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

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Abstract

Cuticular waxes play a pivotal role in limiting transpirational water loss across the plant surface. The correlation between the chemical composition of the cuticular waxes and their function as a transpiration barrier is still unclear. In the present study, intact tomato fruits (Lycopersicon esculentum) are used, due to their astomatous surface, as a novel integrative approach to investigate this composition–function relationship: wax amounts and compositions of tomato were manipulated before measuring unbiased cuticular transpiration. First, successive mechanical and extractive wax-removal steps allowed the selective modification of epicuticular and intracuticular wax layers. The epicuticular film consisted exclusively of very-long-chain aliphatics, while the intracuticular compartment contained large quantities of pentacyclic triterpenoids as well. Second, applying reverse genetic techniques, a loss-of-function mutation with a transposon insertion in a very-long-chain fatty acid elongase β-ketoacyl-CoA synthase was isolated and characterized. Mutant leaf and fruit waxes were deficient in n-alkanes and aldehydes with chain lengths beyond C₃₀, while shorter chains and branched hydrocarbons were not affected. The mutant fruit wax also showed a significant increase in intracuticular triterpenoids. Removal of the epicuticular wax layer, accounting for one-third of the total wax coverage on wild-type fruits, had only moderate effects on transpiration. By contrast, reduction of the intracuticular aliphatics in the mutant to approximately 50% caused a 4-fold increase in permeability. Hence, the main portion of the transpiration barrier is located in the intracuticular wax layer, largely determined by the aliphatic constituents, but modified by the presence of triterpenoids, whereas epicuticular aliphatics play a minor role.

Key words: Cuticle, epicuticular wax, intracuticular wax, tomato, transpiration barrier.

Introduction

All primary aerial surfaces of plants are covered by a cuticle. One of the major functions of the cuticle is the formation of an efficient barrier against unregulated water loss (Kerstiens, 1996a). It is a thin, hydrophobic, and flexible membrane (0.1–10 μm) that is composed of a polymer matrix (cutin) and associated solvent-soluble lipids (cuticular waxes). The cuticular waxes consist of species-, organ- and tissue-specific homologous series of very-long-chain aliphatics (e.g. fatty acids, alcohols, aldehydes, alkanes, and esters) with characteristic chain length distributions and varying proportions of cyclic compounds (e.g. pentacyclic triterpenoids and/or phenyl propanoids).

Despite considerable efforts, the relationship between wax composition and the cuticular barrier properties is
cannot be predicted. To this end, various plant species have been assessed chemically, and quantitative profiling of the wax mixture is required (Baker et al., 1981; Kerstiens, 1996).

Unfortunately, the relatively small organs of Arabidopsis make the functional analysis of its cuticle a difficult task. On the other hand, the cuticular transpiration of several plant species has been assessed in vitro using isolated cuticular membranes (Schönherr and Lendzian, 1981). This approach, however, is restricted to plants with well-developed cuticles, and, for the relatively few species thus investigated to date, wax biosynthetic mutants are not available. For other plant species, cuticular transpiration has to be measured using intact material. To this end, astomatous tissues must be used because even under conditions of minimal permeances (darkness, low air humidity, high CO₂) a residual component of stomatal transpiration is likely (Kerstiens, 1996b).

Considering all these restrictions, tomato (Lycopersicon esculentum cv. Micro-Tom) fruits appear suitable for this approach as they lack stomata. The wax mixture is fairly simple, consisting predominantly of alkanes and triterpenoids (Baker et al., 1982). Furthermore, tomato is an important crop with several plant species has been assessed in vitro using isolated cuticular membranes (Schönherr and Lendzian, 1981). This approach, however, is restricted to plants with well-developed cuticles, and, for the relatively few species thus investigated to date, wax biosynthetic mutants are not available. For other plant species, cuticular transpiration has to be measured using intact material. To this end, astomatous tissues must be used because even under conditions of minimal permeances (darkness, low air humidity, high CO₂) a residual component of stomatal transpiration is likely (Kerstiens, 1996).

In the present investigation, the focus was on how cuticular wax composition is correlated with unbiased cuticular water-barrier properties. The astomatous tomato fruit system cv. Micro-Tom, which offers the possibility to apply new genetic and phytochemical tools to manipulate plant cuticles in an intact system was chosen. The following questions were addressed. (1) Are cuticular waxes on wild-type fruits arranged in layers with distinct chemical composition and how do these layers contribute to the permeation barrier? (2) If wax coverage and composition are altered due to mutation of a particular cuticular wax biosynthetic gene, a fatty acid β-ketoacyl-CoA synthase, how will the overall barrier properties be affected? (3) Employing a combination of genetic tools and selective removal of wax layers, can the transpiration barrier be localized within specific compartments and components of the cuticular waxes?

Materials and methods

Plant material

Lycopersicon esculentum cv. Micro-Tom plants were transformed with Ac/Ds constructs (Meissner et al., 2000). For the present investigation, F1 to F6 progenies of the original F1 plant crosses between Ac-transposase and Ds lines were used. Stabilized Ds lines were selected for the isolation of transposon insertion sites (TIS) by long-range Inverse-PCR (IPCR) adapted from Mathur et al. (1998) as described below. The plants were cultivated in a growth chamber with a 14/10 h day/night regime of 22/18 °C with constant 75% relative humidity.

Identification of the lecer6 insertional mutant

Genomic DNA was extracted from each Ds-insertion line (Dneasy kit, Qiagen, Hilden, Germany), digested with restriction endonucleases and self-circularized with T4 DNA ligase (New England Biolabs Inc., Beverly MA, USA). After heat inactivation the samples were subjected to IPCR amplification using specific primers of the Ds-5′, TTGTATATCCGGTTTCCGGT, and Ds-3′ termini, TTTGCTTTCCGGTCCAAGTTAAA. PCR reactions were performed using the Expand Long Template PCR System (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. PCR fragments were cleaned (Qiaquick PCR purification kit, Qiagen) and used directly as templates for sequencing with Ds-5′ and Ds-3′ specific primers (Gorbunova and Levy, 1997). Each sequence was subjected to similarity searches using the BLAST 2.0 package on the NCBI server, the TIGR database, and the Lab-on-web services (Compugen, Tel Aviv, Israel). The genotype of each individual plant (wild-type, homozygote, or heterozygote for the Ds insertion into the target gene) was tested by PCR using a combination of two gene-specific and one insert-specific primer within one assay (lecer6_forward: 5′-TCCAGC-27TTAGAACGAGTTG-3′; lecer6_reverse: 5′-GCTCTATTCTT-AAGCATCTCC-3′; insert_reverse: 5′-ATTTAAATGAA-27AATGAAAAACGG-3′). In the case of template DNA from wild-type plants, PCR resulted in a single product of 695 bp; homozygote mutant DNA template resulted in a single product of 386 bp, heterozygote lines showed both PCR products. The mutant lines were also characterized by DNA hybridization using a Southern blot analysis as described (Bernatzky and Tanksley, 1986), with a radiolabelled LeCER6 probe and genomic DNA digestion with restriction enzymes (HindIII, BglII, and SpeI).

Expression analysis

Gene expression analysis was performed by semi-quantitative RT-PCR. RNA extractions from Micro-Tom leaves and fruits (epidermis + subepidermal layers) were carried out with the Trizol reagent (Invitrogen, Karlsruhe, Germany). RNA integrity was controlled by
agrose gel electrophoresis. cDNA first-strand synthesis was carried out according to the manufacturer’s instructions (Superscript RTase, Invitrogen).

Template concentration and linear amplification range of PCR cycles were tested in earlier experiments. 27 cycles with an annealing temperature of 54 °C were chosen for LeCER6 amplification. Target gene expression was analysed in parallel to samples 1 × 10⁶ copies of synthetic RNA were added to the plant RNA and checked for identical reverse PCR transcriptase efficiency between different samples. The following PCR primers were used: lecer6_1: 5′-ATTTCTGAACTCTGTAAACGG-3′; lecer6_2: 5′-ACCATAGCACGGCG-3′; actin_1: 5′-CAAGTGCTCATCAGGTTGG-3′; actin_2: 5′-ATACAGTTGTACGACC-3′. To test the reproducibility of reverse PCR amplification, 27 cycles were tested in earlier experiments. 27 cycles with an annealing temperature of 54 °C were chosen for LeCER6 amplification. Target gene expression was analysed in parallel to samples 1 × 10⁶ copies of synthetic RNA were added to the plant RNA and checked for identical reverse PCR transcriptase efficiency between different samples.

Wax analyses of leaves and fruits

An adhesive film of gum arabic was used for the selective preparation and analysis of surface waxes from intact tomato fruits, according to the method of Jetter and Schafer (2001). The whole fruit surface was covered with a dispersion of gum arabic in water (1 g ml⁻¹; Roth, Karlsruhe, Germany). After drying, a thin whitish layer of adhesive could carefully be removed with forceps. This mechanical treatment was repeated once on the same fruit surface. The gum arabic fractions were collected separately and extracted with water/chloroform. After vigorous agitation and phase separation the organic solution was removed and the solvent was removed under reduced pressure. In a similar way, untreated leaf blades and fruits were immersed to a standard and the solvent was removed under reduced pressure. In a similar way, untreated leaf blades and fruits were immersed to a standard and the solvent was removed under reduced pressure. In a similar way, untreated leaf blades and fruits were immersed to a standard and the solvent was removed under reduced pressure. In a similar way, untreated leaf blades and fruits were immersed to a standard and the solvent was removed under reduced pressure.

Prior to gas chromatography (GC) analysis, hydroxyl-containing compounds in all samples were transformed to the corresponding trimethylsilyl derivatives using BSTFA in pyridine (Macherey-Nagel, Düren, Germany). After baseline separation with a temperature controlled capillary GC (8000Top, Fisons Instruments, Rodano-Milan, Italy) with He carrier gas inlet pressure constant at 30 kPa, wax components were identified using a mass spectrometric detector (70 eV, m/z 50–850, MD1000, Fisons). GC was carried out with temperature-programmed injection at 50 °C, over 2 min at 50 °C, raised by 40 °C min⁻¹ to 200 °C, held for 2 min at 200 °C, raised by 3 °C min⁻¹ to 320 °C, and held for 30 min at 320 °C. δ-Amyrin was identified according to Bauer et al. (2002). The quantitative composition of the mixtures was studied by capillary GC (5890 II, Hewlett Packard, Avondale, PA, USA; 30 m DB-1, 0.32 mm i.d., df=1 μm, J&W Scientific, Folsom CA, USA) and flame ionization detection under the same conditions as above, but with H₂ as carrier gas. Single compounds were quantified against the internal standard by manually integrating peak areas.

Characterization of cuticular water transport

Cuticular water permeances were determined for intact, mature, red Micro-Tom fruits. The lack of stomata on the Micro-Tom fruit surface was confirmed microscopically by producing replicas of the epidermis with Collodion 4% (Merck). The attachment site of the pedicel was sealed with paraffin (Merck). The amount of water transpired versus time (5–8 data points per individual fruit; intervals between measurements: 6–10 h) was measured using a balance with a precision of 0.1 mg (Sartorius AC210S, Goettingen, Germany). Cuticular water flow rates (J, in g m⁻² s⁻¹) of individual fruits were determined from the slope of a linear regression line fitted through the gravimetric data. Coefficients of determination (r²) for a representative data set averaged 0.999. Fluxes (J, in g m⁻² s⁻¹) were calculated by dividing F by total fruit surface area as determined from the average of vertical and horizontal diameters by assuming a spherical shape of the fruit. During the measurements, the fruits were stored at constant 25 °C over dry silica gel. Under these conditions, the external water vapour concentration is essentially zero. The vapour phase-based driving force (Δc) for transpiration is therefore 23.07 g m⁻³. For calculating water permeance (p, in m s⁻¹) based on water vapour concentration, J was divided by Δc.

For analysing the contribution of total cuticular waxes on water permeability, the fruits were dipped for 1 min in CHCl₃. The influence of the epicuticular wax layer was measured after mechanical removal with gum arabic.

Results

Identification of a tomato mutant deficient in a gene homologous to CER6 in Arabidopsis

The transposon insertion sites (TIS) from the collection described by Meissner et al. (2000) were subjected to similarity searches on web-localized databases. One line containing one TIS with assigned homology to TC125065, a tentative consensus sequence of tomato ESTs, showed 97% identity on the nucleotide level in the overlapping region (Fig. 1A, B). This TC sequence shows a significant similarity to At1g68530, Arabidopsis very-long-chain fatty acid β-ketoacyl-CoA synthase CER6 (83% identity, 94% similarity). Hence, it was concluded that the tomato gene encoded a CER6-like β-ketoacyl-CoA synthase (condensing enzyme), and it was designated LeCER6.

Based on the expression profile of ESTs identical to LeCER6 clustered in TC125065 (18 ESTs are available in Lycopersicon species as of August 2003), it could be deduced that LeCER6 is expressed in trichomes, flowers, leaves, germinating seeds, and fruits. Gene expression of LeCER6 in Micro-Tom leaves and epidermis of fruits was tested by RT-PCR (Fig. 1C). No expression was detectable in the homozygous mutant, whereas the heterozygous plants did not differ significantly under the experimental conditions used in these RT-PCR experiments. The complete molecular analysis of the mutant, including the complementation of the mutated gene, is currently being completed and will be reported separately.

Fruit characteristics were correlated with the mutant genotype: while 49% (n=343) of the homozygote lecer6 fruits had wrinkled surfaces, only 11% (n=335) and 4% (n=277) of the heterozygotes and the wild-type developed this symptom, respectively (Fig. 2A, C). The wrinkling effect only developed in red, mature fruits. This phenotype was also connected to a reduction in fruit size (wild-type fruit surface area 7.94±2.95 cm² fruit⁻¹, homozygote...
lecer6 6.24±2.66 cm² fruit⁻¹; data are mean ±sd) and in fruit fresh weight (wild-type 2.55±1.34 g fruit⁻¹; homozygote lecer6 1.91±1.20 g fruit⁻¹; data are mean ±sd). These fruit phenotypes were observed in the growth chamber and in the greenhouse with sufficient irrigation of all plants.

Leaf cuticular waxes

The substantial part of solvent-extractable, cuticular leaf waxes consisted of very-long-chain alkanes and of cyclic triterpenoids (Fig. 3). The complete series of n-alkanes with chain lengths between C₂⁷ up to C₃₄ was detected (Fig. 3A). Hentriacontane (C₃₁H₆₄) was found to dominate the wild-type leaf wax. Besides, small amounts of methyl-branched alkanes with branching at the iso- or anteiso

Fig. 1. Characterization of the transposon insertion site (TIS) in Micro-Tom mutant fruits. (A) Comparison of TIS obtained from the IPCR with the tentative consensus sequence TC125065 of tomato ESTs. The dotted line shows the start of translation, the triangle the site of Ds insertion. (B) Nucleotide sequence alignment from the overlapping region of the TIS and TC125065 (Clustal W 1.8). (C) Expression of LeCER6 in leaves and skin of mature fruits determined by RT-PCR for wild-type (WT), heterozygous (+/±), and homozygous mutants (±/±). Expression was compared to actin in the same assay. RT-PCR efficiency in the different samples was monitored by amplification of a synthetic RNA (pAW109) in a separate assay.

Fig. 2. Phenotype of fruits from Micro-Tom wild-type and its mutant lecer6. (A) Wild-type fruits of Micro-Tom keep their spherical and immaculate shape at the state of ripeness. (B) Mature fruits from homozygous mutants tend to have a wrinkled surface. (C) Close-up of mature fruits from lecer6. (D) Micrograph of a replica of the fruit epidermis illustrating the absence of stomata. The bars in (A) to (C) represent a length of 10 mm.

Fig. 3. Composition of leaf cuticular waxes from Micro-Tom wild-type (+/+), from heterozygous (+/±), and from homozygous (±/±) lecer6 mutant plants. (A) Chain length distribution of n-alkanes. (B) Chain length distribution of methyl-branched (iso- and anteiso-) alkanes and (insert to B) total triterpenoids (α-, β-, and δ-ameyrin) within the same samples. (mean ±sd; n=6; statistical evaluation: Student–Newman–Keuls procedure, SPSS 10.0; different letters differ significantly (P<0.05) as determined by one-way ANOVA).
position and the triterpenoids α-, β-, and δ-amyrin were identified (Fig. 3B). The wax composition of heterozygous mutants did not differ significantly from the wild-type. In sharp contrast, homozygous LeCER6-deficient leaves showed a significantly reduced coverage of C$_{31}$ up to C$_{34}$ alkanes, whereas shorter n-alkanes, branched alkanes, and triterpenoids remained unaltered. An increased accumulation of pathway intermediates and of compounds with shorter chain lengths could not be observed. The branched alkane isomers were not affected in the homozygote mutant (Fig. 3B).

**Fruit cuticular waxes**

Total wax load on the Micro-Tom wild-type fruits was approximately 15 µg cm$^{-2}$ and was only slightly reduced for the LeCER6-deficient mutant plants (Fig. 4). With a first gum arabic treatment 4 µg cm$^{-2}$ of wax could be mechanically removed from the wild-type surface. A second adhesive treatment released only small amounts of wax, whereas solvent extraction yielded largely increased quantities of 10 µg cm$^{-2}$. From heterozygous fruits, the same sequence of wax removal steps released similar wax quantities. By contrast, the first gum arabic treatment of homozygous mutant fruits yielded less than 1 µg cm$^{-2}$, while the final extraction step in this case again released 10 µg cm$^{-2}$.

Gum arabic samples from tomato fruits were found exclusively to contain aliphatic components of cuticular waxes (Fig. 5A). n-Hentriacontane dominated the wax mechanically removed from wild-type and heterozygous lecer6 plants, whereas in homozygotes this alkane was only detectable in trace quantities. Longer chain n-alkanes (=31 carbons) and the C$_{32}$ n-aldehyde were also missing, while shorter-chain fatty acids and alkanes, as well as the C$_{31}$ methyl-branched alkane, were unaffected by the mutation.

Wax extraction from wild-type and heterozygous fruits (Fig. 5B) yielded the same aliphatics as the previous gum arabic treatments. In the extracts of homozygous mutants, n-alkanes with chain lengths beyond C$_{30}$ were again missing, while shorter chain-length aliphatics were unaffected. The extracts in all cases were found to contain the pentacyclic triterpenoids α-, β-, and δ-amyrin in high quantities. For homozygous fruits, the decrease in aliphatic components was paralleled by an increase in all three triterpenoids by factors of three to six. To exclude the fact that the higher yields were only due to a difference in the...
extraction efficiency between mutant and wild-type fruits, cuticular membranes were isolated enzymatically (SchoÈnherr and Lendzian, 1981; SchoÈnherr and Riederer, 1986) and extracted exhaustively. The composition of the resulting mixture closely matched the percentages in the extracts from intact fruits (Fig. 5C).

Cuticular permeance for water
Cuticular water permeance (vapour-based) of wild-type and heterozygote fruits were 2.05 ± 0.49 x 10^(-5) m s^(-1) (transpiration rate: 4.73 ± 1.14 x 10^(-4) g m^(-2) s^(-1)), respectively, whereas in LeCER6-deficient plants, cuticular water loss was about four times larger (Fig. 6A). For analysing the contribution of the total cuticular waxes, they were removed by short-term solvent extraction. Permeance for water increased by a factor of 10 for the wild-type plants and a by factor of 2.5 for the homozygote mutant (Fig. 6A). At these higher levels, no significant difference could be observed between the different lines.

In order to analyse the relevance of the epicuticular wax to the overall transpiration barrier, the epicuticular layer was selectively removed with gum arabic. The first mechanical treatment led to a significant increase of cuticular water permeance for the wild-type only, but not for the LeCER6-deficient fruits (Fig. 6B). A consecutive mechanical treatment did not lead to a further increase. After removal of the epicuticular layer, the permeances of wild-type and heterozygous fruits did not reach the level of homozygous fruits.

Discussion
The biosynthesis of aliphatic wax constituents, starting with C_{18} fatty acid precursors, proceeds via sequential elongation steps followed by modification of functional groups (von Wettstein-Knowles, 1993; Hamilton, 1995; Kolattukudy, 1996; Post-Beittenmiller, 1996; Kunst and Samuels, 2003).

Transposon mutagenesis offers a suitable tool to manipulate tomato plants genetically (Emmanuel and Levy, 2002), targeting genes and gene products relevant for wax biosynthetic reactions. In the current study, this reverse genetic approach was employed to identify a mutant deficient in a gene with high sequence homology to Arabidopsis CER6, alias CUT1. The latter had been shown to encode a β-ketoacyl-CoA synthase that is crucial for wax biosynthesis (Millar et al., 1999; Fiebig et al., 2000; Hooker et al., 2002). The enzyme is involved in early elongation steps of very-long-chain fatty acids in Arabidopsis, leading to the production of chain lengths larger than 26 carbons. Based on its high sequence homology, the corresponding tomato gene was designated as LeCER6 and chosen for further investigations into the chemical composition and physiological function of mutant cuticular waxes. Plants carrying a heterozygous mutation in LeCER6 did not differ significantly from the wild-type, neither in the expression level of the gene, nor in the chemical and functional cuticle characteristics. In sharp contrast, the gene was not expressed in the homozygous mutant plants of the cultivar Micro-Tom (Fig. 1C). Therefore, the mutation in LeCER6 is leading to a loss of function and is recessive. This loss of LeCER6-activity caused a reduction in the n-aliphatics with chain lengths of 31 carbons and longer, both in leaf and fruit waxes. The drastic change in chain length patterns clearly indicates that LeCER6 is involved in chain length elongation rather than functional group modification. It should be noted that methyl-branched alkanes with chain lengths beyond C_{30} were not affected by the mutation, thus illustrating the substrate specificity of the LeCER6 condensing enzyme.

Layered structure of tomato cuticular waxes
The chemical composition of tomato fruit cuticular wax had been the topic of previous investigations (Hunt and Baker, 1980; Baker et al., 1982). In accordance with these reports, the current study confirmed that cuticular waxes of Micro-Tom fruits and leaves consist predominantly of very-long chain alkanes and triterpenoids. In addition, in
an attempt to distinguish between intra- and epicuticular wax layers within the tomato fruit cuticle, mechanical and extractive wax-sampling techniques were used sequentially. While consecutive adhesive treatments yielded rapidly declining amounts of waxes, the following extraction step in all cases yielded relatively high amounts of wax (Fig. 4). This proves that both techniques probed different layers within the cuticular wax. The inner layer, being concealed against adhesive sampling, must be located within the mechanically resistant polymer matrix of cutin and can, hence, be interpreted as intracuticular wax. The outer layer, accessible to adhesive sampling, is consequently equivalent to the epicuticular wax film. Because this film was first removed exhaustively, it can be assumed that the intracuticular wax samples contained little residual contamination of epicuticular material. In conclusion, both wax layers were manipulated very selectively for chemical analysis and later for physiological experiments (see below).

The epi- and intracuticular wax layers on wild-type Micro-Tom fruits showed largely differing chemical compositions (Fig. 5A, B). The epicuticular wax film, with a coverage of approximately 5 μg cm⁻², consisted exclusively of aliphatic compounds. A similar pattern of aliphatics (c. 9 μg cm⁻²) was present in the intracuticular wax compartment, but was mixed with 1.5 μg cm⁻² of the triterpenoid alcohols α-, β-, and δ-amyrin. Interestingly, similar gradients between both wax layers, also with triterpenoids restricted to the internal part of the cuticle, had previously been reported for leaves of *Prunus laurocerasus* (Jetter *et al.*, 2000). Homozygous mutant Micro-Tom fruits, deficient in *LeCER6* expression, had reduced amounts of aliphatic wax constituents with chain lengths beyond C₃₀. Consequently, both the overall thickness of the epicuticular film and the relative portion of aliphatics in the intracuticular compartment were reduced proportionally.

In the intracuticular layer, surprisingly, increased extraction yields of pentacyclic triterpenoids (α-, β-, and δ-amyrin) compensated for the reduction in aliphatics. To test whether this finding was due to differential extraction effects, cuticular membranes were isolated from both wild-type and homozygous mutant fruits, and exhaustively extracted with hot chloroform. The resulting triterpenoid yields were similar to those from surface extraction, confirming the accumulation of relatively high amounts of triterpenoids in the mutant cuticle (Fig. 5C). At the moment, it is not clear whether this increase is due to enhanced passive diffusion of pre-formed compounds into the cuticle or to *de novo* synthesis.

**Cuticular permeability of wild-type Micro-Tom fruits**

In the present study, wild-type tomato fruits were found to have a cuticular water permeability of 2.05±0.49×10⁻⁵ m s⁻¹. This value is slightly smaller than the data from four previous investigations, ranging from 2.7×10⁻⁵ m s⁻¹ to 14×10⁻⁵ m s⁻¹ (Schönherr and Lendzian, 1981; Becker *et al.*, 1986; Lendzian and Kerstiens, 1991; Schreiber and Riederer, 1996). The fundamental differences between the current and reported approaches add only little to the overall variability of results. Therefore, this relatively small difference between values clearly illustrates the usefulness of both methods. The relatively large divergence between these results could, on the one hand, be due to differences either in the growth conditions or in the fruit maturation state. However, for tomato fruit cuticles to date none of these factors has been investigated systematically, and hence their potential influence cannot be assessed. On the other hand, the tomato varieties investigated in the various studies might differ in cuticular permeability. To test this possibility, several tomato cultivars were compared in the current study. They all showed fruit cuticular transpiration characteristics very similar to wild-type Micro-Tom (G Vogg, unpublished data), consequently making genetic background variation an unlikely explanation for differences of previous results.

Finally, the methods used to characterize cuticular barrier properties might account for differences in the published results. It might also be expected that the barrier properties will be affected by the harsh conditions during the isolation of cuticular membranes. Accordingly, several studies showed that storage of isolated cuticles prior to physiological experiments decreased permeances significantly, supposedly allowing time to heal defects in the wax barrier (Geyer and Schönherr, 1990; Kirsch *et al.*, 1997).

**Contribution of various cuticle substructures to the transpiration barrier**

A number of previous studies had focused on the correlation between wax characteristics and the transpirational barrier properties of the cuticle. In one approach, the effect of various growth conditions on the composition of *Citrus* leaf cuticles and their water permeability had been analysed (Geyer and Schönherr, 1990; Riederer and Schneider, 1990), but a direct composition/function relationship could not be deduced. In *Sorghum* a negative correlation between wax load and cuticular transpiration rate had been reported (Jordan *et al.*, 1984). In the same plant system, a single-locus mutation with strong negative impact on cuticle thickness and wax deposition (bloom-less) caused a 2.5-fold increase in the epidermal conductance for water vapour (Jenks *et al.*, 1994). However, in leaves, a significant contribution of residual stomatal transpiration cannot be excluded (Kerstiens, 1996b; Muchow and Sinclair, 1989). Even though all these studies showed a general effect of wax composition on permeability, they failed to delineate the contribution of distinct components of cuticular waxes to the transpiration barrier.

In the present investigation, both the genetic manipulation of tomato fruit cuticles and the partial removal of
wax layers offered the opportunity to study the influence of wax composition on water barrier properties. For LeCER6-deficient plants cuticular water loss was more than four times larger than for wild-type fruits (Fig. 6). As this difference could be abolished by exhaustive extraction of total cuticular waxes, the altered permeation properties of the mutant were exclusively due to qualitative and quantitative changes of the cuticular waxes, caused by the knock-out of a single gene involved in their biosynthesis.

The epicuticular wax layer of wild-type tomato fruits was dominated by n-alkanes. When this layer was removed by adhesive treatment, a significant, but only moderate, increase of water permeability resulted (Fig. 6B). This proves that epicuticular waxes contribute to a small extent to the transpiration barrier. This result is in accordance with data on sweet cherry fruit surfaces (Knoche et al., 2000), where the removal of epicuticular waxes led to only a small increase in conductance compared with the effect of removal of the epi- plus intracuticular waxes. Unfortunately, the chemical composition of epi- and intracuticular waxes was not investigated for that species, and, hence, a composition–function correlation could not be deduced.

For lecer6 mutants, an almost complete loss of C₃₁–C₃₄ compounds caused both a drastic reduction in epicuticular wax quantity and substantial changes in surface composition. Homozygous mutant fruits showed significantly higher permeability for water than wild-type (and heterozygous) fruits after removal of their epicuticular layer. The elevated transpiration rates of the mutant fruits can, therefore, only be explained in part by changes in the epicuticular waxes. The larger portion of the transpiration barrier of tomato fruits must be located in the intracuticular wax layer. Because homozygous and wild-type fruits had similar quantities of intracuticular waxes, deficits in the transpiration barrier must be due to changes in the wax composition. Based solely on the chemical results, it can be inferred that the aliphatic constituents of the intracuticular wax layer, especially their chain length profile, play a pivotal role for fruit water relations. Cuticular triterpenoids cannot compensate for the loss of aliphatics.

It has been discussed that the structure of cuticular waxes is a much more important determinant of permeability than either the wax amounts or the composition of the wax mixture (Geyer and Schönherr, 1990). Therefore, from various biophysical and compositional data a model for the molecular structure of plant cuticular waxes was developed (Riederer and Schneider, 1990; Reynhardt and Riederer, 1994). Based on this model the wax barrier consists of impermeable clusters of crystalline zones embedded in a matrix of amorphous material. Water diffusion occurs only in the amorphous volume fractions while crystallites are inaccessible. Based on X-ray diffraction studies it has been postulated that triterpenoids are localized exclusively in the amorphous zones (Casado and Heredia, 1999). Therefore, for Micro-Tom mutant fruits a reduction in n-aliphatics (=31 carbons), together with an increase in cyclic triterpenoids, should create amorphous zones at the expense of crystalline domains. This shift in overall crystallinity might be the actual reason for the increase in cuticular transpiration. Schönherr and Riederer (1988) found that sorption and desorption of lipophilic compounds differs for the inner and outer surfaces of isolated cuticular membranes, respectively. This asymmetry in cuticle properties led to the barrier membrane model (Riederer and Schreiber, 1995), stating that the main portion of the cuticular transport barrier is located at or near the outer surface of the cuticle.

The present findings for tomato fruits not only confirm the existing models, but also add further aspects to it: (i) the cuticle is a mechanically and chemically asymmetric layered barrier, (ii) the main portion of the water barrier is located in the intracuticular wax layer, i.e. underneath the surface, (iii) the relevant barrier properties are due to the specific ratio of aliphatics and triterpenoids of the wax mixture. All the arguments taken together, it is suggested that, at least for tomato fruits, the cuticular transport barrier consists of crystalline aliphatic domains that are located asymmetrically in the intracuticular wax layer.

Conclusions

In conclusion, it could be shown that the tomato system offers the possibility for an integrative approach to correlate cuticle biosynthesis, composition, and its function as a transpiration barrier using an intact system. It has been shown how the mutation of a wax biosynthetic gene (LeCER6), with a known function in very-long-chain fatty acid elongation, leads to an alteration of the chemical cuticular wax composition and, consequently, of the cuticular water permeability. The epidermal cuticular waxes were found to contain a similar composition of aliphatics. In sharp contrast, triterpenoids were located exclusively in the intracuticular layer of the tomato fruit cuticle. Finally, the genetic manipulation of wax composition and the mechanical manipulation (removal) of the epicuticular wax layer were combined to study the contribution of cuticle substructures to the overall transpiration barrier. The main portion of the transpiration barrier of tomato fruits is located in the aliphatic constituents of the intracuticular wax layer, but modified by the presence of variable amounts of triterpenoids. This result substantiates models that describe the cuticular transport barrier as a thin layer, consisting of crystalline aliphatic domains, and located a short distance underneath the cuticle surface.
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