Food Chemistry 354 (2021) 129517

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analysis of composition, morphology, and biosynthesis of cuticular wax in wild type bilberry (*Vaccinium myrtillus* L.) and its glossy mutant

Priyanka Trivedi [a,1], Nga Nguyen [a,1], Linards Klavins [b], Jorens Kviesis [b], Esa Heinonen [c], Janne Remes [c], Soile Jokipii-Lukkari [a], Maris Klavins [b], Katja Karppinen [d], Laura Jaakola [d,e], Hely Häggman [a,*]

[a] Department of Ecology and Genetics, University of Oulu, FI-90014 Oulu, Finland
[b] Department of Environmental Science, University of Latvia, LV-1004 Riga, Latvia
[c] Centre for Material Analysis, University of Oulu, FI-90014 Oulu, Finland
[d] Department of Arctic and Marine Biology, UiT The Arctic University of Norway, NO-9037 Tromsø, Norway
[e] NIBIO, Norwegian Institute of Bioeconomy Research, NO-1431 Ås, Norway

ARTICLE INFO

Keywords:
Cuticular wax
Fruit cuticle
Gene expression
Glossy type mutant
Triterpenoids
Wax composition

Chemical compounds studied in this article:
β-Amyrin (PubChem CID: 73145)
α-Amyrin (PubChem CID: 73170)
Lupeol (PubChem CID: 259846)
Heneicosanone (PubChem CID: 529805)
Octacosanal (PubChem CID: 68406)
Montanic acid (PubChem CID: 10470)

ABSTRACT

In this study, cuticular wax load, its chemical composition, and biosynthesis, was studied during development of wild type (WT) bilberry fruit and its natural glossy type (GT) mutant. GT fruit cuticular wax load was comparable with WT fruits. In both, the proportion of triterpenoids decreased during fruit development concomitant with increasing proportions of total aliphatic compounds. In GT fruit, a higher proportion of triterpenoids in cuticular wax was accompanied by a lower proportion of fatty acids and ketones compared to WT fruit as well as lower density of crystalloid structures on berry surfaces. Our results suggest that the glossy phenotype could be caused by the absence of rod-like structures in GT fruit associated with reduction in proportions of ketones and fatty acids in the cuticular wax. Especially *CER26-like, FAR2, CER3-like, LTP, MIXTA*, and *BAS* genes showed fruit skin preferential expression patterns indicating their role in cuticular wax biosynthesis and secretion.

1. Introduction

Fruit cuticles are lipophilic layers on the aerial parts of plant surfaces composed of cuticular wax and cutin, a polyester polymer matrix. Cuticles have an important role in preventing water loss, protection against UV radiation and pathogen attack in plant organs, including fruits, at different developmental stages and during storage. Cuticular wax is a complex mixture of very long chain fatty acids (VLCFAs) and their derivatives, such as aldehydes, alkanes, ketones, primary and secondary alcohols, esters, and secondary metabolites, including triterpenoids, sterols, and phenolic compounds (Kunst & Samuels, 2009). Fruit cuticular waxes have been shown as good sources of triterpenoids, which are well known for their health-beneficial antioxidant and anti-inflammatory properties and decreasing risk for cardiovascular diseases (Szakiel, Pączkowski, Pensec, & Bertsch, 2012). Previous studies have shown that the composition of cuticular wax varies not only between species, cultivars, and organs, but also with the developmental stage of the same organ (van Maarseveen, Han, & Jetter, 2009). A variable trend in wax deposition rate and alteration in chemical composition of cuticular wax through fruit development in various species has been reported (Trivedi et al., 2019b).

Cuticular wax can be seen as whitish (glaucous) or glossy epicuticular wax, and it is also embedded on the cutin as intracuticular wax (Ensikat, Boese, Mader, Barthlott, & Koch, 2006). The chemical basis for

* Corresponding author.
E-mail addresses: priyanka.priyanka@oulu.fi (P. Trivedi), thi.nguyen@oulu.fi (N. Nguyen), linards.klavins@lu.lv (L. Klavins), jorens.kviesis@lu.lv (J. Kviesis), esa.heinonen@oulu.fi (E. Heinonen), janne.remes@oulu.fi (J. Remes), soile.jokipii-lukkari@oulu.fi (S. Jokipii-Lukkari), maris.klavins@lu.lv (M. Klavins), katja.karppinen@uit.no (K. Karppinen), laura.jaakola@uit.no (L. Jaakola), hely.haggman@oulu.fi (H. Häggman).
[1] Equal contribution.

https://doi.org/10.1016/j.foodchem.2021.129517
Received 15 May 2020; Received in revised form 12 February 2021; Accepted 27 February 2021
Available online 9 March 2021

the difference between glaucous and glossy wax phenotypes is unclear although it has been studied in various species. Glaucous leaf and stem mutants of Arabidopsis showed higher wax load accompanied by higher density of epicuticular wax crystals (Jenks, Rashotte, Tuttle, & Feldmann, 1996). Characterization of naturally occurring glaucous lines has identified β-diketones to be responsible for glaucousness in wheat and barley (Hen-Avivi et al., 2016). Among fruits, glossy mutant fruits of orange showed a decrease in wax load accompanied by reduction in proportion of aldehydes affecting crystalloid formation (Liu et al., 2012; Liu et al., 2015), while in apple fruits, glossiness (or greasiness) was attributed to melting of wax crystalloids and formation of amorphous wax (Yang et al., 2017).

The wax biosynthesis pathway with key genes has been elucidated by studies performed mainly in Arabidopsis. In general, the biosynthesis of aliphatic compounds of cuticular wax starts from the *de novo* fatty acid biosynthesis in plastids producing C_{16}–C_{18} fatty acids by β-ketoacyl-ACP synthase (KAS) as a key enzyme (Fig. S1). The later stages of biosynthesis occur in endoplasmic reticulum (ER), exclusively in epidermal cells, where elongation of VLCFAs (C_{20}–C_{34}) is facilitated by β–ketoacyl-CoA-synthase (KCS). The different classes of aliphatic compounds of the cuticular wax are modified from the VLCFAs by two pathways; acyl reduction pathway (alcohol forming) to produce primary alcohols and wax esters, and decarbonylation pathway (alkane forming) to produce aldehydes, alkanes, ketones, and secondary alcohols (Fig. S1). The wax components are exported through the plasma membrane by heterodimer ABCG transporter family proteins, and further to the cell wall by non-specific lipid transfer protein (LTP; Kunst & Samuels, 2009). Earlier studies of wax biosynthesis in fruits have mostly focused on horticultural plants, such as tomato (*Solanum lycopersicum*), sweet cherry (*Prunus avium* L.), and orange (*Citrus sinensis* L.) fruits (Vogg et al., 2004; Alkio, Jonas, Sprink, van Nocker, & Knoche, 2012; Liu et al., 2015; Wang et al., 2016).

Bilberry (*Vaccinium myrtillus* L.) is a deciduous shrub with wide distribution in cool temperate regions and mountain areas of Europe and Asia. The glossy type (GT) black mutants of bilberry grow along with wild type (WT) bilberry plants and their fruits are harvested together (Fig. 2). As an abundant resource in the Northern forests, wild bilberries are used for the production of juices, jams, and nutraceutical products. Bilberries are an excellent raw material for the extraction of health beneficial compounds, including anthocyanins, and the leftovers of food industry (berry press cakes) can be utilized for the extraction of other bioactive compounds such as wax (Trivedi et al., 2019a).

The aim of the present study was to explore the differences between bilberry WT fruit and its natural GT mutant fruit for chemical composition, morphology, and biosynthesis of cuticular wax during the fruit development. In order to clarify the wax biosynthesis in bilberry, we also identified genes related to cuticular wax biosynthesis from the previously published bilberry transcriptome (Nguyen et al., 2018).

2. Materials and methods

2.1. Plant materials

WT and GT fruits of bilberry (*Vaccinium myrtillus* L.) at four developmental stages, S2 (small unripe green fruit, 21 days after anthesis), S3 (large unripe green fruit, 36 days after anthesis), S4 (ripening red fruit, 48 days after anthesis), and S5 (fully ripe blue fruit, 54 days after anthesis), were utilized in this study (Fig. 2). At least 18 berries per replicate were used for the gene expression analysis, and approximately 100 berries per replicate were collected for the chemical analyses. The fruits were collected using forceps from June to August 2018 from the natural forest stand in Oulu, Finland (65°03′37.0″N 25°28′30.4″E).

2.2. Scanning electron microscopy (SEM)

For SEM analysis, fresh berries were dried immediately after the collection using a vacuum freeze-dryer (Edwards Vacuum, Burges Hill, UK) and fixed on aluminum stubs. The berry surfaces were sputter-coated with 20 nm layer of platinum (Advent Research Materials Ltd, Oxford, UK) using a sputter coater (Agar High Resolution Sputter Coater, Agar Scientific Ltd, Stansted Mountfitchet, UK) followed by investigation of the three-dimensional surface micromorphology by using SEM (Helios Nanolab 600, ThermoFisher Scientific, Hillsboro, USA).

2.3. Cuticular wax extraction and determination of wax amount

Immediately after the collection, the cuticular wax from the four developmental stages of both WT and GT fruits was extracted separately with chloroform (Sigma-Aldrich, St. Louis, USA). Berries were dipped in 15 mL chloroform for 1 min. The extract was evaporated to dryness under nitrogen (AGA, Guildford, UK) flow at room temperature followed by the measurement of dry weight. The cuticular wax extraction was performed in triplicates for each berry developmental stage (except GT S4 stage, where due to the unavailability of GT fruits, the extraction was performed in duplicates). The amount of wax was presented as weight per unit surface area (μg/cm^2). After the wax extraction, images of berries were taken on a white surface. Total surface area of the berries was measured using Image J software v1.50i (NIH, Bethesda, USA) by the formula $S = 4\ \pi r^2$ (r is the radius of berry). Berries were assumed to be spherical.

2.4. GC–MS analysis

GC–MS analysis was done as conducted by Trivedi et al. (2019a) using a modified derivatization procedure according to Dobson et al. (2012). Bilberry wax was weighed into a glass vial and 0.5 mL toluene (Sigma-Aldrich, St. Louis, USA) was added. Sample was dissolved and 3 mL of 14% boron trifluoride-methanol solution (Sigma-Aldrich, St. Louis, USA) was added. The resulting sample was heated at 60 °C for 3 h. The reaction mixture was re-extracted with hexane and the resulting fatty acid methyl esters were analyzed using GC–MS.

GC–MS system PerkinElmer Clarus 580 - Clarus SQ 8C (Waltham, USA) was used for the analysis. Analysis of FAME's was performed using Omegawax 250 column (30 m × 0.25 mm, 0.25 μm, Darmstadt, Germany) and polyfunctional compounds were analyzed as trimethylsilyl derivatives using ELITE 5MS column (30 m × 0.25 mm, 0.25 mm, PerkinElmer, Waltham, USA). Derivatization of samples was done with 60 μL N,O-Bis (trimethylsilyl)trifluoroacetamide (Sigma-Aldrich, St. Louis, USA) and samples were incubated at 60 °C for one hour. Initial column temperature was set to 75 °C for 2 min, then increased from 75 °C to 150 °C at a rate of 20 °C/min. For Omegawax 250 column, temperature was further increased from 150 °C to 270 °C and for Elite 5MS the increase was from 150 °C to 310 °C at 4 °C/min. Final isothermal step was for 5 min at 270 °C for Omegawax 250 and for 10 min at 310 °C for Elite 5MS column. Sample injection volume was set to 0.5 μL with injection and interface temperatures were kept constant at 290 °C. Helium (AGA, Riga, Latvia) was used with a flow rate of 1.0 mL/min and split flow of 10.0 mL/min. Electron impact was set to 70 eV with a scan range from 42 to 750 *m/z*. Quantification of compounds was done using standard solutions in the concentration range of 1.5–500 μg/mL of methyl heptadecanoate (≥99.0%), ergosterol (≥99.0%), hexadecanol (≥99.0%), 1-dodecanal (≥98.0%), (±)-α-tocopherol (99.0%), 1-octadecanol (99.0%), and *n*-tetracosane (≥99.5%) obtained from Sigma-Aldrich, St. Louis, USA. Compounds were identified using NIST MS 2.2 library (Gaithersburg, USA) and Kovat's Retention indices (Kovats, 1958). The analysis was performed in triplicate.

2.5. Identification of candidate genes related to the wax biosynthesis

De novo transcriptome database of bilberry (Nguyen et al., 2018) was utilized for identifying candidate genes related to the wax biosynthetic pathway. The identity of the genes was verified by BLASTX with

threshold E-value cut-off of 1e-5 against reference protein sequences of Arabidopsis (The Arabidopsis Information Resource - TAIR, https://www.arabidopsis.org/) and other fruits (National Centre for Biotechnology Information - NCBI).

2.6. RNA extraction and qRT-PCR

Skin and pulp of both WT and GT fruits were separated at the four developmental stages with three biological replicates by using a razor blade. The samples were immediately frozen in liquid nitrogen and stored at −80 °C until use for RNA extraction. Total RNA was extracted using CTAB-based method developed for bilberry fruit (Jaakola, Pirttilä, Halonen, & Hohtola, 2001). Quantitative reverse transcription PCR (qRT-PCR) analysis was performed as described by Nguyen et al. (2018). Glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*, GenBank accession number AY123769) was used as an internal control to normalize the relative transcript levels. The expression of *GAPDH* has been shown earlier to be stable during the bilberry fruit development (Jaakola et al., 2002). Gene-specific primer sequences used for qRT-PCR analysis are listed in Table S1.

2.7. Statistical analysis

Significant differences in various compound classes between WT and GT fruits at p-value < 5% were analyzed by independent sample *t*-test using SPSS Statistic program v26 (IBM Corporation, New York, USA). SAS JMP®,Version 13 (SAS Institute Inc., Cary, USA) was used to perform Principal component analysis (PCA). Loadings (groups of compounds) situated closely to the scores (growth stage) in the PCA biplot represent highly correlated variables. The relative means of expression of the studied genes in WT and GT fruit were compared with either *t*-test or Mann-Whitney *U* test by using R v3.6.2 (R Core Team, 2019).

3. Results

3.1. Cuticular wax morphology

By visual inspection of the fruit surface, the difference in appearance between WT and GT bilberry can be detected in early stage (S2) of the fruit development (Fig. 1). SEM analysis of fruit surfaces during the WT fruit development showed a dense cover of irregular platelets at S2 stage (Fig. 1A). At S3, S4, and S5 stages a syntopism of dense rod-like structures with irregular platelets was seen in WT fruit. On the GT fruit, an amorphous layer of wax with markedly lower density of crystalloid structures compared to WT bilberry fruit was detected throughout the fruit development (Fig. 1B). Only membranous platelets but no rod-like structures were detected on the GT fruit surface.

3.2. Cuticular wax load

Both WT and GT bilberry fruit had cuticular wax present already in S2 stage of development (Fig. 2). The amount of wax per berry was found to gradually increase during the fruit development, reaching 367.6 μg and 315.5 μg in ripe stage (S5) in WT and GT fruit, respectively (Fig. 2A). No marked differences in the total wax amount between the WT and GT fruit in any developmental stage was detected. The measured surface areas of GT fruits at ripening stages were slightly smaller than in

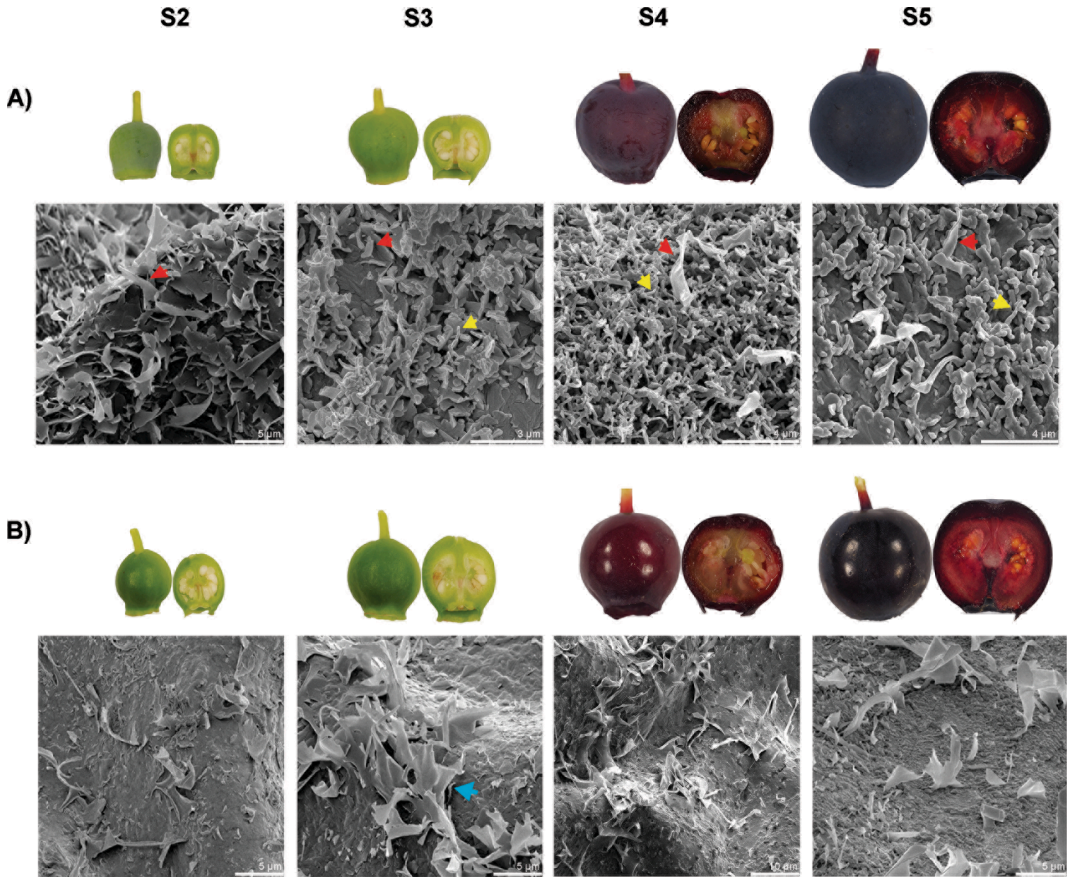

Fig. 1. Changes in epicuticular wax morphology on the surface of (A) wild type (WT) and (B) glossy type mutant (GT) bilberry fruits during development. Red arrows indicate platelet structures, yellow arrows indicate rod-like structures, and blue arrows indicate membranous platelet structures. S2, small green fruits; S3, large green fruits; S4, ripening red fruits; S5, fully ripe blue fruits. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

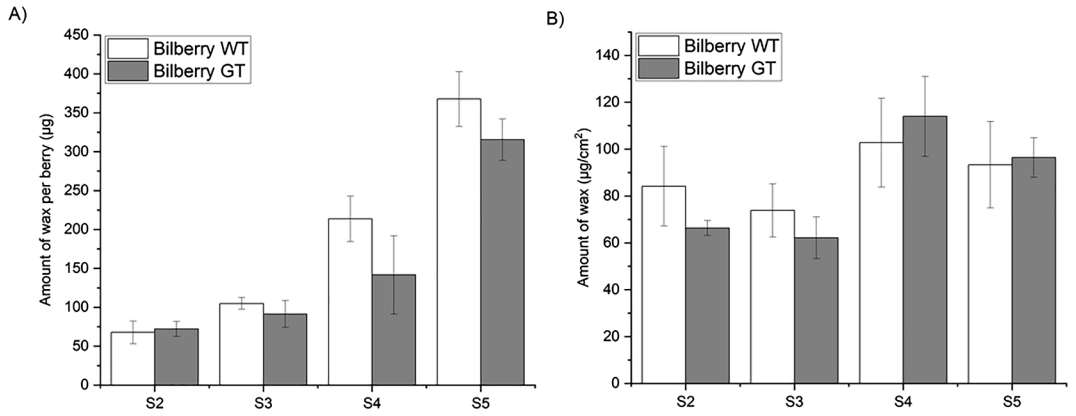

Fig. 2. Accumulation of cuticular wax (A) per berry and (B) per unit surface area (in µg /cm^2) during fruit developmental stages in wild type (WT) and glossy type mutant (GT) bilberry fruits.

WT berries explaining the somewhat higher wax amount per berry in WT berries at S4 and S5 stages. Wax amount per surface area remained more or less constant between S2 and S3 stages, then increasing slightly at S4 stage with a decrease towards S5 stage in both WT and GT fruit (Fig. 2B).

3.3. Composition of cuticular wax

GC–MS analysis showed that the cuticular wax of both WT and GT fruit were mainly composed of triterpenoids, fatty acids, primary alcohols, ketones, aldehydes, and alkanes (Fig. 3A). Triterpenoids followed by fatty acids were found to be the dominant compounds at all developmental stages of both WT and GT fruit cuticular wax. Secondary alcohols and esters were not detected in the cuticular wax of either WT or GT fruit. Our results showed that the cuticular wax of WT fruits consists of higher amounts of fatty acids and ketones than GT fruits, while the cuticular wax of GT fruits contains more triterpenoids at all developmental stages (Fig. 3A). PCA of chemical compounds of cuticular wax revealed that WT and GT fruits formed separate clusters at the early (S2 and S3) development stages and the late (S4 and S5) ripening stages (Fig. 3B). At the early developmental stages, the cuticular wax of both WT and GT fruit contained slightly more alkanes (PC1) than the fruits in the ripening stages (Fig. 3B). However, the berry wax at the ripening stages contained more aldehydes and primary alcohols (PC1) compared to fruits at the early developmental stages. Variance of the first two components accounts for 81% of the total data variability (Fig. 3B).

3.3.1. Cyclic compounds

The proportion of triterpenoids in cuticular wax showed differences through the course of bilberry fruit development and was found to decrease from S2 to S5 stage (from 72.1% to 51.2%) in WT fruit (Fig. 3A). Also in GT fruit cuticular wax, the proportion of triterpenoids was found to decrease during fruit development from S2 to S5 stage (from 84.5% to 65.0%). The triterpenoid proportion was higher in cuticular wax of GT fruit compared to WT fruit at all the stages of bilberry fruit development (Fig. 3A). The relative triterpenoid proportion was found to be higher in GT fruit by 17% in S2, 29% in S3, 29% in S4 and 18% in S5 stage compared to WT fruit.

Generally, oleanolic acid was found to be the predominant triterpenoid in cuticular wax of both WT and GT fruit during the development but was found in higher amounts in GT compared to WT fruit in S3, S4, and S5 stages (Table 1). Ursolic acid, β-amyrin, and α-amyrin were also found in all stages of WT and GT fruit cuticular wax. β-amyrin was present in higher amounts in S2, S3, and S4 stages in GT compared to WT fruit (Table 1). Lupeol was detected in S3, S4, and S5 stages in WT and GT berries. Levels of amyrins and lupeol were found to be highest in

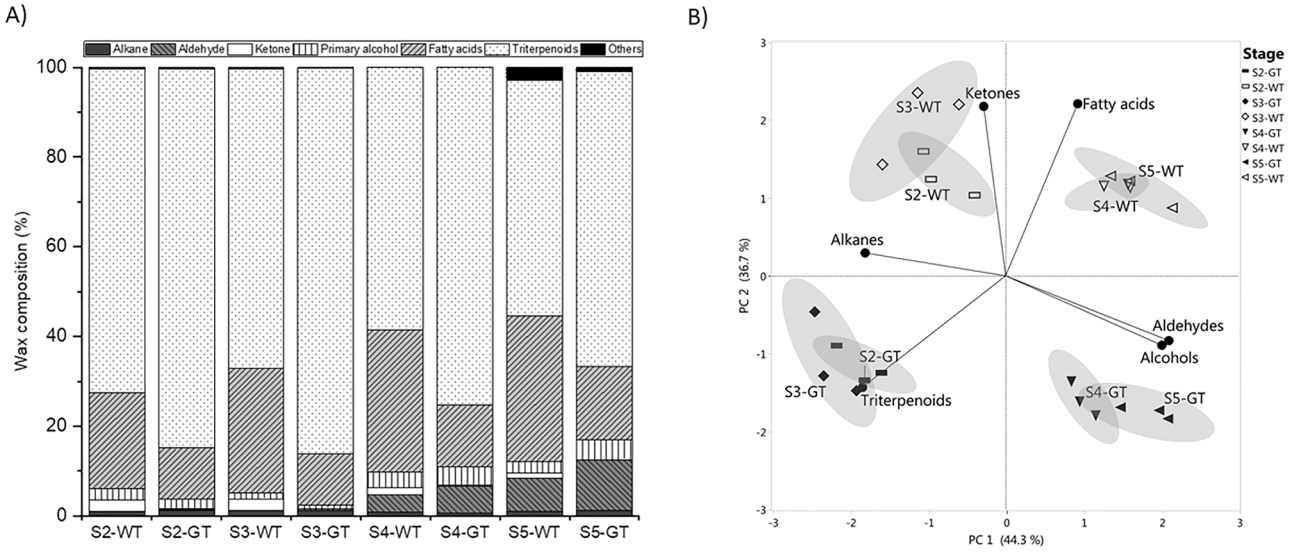

Fig. 3. A) Proportion of chemical compound classes in wild type (WT) and glossy type mutant (GT) bilberry cuticular wax. (B) Principal component analysis and loading plot of cuticular wax composition in wild type (WT) and glossy type mutant (GT) bilberry fruits during development. S2, small unripe green fruits; S3, large unripe green fruits; S4, ripening red fruits; S5, fully ripe blue fruits.

Table 1
Quantities (μg/cm^2) of cyclic compounds during development of wild type bilberry (WT) and glossy type mutant (GT) fruits.

Cuticular wax compounds	Quantity (μg/cm^2) in WT bilberry				Quantity (μg/cm^2) in GT bilberry			
	S2	S3	S4	S5	S2	S3	S4	S5
Triterpenoids								
Oleanolic acid	24.40 ± 6.16	10.76 ± 1.86	11.69 ± 5.79	15.18 ± 3.13*	18.55 ± 4.16	13.30 ± 2.44	24.99 ± 7.00	29.00 ± 5.31*
Ursolic acid	22.47 ± 7.46*	4.33 ± 0.70	6.49 ± 5.70	9.57 ± 1.25	5.30 ± 0.81*	3.69 ± 0.58	11.67 ± 1.56	8.00 ± 1.12
β-Amyrin	2.80 ± 0.91*	2.71 ± 0.91	5.19 ± 2.16	7.24 ± 2.03	6.83 ± 1.45*	3.34 ± 1.27	10.31 ± 2.47	5.22 ± 0.16
α-Amyrin	2.48 ± 0.69	2.16 ± 0.14	4.42 ± 2.03	2.52 ± 0.29	3.25 ± 0.43	2.45 ± 0.22	5.94 ± 1.39	2.80 ± 0.44
Lupeol	nd	2.16 ± 0.25	4.54 ± 2.12	3.28 ± 0.66	nd	2.24 ± 0.05	6.50 ± 0.47	3.37 ± 0.48
28-Norolean-17-en-3-one	2.23 ± 0.73	2.24 ± 0.24	nd	nd	1.06 ± 1.84	2.60 ± 0.25	nd	nd
Olean-2,12-dien-28-oate	nd	nd	9.14 ± 3.25	0.22 ± 0.11	nd	nd	3.64 ± 0.12	nd
Ursa-2,12-dien-28-oate	nd	nd	13.38 ± 2.43	0.21 ± 0.36	nd	nd	6.88 ± 0.85	nd
Unidentified	nd	nd	nd	nd	5.51 ± 0.77	4.00 ± 0.84	1.16 ± 0.17	nd
Phenolic acids								
Benzoic acid	0.12 ± 0.04	0.10 ± 0.02	nd	nd	0.12 ± 0.03	0.09 ± 0.09	nd	nd
p-coumaric acid	0.05 ± 0.00*	0.01 ± 0.01	nd	nd	0.03 ± 0.00*	nd	nd	nd

Data is means ± SD of three replicates, except GT S4 stage, where data is mean ± SD of two replicates
*indicates statistically significant differences between means ($p < 0.05$).

S4 stage in both WT and GT fruit. Esters of oleanane and ursane type triterpenoids were specifically found in the ripening stages (S4 and S5). Phenolic acids (benzoic acid and p-coumaric acid) were found only in S2 and S3 developmental stages in both WT and GT fruit (Table 1).

3.3.2. Aliphatic compounds

Generally, in both WT and GT fruit, the proportion of total aliphatic compounds (including fatty acids, aldehydes, ketones, alkanes, and primary alcohols) increased from S2 to S5 stage during the fruit development (Fig. 3A). A markedly lower proportion of total aliphatic compounds was observed in GT fruit compared to WT fruit at all the developmental stages. This was mainly contributed by the lower percentage of fatty acids and ketones in GT fruit compared to WT fruit (Fig. 3A). Among the fatty acids identified, montanic acid (C28) was the dominant fatty acid in both WT and GT fruit during ripening (S4 and S5) stages (Table 2).

The proportion of ketones was significantly lower in the cuticular wax of GT fruit compared to WT fruit (Fig. 3A). The relative proportion was lower by 8-fold in S2, 19-fold in S3, 6-fold in S4, and 22-fold in S5 stage of GT fruit compared to WT fruit. The proportion of ketones decreased slightly during the WT fruit development. 2-heneicosanone (C21) was the dominant ketone found in both WT and GT fruit at all developmental stages (Table 2).

Aldehydes were detected in high proportions only in S4 and S5 stages in both WT and GT fruit cuticular wax (Fig. 3A). A higher relative proportion of aldehydes was detected in GT compared to WT fruit by 53% in S4 and by 50% in S5 stage of the fruit ripening. Octacosanal was the dominant aldehyde in both ripening WT and GT fruit, followed by hexacosanal and triacontanal (Table 2).

Primary alcohols and alkanes showed variable trends during the development in both WT and GT fruit (Fig. 3A). A lower relative proportion of primary alcohols in GT compared to WT fruit was observed with a decrease in S2 (18%) and S3 (63%), followed by an increase in S4 (11%) and S5 stages (74%). In case of alkanes, a higher relative proportion in GT fruit was observed in S2 and S5 stages, while lower in S3 and S4 stages, when compared to WT fruit.

3.4. Identification and expression of cuticular wax biosynthetic genes

From the bilberry transcriptome database (Nguyen et al., 2018), we identified 335 unigenes encoding enzymes predicted to be involved in the different stages of biosynthesis of aliphatic compounds (Table S2). In the triterpenoid biosynthetic pathway, we identified 21 unigenes encoding two oxidosqualene cyclases (OSCs), namely BAS and LUS (Table S2). 16 unigenes were selected for the gene expression analysis by qRT-PCR based on the high sequence similarity with Arabidopsis and some fruit bearing species (Table S3).

Overall, the genes showed differential expression patterns during the bilberry fruit development of both WT and GT fruit (Fig. 4). Notably, the *CER26-like*, *FAR2*, *CER3-like*, *LTP*, *MIXTA*, and *BAS* genes were expressed at higher levels in the skin compared to pulp in both WT and GT fruit. Among the genes related to VLCFAs formation, the expression level of the fatty acid elongation gene, *CER26-like,* was high at the early developmental stages (S2 and S3) in the GT fruit. In contrast, in the WT fruit, *CER26-like* showed up-regulation at the S4 ripening stage.

In the alcohol forming pathway, the expression of *FAR2* gene, was highest at the early development stages but dropped thereafter in both the WT and GT fruit. In addition, the *FAR2* gene exhibited higher transcript abundance in GT fruit compared to WT fruit. In the alkane-forming pathway, *CER3-like* was markedly up-regulated at the S4 ripening stage in both WT and GT fruit.

In the triterpenoid biosynthesis pathway, the expression pattern of *BAS* was high at the early developmental stage S2, followed by a gradual down-regulation throughout the development of GT fruit. The expression of *BAS* was also down-regulated at the fully ripe stage (S5) in WT fruit.

Among the genes involved in the transportation of wax components, two *ABCG* genes, *ABCG11* and *ABCG15-like,* were expressed at higher levels in skin compared to pulp, especially at the ripening stages S4 and S5. For *LTP* gene, the expression peaked at the early developmental stage (S2) in both WT and GT fruit. Moreover, its expression level was slightly higher in GT compared to WT fruit in S2 and S3 stages.

From the bilberry transcriptome database, we identified a unigene encoding MIXTA, a MYB transcription factor related to the regulation of cuticle formation. The *MIXTA* was up-regulated at the early developmental stages, and showed slightly higher expression level in WT compared to GT fruit in bilberry skin.

4. Discussion

4.1. WT and GT bilberry fruit both show accumulation of cuticular wax

Some previous studies have indicated wax load to decrease in glossy fruit phenotypes. For example, in orange, glossy fruits were reported to contain 44% lower cuticular wax load (Liu et al., 2012). The black GT mutant bilberry fruits have earlier been considered to be waxless (Colak et al., 2017), although no scientific studies concerning the analysis of cuticular wax have been reported. In the present study, we found that both WT and GT bilberry fruit showed almost similar cuticular wax load. Our results support the view that visual phenotype of plant cuticle is not correlated with the wax load (Adamski et al., 2013).

Based on our results, changes in wax accumulation take place during

Table 2
Quantities (μg/cm^2) of very long chain aliphatic compounds during development of wild type bilberry (WT) and glossy type mutant (GT) fruits.

Cuticular wax compounds	Quantity (μg/cm^2) in WT bilberry				Quantity (μg/cm^2) in GT bilberry			
	S2	S3	S4	S5	S2	S3	S4	S5
Fatty acids								
Oleic acid	0.06 ± 0.02	nd	0.15 ± 0.02	0.06 ± 0.01*	0.08 ± 0.01	nd	0.15 ± 0.12	0.13 ± 0.03*
Stearic acid	0.39 ± 0.01*	0.23 ± 0.02*	0.57 ± 0.08	0.28 ± 0.01*	0.17 ± 0.01*	0.12 ± 0.01*	0.25 ± 0.18	0.18 ± 0.03*
Nonadecanoic acid	0.08 ± 0.02	0.05 ± 0.04	nd	0.05 ± 0.04	nd	nd	0.04 ± 0.07	0.00
Arachidic acid	8.34 ± 0.40*	4.97 ± 0.87*	6.40 ± 0.29	3.56 ± 0.21*	1.07 ± 0.20*	0.60 ± 0.15*	1.47 ± 1.25	0.84 ± 0.34*
Behenic acid	0.43 ± 0.01*	0.18 ± 0.04	0.44 ± 0.07	0.29 ± 0.04	0.27 ± 0.09*	0.19 ± 0.05	0.41 ± 0.37	0.36 ± 0.06
Lignoceric acid	0.54 ± 0.09	0.28 ± 0.04	0.62 ± 0.11	0.45 ± 0.09	0.47 ± 0.13	0.30 ± 0.11	0.45 ± 0.34	0.40 ± 0.07
Hyenic acid	0.11 ± 0.04	0.09 ± 0.00	nd	0.13 ± 0.01	0.11 ± 0.00	0.08 ± 0.01	0.00	0.12 ± 0.01
Ceric acid	1.60 ± 0.47	1.50 ± 0.09*	3.40 ± 0.55	5.04 ± 1.30	1.10 ± 0.23	0.99 ± 0.31*	1.88 ± 1.33	3.52 ± 0.68
Carboceric acid	0.07 ± 0.02	0.14 ± 0.07	nd	0.24 ± 0.02*	0.08 ± 0.00	0.12 ± 0.01	nd	0.13 ± 0.01*
Montanic acid	1.40 ± 0.44	1.99 ± 0.23*	14.21 ± 4.04	10.37 ± 2.00*	0.90 ± 0.10*	0.94 ± 0.01*	4.05 ± 0.00	5.03 ± 0.01*
Nonacosanoic acid	0.07 ± 0.02	0.08 ± 0.01	nd	0.12 ± 0.02	0.04 ± 0.07	nd	nd	0.14 ± 0.02
Melissic acid	0.71 ± 0.26	0.60 ± 0.10	1.64 ± 0.53	1.67 ± 0.94	0.54 ± 0.13	0.51 ± 0.26	0.57 ± 0.20	0.92 ± 0.32
Ketones								
2-Nonanone	0.13 ± 0.01*	0.05 ± 0.03	nd	nd	0.01 ± 0.02*	nd	nd	nd
2-Undecanone	0.04 ± 0.01	0.03 ± 0.02	nd	nd	0.04 ± 0.00	nd	nd	nd
2-Tridecanone	nd	nd	0.12 ± 0.11	0.13 ± 0.07	nd	nd	0.04	nd
2-Nonadecanone	0.05 ± 0.01	0.03 ± 0.00	nd	nd	nd	nd	nd	nd
2-Heneicosanone	1.67 ± 0.20	0.92 ± 0.24*	0.97 ± 0.65	0.64 ± 0.05*	0.08 ± 0.01	0.05 ± 0.00*	0.20 ± 0.17	0.04 ± 0.01*
2-Docosanone	nd	nd	nd	nd	nd	nd	nd	nd
Aldehydes								
Octadecanal	nd	nd	0.03 ± 0.03	0.03 ± 0.00	nd	nd	0.01 ± 0.01	0.02 ± 0.02
Tetracosanal	nd	nd	0.05 ± 0.04	0.04 ± 0.01*	nd	nd	0.06 ± 0.03	0.07 ± 0.02*
Pentacosanal	nd	nd	0.07 ± 0.02	0.06 ± 0.02	nd	nd	0.05 ± 0.04	0.08 ± 0.01
Hexacosanal	0.04 ± 0.03	0.02 ± 0.00	1.03 ± 0.07	1.27 ± 0.48*	0.03 ± 0.00	0.03 ± 0.01	1.25 ± 1.13	2.72 ± 0.34*
Heptacosanal	nd	nd	nd	0.16 ± 0.05	nd	0.03 ± 0.00	nd	0.19 ± 0.02
Octacosanal	0.02 ± 0.01	nd	2.11 ± 0.17	3.35 ± 0.72	nd	0.04 ± 0.00	2.15 ± 1.82	4.65 ± 0.45
Triacontanal	nd	nd	0.25 ± 0.24	0.67 ± 0.24	nd	nd	0.28 ± 0.11	0.55 ± 0.15
Primary alcohols								
1-Hexadecanol	0.27 ± 0.09	0.25 ± 0.07	0.07 ± 0.00	nd	0.25 ± 0.22	nd	nd	nd
1-Octadecanol	0.28 ± 0.07*	0.38 ± 0.01*	0.21 ± 0.00	0.29 ± 0.00	0.38 ± 0.02*	0.29 ± 0.03*	nd	nd
2-Octacosen-1-ol	nd	nd	nd	0.27 ± 0.02	nd	nd	nd	0.38 ± 0.06
1-Eicosanol	nd	nd	0.73 ± 0.10	0.23 ± 0.04	nd	nd	0.68 ± 0.06	0.33 ± 0.06
1-Docosanol	0.16 ± 0.16	nd	0.74 ± 0.12	0.24 ± 0.04	nd	nd	0.67 ± 0.04	0.34 ± 0.06
1-Tricosanol	0.15 ± 0.16	nd	nd	nd	nd	nd	nd	nd
1-Tetracosanol	0.17 ± 0.16	nd	0.86 ± 0.20	0.30 ± 0.04	nd	nd	0.73 ± 0.05	0.40 ± 0.06
1-Pentacosanol	0.05 ± 0.09	nd	nd	0.27 ±	nd	nd	nd	0.42 ± 0.07
1-Hexacosanol	0.05 ± 0.09	nd	0.83 ± 0.07	0.44 ± 0.06	nd	nd	0.90 ± 0.09	0.47 ± 0.08
1-Octacosanol	nd	nd	0.29 ± 0.50	nd	nd	nd	0.82 ± 0.06	0.50 ± 0.07
2-Nonacosen-1-ol	nd	nd	nd	0.26 ± 0.02	nd	nd	nd	0.38 ± 0.06
Alkanes								
Tetracosane	nd	0.01 ± 0.02	0.25 ± 0.01	0.20 ± 0.04	nd	0.04 ± 0.00	0.23 ± 0.00	0.24 ± 0.09
Pentacosane	0.13 ± 0.04	0.09 ± 0.01	0.11 ± 0.01	0.04 ± 0.00	0.11 ± 0.00	0.09 ± 0.00	0.10 ± 0.02	0.09 ± 0.06
Hexacosane	0.04 ± 0.01	0.04 ± 0.00	0.51 ± 0.07	0.33 ± 0.24	0.06 ± 0.01	0.04 ± 0.00	0.41 ± 0.09	0.33 ± 0.12
Heptacosane	0.29 ± 0.09	0.18 ± 0.01	nd	0.03 ± 0.02	0.21 ± 0.05	0.16 ± 0.04	nd	0.06 ± 0.06
Octacosane	0.05 ± 0.01	nd	nd	0.11 ± 0.10	nd	nd	nd	0.09 ± 0.16
Nonacosane	0.22 ± 0.03	0.13 ± 0.04	nd	nd	0.18 ± 0.02	0.09 ± 0.00	nd	0.06 ± 0.05
Hentriacontane	0.09 ± 0.02	0.05 ± 0.02	nd	nd	0.08 ± 0.01	0.07 ± 0.02	nd	0.09 ± 0.04

Data is means ± SD of three replicates, except GT S4 stage, where data is mean ± SD of two replicates
*indicates statistically significant differences between means ($p < 0.05$).

the bilberry fruit development. Wax amount per berry increased throughout the fruit development in both WT and GT fruit, indicating constant wax biosynthesis and transportation to berry surface. Wax load per surface area remained somewhat constant during the berry development although there were slight changes that can be attributed to the changes in berry size compared to the wax deposition rate. The decrease in wax load per surface area can be seen at late stage of the ripening (S5) when rapid growth in berry size takes place. In other fruits, variable trends in wax load during the fruit development have been reported (Trivedi et al., 2019b). The increase in wax load throughout the fruit development has been reported in blueberry (Chu, Gao, Chen, Wu, & Fang, 2018), apple (Ju & Bramlage, 2001), pear (Li, Yin, Chen, Bi, & Ge, 2014) and orange fruits (Liu et al., 2012), whereas in grape berry, wax load increased until veraison followed by a decrease in the ripe stage (Pensec et al., 2014).

4.2. Glossy phenotype is attributed to the changes in chemical composition affecting wax morphology

It has been previously reported that Arabidopsis wax mutants showing glossy surface in leaf and stems have reduced density of wax crystals and sometimes also alterations in the crystal shape and size (Jenks et al., 1996). Similar results have been obtained in studies on glossy surfaces of orange fruits (Liu et al., 2012; Liu et al., 2015) and cucumber (Wang et al., 2015). Our study demonstrated a clear decrease in the density of epicuticular wax crystal structures in GT fruit compared to WT fruit. While a dense cover of platelets along with rod-like structures were detected in S3, S4, and S5 stages of WT fruit development, the surface of GT fruit was devoid of rod-like structures and dominated by membranous platelets throughout the fruit development. Our data suggests that the difference in appearance between WT and GT fruit of bilberry is based on the difference in the epicuticular wax morphology,

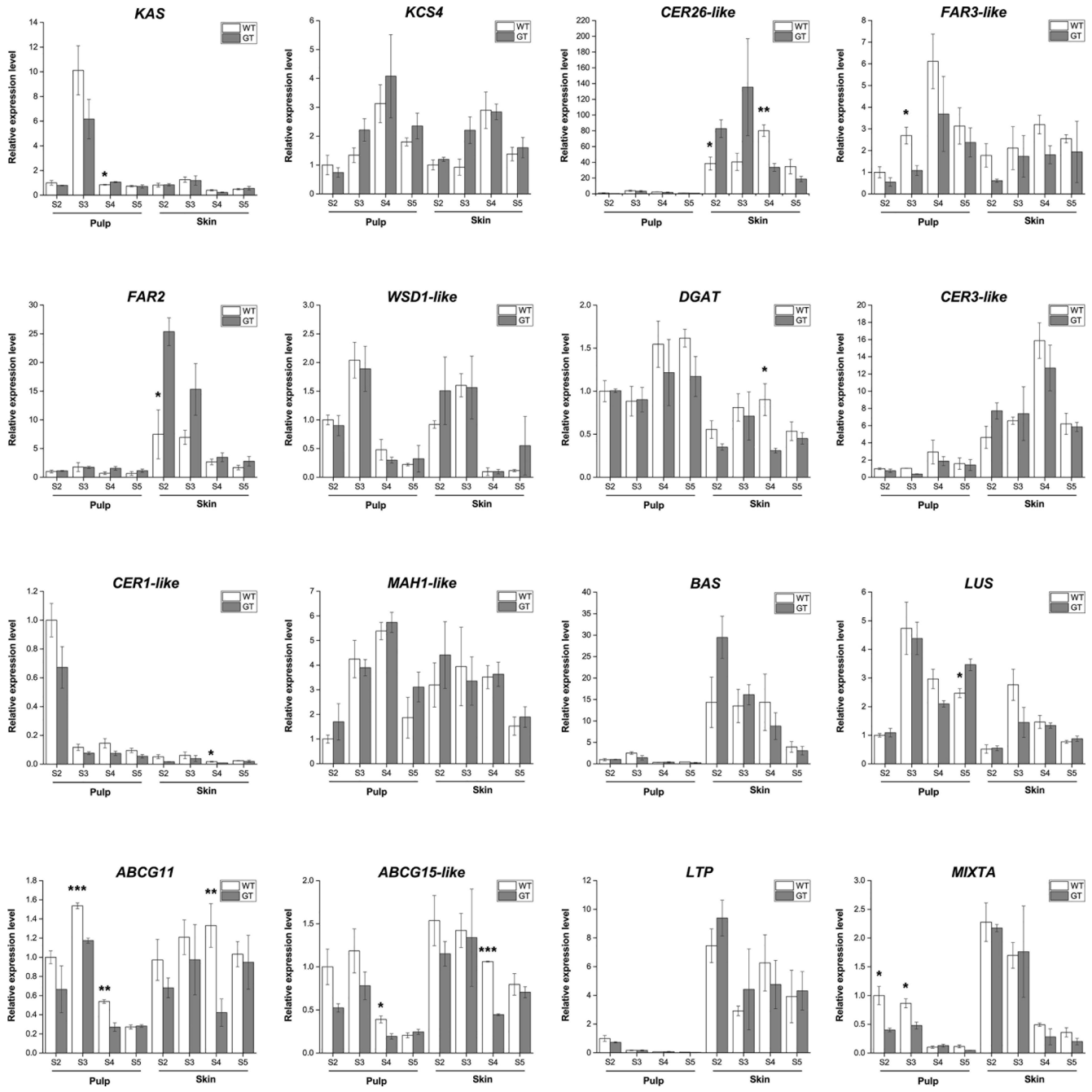

Fig. 4. Expression of wax related genes in wild type bilberry (WT) and glossy type mutant (GT) were studied both in fruit pulp and skin during fruit development. S2, small unripe green fruits; S3, large unripe green fruits; S4, ripening red fruits; S5, fully ripe blue fruits. Error bars represent standard error of three biological replicates. The asterisks denote statistically significant differences between WT and GT (*: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$).

which is due to the differential chemical composition between WT and GT fruit.

Previously, Markstädter, Federle, Jetter, Riederer, and Hölldobler (2000) found a correlation between the glaucous stem phenotype of *Macaranga* species and a high triterpenoid content. In contrast, our study showed a higher proportion of triterpenoids in GT fruits compared to WT fruits. Since triterpenoids generally occur in intracuticular layer of wax (Jetter & Schaffer, 2001), they may not have a significant role in the epicuticular wax crystal formation. Instead, epicuticular wax crystalloids are known to be dominated by aliphatic compounds. Previous studies have also attributed the glaucousness to the presence of β-diketones in wheat flag leaf sheath (Zhang, Wang, & Li, 2013). However, in our study β-diketones were not found. In contrast, among aliphatic compounds we observed consistent differences throughout the fruit development between WT and GT fruits in the proportion of fatty acids and especially ketones. Our result implies that glaucous appearance in WT bilberry fruits could be aided by the presence of ketones and fatty acids. In line with this hypothesis, our previous study showed that glaucous appearing bilberry (rod-like epicuticular morphology) and bog bilberry (coiled rodlet morphology) contain ketones and high amounts of fatty acids while glossy appearing lingonberry and crowberry are devoid of ketones as well as rod-like structures and contain lower amounts of fatty acids (Trivedi et al., 2019a). Especially ketones have earlier been reported to be responsible for the formation of transversely rigid rodlets (Meusel, Neinhuis, Markstädter, & Barthlott, 1999). Also, cuticular waxes containing ketones have been found to form different

types of rodlets in different plant species (Ensikat et al., 2006).

4.3. Chemical composition of cuticular wax changes during bilberry fruit development

The chemical composition of ripe WT bilberry fruit cuticular wax is in accordance with our previous study (Trivedi et al., 2019a). The wax composition showed changes during the course of bilberry fruit development. The proportion of the major compound classes generally varied similarly in WT and GT fruit. The decrease in the proportion of triterpenoids and increase of total aliphatic compounds during the fruit development were detected in bilberry. Increase in the content of terpenoids during fruit development has also been reported in grape (Pensec et al., 2014) and sweet cherry (Peschel, Franke, Schreiber, & Knoche, 2007). However, in blueberry (*V. corymbosum* 'Legacy', *V. ashei* 'Brightwell') fruits, the proportion of triterpenoids increased throughout the development (Chu et al., 2018). A recent study in bilberry also reported the lowest percentage of triterpenoids in the cuticular wax of young fruits with increase during the fruit development (Dashbaldan, Becker, Pączkowski, & Szakiel, 2019). This indicates the differences in wax biosynthesis within the *Vaccinium* species, highlighting the role of genetic and environmental factors in the composition of cuticular wax. Our results indicate that the later stages of berry development (S4 and S5) are the key phases for biosynthesis of aldehydes in bilberry fruit cuticular wax. In the wax biosynthetic pathway (Fig. S1), secondary alcohols are precursors for ketones, however, secondary alcohols were not observed in bilberry cuticular wax. The formation of ketones without the formation of secondary alcohols in bilberry wax remains elusive. This might suggest that secondary alcohols are converted directly to ketones or that ketones are biosynthesized via a different pathway in bilberry compared to Arabidopsis, but this assumption needs further studies.

4.4. Role of wax biosynthetic genes in bilberry fruit cuticular wax formation

The genes proposed to be involved in the wax biosynthesis in bilberry showed differential expression profiles during the fruit development. Few genes showed markedly different expression in skin compared to pulp, indicating their attendance in the cuticular wax biosynthesis. For the fatty acid elongation, several *KCS* genes have been proposed to play a role in determining the specific chain length of VLCFAs in different organs. For example *CER26-like* gene has been characterized for the elongation of specific chain length longer than C_{28} in leaves and stem of Arabidopsis (Pascal et al., 2013). Our result of the skin-specific expression of *CER26-like* gene suggests that it may play an important role in the biosynthesis of VLCFAs and its derivatives in bilberry. In addition, the differential expression of *CER26-like* genes between WT and GT fruit skin suggests that this gene might be responsible for the differential accumulation of very long chain aliphatic compounds.

We observed the skin-specific expression of *FAR2*, a homolog of *AtFAR2,* that produces primary alcohols incorporated into sporopollenin of the pollen exine layer (Chai et al., 2018). Our result suggests a role of *FAR2* gene in alcohol forming pathway in the cuticular wax biosynthesis in bilberry fruit. In bilberry, the accumulation of aldehydes in the cuticular wax corroborated the gene expression of *CER3-like* gene during the berry development. This is in accordance with the expression pattern of *CER3* during the fruit ripening of sweet cherry and orange (Alkio et al., 2012; Wang et al., 2016). Concerning wax transportation, *ABCG11* and *ABCG12* have been identified and characterized for their function in wax deposition in stem of Arabidopsis (McFarlane, Shin, Bird, & Samuels, 2010). In the present study, we found higher expression of *ABCG15-like* gene in fruit skin and a skin-specific expression of *LTP* gene suggesting their role in the transportation of wax compounds into the bilberry cuticle. A regulatory gene of cuticular wax biosynthesis in tomato, *SlMIXTA* has been shown to be down-regulated during the fruit ripening (Lashbrooke et al., 2015). Based on our results, *MIXTA* may play a role in the cuticle formation of bilberry fruit at the early developmental stages.

β-Amyrin synthases (BAS), one of OSCs, has been identified for the formation of β-amyrins in the triterpenoid biosynthetic pathway. Previously, Wang et al. (2011) reported the exclusive localization of two tomato *OSC* genes, *SlTTS1* and *SlTTS2* in the epidermis, demonstrating the production of triterpenoids (β-amyrin, δamyrin and six other triterpenoids) for fruit cuticular wax. We also observed skin-specific expression of *BAS* in bilberry suggesting the production of amyrins for the bilberry fruit cuticular wax. Moreover, the high expression of *BAS* in bilberry at early stage of the fruit development is in line with the high proportion of triterpenoids generally in the early stages of fruit development.

5. Conclusions

Based on our results, bilberry GT fruits have cuticular wax load comparable to WT bilberry fruits. However, the chemical composition and morphology of the cuticular wax along with gene expression of wax biosynthetic genes varied between GT and WT fruit. GT fruits have a higher proportion of triterpenoids accompanied by a lower proportion of fatty acids and ketones compared to WT fruits. Based on our study, the low amount of crystalloids and absence of rod-like structures in the GT fruit cuticular wax is likely due to the reduction in the amount of ketones and fatty acids in bilberry wax. This suggests that the presence of ketones and fatty acids may aid the formation of glaucous phenotype and crystalline structures in bilberry fruit epicuticular wax. The skin-specific expression of *CER26-like*, *FAR2*, *CER3-like*, *LTP*, *MIXTA,* and *BAS* underlines the role of these genes in the wax biosynthesis in bilberry.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was financially supported by I4 future doctoral program, hosted at the University of Oulu: Novel Imaging and Characterization Methods in Bio, Medical, and Environmental Research and Technology Innovations, which is the European Union's Horizon 2020 Research and Innovation Programme under the Marie Sklodowska-Curie action co-funded by international, interdisciplinary and inter-sectoral doctoral programme (grant number 713606 to PT). The research was also funded by InterregNord (Natural Wax of Arctic Berries as Our Treasure – WAX project (number 20201089 to University of Oulu and grant IR16-020 and grant RMF16-026 to Troms Fylkeskommune and NIBIO).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2021.129517.

References

Adamski, N. M., Bush, M. S., Simmonds, J., Turner, A. S., Mugford, S. G., Jones, A., … Uauy, C. (2013). The Inhibitor of wax 1 locus (I w1) prevents formation of β-and OH-β-diketones in wheat cuticular waxes and maps to a sub-c M interval on chromosome arm 2 BS. *The Plant Journal, 74*, 989–1002.

Alkio, M., Jonas, U., Sprink, T., van Nocker, S., & Knoche, M. (2012). Identification of putative candidate genes involved in cuticle formation in *Prunus avium* (sweet cherry) fruit. *Annals of Botany, 110*, 101–112.

Chai, G., Li, C., Xu, F., Li, Y., Shi, X., Wang, Y., & Wang, Z. (2018). Three endoplasmic reticulum-associated fatty acyl-coenzyme a reductases were involved in the production of primary alcohols in hexaploid wheat (*Triticum aestivum* L.). *BMC Plant Biology, 18*, 41.

Chu, W., Gao, H., Chen, H., Wu, W., & Fang, X. (2018). Changes in cuticular wax composition of two blueberry cultivars during fruit ripening and postharvest cold storage. *Journal of Agricultural and Food Chemistry, 66*, 2870–2876.

Colak, N., Primetta, A. K., Riihinen, K. R., Jaakola, L., Grúz, J., Strnad, M., … Ayaz, F. A. (2017). Phenolic compounds and antioxidant capacity in different-colored and nonpigmented berries of bilberry (*Vaccinium myrtillus* L.). *Food Bioscience, 20*, 67–78.

Dashbaldan, S., Becker, R., Pączkowski, C., & Szakiel, A. (2019). Various patterns of composition and accumulation of steroids and triterpenoids in cuticular waxes from screened Ericaceae and Caprifoliaceae berries during fruit development. *Molecules, 24*, 3826.

Dobson, G., Shrestha, M., Hilz, H., Karjalainen, R., McDougall, G., & Stewart, D. (2012). Lipophilic components in black currant seed and pomace extracts. *European Journal of Lipid Science and Technology, 114*, 575–582.

Ensikat, H. J., Boese, M., Mader, W., Barthlott, W., & Koch, K. (2006). Crystallinity of plant epicuticular waxes: Electron and X-ray diffraction studies. *Chemistry and Physics of Lipids, 144*, 45–59.

Hen-Avivi, S., Savin, O., Racovita, R. C., Lee, W.-S., Adamski, N. M., Malitsky, S., … Friedlander, G. (2016). A metabolic gene cluster in the wheat W1 and the barley Cer-cqu loci determines β-diketone biosynthesis and glaucousness. *The Plant Cell, 28*, 1440–1460.

Jaakola, L., Määttä, K., Pirttilä, A. M., Törrönen, R., Kärenlampi, S., & Hohtola, A. (2002). Expression of genes involved in anthocyanin biosynthesis in relation to anthocyanin, proanthocyanidin, and flavonol levels during bilberry fruit development. *Plant Physiology, 130*, 729–739.

Jaakola, L., Pirttilä, A. M., Halonen, M., & Hohtola, A. (2001). Isolation of high quality RNA from bilberry (*Vaccinium myrtillus* L.) fruit. *Molecular biotechnology, 19*, 201–204.

Jenks, M. A., Rashotte, A. M., Tuttle, H. A., & Feldmann, K. A. (1996). Mutants in Arabidopsis thaliana altered in epicuticular wax and leaf morphology. *Plant Physiology, 110*, 377–385.

Jetter, R., & Schaffer, S. (2001). Chemical composition of the Prunus laurocerasus leaf surface. Dynamic changes of the epicuticular wax film during leaf development. *Plant Physiology, 126*, 1725–1737.

Ju, Z., & Bramlage, W. J. (2001). Developmental changes of cuticular constituents and their association with ethylene during fruit ripening in 'delicious' apples. *Postharvest Biology and Technology, 21*, 257–263.

Kovats, V. E. (1958). Gas-chromatographische charakterisierung organischer verbindungen. Teil 1: Retentions indices aliphatischer halogenide, alkohole, aldehyde und ketone. *Helvetica Chimica Acta, 41*, 1915–1932.

Kunst, L., & Samuels, L. (2009). Plant cuticles shine: Advances in wax biosynthesis and export. *Current Opinion in Plant Biology, 12*, 721–727.

Lashbrooke, J., Adato, A., Lotan, O., Alkan, N., Tsimbalist, T., Rechav, K., … Granell, A. (2015). The tomato MIXTA-like transcription factor coordinates fruit epidermis conical cell development and cuticular lipid biosynthesis and assembly. *Plant Physiology, 169*, 2553–2571.

Li, Y., Yin, Y., Chen, S., Bi, Y., & Ge, Y. (2014). Chemical composition of cuticular waxes during fruit development of Pingguoli pear and their potential role on early events of *Alternaria alternata* infection. *Functional Plant Biology, 41*, 313–320.

Liu, D., Yang, L.i., Zheng, Q., Wang, Y., Wang, M., Zhuang, X., … Liu, Y. (2015). Analysis of cuticular wax constituents and genes that contribute to the formation of 'glossy Newhall', a spontaneous bud mutant from the wild-type 'Newhall'navel orange. *Plant Molecular Biology, 88*, 573–590.

Liu, D. C., Zeng, Q., Ji, Q. X., Liu, C. F., Liu, S. B., & Liu, Y. (2012). A comparison of the ultrastructure and composition of fruits' cuticular wax from the wild-type "Newhall"navel orange (*Citrus sinensis* [L.] Osbeck cv. Newhall) and its glossy mutant. *Plant Cell Reports, 31*, 2239–2246.

Markstädter, C., Federle, W., Jetter, R., Riederer, M., & Hölldobler, B. (2000). Chemical composition of the slippery epicuticular wax blooms on Macaranga (Euphorbiaceae) ant-plants. *Chemoecology, 10*, 33–40.

McFarlane, H. E., Shin, J. J. H., Bird, D. A., & Samuels, A. L. (2010). Arabidopsis ABCG transporters, which are required for export of diverse cuticular lipids, dimerize in different combinations. *The Plant Cell, 22*, 3066–3075.

Meusel, I., Neinhuis, C., Markstädter, C., & Barthlott, W. (1999). Ultrastucture, chemical composition, and recrystallization of epicuticular waxes: Transversely ridged rodlets. *Canadian Journal of Botany, 77*, 706–720.

Nguyen, N., Suokas, M., Karppinen, K., Vuosku, J., Jaakola, L., & Häggman, H. (2018). Recognition of candidate transcription factors related to bilberry fruit ripening by de novo transcriptome and qRT-PCR analyses. *Scientific Reports, 8*, 9943.

Pascal, S., Bernard, A., Sorel, M., Pervent, M., Vile, D., Haslam, R. P., … Joubès, J. (2013). The Arabidopsis cer26 mutant, like the cer2 mutant, is specifically affected in the very long chain fatty acid elongation process. *The Plant Journal, 73*, 733–746.

Pensec, F., Pączkowski, C., Grabarczyk, M., Woźniak, A., Bénard-Gellon, M., Bertsch, C., … Szakiel, A. (2014). Changes in the triterpenoid content of cuticular waxes during fruit ripening of eight grape (*Vitis vinifera*) cultivars grown in the Upper Rhine Valley. *Journal of Agricultural and Food Chemistry, 62*, 7998–8007.

Peschel, S., Franke, R., Schreiber, L., & Knoche, M. (2007). Composition of the cuticle of developing sweet cherry fruit. *Phytochemistry, 68*, 1017–1025.

R Core Team. (2019). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. https://www.R-project.org/.

Szakiel, A., Pączkowski, C., Pensec, F., & Bertsch, C. (2012). Fruit cuticular waxes as a source of biologically active triterpenoids. *Phytochemistry Reviews, 11*, 263–284.

Trivedi, P., Karppinen, K., Klavins, L., Kviesis, J., Sundqvist, P., Nguyen, N., … Häggman, H (2019a). Compositional and morphological analyses of wax in northern wild berry species. Food chemistry, 295, 441-448.

Trivedi, P., Nguyen, N., Hykkerud, A. L., Häggman, H., Martinussen, I., Jaakola, L., & Karppinen, K. (2019b). Developmental and environmental regulation of cuticular wax biosynthesis in fleshy fruits. Frontiers in Plant Science, 10, 431.

van Maarseveen, C., Han, H., & Jetter, R. (2009). Development of the cuticular wax during growth of Kalanchoe daigremontiana (Hamet et Perr. de la Bathie) leaves. *Plant, Cell & Environment, 32*, 73–81.

Vogg, G., Fischer, S., Leide, J., Emmanuel, E., Jetter, R., Levy, A. A., & Riederer, M. (2004). Tomato fruit cuticular waxes and their effects on transpiration barrier properties: Functional characterization of a mutant deficient in a very-long-chain fatty acid β-ketoacyl-CoA synthase. *Journal of Experimental Botany, 55*, 1401–1410.

Wang, J., Sun, L., Xie, L., He, Y., Luo, T., Sheng, L., … Cheng, Y. (2016). Regulation of cuticle formation during fruit development and ripening in "Newhall"navel orange (*Citrus sinensis* Osbeck) revealed by transcriptomic and metabolomic profiling. *Plant Science, 243*, 131–144.

Wang, Z., Guhling, O., Yao, R., Li, F., Yeats, T. H., Rose, J. K. C., & Jetter, R. (2011). Two oxidosqualene cyclases responsible for biosynthesis of tomato fruit cuticular triterpenoids. *Plant Physiology, 155*, 540–552.

Wang, W., Zhang, Y., Xu, C., Ren, J., Liu, X., Black, K., … Ren, H. (2015). Cucumber ECERIFERUM1 (CsCER1), which influences the cuticle properties and drought tolerance of cucumber, plays a key role in VLC alkanes biosynthesis. *Plant Molecular Biology, 87*, 219–233.

Yang, Y., Zhou, B., Zhang, J., Wang, C., Liu, C., Liu, Y., … Ren, X. (2017). Relationships between cuticular waxes and skin greasiness of apples during storage. *Postharvest Biology and Technology, 131*, 55–67.

Zhang, Z., Wang, W., & Li, W. (2013). Genetic interactions underlying the biosynthesis and inhibition of β-Diketones in wheat and their impact on glaucousness and cuticle permeability. *PLoS One, 8*, e54129.