, medium and high *Pgβglu-1* expressing lines were chosen for comparative metabolite analysis. GFP represents the average of four independent lines. Error bars represent standard deviation. N=3 (B) To test if acetophenone glucoside could be released in *Pgβglu-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 *Pgβglu-1* overexpressing trees compared very small proportion of acetophenone aglycons released in *gfp* overexpressing tissue. Error bars represent standard error. N=3. (C) The structures of glucosylated acetophenone and their aglycons with the catalytic function of PgβGLU-1.

164

Seedlings overexpressing *Pgβglu-1* accumulate acetophenone aglycons in newly growing

shoot tissue after induced dormancy

Since white spruce is a perennial tree species, acetophenone production may be influenced by

plant development beyond the first growth phase. We tested this possibility by carrying eight-

month old seedlings through a simulated complete growth cycle including bud set, winter

dormancy, and new bud flush, which involved a cold treatment in the dark and subsequent return

to normal light and temperature conditions favorable to active vegetative growth. Bud flush

began three weeks after returning trees to normal growth conditions. Following a gap period of

no detectable levels of acetophenone glucosides and aglycons in the newly flushing shoots,

accumulation of both acetophenone glucosides and the corresponding aglycons was detected at

eight weeks after the beginning of new shoot growth (Figure 4a). While both the acetophenone

glucosides and aglycons were observed in the *Pgβglu-1* overexpressing seedlings, no substantial

quantities of the aglycons were detected in the *gfp* transgenic control seedlings. Under these *in*

planta conditions, overexpression of *Pgβglu-1* led to higher amounts of the picein-derived

aglycon piceol compared to the pungenol aglycon (Figure 4b). Accumulation of piceol also

correlated with the difference of *Pgβglu-1* transcript levels in low, medium and high expressing

lines (Figure 3a and Figure 4b). These results conclusively confirm *in planta* function of

*Pgβglu-1*in the release of acetophenone aglycons and their accumulation in intact plant tissue. In

previous work, we reported the *in vitro* kinetic parameters of the PgβGLU-1 enzyme with picein

as the substrate, but not for pungenin as this substrate is not a commercially available (Mageroy

et al. 2015). The present results suggest that PgβGLU-1 is more active on picein, compared to

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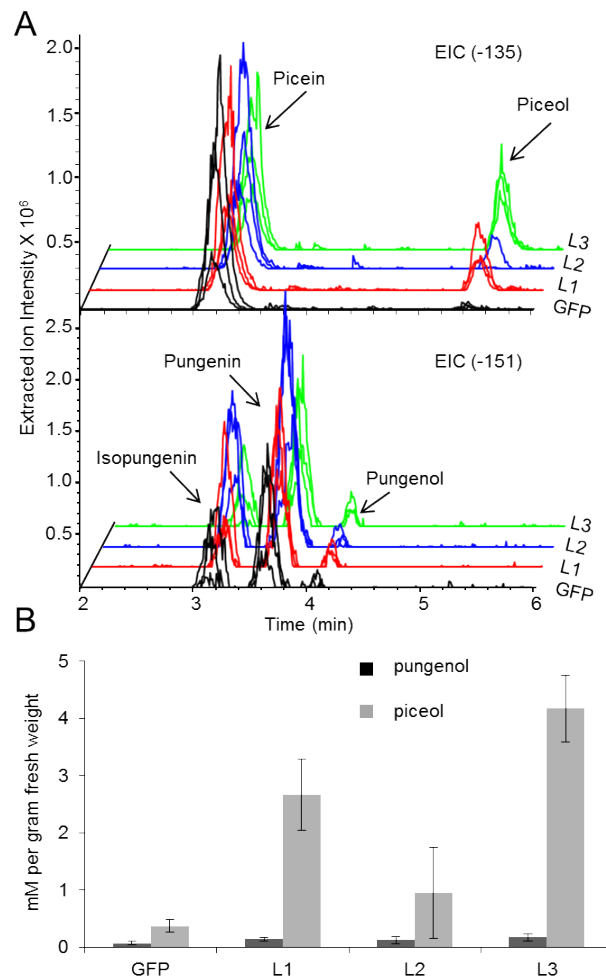
Figure 4. Altered acetophenone glucoside and aglycon profiles in new shoots of white spruce seedlings after bud flush. Eight weeks after the beginning of bud flush, acetophenone aglycons were detected in extraction from intact *Pgßglu-1* overexpressing shoots. (A) The extracted ion chromatogram (EIC)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

187 **CONCLUSIONS**

188 We showed that overexpressing *Pgßglu-1* in a white spruce genotype that does not naturally

189 contain acetophenone aglycons leads to the *in planta* formation of the resistance metabolite

190 piceol, and in disrupted tissues also the additional formation of pungenol. The results validate

191 previously reported *in vitro* function of *Pgßglu-1* and its encoded PgβGLU-1 enzyme. The

192 different results obtained with young seedling before bud flush and seedlings that had passed

193 through bud set and new bud flush point out the need for caution when evaluating phenotypes of

194 young seedling overexpressing a transgene. As we found, the altered metabolite phenotype was

195 not observable in intact tissue until after the first bud flush. Conditions of spruce metabolism that

196 provide the precursors for altered metabolism may vary depending on the developmental stage of

197 seedlings, where precursors for defense metabolism may only become fully accessible after the

198 seedlings have gone through an initial growth phase or a dormancy phase. In future work, effects

199 of the *Pgβglu-1* transgene expression and altered acetophenone profiles in transgenic Pg653 trees

200 will be tested with insect feeding test, which will require production and maturation of a larger

201 number of young trees.

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205

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