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Genotype and provenance-related variation in the leaf surface secondary metabolites of silver birch

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Abstract

Cuticular wax layer of silver birch (*Betula pendula* Roth) leaves is rich in cyclic secondary metabolites that provide defense against various environmental factors. Micropropagated trees from the southern (60°N), central (62°N) and northern (66°N) latitudes of Finland were grown in a common garden setup and quantified for the variation in leaf surface secondary metabolites and other leaf traits, and their association with genotype and provenance was studied.

The studied 12 genotypes differed greatly in the quantity of surface secondary metabolites, both for individual flavonoid and triterpenoid aglycones and for the overall metabolite profile. Qualitative differences were observed for one triterpenoid that was present in a single genotype (R3). The variance explained by the provenance was low (between 1 – 36 %) for most metabolites, but the profile showed a clear separation by the provenance. The contents of two alkyl coumarates, reported for the first time in silver birch leaf waxes, displayed difference among the provenances. The correlations between the surface secondary metabolites and damage by insect herbivores suggest an association between the studied surface compounds and herbivore resistance. Altogether, the content of leaf surface secondary metabolites varied strongly among the silver birch genotypes and the profile clearly among the provenances.

Keywords:

genotype, provenance, cuticular wax, flavonoids, triterpenoids

INTRODUCTION

Climate change scenarios predict increasing temperature, precipitation and atmospheric CO₂ concentration in northern Europe (IPCC 2013). In boreal forests, this is expected to cause an increasing pressure by pests and pathogens along with more frequent heat waves and summer drought (Kirilenko & Sedjo 2007; IPCC 2013). The ability of a tree species to adapt to changing environmental conditions depends on the genetic diversity within the populations, especially in defensive traits relevant to the stress factors involved (Aitken et al. 2008).

The primary function of cuticular wax layer covering the plant surface is to limit nonstomatal water loss (Samuels et al. 2008). The wax layer consists of very long chain fatty acid derivatives but depending on the species, it can also contain cyclic secondary compounds (Jetter et al. 2006). The cuticular wax extracts of silver birch (*Betula pendula* Roth) leaves contain high amounts of cyclic secondary metabolites, such as flavonoid aglycones, triterpenoid aglycones and sterols (Keinänen and Julkunen-Tiitto 1998; Martemyanov et al. 2015a; Lihavainen et al. 2017). In addition to the cuticular compounds, silver birch leaves contain a diversity of intracellular phenolics, including flavonoid glycosides (Keinänen and Julkunen-Tiitto 1998; Ossipov et al. 1996; Laitinen et al. 2000) that are hydrophilic compounds and stored mainly in the vacuoles of the epidermal cells. Surface flavonoid and triterpenoid aglycones are lipophilic and they can be extracted from the leaf surface by submerging the leaves in an appropriate solvent (Keinänen and Julkunen-Tiitto 1998), when the extraction procedure excludes intracellular phenolics. The place of synthesis of leaf surface secondary metabolites remains unclear in silver birch: they can be synthesized and excreted to the leaf surface either by epidermal cells, or by glandular trichomes that cover the leaves, or by both. A positive correlation between glandular trichome density and leaf surface secondary metabolite concentrations supports the role of glandular trichomes in their production (Valkama et al. 2004; Lihavainen et al. 2017). The concentration of birch surface flavonoids has been reported to decrease as leaves expand (Valkama et al. 2004; Martemyanov et

al. 2015b). This could be due to the dilution effect as trichome density decreases during the leaf expansion, but also due to aging as trichomes cease to exude secondary metabolites to the leaf surface (Valkama et al. 2004).

Secondary metabolites of the cuticular wax layer play an important role as the first defense barrier against herbivores and pathogens (Samuels et al. 2008). Flavonoids show high antioxidant capacity and are widely regarded as defense against abiotic (UV radiation, excess light) and biotic (herbivore and pathogen attacks) stress factors (Ferreira et al. 2012). The chemical properties and light absorption capacity of leaf surface flavonoid aglycones depend on their structure, such as the number of methoxy groups and free hydroxyl groups (Rice-Evans et al. 1995). Flavonoid aglycones were previously reported to be involved in resistance against moth larvae and fungal diseases in birch species (Valkama et al. 2005a; Lahtinen et al. 2004; Martemyanov et al. 2012a). On the other hand, even though the previous year's defoliation on *B. pendula* leaves resulted in an increase of flavonoid aglycones, there was no correlation with gypsy moth (*Lymantria dispar*) larval survival (Martemyanov et al. 2012b). Triterpenoids are a highly diverse group of secondary metabolites and several different triterpenoids (tetracyclic and pentacyclic structures) have been reported from bud or leaf surface extracts of *Betula* species (e.g., Pokhilo and Uvarova 1988; Fuchino et al. 1996). Many triterpenoids, such as papyriferic acid in the resin glands of silver birch twigs (Tahvanainen et al. 1991), exhibit efficient antifeedant properties against herbivores (Reichardt et al. 1984). Cuticular wax compounds, aliphatic phenylethyl and benzyl wax esters, have been associated with the resistance to autumn gum moth in *Eucalyptus globulus* (Jones et al. 2002). Thus, high content of surface wax metabolites can be considered as a desirable trait in tree leaves.

Silver birch genotypes have been shown to vary widely in the content and composition of intracellular flavonoid glycosides (Keinänen et al. 1999; Laitinen et al. 2000) and in the content of triterpenoid aglycones of twigs (Laitinen et al. 2005). A recent study showed that there is a high intrapopulation genotypic variation in silver birch leaf surface secondary metabolites, including

flavonoid and triterpenoid aglycones, and that the genotypic variation remained in senescent leaves and was persistent through the leaf litter decomposition (Paaso et al. 2017). Intraspecific genotypic variation in the concentrations of secondary metabolites has been shown for other deciduous tree species as well, for example leaves of *Salix myrsinifolia* (Paunonen et al. 2009) and *Populus* (Barchet et al. 2013).

Geographical studies on winter savory (*Satureja montana*), juniper (*Juniperus communis*), silver birch and downy birch (*B. pubescens*) have shown that there is variation in cuticular wax constituents, such as hydrocarbons (Rajcevic et al. 2014), triterpenoids (Makhnev et al. 2012) and flavonoids (Stark et al. 2008). The triterpenoid content in silver birch leaves was reported to be higher in northern latitudes than in southern latitudes in the Ural region (Makhnev et al. 2012). Downy birch (*B. pubescens*) populations in different latitudes in Finland varied in flavonoid profiles, but not in total flavonoid content (Stark et al. 2008). Common garden studies based on plants of different provenances growing in the same environment are needed to provide insights into the extent and persistence of geographical differences. There are a few common garden studies focusing on chemical variation (Slimestad 1998; Pratt et al. 2014), but studies on leaf wax compounds are scarce (Ramirez-Herrera et al. 2011) despite the ecological significance of the wax layer.

We studied the natural variation in the leaf surface secondary metabolites of silver birch across Finland. Silver birch exhibits a broad geographic distribution range and that, together with high genetic variation in surface flavonoid and triterpenoid aglycones, makes it a good model species to study the natural variation in leaf surface secondary metabolites. The aims of this research were to study the genotypic diversity in leaf surface secondary metabolites and the variation in secondary metabolites related to provenances utilizing 12 genotypes originating from the south (60°36'N), central (62°45'N) and north (66°27'N) of Finland growing at the common garden field site in Joensuu (62°35'N). Since the genomes of the studied genotypes have been

sequenced (Salojärvi et al. 2017), we were able to quantify the relatedness of the genotypes by identity by descent (IBD) analysis of single nucleotide polymorphism data. In addition, we investigated whether the high content of particular surface secondary metabolites was associated with specific leaf traits or with herbivore resistance.

We hypothesize (i) that there is a high variation (both quantitative and qualitative) among silver birch genotypes in the leaf surface secondary metabolites. Provenance variation in leaf surface flavonoid and triterpenoid aglycones has attained little attention, despite their prominent role in abiotic stress responses and plant-herbivore-interactions. Thus, we hypothesize that (ii) surface secondary metabolite contents vary among the provenances indicating adaptation to the prevailing environmental conditions in the latitude of origin, and that (iii) the high content of surface secondary metabolites is associated with herbivore resistance.

MATERIAL AND METHODS

Plant material and experimental field site

Micropropagated plantlets of silver birch (*Betula pendula* Roth) were planted in a botanical garden in Joensuu, Finland (62°35' N, 29°46' E), in 2010. Three provenances originating from 60°36'N (southern Finland, Loppi), 62°45'N (central Finland, Vehmersalmi) and 66°27'N (northern Finland, Rovaniemi) were selected for this study (Table 1). We studied four genotypes from each of the three provenances (12 genotypes in total). Two micropropagated saplings of the same genotype were planted randomly in each of the five blocks in the field site, thus there were ten individual trees for each genotype. Details of the common garden experimental design and setup are described in Heimonen et al. (2015a).

The places of origin of the provenances differ from each other for their mean annual temperature, temperature sum (the sum of daily mean temperatures above 5°C), precipitation and

the length of the growing season that all decrease by increasing latitude (Table 1). Day length increases by latitude and reaches continuous daylight at 66°N (Fig. S1).

Leaf sampling

Silver birch trees were three years old when leaves were collected on 25th and 26th of June (176 - 177 day of year (doy) in 2013 for surface metabolite analysis. Four genotypes from each provenance were sampled: L1, L6, L14, L15 (60°N), V1, V4, V5, V14 (62°N) and R3, R8, R11 and R15 (66°N). Samples were collected from ten individual trees of each genotype, except nine for R8 and V5 and eleven for V14 (n=9-11). A fully developed short shoot leaf was collected from the southern part of each tree from the upper third of the canopy, put in a plastic bag, kept in ice and transported to the laboratory where extracted without delay.

Wax extraction

Fresh leaves were extracted as in Lihavainen et al. 2017 by submerging them in 10 ml of dichloromethane for 30 s in a decanter glass after which the extracts were poured to test tubes. Dichloromethane contained 0.7% of hexane and internal standards: 1.99 mg l⁻¹ lupeol, 2.98 mg l⁻¹ of cholesteryl acetate and 1.99 mg l⁻¹ of oleyl palmitate. An aliquot of 100 µl of the extract was transferred from the test tube to 2 ml autosampler vials, and evaporated at room temperature. All the chemicals were purchased from Sigma-Aldrich. Quality control (QC) samples were prepared by combining extracts from each genotype from one experimental block, mixed and aliquots of 100 µl for QCs were included in all subsequent sample preparation steps. To each vial, 100 µl of dichloromethane was added, and the aliquots were dried in vacuum at 35°C for 10 min. Sample vials were then purged with gaseous nitrogen and stored at -70°C.

Analysis of leaf wax secondary metabolites

Frozen samples were allowed to reach the room temperature for 1 h before opening the vials and were then dissolved in 100 μl of 80 % acetonitrile (Merck). Two QC samples were included in sample set on each day of chemical analyses. HPLC system (UltiMate 3000RS Dual System, Dionex) was connected to charged aerosol detector (CAD, Corona *ultra*, ESA) and LTQ mass spectrometer with positive APCI ionization mode (Thermo Scientific). Injection volume was 5 μl and column oven temperature was 40°C. A precolumn (SecurityGuard™ cartridge C18, Phenomenex) and a Kinetex column (XB-C18, 3.0 mm ID x 150 mm, particle size 2.6 μm , Phenomenex) were employed for the separation of metabolites. Mobile phases consist of (A) water containing 0.5% of formic acid and (B) 100% acetonitrile. An inverse gradient system maintained constant mobile phase composition at the two detectors (50% acetonitrile). Gradient flow (1.0 ml min^{-1}) was as follows: from 15% to 50% of acetonitrile in 7 min, increasing to 70% at 20 min, reaching 100% acetonitrile at 31 min, and maintaining it for 6 min. Additional washing step with 100% acetonitrile with flow rate of 1.5 ml min^{-1} was maintained for 4 min, after which the solvent composition and flow rate returned to starting conditions followed by 7 min of equilibration. Inverse gradient with 0.51 min time compensation was employed by the other pump. Total flow (2 ml min^{-1}) was divided between CAD and MS in the proportion of 1.3 and 0.7 ml min^{-1} , respectively.

Peak areas were determined from charged aerosol detector (CAD) signal with XCalibur software (Thermo Scientific). CAD was used for quantification, because the analysis is mass dependent and thus, allows quantification of unknown compounds (Vehovec & Obreza 2010). Metabolite peak area was normalized by the peak area of the internal standard, lupeol (mw 426.7 g mol^{-1}). Standard curves were produced for fisetin (mw 286.2 g mol^{-1} , Extrasynthese) and betulinic acid (mw 456.7 g mol^{-1} , Extrasynthese) from the CAD signal. Thus, the content of flavonoids was calculated as nanomoles based on fisetin, and the content of triterpenoids, alkyl coumarates and sitosterol based on betulinic acid. The content was then normalized by twice the leaf area (cm^2),

since both abaxial and adaxial leaf surface secondary metabolites were included in the extraction. Content is thereby expressed as nmol cm^{-2} and the normalized data was used for all further analyses. In addition, the relative contents of each individual metabolite within the particular metabolite group was calculated for flavonoids and triterpenoids separately. Total content of the metabolites and the mean content of metabolite groups were determined also on the basis of leaf dry mass (mg g^{-1} DW).

Metabolites were annotated based on MS data (ESI+ Table S1, APCI+ Table S2), available standard compounds and literature. In addition to HPLC-CAD/APCI+ analysis, samples were analyzed with Acquity UPLC-PDA/QTOF/MS (Waters) for metabolite annotation purposes. Accurate mass and predicted molecular formula were acquired for Na-adducts $[\text{M}+\text{Na}]^+$ or protonated molecule $[\text{M}+\text{H}]^+$ (Table S1). UV absorption spectra was acquired for flavonoid aglycones and alkyl coumarates (Table S1). UPLC-PDA/QTOF/MS analysis method is described in Supplementary Table S1. Flavonoid aglycones were annotated based on the number of hydroxyl and methyl groups $[\text{M}+\text{H}-\text{CH}_3]^+$ (Table S1). Triterpenoid aglycones were annotated as dammaranes or epoxydammaranes, as acetyl and/or malonyl derivatives, and as triols, tetraols or pentanols (Table S1, S2). Epoxydammaranes display an intensive ion m/z 143 corresponding to oxidized side chain at C17 position (Table S2). Triterpenoids were annotated as malonyl $[\text{M}+\text{H}-\text{C}_3\text{H}_4\text{O}_4]^+$ and/or acetyl derivatives $[\text{M}+\text{H}-\text{CH}_3\text{COOH}]^+$ based on their fragmentation patterns, and the number of hydroxyl groups was determined on the basis of fragmentation pattern and molecular formula (Table S1, S2). Peaks T2, T3 and T4 consisted of two triterpenoids (Table S1, S2). Two alkyl coumarates were annotated as (E) and (Z) isomers of hexadecyl-*p*-coumarate (Table S1, S2).

Leaf traits

After weighing the sample leaves fresh (FW, g) and dry (DW, g), leaf water content (LWC, %) was determined as $\text{LWC} = ((\text{FW}-\text{DW})/\text{FW}) \times 100$. Both dry weight and leaf area were measured after

the wax extraction. Sample leaf area (cm^2) was determined with LAMINA software (Bylesjö et al. 2008). Specific leaf area (SLA, $\text{cm}^2 \text{g}^{-1}$) was determined as leaf area (cm^2) divided by the dry weight of leaf. Leaf age at the time of sampling was determined as a difference in days between bud burst (doy) and sampling (doy). Chlorophyll content index (CCI) was measured with a chlorophyll meter (CCM-200, Opti-Sciences) from 3-6 short shoot leaves from the south side of the tree, from the upper third of the canopy on 19th and 20th of June (170-171 doy).

Herbivore damage

Herbivore feeding was assessed by visual scoring as a percentage (0, 1, 5, 10, 20, 30 up to 100%) of leaf area eaten by natural insect herbivores at the common garden field site in 2011 and in 2012 as described in Heimonen et al. (2015b). The area eaten included all damage by chewing, mining and galling insects as described in Heimonen et al. (2015a). Herbivory index was determined as a mean of 2011 and 2012 for early summer (May), midsummer (July) and a total mean of early and midsummer.

Relatedness of the genotypes

Identity by descent (IBD) analysis was carried out using PLINK v.1.9 software (Chang et al. 2015). Single nucleotide polymorphism (SNP) data for the twelve individuals was extracted from SNP dataset estimated from low coverage Illumina whole genome sequencing of 86 silver birch individuals and the GATK pipeline, as described in (Salojarvi et al. 2017). The SNP set was filtered for linkage disequilibrium (LD) using overlapping windows of width 50 variants with 10 variant step size, and variants with squared allele count correlations $R^2 > 0.1$ were pruned. Using the pruned set of SNPs, genetic distance between each individual was calculated with identity-by-state distance, defined as $\text{DST} = \text{IBS2} + 0.5 * \text{IBS1} / (\text{IBS0} + \text{IBS1} + \text{IBS2})$, where IBS0, IBS1 and IBS2 are the number of loci with 0, 1 or 2 alleles in common.

249

250 **Data processing and statistics**

251 The effects of genotype and provenance on metabolite means, absolute content, relative content and
252 leaf traits were tested with linear mixed model (IBM SPSS Statistics version 21 and R version
253 3.1.3). Provenance was treated as a fixed factor and genotype as a random factor nested within the
254 provenance. In addition, linear mixed model was performed in *R* (R Core Team 2014) to determine
255 the variance explained using *lme4* package treating the variables similarly as in SPSS. Marginal R^2
256 (R^2_m , variance explained only by fixed factor) and conditional R^2 (R^2_c , variance explained by both
257 fixed and random factors) (Nakagawa and Schielzeth 2013) were calculated from *MuMIn* package
258 (Barton 2014) using *r.squaredGLMM* function. Log₁₀-transformed data were used for all the
259 analysis.

260 Multivariate statistics by principal component analysis (PCA; Simca P+ 12.0.1.0, Umetrics)
261 was performed to visualize the pattern of variation in the chemical profile of leaf surface secondary
262 metabolites to provide an overview of the data. Since PCA showed clustering of samples that was
263 interpretable to the groupings of genotypes and provenances, further examination by discriminant
264 analysis was justified. Linear discriminant analysis (LDA, R version 3.1.3) was performed
265 separately for genotype and provenance to investigate the variance in the dataset that defines the
266 differences among genotypes and provenances, respectively. LDA provides linear combinations of
267 variables (metabolites) that best explain the discrimination among the classes (genotypes or
268 provenances). The *lda* function from R packages MASS and caret was used to calculate the
269 accuracy, *k*-fold cross validation and p-values. The data was mean centered and scaled by unit
270 variance. Missing values were replaced with an arbitrary value of 0.001. One triterpenoid that was
271 present only in one genotype (R3) was excluded from analysis. Relationships between the surface
272 metabolites, herbivory index and leaf traits were studied by means of correlation analysis (either
273 Pearson or Spearman, SPSS).

274

275 **RESULTS**

276 Eight flavonoid peaks, twelve triterpenoid peaks, two alkyl coumarate peaks and β -sitosterol were
277 detected and quantified from the leaf wax extracts (Tables S1, S2). Total flavonoid content varied
278 between 7.1 and 21.2 nmol cm⁻² (Fig. 5) and between 0.59 and 1.64 mg g⁻¹ DW with a mean content
279 of 1.0 mg g⁻¹ DW. The most abundant flavonoid was tetrahydroxyflavone dimethyl ether 2 (F7)
280 comprising 40% of the total flavonoids (Fig. 1, Table S3). Three of the flavonoids were identified
281 as chrysoeriol (F1), diosmetin (F2) and Genkwanin (F6) and others were tentatively annotated as
282 hexahydroxyflavone trimethyl ether (F3), tetrahydroxyflavone dimethyl ether 1 (F4),
283 pentahydroxyflavone trimethyl ether 1 (F5) and pentahydroxyflavone trimethyl ether 2 (F8).
284 Triterpenoid aglycones were the major constituents among the leaf wax secondary metabolites.
285 Triterpenoid content ranged from 12.8 to 155.6 nmol cm⁻² (Fig. 5) and from 1.6 to 19.3 mg g⁻¹ DW
286 with a mean content of 8.56 mg g⁻¹ DW. The most abundant triterpenoid aglycone was tentatively
287 annotated as 12-*O*-acetyl-3-*O*-malonyl-betulafolienetriol (T10) comprising 77 % of the total
288 triterpenoid fraction (Fig. 2, Tables S1, S2, S3). The presence of papyriferic acid (T7) was
289 confirmed with a standard. The spectrum of betulafolienetriol (T9) was similar to the spectrum of
290 its isomer protopanaxadiol. Other triterpenoids were tentatively annotated as acetyl-malonyl-
291 betulafolienetetraols (T1, T3, T4, T8), acetyl-malonyl-betulafolienepentanol (T2, T5), papyriferic
292 acid derivative (T6) and 12-*O*-acetyl-betulafolienetriol (T11) (Table S1). Two alkyl coumarates
293 were tentatively annotated as (E) and (Z) isomers of hexadecyl-*p*-coumarate (Fig. 3, Tables S1, S2),
294 and its total content ranged from 0.3 to 2.1 nmol cm⁻² (Fig. 3) (0.03-0.28 mg g⁻¹ DW with a mean
295 content of 0.14 mg g⁻¹ DW). β -sitosterol content showed the largest quantitative variation of the
296 surface metabolites, ranging from 0.01 to 0.7 nmol cm⁻² (Fig. 4) (1.2-79.7 μ g g DW⁻¹). Total
297 content of secondary metabolites in leaf wax extracts ranged from 22.8 to 170.6 nmol cm⁻² (Fig. 5)
298 (2.49 to 20.59 mg g⁻¹ DW with a mean of 9.7 mg g⁻¹ DW).

Genotype affected significantly the mean contents of 14 individual leaf surface metabolites: four flavonoid aglycones, 9 triterpenoids and sitosterol (Figs. 1, 2, 3, Table S2). Thus, there was a considerable variation in the metabolite levels within each provenance. For example, one of the southern genotypes (L15) had consistently low content of most metabolites, while another (L6) had high contents in total flavonoids and triterpenoids (Fig. 5). Qualitative difference among the genotypes was observed only in the triterpenoids. Triterpenoid T8 was present only in one genotype (R3) (Table S3, Fig. 2). The total contents of flavonoid ($p=0.051$) and triterpenoid aglycones ($p=0.041$) and the content of β -sitosterol were significantly associated with the genotype (Figs. 4, 5, Table S3).

Provenance affected the mean content of the two alkyl coumarates (AC1 and AC2) and β -sitosterol (Figs. 3, 4, Table S3). The contents of alkyl coumarates and β -sitosterol were highest in the southern provenance and lowest in the central provenance. The variance explained by provenance was relatively low for most of the compounds in comparison to the total variance explained by both genotype and provenance (Table S3). Only for the contents of AC1 ($R^2_m=0.15$), AC2 ($R^2_m=0.23$) and β -sitosterol ($R^2_m=0.36$) the variation explained by the provenance was significant. Coefficient of variation for the contents of leaf surface secondary metabolites was 49.3% in southern (60°N), 28.6% in central (62°N) and 42.5% in northern (66°N) provenance.

Genotypic and provenance-related variation in chemical profiles of leaf surface secondary metabolites was also evident in PCA (Figs. 6, 7). Four major principal components comprised altogether 81.6% of total variance (see Table S4 for model diagnostics, Fig. S4 for loadings). The first principal component (PC1) explained 53% of the variation and it was related to the differences among the genotypes and the overall content of leaf surface secondary metabolites (Figs. 6 a, b). Quality control (QC) samples of the nine analysis days formed a tight cluster in PCA (data not shown). The second (PC2) and the fourth principal components (PC4) explained 14 % and 6% of variation, respectively, attributable to the differences among the provenances (Figs. 7 a,b). The PC2

separated mainly the southern and northern genotypes, whereas PC4 separated the central and northern ones (Fig. 7a).

The surface metabolites accounting for provenance variation in PCA included eight flavonoid aglycones (F1-F8), the major triterpenoid aglycone, 12-*O*-acetyl-3-*O*-malonyl-betulafolienetriol (T10), one unknown triterpenoid (T12), alkyl coumarates (AC1 and AC2) and β -sitosterol (S) (Fig. S2). The relative contents of the most hydrophobic flavonoid aglycones, tetrahydroxyflavone dimethyl ether 2 (F7) and pentahydroxyflavone trimethyl ether 2 (F8), displayed clinal patterns with the highest content in the southern provenance (Fig. S2a). Flavonoid aglycones, such as diosmetin (F2), hexahydroxyflavone trimethyl ether (F3), tetrahydroxyflavone dimethyl ether 1 (F4) and pentahydroxyflavone trimethyl ether 1 (F5) showed an opposite pattern and were at high level in the northern provenance (Fig. S2a). The content of 12-*O*-acetyl-3-*O*-malonyl-betulafolienetriol (T10) was at the highest level in the northern provenance (Fig. S2b).

A supervised classification method, linear discriminant analysis (LDA) was performed to model the differences in metabolite profiles among genotypes (Figs. 6c, d) and provenances (Figs. 7c, d). A good class separation was attained in LDA for both genotype (Fig. 6) and provenance (Fig. 7). Genotypes were separated by the first axis (34%) and second axis (21%), with several of the genotypes forming tight clusters (e.g., L1, L15, R3, and L14) (Fig. 2c). For the provenance, LDA formed rather tight groups (LD1 70% and LD2 30%, Fig. 7c). LDA discriminated the genotype with an accuracy of 97% and provenance with 94%. Using *k*-fold (7-fold) cross validation, a prediction accuracy of 89% for genotype and 88% for provenance were achieved, with p -value < 0.001 for both genotype and provenance. In general, the loadings of the PCA and LDA indicated that the metabolites contributing to the grouping of the genotypes and provenances in the PCA also contributed to the grouping in LDA (Figs. 6d, 7d). However, PCA suggests that the differences among the genotypes are mainly due to the overall content of all leaf surface secondary

metabolites, whereas more detailed information on the metabolites can be obtained in LDA, for example, R3 was separated from the other genotypes by its high content of F5 (Fig. 6).

The sequence data showed that among the 12 genotypes, two of them (L15 and R3) were the most distant from the others, whereas three of the genotypes (V1, V4 and V5) from central Finland were closely related to each other (Fig. 8).

Significant positive correlation was observed between the flavonoid and triterpenoid contents in most of the genotypes, except in V14 and L15 (Fig. S3). Alkyl coumarate content correlated positively with flavonoid ($r = 0.476$, $p < 0.001$), triterpenoid ($r = 0.420$, $p < 0.001$) and β -sitosterol ($r = 0.411$, $p < 0.001$) contents.

Genotype was a significant determinant for chlorophyll content (CCI) and leaf age (Fig. 9). Leaf age did not show correlation with the content of any of the studied metabolite groups (Fig. S5), whereas leaf area ($r = -0.260$, $p = 0.004$) and SLA ($r = -0.325$, $p < 0.001$) correlated negatively with the total flavonoid content. Herbivore feeding was determined in early (May) and midsummer (July) in 2011 and 2012 and the content of the leaf surface metabolites in June 2013. Herbivory indices showed mainly negative correlation with the contents of the secondary metabolite groups (Table 2). The mean herbivory index of early and midsummer showed a significant provenance related variation (Fig. 9).

Discussion

The leaf surface secondary metabolites of silver birch

The total content of secondary metabolites in the wax layer of silver birch leaves varied widely among the genotypes (2.5-20.6 mg g⁻¹ DW). Compared with intracellular secondary metabolites in silver birch, such as condensed tannins (20-50 mg g⁻¹) or phenolic glycosides (20-30 mg g⁻¹) (Keinänen et al. 1999), the secondary metabolites on the leaf surface can be less or equally abundant. The main secondary metabolites in the silver birch leaf surface extract were triterpenoid

aglycones as reported by Martemyanov et al. (2015a) and Lihavainen et al. (2017). The content of triterpenoid aglycones in the leaf wax extracts was higher than on the twigs of mature silver birch trees ($<1 \text{ mg g}^{-1}$), but lower than in the twigs of juvenile birch saplings ($10.2\text{--}64.6 \text{ mg g}^{-1}$) (Laitinen et al. 2005). Most of the detected triterpenoids were acetyl and/or malonyl esters of betulafolienetriol, betulafolienetetraol and betulafolienepentanol that have been found in silver birch or other *Betula* species (Pokhilo and Uvarova 1988; Rickling and Glombitza 1993; Fuchino et al. 1996; Makhnev et al. 2012). The main triterpenoid aglycone was tentatively annotated as 12-*O*-acetyl-3-*O*-malonyl-betulafolienetriol, reported from leaves of silver birch (Rickling and Glombitza 1993) and Japanese white birch (*Betula platyphylla* var. *japonica*) (Fuchino et al. 1996). The other secondary metabolites of leaf surface wax extract were polymethylated flavonoid aglycones, alkyl coumarates and β -sitosterol. The content and composition of flavonoid aglycones in the wax extracts of silver birch leaves was consistent with previous studies (Keinänen et al. 1999; Valkama et al. 2003; Martemyanov et al. 2015a).

To our knowledge, this is the first study to report alkyl coumarates from silver birch. These were tentatively annotated as hexadecyl-*p*-coumarates. Alkyl hydroxycinnamates, such as alkyl coumarates, ferulates and caffeates have been previously detected in the wax layer of leaves (He et al. 2015) and roots () of cattails (*Typha domingensis* and *Typha latifolia*). The suggested function of alkyl hydroxycinnamates in the cuticular layer is to fortify cell walls and polyphenolic barrier, since they are associated with suberization (Kosma et al. 2015). However, these compounds are known to have antimicrobial and antioxidant properties (Domergue and Kosma et al. 2017). In addition, alkyl coumarates have been shown to inhibit insect feeding in sweet potato (*Ipomoea batatas*) (Snook 1994).

Strong genotypic variation in the leaf surface secondary metabolites

This study showed that the accumulation of secondary metabolites on the leaf surface varied strongly among the silver birch genotypes. Our results agree with the previous studies that show strong genotypic variation in flavonoid and triterpenoid aglycones in leaves (Paaso et al. 2017) and twigs (Laitinen et al. 2005) of silver birch, as well as in intracellular flavonoid glycosides and phenolic acids in leaves (Keinänen et al. 1999; Laitinen et al. 2000). In this study, the genotypic variation was clear for the concentrations of individual metabolites as well as for the chemical profiles. The heritable genotypic variation within a species can affect ecosystem processes, and species interactions in a community (Whitham et al. 2006). Furthermore, genotypes differ in their responses to environmental conditions (Keinänen et al. 1999; Laitinen et al. 2005), which in turn results in additional variation in the concentrations of secondary metabolites.

We found surprisingly little qualitative differences in the surface secondary metabolites among the 12 Finnish silver birch genotypes originating from three different latitudes. The northern genotype R3 accumulated a high content of one triterpenoid (T8) that was not detected in any of the other genotypes. Previously, silver birch genotypes representing one (Laitinen et al. 2000) or two provenances (Keinänen et al. 1999) have displayed clear qualitative differences in foliar phenolic compounds. However, the qualitative differences have been due to glycosylation patterns of flavonoids, and therefore equal diversity may not be expected for the flavonoid aglycones. Nevertheless, the qualitative variation present in R3 suggests that the biosynthesis of triterpenoid aglycones can differ among the genotypes. The enzyme that performs the specific modification to constitute the structure of T8 can be absent or inactive in all other silver birch genotypes but R3, similarly as proposed for a glycosylation step for flavonol glycosides (Keinänen et al. 1999).

The genome sequencing data showed varying levels of relatedness that was also reflected in the variation of the leaf surface secondary metabolites. The two genotypes that were the most unrelated were clearly separated by their metabolite profiles as well; L15 exhibited the lowest content of leaf surface secondary metabolites and R3 displayed one specific triterpenoid. The three

genotypes from central provenance were closely related and those genotypes did not differ in their secondary metabolites. The central provenance displayed also the lowest coefficient of variation (CV %) in the content of leaf surface secondary metabolites, implying a connection between the genotypic and secondary metabolite variation.

Provenance variation in the leaf surface secondary metabolites

The contents of alkyl coumarates and β -sitosterol were the highest in the southern provenance and the lowest in the central provenance. This clear provenance effect may be related to the high hydrophobicity of these compounds. Closer investigation of flavonoid and triterpenoid profiles with multivariate analysis revealed provenance-related patterns, and groups of metabolites exhibiting similar patterns. Flavonoid profiles showed clinal variation: the contents of two highly hydrophobic flavonoids were higher in the southern than in the northern provenance, but an opposite trend was found for some less hydrophobic flavonoids. The hydrophobicity of flavonoid aglycones is nearly proportional to their degree of methylation. The exudation of highly methylated flavonoid aglycones has been reported to co-occur with drought and high temperature during the summer season in *Cistus ladanifer* (Chaves et al. 1997). The prevailing temperature and water conditions of *C. ladanifer* populations can affect leaf surface flavonoid profiles: the response of compounds depended, for example, on the position of methoxy group (Sosa et al. 2005). In low latitudes, silver birch may benefit from highly hydrophobic flavonoid profile by gaining protection against high temperature. Trees from the high latitudes growing in the continuous light during summer season may benefit from flavonoids of a rather low methylation level that are likely to have a high antioxidant activity (Rice-Evans et al. 1995).

Our study is in accordance with other studies that have shown provenance-related variation in leaf secondary metabolites (Slimestad 1998; Pratt et al. 2014). The chemical profiles of plants have been associated with provenances differing from each other in their precipitation level (Pratt et al.

2014) or temperature (Virjamo and Julkunen-Tiitto 2016). Southern and northern latitudes of Finland differ from each other in temperature, precipitation, photoperiod and light quality, the length of growing season and herbivore pressure. It is generally assumed that herbivore pressure is higher in low latitudes than in high latitudes (Schemske et al. 2009), even though contradictory views have been proposed (Moles et al. 2011). The variation in leaf surface secondary metabolites among provenances in our study may thus be due to adaptation to their latitudes of origin, and be related to the differences in the temperature, water and light conditions among their latitudes of origin. On the other hand, the variation in leaf surface secondary metabolites among provenances may be affected by acclimation to the prevailing conditions of the common garden field.

Association of the surface secondary metabolites and other leaf traits

Leaf chlorophyll content and leaf age at the time of sampling varied among the genotypes, but none of the studied leaf traits showed significant variation among the provenances. In contrast, other common garden studies with broadleaved tree species have demonstrated that provenances differ in leaf traits, such as leaf size, SLA and chlorophyll content (reviewed in Bussotti et al. 2015; Soolanayakanahally et al. 2015). For example, in common garden studies with aspen (*Populus tremula*) and balsam poplar (*P. balsamifera*), trees originating from higher latitudes had higher chlorophyll content than those from lower latitudes (Soolanayakanahally et al. 2015).

The positive correlations among the contents of different secondary metabolite groups imply that the overall production and deposition to the leaf surface of different compound groups were coordinated with each other in silver birch leaves. However, only flavonoid content correlated negatively with leaf area and SLA, which indicates that the flavonoid content on the leaf surface was closely intertwined with the leaf morphology. A decline in the concentration of flavonoid aglycones with leaf expansion during the growing season has been reported in previous studies (Valkama et al. 2004; Martemyanov et al. 2015b). There was no correlation between leaf age and

the secondary metabolites, regardless of the significant genotypic variation in leaf age. This was not unexpected, since the genotypes with a late budburst have been shown to exhibit a fast leaf expansion in silver birch, compensating for the differences in the timing of budburst (Possen et al. 2014).

Leaf surface secondary metabolites and herbivore resistance

Our results indicated that the secondary metabolites on the leaf surface are associated with the herbivore resistance in silver birch. The genotypes displaying the highest triterpenoid content and total secondary metabolite content on their leaf surface in early summer 2013 exhibited the lowest herbivore damages in the two previous study years. This is in line with an artificial feeding experiment that has shown that the high content of surface lipophilic compounds in silver birch leaves reduced the weight and the survival of *L. dispar* larvae (Martemyanov et al. 2015a). Additionally, tree-specific content of flavonoid aglycones correlated strongly with the mortality of neonate autumnal moth (*Epirrita autumnata*) larvae, and the experimental removal of the leaf surface compounds enhanced the development and growth rate of the 1st instar larvae (Lahtinen et al. 2004). Birch leaf wax extracts contained triterpenoids including papyriferic acid, which is found also in birch twigs and known to restrict feeding by mammalian herbivores (Reichardt et al. 1984; Tahvanainen et al. 1991). A previous study with birch trees has shown that induced triterpenoid content had a negative correlation with leaf damage by chewers, but a positive correlation with leaf damage by gall mites (Valkama et al. 2005b). This is noteworthy since triterpenoids that are present on the leaf surface can affect the feeding behavior of external feeders, but are not encountered by internal feeders. Thus, our estimates for the correlation between herbivory index and secondary metabolite contents are likely to be conservative as the data included both miners and chewers. Moreover, the herbivore surveys and surface metabolite analysis were performed in different years, and presumably the association would be stronger if analysed during the same summer. Herbivore

damage may be intertwined with abiotic factors varying between years and seasonal trends (early versus late summer), as well as with the leaf traits and phenology of trees (Heimonen et al. 2016). Other secondary metabolites in silver birch leaves, particularly condensed tannins, can also affect herbivory (Mutikainen et al. 2000). Although the association between the leaf surface secondary metabolites and herbivore damage found in this and other studies is not a direct evidence of a causal relationship, it implies that the triterpenoid and flavonoid aglycones on the leaf surface have a role in chemical defense.

Conclusions

The contents of the leaf surface secondary metabolites showed higher variation among the genotypes than among the provenances. However, only one northern genotype accumulated a triterpenoid aglycone that was not present in the other genotypes indicating genotypic variation in the biosynthesis of triterpenoid aglycones. Provenance-related patterns were found particularly in the contents of the most hydrophobic metabolites, with the highest content in the southern provenance. This could be due to adaptation to the environmental conditions in different latitudes of origin or acclimation to prevailing conditions of the common garden site. The genotypic and provenance-related metabolite variation may further affect the plant responses to temperature, light, drought and herbivory. The genotypes displaying high content of triterpenoids and thereby high total content of surface secondary metabolites on their leaf surface exhibited low herbivore damage in the previous study years. These compounds are among the first encountered factors by insect herbivores on leaf surfaces. Thus, the potential role of surface secondary metabolites needs to be considered in further studies of plant–herbivore interaction and resistance breeding.

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Supplementary data:

Table S1. Annotation details of secondary metabolites in silver birch (*Betula pendula* Roth) leaf wax extracts by UPLC-PDA/QTOF/MS (ESI+).

Table S2. Annotation details of secondary metabolites in silver birch (*Betula pendula* Roth) leaf wax extracts by HPLC-CAD/MS (APCI+).

Table S3. Variance components and the effects of genotype and provenance on the contents of surface secondary metabolites in silver birch leaves.

Table S4. Principal component analysis (PCA) model diagnostics.

Figure S1. Mean monthly day length (h) in the latitude of origin of the provenances and common

547 garden field site.

548 **Figure S2.** Relative content of individual leaf surface metabolites.

549 **Figure S3.** Correlation of total triterpenoid and total flavonoid contents.

550 **Figure S4.** Loadings of PC1, PC2, PC3 and PC4 of principal component analysis (PCA).

551 **Figure S5.** Correlation of leaf age and the contents of leaf surface secondary metabolite groups.

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- 752

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Table 1. Location and climate parameters of provenances and common garden field site.

Provenance	Coordinates	Mean annual temperature (°C) ^a	Mean annual temperature sum (°C) ^a	Mean annual precipitation (mm) ^b	Length of growing season (days) ^c
Loppi	60°36'N, 24°25'E	4.2	1287	630	175-185
Vehmersalmi	62°45'N, 28°10'E	3.1	1258	597	155-165
Rovaniemi	66°27'N, 25°14'E	0.8	957	540	135-145
Joensuu (Common garden)	62°35'N, 29°46'E	3.0	1255	591	155-165

^a Period 1959-2012, Finnish Meteorological institute^b Period 1959-2012, European Climate Assessment & Dataset^c Period 1981-2010, Finnish Meteorological institute

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Table 2. Correlation coefficients between herbivory indices and the content of secondary metabolite groups. Relationship between herbivory indices and leaf surface secondary metabolites was determined by the means of spearman correlation, * for $P<0.05$, ** for $P<0.01$. Herbivore surveys were performed in May and July in 2011 and 2012, and leaf surface secondary metabolite content was determined in June 2013. Early summer, midsummer, and early and midsummer combined were calculated from mean of 2011-2012.

Secondary metabolites	Herbivory Index		
	Early summer	Midsummer	Early and midsummer
Triterpenoids	-0.198*	-0.087	-0.183*
Flavonoids	-0.213*	-0.033	-0.136
Alkyl coumarates	-0.092	0.079	-0.021
β -Sitosterol	-0.039	-0.089	-0.072
Total	-0.249**	-0.084	-0.180

785

786 **Figure 1.** Contents of flavonoid aglycones in silver birch leaf wax extracts based on surface
 787 leaf area (nmol cm^{-2}), mean $\pm$ SE ($n=9-10$). Significant effects for genotype (G) and
 788 provenance (P) tested by linear mixed model, P as a fixed factor and G as a random factor
 789 nested within the provenance (* for $P<0.05$). The genotypes within each provenance are in
 790 decreasing order based on the total content of secondary metabolites.

791

792 **Figure 2.** Contents of triterpenoid aglycones in silver birch leaf wax extracts based on surface
 793 leaf area (nmol cm^{-2}), mean $\pm$ SE ($n=9-10$). Significant effects for genotype (G) and
 794 provenance (P) and ordering of genotypes as in Fig. 1.

795

796 **Figure 3.** Contents of individual alkyl coumarates in silver birch leaf wax extracts on surface
 797 leaf area (nmol cm^{-2}), mean $\pm$ SE ($n=9-10$). Significant effects for genotype (G) and
 798 provenance (P) and ordering of genotypes as in Fig. 1.

799

800 **Figure 4.** Content of β -sitosterol in silver birch leaf wax extracts on surface leaf area (nmol
 801 cm^{-2}), mean $\pm$ SE ($n=9-10$). Significant effects for genotype (G) and provenance (P) and
 802 ordering of genotypes as in Fig. 1.

803

804 **Figure 5.** Total contents of flavonoid aglycones, triterpenoid aglycones, alkyl coumarates and
 805 secondary metabolites in silver birch leaf wax extracts based on surface leaf area (nmol cm^{-2}),
 806 mean $\pm$ SE ($n=9-10$). Significant effects for genotype (G) and provenance (P) and ordering of
 807 genotypes as in Fig. 1.

808

809 **Figure 6.** Principal component analysis (PCA) scatter plot (a), loading plot (b), and linear
 810 discriminant analysis (LDA) scatter plot (c), loading plot (d) of the genotypes, based on leaf
 811 surface metabolites ($n=9-10$ replicates for genotype). In scatter plots, genotypes indicated by
 812 symbols and provenances by colors. In loading plots, metabolite groups indicated by colors;
 813 T=triterpenoid, F=flavonoid, AC=alkyl coumarate, S= β -sitosterol. Shown are PC1 and PC2
 814 and LD1 and LD2.

815

816 **Figure 7.** Principal component analysis (PCA) scatter plot (a), loading plot (b), and linear
 817 discriminant analysis (LDA) scatter plot (c), loading plot (d) of the provenances, based on
 818 leaf surface metabolites ($n=37-40$ replicates for provenance). In scatter plots, provenances
 819 indicated by colors. In loading plots, metabolite groups indicated by colors; T=triterpenoid,
 820 F=flavonoid, AC=alkyl coumarate, S= β -sitosterol. Shown are PC2 and PC4 and LD1 and
 821 LD2.

822

823 **Figure 8.** A heatmap of pairwise identity by descent (IBD) values for the genotypes,
 824 illustrating the genetic relatedness. High value means high similarity.

825

826 **Figure 9.** Leaf traits and herbivory index; leaf area, specific leaf area, leaf water content, leaf
 827 age at the time of sampling, chlorophyll content index (CCI) and herbivory index, mean $\pm$ SE
 828 ($n=9-10$). Significant effects for genotype (G) and provenance (P) tested by linear mixed
 829 model, P as a fixed factor and G as a random factor nested within the provenance (* for
 830 $P<0.05$). The genotypes within each provenance are in decreasing order based on the total
 831 content of secondary metabolites. Herbivory index is determined as a mean of herbivore
 832 damage determined in early and midsummer 2011 and 2012.

833

Figure 1. Contents of flavonoid aglycones in silver birch leaf wax extracts based on surface leaf area (nmol cm-2), mean±SE (n=9-10). Significant effects for genotype (G) and provenance (P) tested by linear mixed model, P as a fixed factor and G as a random factor nested within the provenance (* for P<0.05). The genotypes within each provenance are in decreasing order based on the total content of secondary metabolites.

67x40mm (300 x 300 DPI)

Figure 2. Contents of triterpenoid aglycones in silver birch leaf wax extracts based on surface leaf area (nmol cm^{-2}), mean $\pm$ SE (n=9-10). Significant effects for genotype (G) and provenance (P) and ordering of genotypes as in Fig. 1.

90x69mm (300 x 300 DPI)

Figure 3. Contents of individual alkyl coumarates in silver birch leaf wax extracts on surface leaf area (nmol cm⁻²), mean±SE (n=9-10). Significant effects for genotype (G) and provenance (P) and ordering of genotypes as in Fig. 1.

36x17mm (300 x 300 DPI)

Figure 4. Content of β -sitosterol in silver birch leaf wax extracts on surface leaf area (nmol cm^{-2}), mean $\pm$ SE (n=9-10). Significant effects for genotype (G) and provenance (P) and ordering of genotypes as in Fig. 1.

38x35mm (300 x 300 DPI)

Figure 5. Total contents of flavonoid aglycones, triterpenoid aglycones, alkyl coumarates and secondary metabolites in silver birch leaf wax extracts based on surface leaf area (nmol cm⁻²), mean±SE (n=9-10). Significant effects for genotype (G) and provenance (P) and ordering of genotypes as in Fig. 1.

33x9mm (300 x 300 DPI)

Figure 6. Principal component analysis (PCA) scatter plot (a), loading plot (b), and linear discriminant analysis (LDA) scatter plot (c), loading plot (d) of the genotypes, based on leaf surface metabolites ($n=9-10$ replicates for genotype). In scatter plots, genotypes indicated by symbols and provenances by colors. In loading plots, metabolite groups indicated by colors; T=triterpenoid, F=flavonoid, AC=alkyl coumarate, S= β -sitosterol. Shown are PC1 and PC2 and LD1 and LD2.

83x74mm (300 x 300 DPI)

Figure 7. Principal component analysis (PCA) scatter plot (a), loading plot (b), and linear discriminant analysis (LDA) scatter plot (c), loading plot (d) of the provenances, based on leaf surface metabolites (n=37-40 replicates for provenance). In scatter plots, provenances indicated by colors. In loading plots, metabolite groups indicated by colors; T=triterpenoid, F=flavonoid, AC=alkyl coumarate, S= β -sitosterol. Shown are PC2 and PC4 and LD1 and LD2.

82x73mm (300 x 300 DPI)

Figure 8. A heatmap of pairwise identity by descent (IBD) values for the genotypes, illustrating the genetic relatedness. High value means high similarity.

58x51mm (300 x 300 DPI)

Figure 9. Leaf traits and herbivory index; leaf area, specific leaf area, leaf water content, leaf age at the time of sampling, chlorophyll content index (CCI) and herbivory index , mean $\pm$ SE (n=9-10). Significant effects for genotype (G) and provenance (P) tested by linear mixed model, P as a fixed factor and G as a random factor nested within the provenance (* for P<0.05). The genotypes within each provenance are in decreasing order based on the total content of secondary metabolites. Herbivory index is determined as a mean of herbivore damage determined in early and midsummer 2011 and 2012.

64x38mm (300 x 300 DPI)