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ORIGINAL PAPER

Effect of sweet cherry genes *PaLACS2* and *PaATT1* on cuticle deposition, composition and permeability in *Arabidopsis*

Myriam Declercq · Merianne Alkio · Thorben Sprink · Lukas Schreiber · Moritz Knoche

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Abstract The cuticular membrane (CM) of sweet cherry (*Prunus avium* L.) fruit is severely strained during development. Strain results from a cessation of CM deposition during early development and is possibly caused by a downregulation of genes involved in CM synthesis. The objectives of our study were to investigate the effects of ectopic expression of two sweet cherry genes, *PaLACS2* (a putative long-chain acyl-CoA synthetase) and *PaATT1* (a putative cytochrome P450 monooxygenase), in *Arabidopsis thaliana* (L.). Effects on the expression of endogenous *LACS2*, *ATT1* and *LACS1* genes, wax and cutin composition, and cuticle permeability were investigated in 13 transgenic lines. Of these, six lines are selected for presentation based on the magnitude of the response. The amount of cutin increased in the *PaLACS2* overexpression line *C-L-29* and in the complemented *lacs2-1* knockout mutant line *l-L-14*, but overexpression had no effect on cutin composition or wax. Wax deposition decreased in the complemented knockout lines *l-L-14* and *l-L-21*. Overexpressing *PaATT1* in *A. thaliana* line *C-A-6* had no significant effect on cutin and wax deposition. In the complemented knockout lines *a-A-7* and *a-A-12*, cutin deposition increased, whereas wax deposition was unaffected. The permeability of the cuticle for water and toluidine blue decreased in the *PaLACS2* and *PaATT1* complemented knockout lines. The results suggest that (1) *PaLACS2* and *PaATT1* expressed in *A. thaliana* are involved in cutin biosynthesis, and (2) their functions are consistent with those of a typical long-chain acyl-CoA synthetase (*PaLACS2*) and of a cytochrome P450 monooxygenase (*PaATT1*).

Keywords *Prunus avium* · Cutin · Wax · Long-chain acyl-CoA synthetase 2 (LACS2) · Aberrant Induction of Type Three 1 (ATT1) · Ectopic expression

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Introduction

The cuticular membrane (CM) is a hydrophobic polymer film that covers the outer surface of all primary aerial organs of higher plants (Esau 1977). It serves as a barrier against uncontrolled water loss and gas exchange and protects against biotic and abiotic stresses (Riederer 2006; Schreiber 2010). The CM comprises a cutin matrix and wax embedded in or deposited on the surface of the matrix (Heredia 2003; Jeffree 2006). Cutin is a polyester of cross-linked hydroxy and epoxy fatty acids (Kolattukudy 2001; Pollard et al. 2008). Cuticular wax mainly consists of very long chain aliphatics (Samuels et al. 2008); the fruit wax of *Rosaceae* species often being rich in terpenoids (Belding et al. 1998; Peschel et al. 2007). It is the wax fraction that is thought to form the primary penetration barrier within the cuticle (Reynhardt and Riederer 1994).

An intact CM is essential in maintaining the barrier function. This is particularly important for the CM of fruit that

often expands considerably until late in development. In sweet cherry fruit, the CM and underlying cell layers are considerably strained in the course of development (Knoche et al. 2004; Grimm et al. 2012), and strain promotes the formation of microscopic fractures (microcracks) (Peschel and Knoche 2005). Microcracks, in turn, impair the barrier function of the CM causing uncontrolled water transport and an increased incidence of fruit rot (Børve et al. 2000; Riederer 2006). Furthermore, microcracks are considered an early event in rain-induced fruit cracking (Peschel and Knoche 2005; Knoche and Peschel 2006), a serious limitation in sweet cherry production worldwide (Simon 2006).

Strain of the CM results from a mismatch of surface expansion and CM deposition caused by an early cessation of CM deposition while expansion continues (Knoche et al. 2004). To better understand CM deposition in sweet cherry, expression patterns of selected genes potentially involved in cuticle formation were recently analysed in developing fruit (Alkio et al. 2012). Candidate genes were characterised by high expression levels in the fruit skin compared to the flesh and expression levels that paralleled the rate of CM deposition (Alkio et al. 2012). A downregulation of the candidate genes that coincides with the cessation of CM deposition was regarded as evidence for a role in CM formation.

The sweet cherry genes *PaLACS2* and *PaATT1* represent such candidate genes. First, the corresponding gene in *Arabidopsis*, *LACS2*, a long-chain acyl-CoA synthetase, catalyses the esterification of fatty acids to coenzyme A, an early step in the synthesis of cutin (Schnurr et al. 2004; Li-Beisson et al. 2013). *ATT1*, a cytochrome P450 monooxygenase, oxidises the resulting acyl chains to ω-hydroxy fatty acids that represent monomers of the cutin polymer (Xiao et al. 2004; Bak et al. 2011). Second, the expression of the sweet cherry *PaLACS2* and *PaATT1* is restricted to the fruit skin and closely related to the rate of CM deposition (Alkio et al. 2012). Third, LACS2 has a substrate specificity for 16:0, 18:X, 20:0 and 24:0 fatty acids (Shockey et al. 2002), with 16:0 and 18:X being primary substrates for cutin synthesis (Kolattukudy 2001). The corresponding hydroxy fatty acids are major constituents of the sweet cherry CM (Peschel et al. 2007). Fourth, studies using *Arabidopsis thaliana LACS2* knockout mutants revealed pleiotropic phenotypes (Schnurr et al. 2004; Bessire et al. 2007; Tang et al. 2007; Weng et al. 2010). For example, the *A. thaliana* knockout mutant *lacs2-1* is characterised by decreased cutin, increased wax deposition and reduced growth (Schnurr et al. 2004; Xia et al. 2009). Fifth, ATT1 has a substrate specificity for 18:X fatty acids (Rupasinghe et al. 2007) and *ATT1* is required for the production of 18:X α,ω-dicarboxylic acids in seeds (Xiao et al. 2004; Molina et al. 2008). Last, the *A. thaliana* knockout mutant *att1-1* is characterised by decreased cutin deposition and sensitivity to water stress (Xiao et al. 2004). Thus, *PaLACS2* and *PaATT1* merit further investigations. To obtain a better understanding of their role in cutin formation in sweet cherry, a functional characterisation in a suitable model system is required.

The aims of this study therefore were to (1) clone and express *PaLACS2* and *PaATT1* from sweet cherry in *A. thaliana*, (2) analyse their effects on cutin and wax deposition and composition, and (3) establish consequences for cuticle permeability.

Materials and methods

Plant material

Sweet cherry (Regina) fruits from trees grafted on Gisela 5 (*Prunus cerasus* L. *x Prunus canescens* Bois) rootstocks were sampled in a commercial orchard in Gleidingen (52° 16′ N, 9° 50′ E), Germany, and processed as described in Alkio et al. (2012).

PaLACS2 was studied in the *A. thaliana* wild-type line Columbia 6 (N8155) and the T-DNA insertion line *lacs2-1* (N19786) (Schnurr et al. 2004). *PaATT1* was studied in the *A. thaliana* wild-type line Columbia 0 (N1093) and the T-DNA insertion line SALK_128714C (N673472) (Alonso et al. 2003). The latter has an insertion in the second exon of the *ATT1* gene and will subsequently be referred to as *att1-3*. All seeds were obtained from the European Arabidopsis Stock Centre (NASC, Nottingham, UK) and their genotypes verified by PCR.

Seeds were surface-sterilized (Clough and Bent 1998), sown into petri dishes and stratified for 3 to 5 days. A half-strength Murashige and Skoog medium including vitamins supplemented with 100 μl l^{-1} Timentin (ticarcillin disodium/clavulanate potassium, Duchefa Biochemie, Haarlem, The Netherlands) was used for germination. After 10 days, plantlets were transferred to soil (Einheitserde Typ Pikier+Perlite, 4:1, Stangenberg, Sinntal, Germany) and cultivated at 20 to 23 °C, 65 % relative humidity and a day/night cycle of 8/16 h. Four weeks after germination, plants were used for analyses, except for the measurement of the plant size where surface areas were quantified 6 weeks after germination.

Cloning and transformation

Total RNA was isolated from sweet cherry fruit using the InviTrap Spin Plant RNA Mini Kit (RP buffer, Stratec Molecular, Berlin, Germany). This and all other reagents or kits were used according to the manufacturer's instructions. Quantity and quality of the RNA were determined by quantifying the absorbance at 230, 260 and 280 nm (BioPhotometer plus spectrophotometer, Eppendorf, Hamburg, Germany; microliter tray cell, Hellma Analytics, Müllheim, Germany). Total RNA was treated with DNase I (Fermentas Germany, Schwerte, Germany). For first-strand complementary DNA (cDNA) synthesis, an amount of 1,500 ng of total RNA was

reverse transcribed using the First Strand cDNA Synthesis Kit (Fisher Scientific Germany, Schwerte, Germany) with anchored oligo(dT) primers. The coding sequences (CDSs) of *PaLACS2* (JU090714.1) and *PaATT1* (JU090723.1) were amplified from the resulting cDNA using the Phusion polymerase (Finnzymes Germany, Schwerte, Germany) in an Adapter-PCR reaction for *PaLACS2* (Gateway™ Technology, Life Technologies, Darmstadt, Germany) and standard PCR for *PaATT1*. These and all other primers as well as their reaction conditions are listed in Online Resource 1. The CDSs of *PaLACS2* and *PaATT1* were introduced into the entry vector pDONR221 (Life Technologies) and transformed into *Escherichia coli*. The entry vectors were sequenced using the Sanger method (Seqlab Sequence Laboratories, Göttingen, Germany).

PaLACS2 entry vectors always contained single point mutations all over the CDS. Mutagenesis using QuickChange PCR (Agilent Technologies, Böblingen, Germany) eliminated a point mutation at position 1343 within the CDS but produced two silent mutations in the same sequence domain (for details, see Online Resource 2). Further cloning transferred the CDSs of *PaLACS2* and *PaATT1* into the destination vector pGWB2 (Nakagawa et al. 2007). The resulting expression vectors contained either the *PaLACS2* or *PaATT1* CDS under the control of a CaMV 35S promoter and a hygromycin resistance gene.

Expression vectors were transformed into *Agrobacterium tumefaciens* strain GV3101::pMP90. Competent cells were prepared using a modified protocol with calcium chloride (Hanahan et al. 1991; Bloom et al. 1997) and transformed according to the freeze and thaw protocol as described by Hofgen and Willmitzer (1988).

The secondary inflorescences of *A. thaliana* were transformed following the floral dip protocol (Clough and Bent 1998). Seeds of the T_1 generation were sown onto selection medium containing 30 mg l^{-1} hygromycin B.

Molecular analysis

The isolation of genomic DNA from *A. thaliana* followed the protocol of Doyle and Doyle (1990). For extraction of total RNA, the InviTrap Spin Plant RNA Mini Kit (DCT buffer, Stratec Molecular) was used. The treatment with DNase I and the synthesis of cDNA was performed as described above. Gene-specific primers were designed (Beacon Designer, Premier Biosoft International, Palo Alto, CA, USA) for reverse transcriptase quantitative PCR (RT-qPCR) and synthesized by Eurofins MWG Operon (Ebersberg, Germany). Beside *PaLACS2* and *PaATT1*, further genes involved in cuticle synthesis in *A. thaliana* were analysed: *LACS2* (At1G49430) and *ATT1* (At4G00360), the homologous genes in *A. thaliana*, and *LACS1* (At2G47240), a gene involved in wax synthesis (Lü et al. 2009). RT-qPCR was performed as described by Alkio et al. (2012) with the following modifications: The 15-µl reaction mixture contained 1 µl of cDNA sample corresponding to 75 ng of total RNA. PCR amplification efficiencies were determined from a fivefold serial dilution. The RT-qPCR was performed with two biological replicates consisting of two technical repeats each.

Abundance of transcripts in all lines was normalised against the transcript abundances of At2G28390, At4G34270 and At4G33380 (Czechowski et al. 2005) using qbase^{PLUS} software (Biogazelle, Ghent, Belgium; Vandesompele et al. 2002; Hellemans et al. 2007). Subsequently, the transcript abundances were calculated relative to the Columbia wild-types for *LACS2*, *ATT1* and *LACS1* and relative to the lowest value for *PaLACS2* and *PaATT1*. Expression stability values M of the reference genes were always below the critical threshold of 0.5, indicating a stable expression in the samples analysed (Hellemans et al. 2007).

Analysis of cutin and wax composition

Cutin and wax analyses were performed as described by Höfer et al. (2008) and Franke et al. (2005). Cuticular wax was extracted from *A. thaliana* plants by dipping for 10 s in chloroform. The leaf surface area was determined by image analysis. An aliquot of 10 µg tetracosane (C_{24}) was added as an internal standard to the chloroform extract (Höfer et al. 2008). Subsequently, the dewaxed leaves were exhaustively extracted at ambient temperature to remove all soluble lipids using chloroform/methanol (1:1, *v*/*v*). The extraction solutions were replaced every 24 h for 3 days and the extracted leaves dried. Four biological replicates were prepared per plant line, each consisting of leaves of three to five plants.

CMs were depolymerized by transesterification with 1–2 ml 1 M methanolic HCl for 2 h at 80 °C. After addition of 1–2 ml saturated aqueous NaCl and 10 µg dotriacontane (C_{32}) as internal standard, aliphatic monomers were extracted (three times with 1 ml each) using hexane (Franke et al. 2005). Following evaporation of the solvents in a stream of nitrogen to a final volume ≤150 µl, all samples were treated with 20 µl bis-(N,N,-trimethylsilyl)-tri-fluoroacetamide (BSTFA; Macherey-Nagel, Düren, Germany) and 20 µl pyridine for 40 min at 70 °C to convert free hydroxyl and carboxyl groups into their corresponding trimethylsilyl (TMS) ethers and esters.

Cutin monomers were separated by capillary GC (DB-1 column, 30 m×0.32 mm, 0.1 µm, Agilent Technologies, Böblingen, Germany) using a gas chromatograph (Agilent 6890 N, Agilent Technologies) coupled to a quadrupole mass selective detector (Agilent 5973 N, Agilent Technologies). The following temperature program was run: on-column injection at 50 °C, 2 min at 50 °C, 10 °C min^{-1} to 150 °C, 1 min at 150 °C, 3 °C min^{-1} to 315 °C, and 20 min at 315 °C. For separation of wax constituents, the following program was run: on-column injection at 50 °C, 2 min at 50 °C, 40 °C min^{-1} to

200 °C, 2 min at 200 °C, 3 °C min^{-1} to 315 °C, and 30 min at 315 °C. Monomers were identified based on their EI-MS spectra. Monomers were quantified using an identical GC system equipped with a flame ionization detector. The analyses were conducted with four replicates for each plant line, except for Columbia 0 where the number of replicates was three.

Transpiration assay

Well-watered *A. thaliana* plants were equilibrated in the dark for a minimum of 6 h. Subsequently, entire rosettes were excised above the hypocotyl and held at ambient temperature (21–22 °C) and relative humidity (38–41 %). Following an initial period of about 20 min to ensure stomatal closure (Kerstiens et al. 2006; Bessire et al. 2007), rosettes were weighed in 20-min intervals for up to 120 min. Thereafter, surface areas of rosettes were determined by image analysis (Kerstiens et al. 2006; Bessire et al. 2007). The number of replications per plant line was five. The flux in transpiration J (kg m^{-2} s^{-1}) was calculated as the mass of water transpired ΔW (kg) per unit surface area A (m^2) and time Δt (s) using the following equation (Burghardt and Riederer 2006):

$$J = \frac{\Delta W}{\Delta t A}$$

Toluidine blue staining

Plants of *A. thaliana* were submerged for 30 min in 0.05 % aqueous toluidine blue following a modified protocol of Tanaka et al. (2004). Thereafter, plants were removed from solution, rinsed, spread on a glass plate and photographed. The minimum number of replicates was four.

Data analysis

Data in figures are presented as means ± standard error of the means (SigmaPlot 12.3, Systat Software, Erkrath, Germany). Data were subjected to analysis of variance (SAS version 9.1.3, SAS Institute, Cary, NC, USA). Mean separation was performed by comparing the overexpression lines to the Columbia wild types or the complemented lines to the respective knockout mutants using Dunnett's test ($P \leq 0.05$).

Results

Molecular characterisation

A total of eight (*PaLACS2*) and five transgenic lines (*PaATT1*) were investigated (see Online Resources 3, 4, 5 and 6). For presentation in the manuscript, one overexpression line of a Columbia wild-type and two complemented knockout mutant lines per gene were selected. This selection was based on (1) the magnitude of the response and (2) the likelihood of identifying effects on the peculiar composition of the *Arabidopsis* cutin (which—unlike cuticles from other species—contains major amounts of 18:2 α,ω-dicarboxylic acids and low amounts of mid-chain hydroxy fatty acids; Franke et al. 2005). In general, all lines responded qualitatively similar, but their quantitative response differed. The genotypes of all transgenic *A. thaliana* lines and the successful insertion of both sweet cherry genes were confirmed by PCR.

The expression level of *PaLACS2* in the complemented knockout line *lacs2-PaLACS2-21* (*l-L-21*) was threefold higher compared to that in the overexpression line Col6-*PaLACS2-29* (*C-L-29*) and *lacs2-PaLACS2-14* (*l-L-14*). No expression of *PaLACS2* was detected in *A. thaliana* Columbia 6 and *lacs2-1* (Fig. 1 and Online Resource 3). Expression of *LACS2* was 1,000-fold lower in *lacs2-1* and the complemented knockout lines as compared to that in Columbia 6 and the overexpression line. *PaLACS2* had no consistent effect on *LACS2* expression. *PaLACS2* increased the expression of *ATT1* in the overexpression line *C-L-29* and decreased it in the complemented knockout lines, including *l-L-14* and *l-L-21*. The expression of *LACS1* was increased in the *A. thaliana lacs2-1* mutant and decreased to a varying extent in the complemented lines.

PaATT1 was expressed in the transgenic *A. thaliana* lines with the expression level being 1,000-fold higher in the complemented knockout mutant *att1-PaATT1-7* (*a-A-7*) than in the overexpression line Col0-*PaATT1-6* (*C-A-6*) and *att1-PaATT1-12* (*a-A-12)* (Fig. 2 and Online Resource 4). *PaATT1* decreased the expression of *ATT1* in the complemented knockout lines *a-A-7* and *a-A-12* and the expression of *LACS2* in the overexpression line *C-A-6*. It had no consistent effect on the expression of *LACS2* in the complemented knockout lines *a-A-7* and *a-A-12* when compared to the corresponding background line.

Cutin and wax composition

The amount of cutin per unit area in the *PaLACS2* overexpression line *C-L-29* was increased 2.5-fold compared to that in Columbia 6 (Fig. 3a) whereas the amount of wax remained largely unchanged (Fig. 3b). There were essentially no qualitative changes in cutin or wax composition (Online Resources 5, 6). The complemented knockout line *l-L-14* had more cutin per unit surface area than the *A. thaliana lacs2-1* mutant or Columbia 6 (Fig. 3a). Total amount of cutin was doubled compared to that of Columbia 6 and increased fourfold compared to *lacs2-1*. In contrast, the amount and composition of cutin in the complemented knockout line *l-L-21* remained largely unchanged. Amounts of total wax and alkanes were significantly reduced in the complemented lines compared to *A. thaliana lacs2-1* (Fig. 3b). The effects on

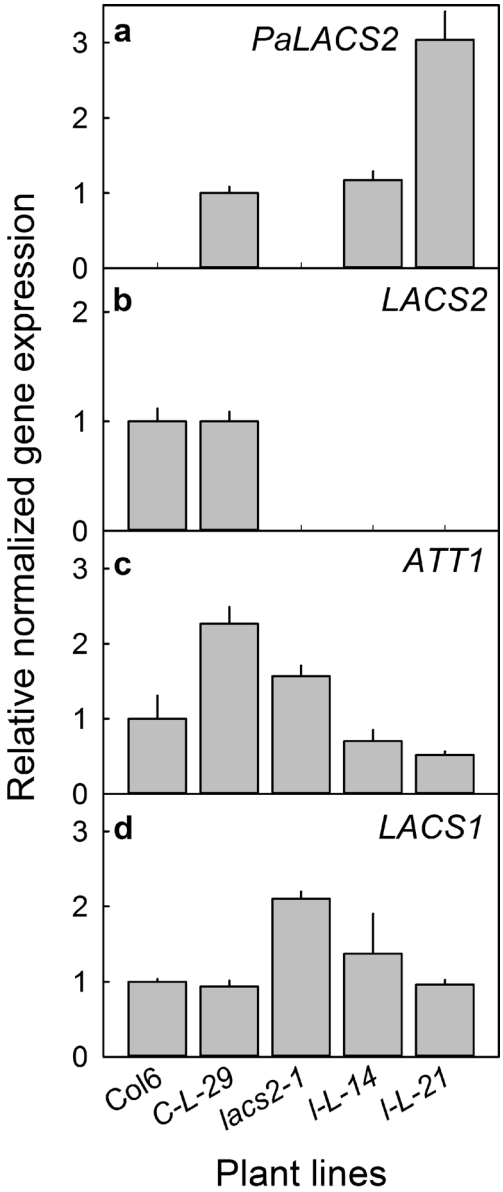

Fig. 1 Relative normalised expression levels for *PaLACS2* (**a**), *LACS2* (**b**), *ATT1* (**c**) and *LACS1* (**d**) in leaves of *A. thaliana* Columbia 6, the *PaLACS2* overexpression line *C-L-29*, the *lacs2-1* knockout mutant and the *PaLACS2* complemented knockout lines *l-L-14* and *l-L-21*. Results were normalised using three reference genes. The gene expression was calculated relative to Columbia 6 for *LACS2*, *ATT1* and *LACS1*. Expression of *PaLACS2* was calculated relative to the smallest value. Columbia 6 and *lacs2-1* did not express *PaLACS2*. *Bars* represent means ± SE of four replicates

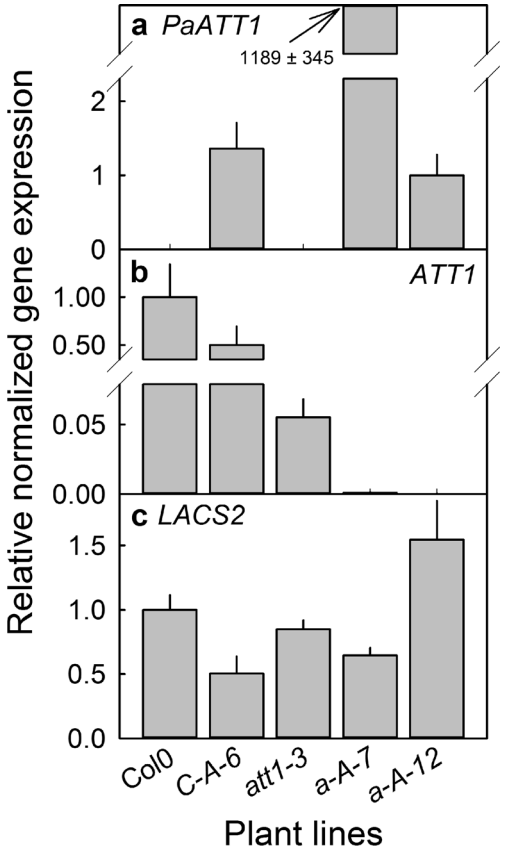

Fig. 2 Relative normalised expression levels for *PaATT1* (**a**), *ATT1* (**b**) and *LACS2* (**c**) in leaves of *A. thaliana* Columbia 0, the *PaATT1* overexpression line *C-A-6*, the *att1-3* knockout mutant and the *PaATT1* complemented knockout lines *a-A-7* and *a-A-12*. Results were normalised using three reference genes. The gene expression was calculated relative to Columbia 0 for *ATT1* and *LACS2* and relative to smallest value for the expression of *PaATT1*. Columbia 0 and *att1-3* did not express *PaATT1*. *Bars* represent means ± SE of four replicates

alcohols, aldehydes, and fatty acids were generally not significant. Notably, the *A. thaliana lacs2-1* knockout mutant had less cutin but more wax per unit leaf area—in particular, more alkanes—than Columbia 6 (Fig. 3 and Online Resource 6).

In the *PaATT1* overexpression line *C-A-6*, total amounts of cutin (+28 %) and wax (+24 %) tended to increase compared to those of Columbia 0 (Fig. 4); the cutin and wax compositions, however, remained unaffected (Online Resources 5, 6).

The total amount of cutin per unit area was increased in the complemented knockout lines *a-A-7* and *a-A-12* compared to the *A. thaliana att1-3* mutant (Fig. 4a). In particular, the amounts of 18:1, 18:2 and 18:3 ω-hydroxy fatty acids as well as of the 18:1 and 18:2 α,ω-dicarboxylic acids were higher in the complemented knockout lines *a-A-7* and *a-A-12* compared to those in the *A. thaliana att1-3* mutant. There was no effect on amount and composition of wax (Fig. 4b).

Physiological characterisation

The *A. thaliana lacs2-1* plants (6±1 cm^2) were small compared to those of Columbia 6 (32±3 cm^2) and leaves were darker and more curled (Fig. 5a). Flowering began earlier. Plants of the *PaLACS2* overexpression line *C-L-29* were similar in size (33± 2 cm^2) to those of Columbia 6, but more compact. The plants of the *PaLACS2* complemented knockout lines *l-L-14* (12±1 cm^2) and *l-L-21* (19±2 cm^2) were larger and less curled compared to the *A. thaliana lacs2-1* knockout mutant.

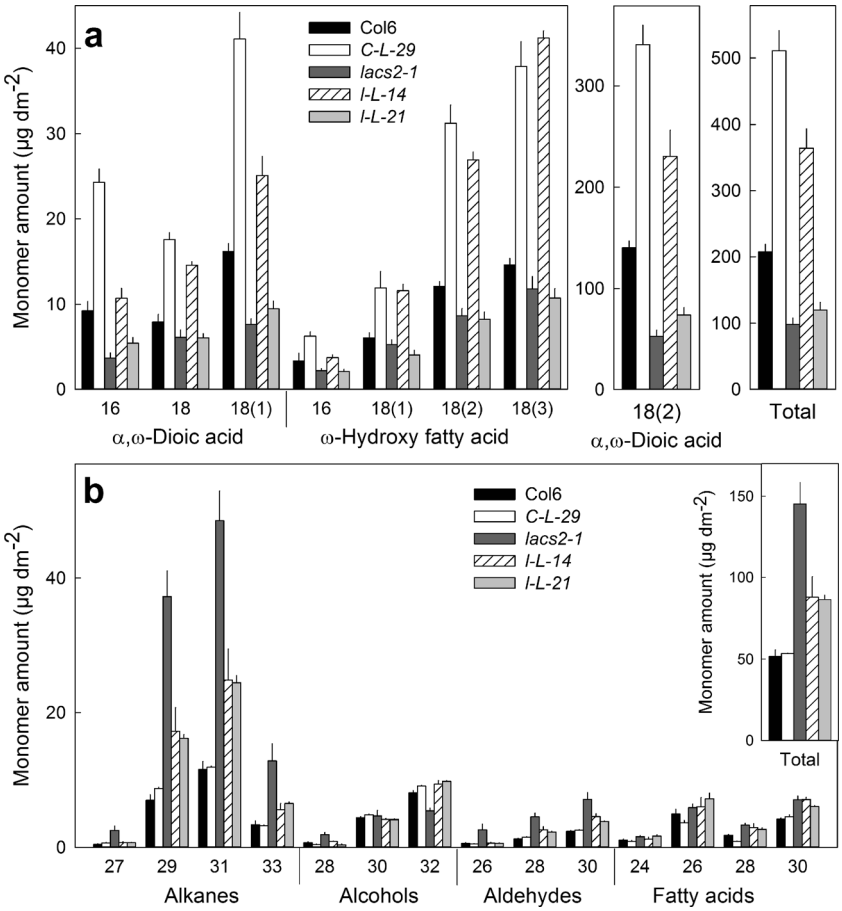

Fig. 3 Major constituents of cutin (**a**) and wax (**b**) in leaves of *A. thaliana* Columbia 6, the *PaLACS2* overexpression line *C-L-29*, the *lacs2-1* knockout mutant and the *PaLACS2* complemented knockout lines *l-L-14* and *l-L-21*. *Numbers* on *x*-axis indicate carbon chain lengths. *Bars* represent means ± SE of four replicates. α,ω-Dioic acid, α,ω-dicarboxylic acid

Plants of *A. thaliana att1-3* (28±3 cm^2) were, on average, smaller compared to those of Columbia 0 (44±2 cm^2) and their leaves were shorter (Fig. 5c). Also, individual plants within the population of *att1-3* varied in size. Plants of the *PaATT1* overexpression line *C-A-6* (41±2 cm^2) were morphologically similar to those of Columbia 0; those of the complemented knockout lines *a-A-7* (30±2 cm^2) and *a-A-12* (39±4 cm^2) were similar in size to the *A. thaliana att1-3* mutant. Plants of *a-A-7* and *a-A-12* were more uniform than those of the *A. thaliana att1-3* mutant.

Plants of Columbia 6 and of the *PaLACS2* overexpression line *C-L-29* did not stain with toluidine blue (Fig. 5b). In the complemented knockout lines *l-L-14* and *l-L-21*, plants stained in a patchy pattern, whereas those of the *lacs2-1* knockout mutant were completely stained. Similar data were obtained for *PaATT1* where plants of Columbia 0 and those of the *PaATT1* overexpression line *C-A-6* did not stain with toluidine blue, but those of the *A. thaliana att1-3* mutant and—to a somewhat lesser extent—the complemented knockout lines *a-A-7* and *a-A-12* stained in a patchy pattern (Fig. 5d).

Flux in transpiration decreased with time in all lines (Fig. 6 and Online Resource 5). There was no difference in transpiration between Columbia 6 and the *PaLACS2* overexpression line *C-L-29* (Fig. 6a). Transpiration of the *A. thaliana lacs2-1* mutant was higher compared to that of Columbia 6, but decreased in the complemented knockout lines *l-L-14* and *l-L-21*. There was no effect of overexpressing *PaATT1* in line *C-A-6* as compared to Columbia 0. However, the complemented knockout lines *a-A-7* and *a-A-12* had a decreased transpiration flux as compared to the *A. thaliana att1-3* mutant (Fig. 6b).

Discussion

The data presented demonstrate that expressing *PaLACS2* and *PaATT1* in *A. thaliana* generally (1) increased cutin deposition, (2) had no effect on or decreased wax deposition, and (3) decreased cuticle permeability in the complemented mutant lines, but not in the respective overexpression lines.

Cutin and wax deposition

Constitutive expression of *PaLACS2* and *PaATT1* resulted in increased amounts of cutin monomers in 12 out of 13 transgenic *A. thaliana* lines (Figs. 3a and 4a and Online Resource 5, 6). For 6 of the 12 lines, this effect was significant at the 5 % level. These observations indicate that *PaLACS2* and *PaATT1*

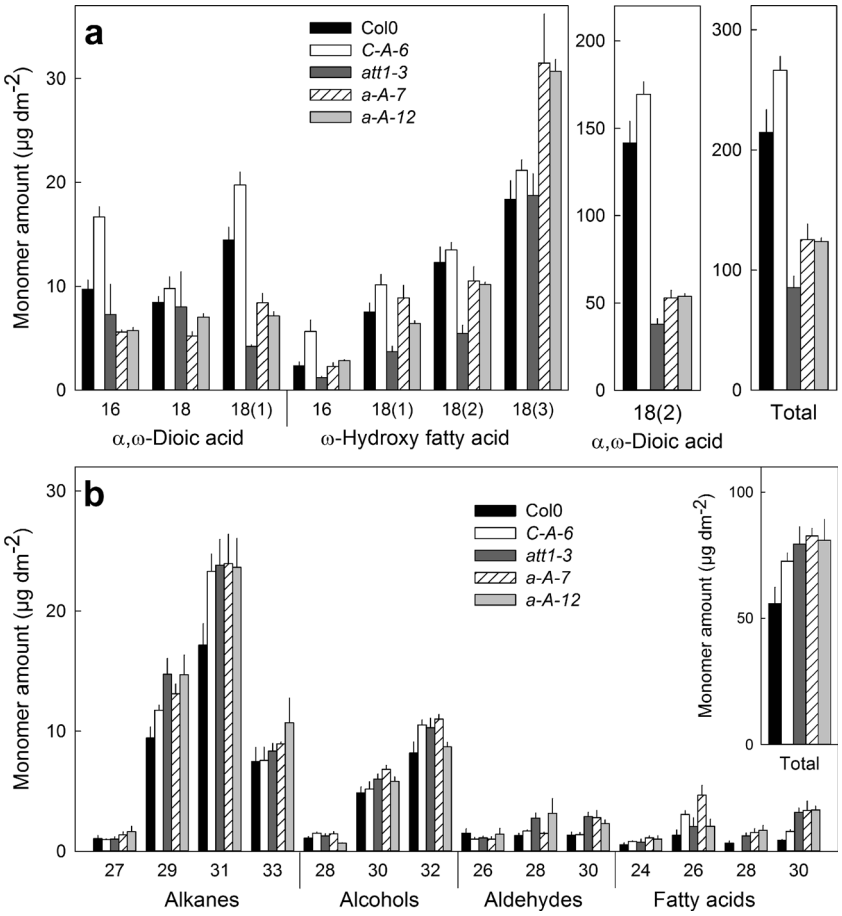

Fig. 4 Major constituents of cutin (**a**) and wax (**b**) in leaves of *A. thaliana* Columbia 0, the *PaATT1* overexpression line *C-A-6*, the *att1-3* knockout mutant and the *PaATT1* complemented knockout lines *a-A-7* and *a-A-12*. *Numbers* on *x*-axis indicate carbon chain length. *Bars* are means ± SE of four replicates except for Columbia 0 where three replicates were analysed. α,ω-Dioic acid, α,ω-dicarboxylic acid

can increase cutin biosynthesis. The only exception was the lack of an effect of *PaLACS2* on the amount and/or composition of cutin in the overexpression line *C-L-15* (Online Resources 5, 6). Because cutin deposition was increased in all other *PaLACS2* transgenic lines including the overexpression line *C-L-29*, and both, *C-L-15* and *C-L-29*, originated from the same *A. thaliana* wild-type Columbia 6, the presence or absence of an effect of *PaLACS2* might be related to the site of the T-DNA integration in the genome (Butaye et al. 2005; De Buck et al. 2013).

Comparing the effects of *PaLACS2* and *PaATT1* on cutin composition of *A. thaliana* revealed that *PaLACS2* increased the individual constituents to a largely similar extent (Fig. 3a and Online Resource 6). In contrast, *PaATT1* resulted in an increase particularly of the 18:1, 18:2 and 18:3 ω-hydroxy fatty acids and the 18:1 and 18:2 α,ω-dicarboxylic acids (Fig. 4a and Online Resource 6). These effects are consistent with the functions assigned to *LACS2* and *ATT1* in *A. thaliana*. *LACS2* catalyses the esterification of fatty acid precursors for the cutin synthesis, whereas *ATT1* encodes a cytochrome P450 monooxygenase catalysing the ω-hydroxylation of fatty acids in the cutin synthesis pathway (Schnurr et al. 2004; Xiao et al. 2004). The increase of 18:1 and 18:2 α,ω-dicarboxylic acids caused by *PaATT1* may be viewed as an indirect effect, since the α,ω-dicarboxylic acids are derived from the corresponding ω-hydroxy fatty acids (Kolattukudy 2001; Rupasinghe et al. 2007). Furthermore, increased amounts of the 18:3 ω-hydroxy fatty acids in the *PaATT1* complemented knockout lines imply a wider substrate specificity for *PaATT1* than that known for *ATT1*. Similar effects have been described for *CYP77A4* in *A. thaliana* (Sauveplane et al. 2009) and *CYP704B2* in rice (Li et al. 2010). This specific function of *PaATT1* may also explain the absence of a significant effect on amount and composition of wax in the *PaATT1* transgenic lines (Online Resources 5, 6).

Expression of *PaLACS2* had no effect on wax deposition and composition in the overexpression line *C-L-29* and the complemented knockout lines *l-L-2*, *l-L-3*, *l-L-6* and *l-L-7*, but in the complemented knockout lines *l-L-14* and *l-L-21*, wax deposition was decreased relative to the *A. thaliana lacs2-1* mutant (Fig. 3b and Online Resources 5, 6). The absence of an effect of *PaLACS2* on wax in the overexpression line *C-L-29* may simply result from the absence of defects in cutin and/or wax synthesis in Columbia 6. Also, like *LACS2* in *Arabidopsis*, *PaLACS2* is assumed to be primarily involved in cutin rather than wax synthesis (Schnurr et al. 2004; Online Resource 6).

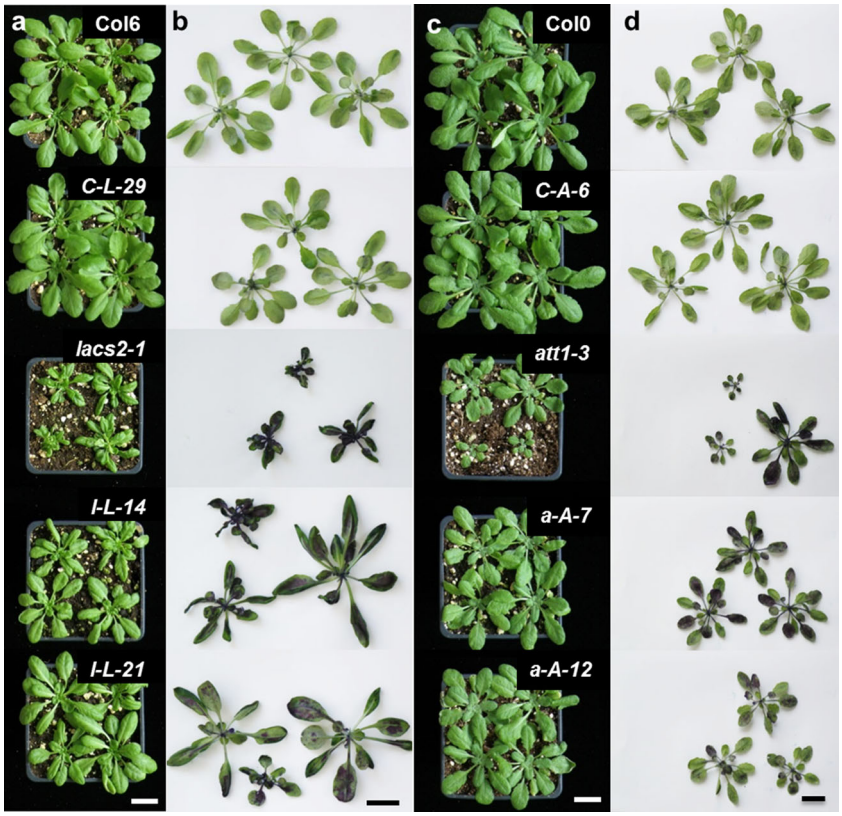

Fig. 5 Habitus of *A. thaliana* plants in top view without (**a**, **c**) and with (**b**, **d**) toluidine blue staining (0.05 % for 30 min). Column **a** and **b** from *top* to *bottom*: Columbia 6, the *PaLACS2* overexpression line *C-L-29*, the *lacs2-1* knockout mutant, and the *PaLACS2* complemented knockout lines *l-L-14* and *l-L-21*. Column **c** and **d** from *top* to *bottom*: Columbia 0, the *PaATT1* overexpression line *C-A-6*, the *att1-3* knockout mutant, and the *PaATT1* complemented knockout lines *a-A-7* and *a-A-12*. Images were taken 4 weeks after germination

The decreased amounts of wax constituents in the *PaLACS2* complemented knockout mutant lines *l-L-14* and *l-L-21* deserve some further comment (Fig. 3b). The decreased wax deposition partly reversed the increased wax deposition in the *A. thaliana lacs2-1* knockout mutant. Both the decrease of wax deposition in the *PaLACS2* complemented knockout lines and the increase of wax deposition in the *lacs2-1* mutant may be accounted for by a redistribution of precursors between the cutin and wax synthesis pathways depending on the extent of inhibition of cutin synthesis (Jenks et al. 2002; Goodwin et al. 2005; Lü et al. 2009; Wang et al. 2011). The cutin and wax synthesis pathways share 16:0 and 18:X fatty acids and corresponding acyl-CoAs as common precursors (Li-Beisson et al. 2013). Further support for this hypothesis comes from the increased expression of *LACS1* observed in the *A. thaliana lacs2-1* mutant as compared to that in Columbia 6 (Fig. 1d). *LACS1* modifies very long chain fatty acids for wax synthesis (Lü et al. 2009; Weng et al. 2010) and has overlapping functions with *LACS2* in cutin biosynthesis in *A. thaliana* (Lü et al. 2009).

Expression of *PaATT1* had generally no effect on wax deposition and composition (Fig. 4b and Online Resources 5, 6).

Permeability of the cuticle

The permeability of the cuticle decreased in complemented knockout lines expressing *PaLACS2* or *PaATT1* as indexed by toluidine blue staining (Fig. 5) and the transpiration assay (Fig. 6). This decrease was significant in five of ten complemented lines (Online Resource 5). This finding suggests that the barrier function of the cuticle was partially restored by *PaLACS2* and *PaATT1* when expressed in the corresponding *A. thaliana* knockout mutants. That the decreased permeability, however, was accompanied by the lack of an effect on or even a decrease in wax deposition in the complemented knockout mutants was somewhat surprising (Figs. 3b and 4b and Online Resources 5, 6). However, similar effects were also reported earlier for *Arabidopsis* (Jenks et al. 2002; Kurdyukov et al. 2006; MacGregor et al. 2008; Wang et al. 2011). For a homogenous membrane, one would expect decreased CM permeability to be caused by increased wax deposition (Riederer and Schreiber 2001). A lack of a close relationship between permeability and wax deposition may result from one or several of the following factors. First, the cuticle is a complex heterogeneous membrane, and therefore, its permeability is not a simple function of the amounts of wax present (Riederer and Schreiber 2001). Second, within the wax fraction, the crystalline portion of the embedded wax is considered to be impermeable to water (Reynhardt and Riederer 1994), and crystalline wax may not simply be a function of the total amount present. That implies that the observed effect on the permeability is likely to be the net effect of increased cutin deposition, an unaffected or decreased wax deposition, and any possible changes in wax crystallinity associated with them.

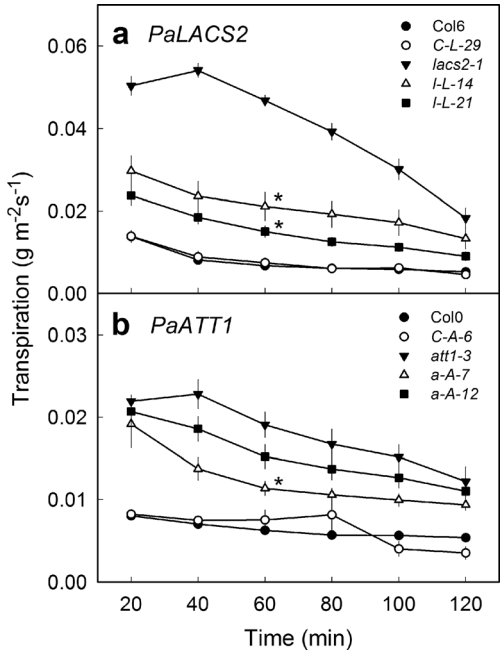

Fig. 6 Effects of *PaLACS2* (**a**) and *PaATT1* (**b**) on flux in transpiration of *A. thaliana* plants. **a** Columbia 6, the *PaLACS2* overexpression line *C-L-29*, the *lacs2-1* knockout mutant, and the *PaLACS2* complemented knockout lines *l-L-14* and *l-L-21*. **b** Columbia 0, the *PaATT1* overexpression line *C-A-6*, the *att1-3* knockout mutant, and the *PaATT1* complemented knockout lines *a-A-7* and *a-A-12*. The flux in transpiration was calculated as the amount of water lost per unit leaf surface area and time. Data points represent means ± SE of five replicates. *Asterisk* (*) indicates significant differences when comparing the overexpression lines to the Columbia wild-type lines or the complemented knockout lines to the respective knockout mutants at 60 min, Dunnett's test, $P \leq 0.05$

Potential consequences of modifying cutin and wax deposition for fruit cracking

The objective in studying the molecular background of cuticle deposition in sweet cherry is ultimately to reduce fruit cracking. Cuticle deposition and cracking may be related in several ways. First and most importantly, we expect to reduce strain and stress in the cuticle by synchronizing cuticle deposition and surface expansion. Earlier studies have established that cuticle deposition essentially ceases within 4 weeks after full bloom, whereas most surface expansion occurs thereafter (Knoche et al. 2004). The cessation of cuticle deposition causes stress, strain, and, consequently, formation of microcracks in the cuticle (Peschel and Knoche 2005). Microcracks impair the barrier function of the cuticle (Knoche and Peschel 2006) and therefore are an important determinant of its water permeability. Second, continuing wax deposition in the cutin network on the expanding fruit surface fixes strain (Khanal et al. 2013). This strain fixation reduces stress and, hence, could reduce microcracking. Also, in apple, wax deposition fills microcracks, thereby restoring the cuticle's barrier function. Whether this also holds for sweet cherry is currently unknown. Third, amounts and composition of cutin and wax may affect the permeability of the cuticle. However, it is important to note that because the cuticle is heterogeneous, relationships between permeability and composition or amounts of constituents will not be a simple function of the amount of its constituents. Finally, we would like to point out that effects on mechanical properties of the fruit skin such as fracture strains, fracture pressures or the modulus of elasticity are unlikely to result from altering cuticle deposition. In sweet cherry, the epidermal and hypodermal cell layers and not the cuticle form the structural backbone of the fruit skin (Brüggenwirth et al. 2014). Unfortunately, sweet cherry is not easy to transform, and therefore, our studies—at present—are limited to the *Arabidopsis* model where only some of these strategies can be investigated.

Conclusion

PaLACS2 and *PaATT1* are involved in the biosynthesis of cutin when expressed in *A. thaliana*. This and the high sequence similarity with peach [*Prunus persica* (L.) Batsch] and *A. thaliana* (Alkio et al. 2012) suggest that *PaLACS2* presumably codes for a long-chain acyl-CoA synthetase that catalyses the esterification of fatty acid precursors whereas *PaATT1* likely encodes a cytochrome P450 monooxygenase which catalyses the ω-hydroxylation of fatty acids in cutin biosynthesis in sweet cherry.

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Data archiving statement *PaLACS2* (accession number JU090714.1) and *PaATT1* (accession number JU090723.1) cDNA sequences were previously deposited in GeneBank (Alkio et al. 2012).

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