Identification of putative candidate genes involved in cuticle formation in *Prunus avium* (sweet cherry) fruit

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INTRODUCTION

The cuticle, or the cuticular membrane (CM; Kerstiens, 1996a), is a non-cellular, non-living, lipoidal membrane covering aerial parts of the primary plant body (Bukovac et al., 1981). It forms the interface between the plant and its environment and serves as a protective barrier against uncontrolled water loss or uptake, leaching of nutrients, invasion by pathogens and mechanical damage (Kolattukudy, 1980; Kerstiens, 1996b; Schreiber, 2010). Maintaining these functions requires an intact CM.

The CM is a heterogeneous composite comprising cutin, cutan and waxes as major constituents (Jeffree, 1996). Cutin is a polyester of cross-linked hydroxy and epoxy fatty acids that renders the cuticle chemically and mechanically stable (Kolattukudy, 1980; Schreiber, 2010). Cutan is also a polymer, but one that is more resistant to alkaline hydrolysis and more hydrophobic than cutin (Jeffree, 1996). Waxes are a mixture of various very long chain fatty acid (VLCFA) derivatives, terpenes and phenolic lipids, which makes the cuticle waterproof (Kerstiens, 1996b; Schreiber, 2010).

Recent studies established that the CM of fleshy fruit is often under considerable stress. Examples include *Prunus* species (e.g. *P. avium*, sweet cherry; *P. domestica*, European plum), *Ribes* berries (*R. uva-crispa*, gooseberry; *R. nigrum*, blackcurrant; *R. nigrum x uva-crispa*, jostaberry) and *Vitis vinifera* (grape berry; Knoche et al., 2004; Knoche and Peschel, 2007a; Khanal et al., 2011; Becker and Knoche, 2012). The resulting strain of the cuticle represents the driving force in formation of microscopic cracks (Peschel and Knoche, 2005). These ‘microcracks’ are limited to the cuticle and do not traverse epidermal or hypodermal cell layers (Glenn and Poovaiah, 1989; Peschel and Knoche, 2005). The consequences of microcracks in the CM are three-fold. First, microcracks impair the barrier function in pathogen defence, increasing the incidence of fruit rots (Borve et al., 2000). Second, microcracks allow for rapid and uncontrolled water movement into and out of the fruit that bypasses the cuticle as the penetration barrier (Glenn and Poovaiah, 1989; Knoche and Peschel, 2006). Third, macroscopic cracks were reported to develop along microcracks and progress along their axis (Glenn and Poovaiah, 1989). Thus,
Microcracks essentially predispose fruit to subsequent rain-induced cracking.

Circumstantial evidence suggests that strain of the cuticle results from an increase in fruit surface area in the absence of CM deposition.

First, the onset of strain coincides with the cessation of CM deposition, which in turn marks the beginning of the decrease in CM thickness in developing sweet cherry fruit (Knoche et al., 2004). Second, sweet cherry development follows a double sigmoidal growth pattern with time characterized by two maxima of similar magnitude in relative growth rate. The first maximum, which occurs when CM deposition is high, does not cause stress and strain of the CM. In contrast, the second maximum that occurs after CM deposition has ceased causes significant stress and strain of the CM. This would be expected if strain resulted from a mismatch of fruit surface expansion and CM deposition (Knoche et al., 2004). Third, there is little strain in the CMs of fruit of Solanum lycopersicum (tomato; 10%, M. Knoche, unpubl. res.) or Malus × domestica (apple; 6% strain, Knoche et al., 2011), species that continuously deposit CM during fruit development until maturity (Knoche and Peschel, 2007b; Dominguez et al., 2008; Knoche et al., 2011). In contrast, in sweet cherry fruit that develop considerable strain (up to 108%; Knoche and Peschel, 2006), CM mass per unit area and hence CM thickness decreases during development, indicating that CM deposition does not keep pace with surface expansion (Knoche et al., 2004). Interestingly, the CM of leaves of sweet cherry maintains a constant thickness throughout most of its development and strain is practically absent (M. Knoche, unpubl. res.). Similarly, developing leaves of Clivia miniata (Riederer and Schönherr, 1988), Hedera helix (Viougeas et al., 1995) and Arabidopsis thaliana (Franke et al., 2005) maintain a constant CM thickness or the CM even increases in thickness. Thus, there is no indication of stretching in the CM of leaves (Jeffree, 1996).

To demonstrate causal relationships, CM deposition must be varied and the resulting CM stress, strain and frequency of microcracks quantified. However, manipulating CM deposition requires a better understanding of the molecular biology of CM formation in sweet cherry fruit. In Arabidopsis, a number of genes involved in the biosynthesis of cutin and wax in vegetative tissues and petals have been characterized over the past decade (for reviews see Pollard et al., 2008; Samuels et al., 2008; Li-Beisson et al., 2010; Javelle et al., 2011). Recently, genes involved in CM deposition were characterized in tomato fruit (Leide et al., 2007; Isaacson et al., 2009; Wang et al., 2011), but no information is available for sweet cherry or other tree fruits.

The purpose of our study was to (1) identify sweet cherry genes potentially relevant for CM formation based on sequence similarity with Arabidopsis, (2) quantify expression levels of selected genes in the exocarp (fruit skin) and the mesocarp (fruit flesh) and (3) relate their expression level to the rate of cuticle deposition during development. Criteria for the selection of CM target genes were that the functions of respective genes in Arabidopsis should (1) collectively cover different sub-steps in CM formation and (2) be supported by experimental evidence. Functions of the selected target genes in Arabidopsis include delivery of activated fatty acids for cutin and wax biosynthesis ( FATB and LACS9; references are given in Table 1; for review, see Li-Beisson et al., 2010), biosynthesis of cutin monomers (LACS2, LACS1, AT1, LCR, GPAT4 and GPAT8) and VLCFA waxes (LACS1, KC56, KCS1, KCR1, CER3 and CER1), export of cuticular lipids (CER5, WBC11 and LTPG1) and transcriptional regulation (WIN1). Except for LACS9 and WIN1, all Arabidopsis homologues of the putative CM candidate genes analysed here were upregulated in the Arabidopsis stem epidermis during the stage of most active cuticle deposition (Suh et al., 2005).

MATERIALS AND METHODS

Plant material and determination of fruit fresh mass and surface area

Sweet cherry (Prunus avium ‘Regina’ grafted on Gisela 5 rootstock) fruit were collected weekly from anthesis to maturity from trees grown under rain cover at the Obstbauversuchsanstalt Jork in Jork (59°37′E, 53°50′N) and a commercial orchard in Gledingen (59°84′E, 52°27′N), Germany. On each sampling date, fresh mass and diameter of 30 representative ovaries or fruits were determined. If present, the pedicel, receptacle, petals, stamens and style were removed prior to weighing. Mean fruit diameter was calculated from the polar and two perpendicular, equatorial diameters and used to calculate fruit surface area assuming a spherical shape of the fruit as a first approximation.

Cuticle isolation

Five replicate samples were collected, each consisting of 20 fruits or exocarp discs excised from the cheek region using a cork borer. The CMs were isolated enzymatically as described by Peschel et al. (2007) except that after tissue removal CMs were rinsed ten times in deionized water in the absence of borax buffer. Isolated CMs were dried at 40°C for at least 24 h and weighed. Mass of the CMs per unit surface area was calculated by dividing CM mass by the cross-sectional area of the cork borer. An average ‘gravimetric’ CM thickness was estimated by dividing CM mass per unit surface area by CM density (1200 kg m⁻³ for tomato fruit CM; Petracek and Bukovac, 1995). The CMs of fruit younger than 14 d after full bloom (DAFB) were too fragile to be isolated.

Total RNA extraction

In sweet cherry fruit, exocarp and mesocarp cannot be physically separated. Therefore, at full bloom only one type of tissue, i.e. the entire ovary, was sampled. At all other sampling dates two tissue fractions were collected. From 5 to 96 DAFB ‘exocarp’ samples containing exocarp and some adhering mesocarp tissue were cut tangentially as slices (<2 mm in thickness) using a sterile scalpel. From 19 to 96 DAFB ‘mesocarp’ was sampled analogously after removal of the exocarp. The exocarp sample thus represents an enriched exocarp fraction that contains some mesocarp, but the mesocarp fraction contains mesocarp tissue only. From very small fruitlets the whole ovary with (5 DAFB) or without ovule (12 DAFB)
### Table 1. Characterization of the cDNA sequences analysed in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Ref.</th>
<th>Contig length (bp)</th>
<th>CDS length (bp)</th>
<th>Best hit ACC</th>
<th>Similarity (%)</th>
<th>Best hit ID</th>
<th>Alignment length</th>
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<td>PaWINA</td>
<td>TF</td>
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Sweet cherry genes investigated in this work were named after the most similar Arabidopsis gene and a prefix Pa for P. avium. Contig sequences were deposited at the NCBI Transcriptome Shotgun Assembly Sequence Database under accession numbers JU090712 to JU090733. The functions of the corresponding Arabidopsis genes (TF, transcription factor; Unkn., unknown function; Precurs., delivery of precursors for cuticular lipids; W, wax deposition; C, cutin deposition; Anthoc., anthocyanin synthesis; Ref., reference genes for RT-qPCR) are indicated together with references for the functions (1, Broun et al., 2004; 2, Kannangara et al., 2007; 3, Bonaventure et al., 2003; 4, Bonaventure et al., 2004; 5, Schnurr et al., 2002; 6, Pighin et al., 2004; 7, Bird et al., 2007; 8, SB Lee et al., 2009; DeBono et al., 2009; 9, Xiao et al., 2004; 10, Wellesen et al., 2001; 11, Li et al., 2007; 12, Schnurr et al., 2004; 13, Weng et al., 2010; 14, Millar et al., 1999; 15, Todd et al., 1999; 16, Beaudoin et al., 1999; 17, Aarts et al., 1995; 18, Hannoufa et al., 1993; 19, Rowland et al., 2007; 20, Y Lee et al., 2005). Accession numbers for best BLASTX hits for the cherry sequences in peach predicted peptides database (e-values 0.0–1.2E-73) and in Arabidopsis TAIR9 predicted peptides database (e-values 0.0–1.2E-41) are given together with the percentage of similar amino acids between the predicted protein sequences, and the length of the alignment between predicted peptides in amino acids. CDS, coding sequence.
was taken as the mesocarp fraction. On each sampling date at least four biological replicates from a minimum of ten fruits each were flash frozen in liquid nitrogen within 10 min of removal from the tree and stored at −80 °C until RNA extraction. For high-throughput sequencing of cDNA (RNA-Seq), total RNA was extracted from exocarp collected 14 and 21 DAFB using the Concert Plant RNA Extraction Reagent (Invitrogen GmbH, Darmstadt, Germany; this and other reagents and kits were used according to the manufacturer’s instructions). The method did not yield RNA in high quality from sweet cherries sampled at 56 and 77 DAFB. Therefore, total RNA was extracted from the latter samples following the procedure of Hunter and Reid (2001). For reverse transcriptase–quantitative PCR (RT-qPCR), total RNA was extracted using an InviTrap Spin Plant RNA Mini Kit (Invitrogen GmbH, Berlin, Germany) with lysis solution RP. All RNA samples were treated with DNase I (Fermentas, St. Leon-Rot, Germany), purified using the RNasy MiniElute Cleanup Kit (QIAGEN GmbH, Hilden, Germany) and eluted in RNase-free water. The quality of all RNA samples was assessed using agarose gel electrophoresis and by measuring absorbance at 230, 260 and 280 nm (BioPhotometer plus spectrophotometer; Eppendorf, Hamburg, Germany; microlitre tray cell; Hellma Analytics, Müllheim, Germany). Samples used for RNA-Seq were also analysed using a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). RNA concentration was estimated from the absorbance at 260 nm. None of the samples contained genomic DNA as verified by PCR using non-reverse transcribed RNA as template and primers for PaC (primer sequences given in Supplementary Data Table S1).

RNA-Seq sample preparation and sequencing

RNA samples from exocarp at 14, 21 and 56 DAFB (two biological replicates each), and at 77 DAFB (one sample), were processed for sequencing in a Genome Analyzer II (Illumina Inc., San Diego, CA, USA) as described by Marioni et al. (2008) with minor modifications. Briefly, mRNA was purified from 10 μg of total RNA per sample using the MicroPoly(A)Purist Kit (Ambion, Austin, TX, USA). The concentration of mRNA was determined using a spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Approximately 300 ng of mRNA was fragmented by incubating the sample in the presence of Zn²⁺ (RNA Fragmentation Buffer; Ambion) at 70 °C for 30 s. First-strand cDNA was synthesized using reverse transcriptase and random hexamer primers (Superscript III first-strand cDNA synthesis kit; Invitrogen), and second-strand cDNA using DNA polymerase I and RNase H (both Invitrogen). Further sample preparation was carried out using the Genomic DNA Sample Prep Kit (Illumina). cDNA fragments were sequenced in an Illumina Genome Analyzer II, yielding single reads of 36 bp per fragment. After removing poor quality reads, homopolymer sequences and adapter sequences, filtered Illumina reads were used for assembly and mapping. The filtered reads were deposited in the NCBI Sequence Read Archive, accession SRP011083.

Assembly of contiguous sequences and sequence annotation

Illumina reads were assembled to contiguous sequences (contigs) using Velvet 1.0.16 (Zerbino and Birney, 2008; Martin et al., 2010), TGICL 2.0 (Pertea et al., 2003) and CAP3 (Huang and Madan, 1999). Assembly parameters are given in Supplementary Data Methods 1a. Contigs shorter than 100 bp were removed. For contig annotation, homology analysis was performed using the BLAST program (Altschul et al., 1997). BLASTX analysis was conducted with the sweet cherry contigs as query against predicted peptides of P. persica (peach v1.0; International Peach Genome Initiative) and A. thaliana (TAIR 9.0; The Arabidopsis Information Resource). Contigs without any hits of e-value < 10⁻⁶ in P. persica or Arabidopsis predicted peptides were subjected to BLASTX searches against the non-redundant protein database at NCBI. Contigs with best hits against viral or bacterial sequences were removed from further analyses. To identify sweet cherry orthologues of selected Arabidopsis genes, TBLASTN searches were conducted in the sweet cherry contig database with the Arabidopsis peptide sequences as probes. Sequence similarity was verified by BLASTX searches with the best sweet cherry hit back to the Arabidopsis- and to peach-predicted protein databases. For detailed analyses, 18 sweet cherry contigs were selected as CM target genes based on their similarity to Arabidopsis genes involved in cuticle formation. Additional sequences were retrieved as a “negative control” gene, whose expression was expected to be upregulated with progressing maturity (anthocyanidin glucosyltransferase, PaUGT78D2) and three potential reference genes, whose expression levels were expected to be constant over development (PaSRP19, PaPP2A-2 and PaC). The Illumina reads were mapped to the sweet cherry contigs using the short read aligner tool Bowtie (Langmead et al., 2009) allowing for two mismatches per read (Supplementary Data Methods 1a).

For all 22 contigs analysed, alignments of the mapped reads to each contig were visually inspected using Tablet (Milne et al., 2010). If a base in the contig was different from the base in the majority of the reads that mapped to that position, that base in the contig was manually corrected to the one present in the mapped reads. Eleven bases in six contigs were subjected to such correction (Supplementary Data Methods and Fig. S1A). Lengths of open reading frames in the sweet cherry contigs were determined using the CLC Sequence Viewer (CLC bio, Aarhus, Denmark) and by comparing the sequences with the predicted peptide sequences in the peach v1.0 database. Protein signatures in the target contigs were analysed using the InterProScan tool (Zdobnov and Apweiler, 2001).

Estimating transcript levels using RNA-Seq data

Gene expression levels were quantified for each contig using the mapped Illumina reads by calculating the number of expected fragments (reads) per kilobase of transcript per million fragments mapped (FPKM; Trapnell et al., 2010). Reproducibility between two biological replicate samples collected at 14, 21 and 56 DAFB was assessed by comparing the FPKM values for each contig. For correlations between the
FPKM values of all contigs in each of the two replicates $r > 0.977$ ($n = 54,883, P < 0.0001$) and of the 22 contigs analysed in this paper $r > 0.994$ ($n = 22, P < 0.0001$; Supplementary Data Methods 1b).

Estimating transcript levels using RT-qPCR

To determine target gene expression in developing fruit via RT-qPCR, 1.5 µg of total RNA per sample was reverse transcribed using the Transcripter High Fidelity cDNA Synthesis Kit (Roche Diagnostics Deutschland GmbH, Mannheim, Germany) with anchored oligo(dT) primers. Gene-specific primers were designed to amplify 100–200-bp fragments from the cDNA (software Beacon Designer; Premier Biosoft International, Palo Alto, CA, USA) and synthesized by Eurofins MWG Operon (Ebersberg, Germany). PCR amplification efficiencies were determined from ten-fold serial dilution series with a minimum of four data points per standard curve. Primer sequences, annealing temperatures ($T_a$) and efficiencies are given in Supplementary Data Table S1. Quantitative real-time PCR was performed using the CFX96 Real-Time PCR Detection System (Bio-Rad). Each 15-µL reaction contained 1 µL of the cDNA sample (corresponding to 20 ng of total RNA) as template, gene-specific primers at a final concentration of 0.2 mm each and SYBR Green Supermix (Bio-Rad Laboratories GmbH, Munich, Germany); reagents and final concentrations in the mix were 10 mm KCl, 0.4 mm Tris-HCl, 40 nm of each dNTP, 5 U µL$^{-1}$ iTaq DNA polymerase, 0.6 mm MgCl$_2$, SYBR Green I, 2 nm fluorescein and stabilizers; pH 8.4). Two biological replicates were analysed with at least two technical replicates each. An inter-run calibrator consisting of pooled aliquots of all cDNA samples was included in each 96-well plate to enable calculation of relative transcript levels in separate runs. The PCR cycle conditions were: polymerase activation at 95 °C for 3 min, followed by 41 cycles of 95 °C for 10 s, at $T_a$ for 20 s and at 72 °C for 30 s. Subsequently, melting curves were recorded to detect non-specific amplification by increasing the temperature from 65 to 95 °C in 0.5 °C increments. Primer specificity was further verified by sequencing the amplicons (SeqLab Sequence Laboratories Göttingen GmbH, Göttingen, Germany; Supplementary Data Fig. S1). For determination of quantification cycle, threshold fluorescence was manually set to 80 relative fluorescence units. Abundance of each transcript at different stages of fruit development was measured relative to the abundance in the ovary at full bloom and normalized to 80 relative fluorescence units. Abundance of each transcript was determined from ten-fold serial dilution series with a minimum of four data points per standard curve. The calculated rate of CM deposition reached a maximum ($\pm$ s.d.) of 92.5 ± 5.1 µg per fruit d$^{-1}$ at 18.5 ± 1.3 DAFB (Fig. 1C, dashed line). The phase of high rates of CM deposition thus corresponded to stage I followed by a rapid decline in stages II and III. At the onset of stage III (approx. 40 DAFB) the rate of CM deposition amounted to only one-third of the maximum rate, 34.6 ± 1.4 µg per fruit d$^{-1}$, and further declined to 2.3 ± 0.6 µg per fruit d$^{-1}$ at maturity (96 DAFB). Qualitatively and quantitatively similar data were obtained in two subsequent years.

Identification of sweet cherry orthologues of Arabidopsis genes involved in cuticle formation

Contigs representing cDNA sequences of genes expressed in sweet cherry fruits were assembled from RNA-Seq data. In the following we also use the term ‘cDNA’ for ‘contig’. The assembled contigs are not identical to full-length cDNAs, because they lack bases in the 3’ and 5’ regions and may contain combinations of single nucleotide polymorphisms which do not occur naturally. Filtered 36-bp reads (30-6 × 10$^6$) were assembled de novo to 54,966 contigs of 100–5849 bp each. Eighteen putative sweet cherry orthologues of Arabidopsis genes with documented functions in cuticular lipid synthesis and four control genes were selected for detailed analyses (Table 1). Contigs of all 22 genes contain the complete coding sequence and a number of bases from the 5’ and 3’ untranslated regions (Table 1; NCBI Transcriptome Shotgun Assembly Sequence Database accessions JU090712–JU090733). Sequence similarity between transcripts from $P$. avium and $P$. persica was generally 95–100 % at the predicted amino acid level (Table 1). Only
the putative *P. avium* β-ketoacyl-CoA synthase (KCS) 6 and its best *P. persica* hit, ppa006758, were less similar (82% similarity). Here, sequence alignment indicated a gap of 53 amino acids in the ppa006758 sequence as compared with the translated *PaKCS6*. Sequence similarity outside of this gap was 99%. In the *Arabidopsis* KCS6 sequence, which is 94% similar, the corresponding gap is absent. Overall, similarity between sweet cherry and *Arabidopsis* sequences was 67–98% (Table 1). The translated amino acid sequence of the sweet cherry cDNA was always most similar to the respective *Arabidopsis* sequence used as a query in the BLAST search except for the transcription factor *WIN1*, the cytochrome p450 genes *ATT1* (CYP86A2) and *LCR* (CYP86A8), and the glycerol-3-phosphate acyltransferases (GPAT) 4 and 8. In the case of *WIN1*, two sweet cherry contigs were equally similar to the *Arabidopsis* AP2/EREBP transcription factors *WIN1/SHN1, SHN2* and *SHN3*. This was denoted by naming the corresponding contigs *PaWINA* and *PaWINB*. For *ATT1* and *LCR*, two cherry contigs were identified with *LCR* as the best *Arabidopsis* hit at the predicted amino acid level. However, at the nucleotide level each transcript was more similar to either *ATT1* or *LCR*, and the transcripts were named accordingly. In the case of *GPAT4* and *GPAT8*, one cherry contig was equally similar to both *Arabidopsis* sequences, but clearly different from other putative acyltransferases. This contig was named *PaGPAT4/8*.

InterProScan analysis detected signatures indicating functions and cellular localization of the predicted gene products in all analysed contigs (Supplementary Data Fig. S2). For instance, motifs detected in *PaATT1* and *PaLCR* (Fig. S2I) indicate membership in the cytochrome P450 subfamily 86A. In *PaCER5* (Fig. S2F) and *PaWBC11* (Fig. S2G), motifs characteristic for the ABC transporter gene family were found. In *PaWINA* and *PaWINB*, DNA binding domains of the AP2/ERF element type were detected, suggesting roles in transcriptional regulation. Transmembrane regions were detected in several contigs (*PaCER5, PaWBC11, PaATT1, PaLCR, PaGPAT4/8, PaKCS6, PaKCS1, PaKCR1, PaCER1 and PaCER3*; Figs S2F–J, M–R) suggesting localization in a membrane. In all instances the detected features were consistent with the functions of the respective *Arabidopsis* gene used to identify the sweet cherry cDNA.

Expression of genes putatively involved in cuticle formation in developing sweet cherry fruit

The genes involved in CM deposition are likely to exhibit higher expression levels when the rate of CM deposition is high. Therefore, we compared mRNA levels of the selected sweet cherry genes with the rate of CM deposition in developing fruit.

First, RNA-Seq data were used to determine gene expression levels in the exocarp at two developmental stages representing high and low CM deposition rates, i.e. at 21 DAFB (stage I) with CM deposition rate (± s.d.) of 101.8 ± 5.3 µg per fruit d−1 and at 56 DAFB (stage III) with 12.7 ± 1.6 µg per fruit d−1, respectively (Table 2). When rates of CM deposition were high, the most abundant of the 18 CM target transcripts were *PaLipase, PaATT1, PaCER1, PaGPAT4/8* (for all, FPKM > 300), *PaLTPG1* (FPKM = 180) and *PaWINA and PaWINB* (for both, FPKM ~ 100). However, when the CM deposition rate was low, expression levels of *PaLipase, PaLTPG1, PaATT1, PaLCR, PaGPAT4/8, PaLACS2, PaLACS1, PaWINA and PaWINB* were considerably lower. Transcript levels of *PaUGT78D2* were low during high CM deposition and vice versa. There was no change in expression between the two stages for *PaCAC, PaSRP19* or *PaPP2A-2*.

Second, we analysed gene expression levels at weekly intervals in two types of fruit tissue, i.e. in the exocarp and mesocarp, using RT-qPCR (Figs 2 and 3). *PaCAC, PaSRP19* and *PaPP2A-2* proved suitable for normalization of gene expression levels because (1) their transcript levels were constant in exocarp and mesocarp from anthesis to maturity (Fig. 2I for *PaSRP19*; data for *PaCAC* and *PaPP2A-2* not shown),
Table 2. Expression levels of selected sweet cherry genes in the exocarp during high (21 DAFB) and low (56 DAFB) cuticle deposition rate.

<table>
<thead>
<tr>
<th>Gene</th>
<th>21 DAFB (FPKM; mean ± s.e.)</th>
<th>56 DAFB (FPKM)</th>
<th>Ratio 21/56 DAFB</th>
</tr>
</thead>
<tbody>
<tr>
<td>PaWINA</td>
<td>87 ± 1</td>
<td>0 ± 0</td>
<td>--</td>
</tr>
<tr>
<td>PaWINB</td>
<td>103 ± 4</td>
<td>2 ± 0</td>
<td>70.3</td>
</tr>
<tr>
<td>PaLipase</td>
<td>546 ± 35</td>
<td>2 ± 0</td>
<td>258.1</td>
</tr>
<tr>
<td>PaFATB</td>
<td>66 ± 7</td>
<td>57 ± 1</td>
<td>1.2</td>
</tr>
<tr>
<td>PaLACS9</td>
<td>16 ± 0</td>
<td>16 ± 1</td>
<td>1.0</td>
</tr>
<tr>
<td>PaCER5</td>
<td>12 ± 1</td>
<td>25 ± 1</td>
<td>0.5</td>
</tr>
<tr>
<td>PaWBC11</td>
<td>25 ± 1</td>
<td>22 ± 0</td>
<td>1.2</td>
</tr>
<tr>
<td>PaLTPG1</td>
<td>182 ± 8</td>
<td>68 ± 1</td>
<td>2.7</td>
</tr>
<tr>
<td>PaATT1</td>
<td>409 ± 2</td>
<td>18 ± 6</td>
<td>23.0</td>
</tr>
<tr>
<td>PaLCR</td>
<td>11 ± 1</td>
<td>0 ± 0</td>
<td>--</td>
</tr>
<tr>
<td>PaGPAT4/8</td>
<td>364 ± 2</td>
<td>71 ± 7</td>
<td>5.2</td>
</tr>
<tr>
<td>PaLAC2S</td>
<td>57 ± 3</td>
<td>5 ± 1</td>
<td>11.3</td>
</tr>
<tr>
<td>PaLACSI</td>
<td>19 ± 1</td>
<td>2 ± 1</td>
<td>7.8</td>
</tr>
<tr>
<td>PaKCS6</td>
<td>105 ± 1</td>
<td>80 ± 1</td>
<td>1.3</td>
</tr>
<tr>
<td>PaKCS1</td>
<td>99 ± 13</td>
<td>80 ± 13</td>
<td>1.2</td>
</tr>
<tr>
<td>PaKCR1</td>
<td>40 ± 5</td>
<td>70 ± 5</td>
<td>0.6</td>
</tr>
<tr>
<td>PaCER1</td>
<td>380 ± 23</td>
<td>416 ± 15</td>
<td>0.9</td>
</tr>
<tr>
<td>PaCER3</td>
<td>44 ± 9</td>
<td>86 ± 1</td>
<td>0.5</td>
</tr>
<tr>
<td>PaUGT78D2</td>
<td>6 ± 2</td>
<td>1430 ± 151</td>
<td>0.0</td>
</tr>
<tr>
<td>PaSRP19</td>
<td>82 ± 1</td>
<td>94 ± 6</td>
<td>0.9</td>
</tr>
<tr>
<td>PaPP2A-2</td>
<td>79 ± 3</td>
<td>81 ± 2</td>
<td>1.0</td>
</tr>
<tr>
<td>PaAC</td>
<td>25 ± 0</td>
<td>24 ± 2</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Expression levels were calculated from RNA-Seq data. Putative gene functions and other information are given in Table 1 and Fig. 4. Ratio is the relative expression level at 21 DAFB divided by the expression level at 56 DAFB. DAFB, days after full bloom; FPKM, number of expected fragments per kilobase of transcript per million mapped fragments.

(2) relative expression levels differed between the genes and covered the range of expression of the CM target genes (Table 2), and (3) they were all expected to be involved in different cellular processes (Table 1 and Supplementary Data Figs S2T–V). Transcript levels of the putative anthocyanin 3-O-glucosyltransferase PaUGT78D2 were undetectable until approx. 60 DAFB. Thereafter, relative expression increased in exo- and mesocarp up to 800- and 300-fold, respectively (Fig. 2F). Increased PaUGT78D2 expression coincided with the onset of colour change (Fig. 1A arrow).

Expression of 15 of the 18 CM target genes was exocarp-specific throughout development, i.e. their mRNA was only detected in the exocarp but not in the mesocarp (PaWINA, PaWINB, PaLipase, PaLTPG1, PaWBC11, PaCER3, PaATT1, PaLCR, PaGPAT4/8, PaLAC2S, PaLACSI, PaKCS6, PaKCS1, PaCER1, PaCER3; Figs 2 and 3).

Thirteen of the exocarp-specific genes showed prominent expression maxima during stage I when CM deposition was high (Figs 2A–C, F, G and 3A–G, I). Expression peaks for PaLACSI (Fig. 3F), PaGPAT4/8 (Fig. 3C), PaLAC2S (Fig. 3E), PaKCS6 (Fig. 3F) and PaKCS1 (Fig. 3G) were detected around 12 DAFB, while expression of PaWINA (Fig. 2A), PaWINB (Fig. 2B), PaLipase (Fig. 2C), PaLTPG1 (Fig. 2F), PaWBC11 (Fig. 2G), PaATT1 (Fig. 3A), PaLAC2S (Fig. 3D) and PaCER1 (Fig. 2I) peaked between 20 and 30 DAFB. Significant positive correlations between transcript levels in the exocarp and the rate of CM deposition were found for PaCER1 (r = 0.97, P < 0.0001), PaWINA (r = 0.96, P < 0.0001), PaLACSI (r = 0.95, P < 0.0001), PaLipase (r = 0.94, P < 0.0001), PaLAC2S (r = 0.93, P < 0.0001), PaWINB (r = 0.90, P < 0.0001), PaATT1 (r = 0.87, P < 0.001) and PaLCR (r = 0.82, P < 0.05) (n = 12 for all). Expression levels of PaLTPG1 were closely related to the CM deposition rate until approx. 70 DAFB (Fig. 2F). However, after 70 DAFB PaLTPG1 expression increased again. Similarly, the expression of PaWBC11, PaGPAT4/8, PaKCS6 and PaKCS1 increased temporarily at 80 DAFB in the exocarp.

Expression of PaCER5 and PaCER3 was also exocarp-specific at all developmental stages but did not correlate significantly with the CM deposition rate. In contrast to the genes described above, relative mRNA levels were low during the first weeks after anthesis and increased more (PaCER3) or less (PaCER5) rapidly until 25 DAFB. Thereafter, mRNA from both genes was detected at variable levels including a transient peak at 80 DAFB.

Expression of PaKCR1 and PaFATB peaked in the exocarp during stage I and again approx. 80 DAFB. However, unlike the above genes, expression was detected also in the mesocarp from 30 DAFB onwards.

Expression of PaLACSI was not tissue-specific at any stage. Maximum mRNA abundance was detected at 5 DAFB.

**DISCUSSION**

The present study established that (1) the sweet cherry genome contains sequences similar to genes involved in cuticle formation in Arabidopsis and (2) transcription of 13 of these genes in the exocarp-enriched fraction was closely correlated with the rate of CM deposition indicating a potential role in CM deposition in sweet cherry fruit.

**Identification of sweet cherry genes putatively involved in cuticle formation**

Sequence analyses and gene expression patterns allowed identification of several promising candidate genes for CM formation in sweet cherry fruit. The evidence demonstrating that the de novo assembled cDNA sequences indeed represent genes expressed in sweet cherry fruit includes (1) the high similarities with peach and Arabidopsis sequences (Table 1), (2) the usefulness of the sweet cherry contigs for designing RT-qPCR primers (Figs 2 and 3), (3) the 100% identity of almost all of the sequenced amplicons with the contigs (Supplementary Data Fig. S1), (4) the consistency of the protein signatures of the translated cDNA sequences with the expected functions in CM formation (Supplementary Data Fig. S2) and (5) the increased expression level of PaUGT78D2, a putative key gene in the anthocyanin biosynthetic pathway that essentially mirrored the colour change during stage III (Fig. 2F; Fig. 1A arrow).

**Potential roles of the genes in sweet cherry fruit**

CM deposition is a multi-step process comprising the synthesis of fatty acids as the precursors for cuticle constituents, the synthesis of cutin monomers and wax, their transport to and...
through the plasma membrane, their subsequent apoplastic transport across the cell wall, polymerization and assembly. A number of genes involved in the biosynthesis of cutin monomers and waxes have been identified in Arabidopsis, but little is known about the trafficking and assembly of these components (Pollard et al., 2008; Samuels et al., 2008; Li-Beisson et al., 2010; Javelle et al., 2011). Furthermore, there is essentially no published information on the molecular biology of CM deposition in tree fruit crops in general and in sweet cherry fruit in particular. This is not surprising considering the effort required for functional analysis of genes involved in reproductive development of a perennial woody plant species (Petri and Burgos, 2005). However, the high degree of sequence conservation of CM-related genes between Arabidopsis and sweet cherry observed in our experiments (Table 1 and Supplementary Data Fig. S2) justifies some preliminary speculation about their...
putative roles in sweet cherry (Fig. 4). Our reasoning is based on two criteria. First, expression of an essential, rate-limiting gene should parallel the rate of CM deposition. Second, expression must occur in the exocarp. These conditions are best met by PaWINA, PaWINB, PaLipase, PaATT1, PaLCR, PaLACS2, PaLACS1 and PaCER1 (Figs 2A–C, 3A, B, D, E, I and Supplementary Data Fig. S2A–C, I–L, Q). For these genes, transcription levels were high when the CM deposition rate was high and low when CM deposition was low. For PaLTPG1, PaWBC11, PaGPAT4/8, PaKCS6 and PaKCS1, an additional transient, exocarp-specific expression peak at 80 DAFB was observed that was not paralleled by increased CM deposition (Figs 2F, G and 3C, F, G; Supplementary Data Fig. S2G, H, M–O). At present we do not have a conclusive explanation for this observation.

Tentative function that may be assigned to PaLACS2 (Fig. 2D and Supplementary Data Fig. S2K; Schnurr et al., 2004), PaLACS1 (Fig. 2E and Supplementary Data Fig. S2L;
Fig. 4. Hypothetical biosynthetic pathways for cutin monomers and wax constituents in the sweet cherry fruit epidermis (solid arrows). Biosynthesis of cutin and fatty acyl waxes starts with C16 and C18 fatty acids, which are produced in the plastids (1) and chemically modified to cutin monomers (2) and wax constituents (3) in the endoplasmatic reticulum prior to transport through the cell wall (4). Sweet cherry genes with exocarp-specific expression maxima during the most active CM deposition are highlighted in boxes. Dashed arrows indicate that PaLiPase may participate in cutin and wax production. Pa, Prunus avium; CM, cuticular membrane; FA, fatty acid or fatty acyl; 16C, 18C, 20C, FAs with chain lengths of 16, 18 or 20 carbons; ACP, acyl carrier protein; CoA, coenzyme A; glycerol-3-phosphate acyltransferase; CER5 and WBC11, ABC-transporters; Lipase, a GDSL/SGNH motif lipase; VLCFA, very long chain fatty acid; FA elongase; CER, eceriferum; LTPG, glycosylphosphatidyl-inositol-anchored lipid transport protein; WIN, WAX INDUCER, an AP2/EREBP family transcription factor. For further explanations see text.

Lü et al., 2009; Weng et al., 2010), PaATT1, PaLCR (Fig. 3A, B and Supplementary Data Fig. S2L; J; Wellesen et al., 2001; Xiao et al., 2004) and PaGPAT4/8 (Fig. 3C and Supplementary Data Fig. S2M; Li et al., 2007) is the synthesis of 9(10), 16-dihydroxy-hexadecanoic acid and 9, 10, 18-trihydroxy-octadecanoic acid, the most abundant cutin monomers in sweet cherry (Peschel et al., 2007). Alkanes make up the second largest group of cuticular waxes in sweet cherry fruit (19%, w/w, of total wax at maturity; Peschel et al., 2007). Alkane synthesis in sweet cherry fruit continues after the synthesis of other cuticular lipids has essentially ceased (Peschel et al., 2007). PaCER1 is a promising candidate gene for alkane synthesis in sweet cherry, as expression of this gene remained elevated following the peak at 26 DAFB (Fig. 3I and Supplementary Data Fig. S2Q; Hannoufa et al., 1993; Aarts et al., 1995). Other putative candidate genes in wax biosynthesis are PaLACS1 (Fig. 3L and Supplementary Data Fig. S2L; Weng et al., 2010), PaKCS6 (Fig. 3F, Supplementary Data Fig. S2N; Millar et al., 1999) and PaCER3 (Fig. 3J and Supplementary Data Fig. S2R; Hannoufa et al., 1993; Rowland et al., 2007). PaLPTG1 may play a role in apoplastic wax transport (Fig. 2F and Supplementary Data Fig. S2H; DeBono et al., 2009; SB Lee et al., 2009), while PaWBC11 and PaCER5 may export cuticular lipids through the plasma membrane (Fig. 2G, H and Supplementary Data Fig. S2F, G; Pighin et al., 2004; Bird et al., 2007). Finally, in Arabidopsis the SHINE clade genes of the AP2/EREBP transcription factor family regulate expression of several genes in cutin and wax biosynthesis (Aharoni et al., 2004; Broun et al., 2004; Kannangara et al., 2007). In sweet cherry fruit, PaWINA and PaWINB may have a similar function (Fig. 2A, B and Supplementary Data Fig. S2A, B).

Conclusions

Our data indicate that the cessation of CM deposition during early sweet cherry development is associated with downregulation of several putative CM candidate genes, similar to those identified as being involved in the synthesis or transport of cuticle constituents in Arabidopsis. The tentatively assigned functions of these genes in sweet cherry can now be verified. When confirmed, the hypothesis of a cause/effect relationship between CM deposition, fruit surface expansion, strain and formation of microcracks in the CM can be tested. Given the economic importance of fruit surface defects in horticulture...
and the incomplete understanding of cuticle formation in fruit this subject merits further study.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: primers used in the RT-qPCR analyses. Fig. S1: quality controls of the de novo assembled sweet cherry cDNA sequences. Fig. S2: visual output of the InterProScan analyses of sweet cherry cDNA sequences for characteristic motifs in the predicted peptide sequences. Supplementary Methods: workflow of de novo contig assembly, read counts and FPKM values of the 18 CM target genes and four reference genes calculated from RNA-Seq data.

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LITERATURE CITED


