

RESEARCH PAPER

Mining the surface proteome of tomato (*Solanum lycopersicum*) fruit for proteins associated with cuticle biogenesis

Trevor H. Yeats¹, Kevin J. Howe², Antonio J. Matas¹, Gregory J. Buda¹, Theodore W. Thannhauser² and Jocelyn K. C. Rose^{1,*}

¹ Department of Plant Biology, Cornell University, Ithaca, NY 14853, USA

² USDA-ARS, Robert W. Holley Center for Agriculture and Health, Ithaca, NY 14853, USA

* To whom correspondence should be addressed: E-mail: jr286@cornell.edu

Received 20 March 2010; Revised 26 May 2010; Accepted 3 June 2010

Abstract

The aerial organs of plants are covered by the cuticle, a polyester matrix of cutin and organic solvent-soluble waxes that is contiguous with the polysaccharide cell wall of the epidermis. The cuticle is an important surface barrier between a plant and its environment, providing protection against desiccation, disease, and pests. However, many aspects of the mechanisms of cuticle biosynthesis, assembly, and restructuring are entirely unknown. To identify candidate proteins with a role in cuticle biogenesis, a surface protein extract was obtained from tomato (*Solanum lycopersicum*) fruits by dipping in an organic solvent and the constituent proteins were identified by several complementary fractionation strategies and two mass spectrometry techniques. Of the ~200 proteins that were identified, a subset is potentially involved in the transport, deposition, or modification of the cuticle, such as those with predicted lipid-associated protein domains. These include several lipid-transfer proteins, GDSL-motif lipase/hydrolase family proteins, and an MD-2-related lipid recognition domain-containing protein. The epidermal-specific transcript accumulation of several of these candidates was confirmed by laser-capture microdissection and quantitative reverse transcription-PCR (qRT-PCR), together with their expression during various stages of fruit development. This indicated a complex pattern of cuticle deposition, and models for cuticle biogenesis and restructuring are discussed.

Key words: Cuticle, cutin, lipid, proteome, tomato fruit, wax.

Introduction

The plant cuticle is a hydrophobic membrane that covers the aerial organs of land plants and provides protection against desiccation, pathogens, UV radiation, and herbivory (Riederer, 2006). It is continuous with the outer periclinal polysaccharide cell wall of the epidermis and consists of organic soluble waxes embedded in, and layered on, a non-soluble polyester matrix of ω -substituted fatty acids. The waxes include both aliphatic compounds, derived

from very long chain fatty acids, and secondary metabolites, such as triterpenoids and flavonoids (Jetter *et al.*, 2006). In the majority of species analysed to date, cutin is composed primarily of polymerized mid-chain-substituted ω -hydroxy fatty acids, although *Arabidopsis thaliana* is a notable exception in that α,ω -dicarboxylic fatty acids predominate in stems and leaves (Bonaventure *et al.*, 2004; Franke *et al.*, 2005) and ω -hydroxy fatty acids only contribute

Abbreviations: BDG, BODYGUARD; DPA, days post-anthesis; DTT, dithiothreitol; EDTA, ethylenediaminetetra-acetic acid; ER, endoplasmic reticulum; GMC, glucose-methanol-choline; GPAT, glycerol-3-phosphate acyltransferase; HTH, HOTHEAD; LC-ESI-MS/MS, liquid chromatography–electrospray ionization–tandem mass spectrometry; LC-MALDI-TOF/TOF, liquid chromatography–matrix-assisted laser desorption ionization time of flight tandem mass spectrometry; LPS, lipopolysaccharide; LTP, lipid-transfer protein; PBS, phosphate-buffered saline; ML, MD-2-related lipid recognition domain-containing protein; SP, signal peptide; XEGIP, xyloglucan-specific endoglucanase inhibitor protein.

© 2010 The Author(s).

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/2.5>), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

significantly to the cutin of flowers (Beisson *et al.*, 2007; Li-Beisson *et al.*, 2009; Panikashvili *et al.*, 2009). In addition, the presence of glycerol in the cutin polymer is now well established (Graca *et al.*, 2002).

Both cutin monomers and waxes are produced within the epidermal cells, and a clear picture of the molecular biology of their synthesis is emerging. This has been largely a result of the characterization of *Arabidopsis* mutants (Pollard *et al.*, 2008; Samuels *et al.*, 2008), a species whose cutin is probably rich in glycerol (Pollard *et al.*, 2008). For example, the glycerol-3-phosphate acyltransferases GPAT4, GPAT6, and GPAT8 have been shown to be required for cutin synthesis (Li *et al.*, 2007; Li-Beisson *et al.*, 2009), and it was recently reported that an acyltransferase of the BAHD family, DCR, is required for cutin synthesis in *Arabidopsis* floral organs (Panikashvili *et al.*, 2009). Both classes of enzymes appear to be intracellular: GPAT8 was localized to the endoplasmic reticulum (ER) (Gidda *et al.*, 2009) and DCR was shown to be in the cytoplasm (Panikashvili *et al.*, 2009). However, the subsequent extracellular aspects of cuticle biogenesis, including trafficking of the constituents and their assembly into a mature cuticle, as well as restructuring of cuticle architecture during growth and development, are far less well understood.

Current models hypothesize the involvement of several classes of extracellular proteins and enzymes, although few examples have yet been identified. Following biosynthesis in the ER, wax and cutin monomers, or oligomers, are exported across the plasma membrane to the apoplast, in a process dependent on ABC transporters such as the *Arabidopsis* proteins CER5 (Pighin *et al.*, 2004) and WBC11 (Bird *et al.*, 2007). Recently, a glycosylphosphatidylinositol (GPI)-anchored lipid-transfer protein, LTPG, was shown to be required for wax secretion, possibly by acting as a membrane-anchored lipid-binding protein that receives waxes as they are extruded by ABC transporters (Debono *et al.*, 2009). Trafficking of hydrophobic lipids across the polar environment of the polysaccharide cell wall is then often attributed to soluble extracellular lipid transfer proteins (LTPs). However, their ability to bind wax or cutin monomers has not been confirmed, and no cuticle mutant has been attributed to a lesion in a gene encoding a soluble LTP (Yeats and Rose, 2008).

Polymerization of the cutin polymer during development and organ expansion may also involve extracellular proteins. The protein BODYGUARD (BDG) is secreted by epidermal cells and is required for normal cuticle development in *Arabidopsis*, although the *bdg* mutant paradoxically accumulates a larger amount of cutin (Kurdyukov *et al.*, 2006a). While no biochemical activity for BDG has been identified, the protein is a member of the α/β -hydrolase superfamily, leading the authors to suggest that it is a putative cutin synthase. A similar function has been proposed for AgaSGNH, a GDSL-motif lipase/hydrolase family protein from *Agave americana*, which was reported to have protein localization and gene expression patterns that correlated with cutin biosynthesis, although it was not

associated with a genetic phenotype or biochemical activity (Reina *et al.*, 2007).

Thus, remarkably little is known as yet about key mechanisms of cuticle biogenesis, and experimental strategies to identify new proteins that associate with cutin and waxes could provide a valuable means to identify new candidates. Cuticular waxes are easily extracted free of cellular lipid contamination by brief immersion of plant organs in organic solvents such as chloroform (Jetter *et al.*, 2006), while a small additional fraction of the recovered material is comprised of proteins (Martin and Juniper, 1970). Edman degradation peptide sequencing has previously been used to identify three proteins in plant cuticular waxes: an LTP from *Brassica oleracea* (Pyee *et al.*, 1994), and an endo- β -1,3-glucanase and a chitinase (glycosyl hydrolase family 17 and 18, respectively; www.cazy.org) from the wax of *Copernicia cerifera* (Cruz *et al.*, 2002). However, it was hypothesized that generating a more comprehensive inventory of proteins that are associated with the outermost surface tissues of plant organs, using a range of complementary protein fractionation strategies coupled with modern sensitive mass spectrometry-based methods, would help identify new candidate proteins with a potential role in cuticle biosynthesis. To this end, the surface proteome of developing tomato (*Solanum lycopersicum*) fruit was targeted as a model system. Although *Arabidopsis* research has greatly accelerated the discovery of new cuticle-related genes, its cuticle poses some experimental limitations since it is relatively thin, fragile, and difficult to isolate in substantial quantities. Conversely, tomato fruit cuticles are astomatous and large amounts of intact cuticular material can be isolated for chemical and biomechanical analyses. For example, the fruit accumulate of the order of 1 mg cm⁻² cutin (Baker *et al.*, 1982), compared with the stem of *Arabidopsis*, which has 0.5–10 μ g cm⁻² (Franke *et al.*, 2005; Suh *et al.*, 2005). Thus, the typical 6 week period of tomato fruit development represents a remarkably rapid and extensive phase of cuticle biosynthesis, in a genetically tractable species for which there are now also many genomic resources (Mueller *et al.*, 2005; www.solgenomics.net).

The proteomic analysis of tomato fruit cuticle extracts and the identification of several secreted proteins with lipid-related domains are described. The expression patterns of the genes encoding these proteins are further analysed as to the specificity of their expression in the epidermis and during the time course of fruit development. Finally, based on these expression patterns and current models of cuticle biosynthesis, potential roles for these candidates in extracellular cutin and wax deposition and metabolism are discussed.

Materials and methods

Plant materials

Solanum lycopersicum (cv. M82) plants were grown in the field (Freeville NY, summer 2007 and 2008) and 500 immature green fruits were harvested for protein extraction. To avoid bruising and

damage during handling, fruits were harvested from all stages of expansion after the fruits had lost their visible trichomes and became glossy in appearance, at ~15–40 days post-anthesis (DPA). Prior to protein extraction, fruits were washed with deionized water and left to dry overnight. By first rinsing the fruits, it is believed that the analysis excluded phylloplane proteins that are secreted to the outer surface of the cuticle by mechanisms discussed by Shepherd and Wagner (2007). Fruits used for confocal microscopy, laser-capture microdissection, and developmental gene expression time course experiments were harvested from plants grown in the greenhouse (Ithaca, NY). To define the developmental stage of fruits during expansion, flowers were tagged at anthesis. The ripening stages were determined visually by colour change according to standard conventions (Gonzalez-Bosch *et al.*, 1996). For RNA isolation, pericarp tissue from 3–10 fruits at each developmental stage was manually dissected, flash-frozen, ground in liquid nitrogen, and stored at -80°C .

Microscopy

Confocal microscopy was performed as previously described (Buda *et al.*, 2009). To illustrate the different pericarp cell types harvested by laser-capture microdissection, 10 μm paraffin sections of immature green fruits were prepared and stained with Toluidine blue O according to standard protocols (Ruzin, 1999).

Wax extraction and protein isolation

Wax extraction and purification of polar components from the wax was conducted essentially as previously described (Pye *et al.*, 1994). Fruits were dipped, without submerging the calyx scar, for 10 s in ~500 ml of chloroform:methanol (2:1) that was gently stirred by a magnetic stir bar. For each set of extractions, 2–3 aliquots of 500 ml of fresh solvent were used and the extracts were pooled. The extract was then evaporated to dryness by rotary evaporation at 50°C with reduced pressure. The residue was resuspended in 80 ml of chloroform and 40 ml of distilled water, and transferred to a separatory funnel. The upper aqueous phase was recovered and lyophilized and the residue resuspended in 500 μl of buffer [0.7 M sucrose, 0.1 M KCl, 0.5 M TRIS-HCl pH 7.5, 50 mM ethylenediaminetetra-acetic acid (EDTA), 2% β -mercaptoethanol, and 1 mM phenylmethylsulphonyl fluoride]. The protein component was then extracted into phenol and precipitated with 0.1 M ammonium acetate in methanol (Isaacson *et al.*, 2006). Calculation of approximate protein yield by densitometry of the gel-separated samples (see below) indicated that each extraction yielded ~8 μg of protein. Thus, assuming an average fruit surface area of 50 cm^2 , the yield of protein was of the order of 0.3 ng cm^{-2} of surface. For comparison, the wax coverage of immature green tomato fruit is of the order of 5 $\mu\text{g cm}^{-2}$ (15 000-fold greater).

Fractionation and proteomic analysis of protein extracts

Three independent extractions were analysed using three different pre-fractionation schemes.

Isolation of individual bands from 1D polyacrylamide gels

The pelleted protein extract was resuspended in 30 μl of 1 \times LDS sample buffer (Invitrogen, Carlsbad, CA, USA) and separated on a 10% polyacrylamide gel (Novex 10% Bis-Tris Gel, 1.0 mm; Invitrogen) using MOPS running buffer, according to the manufacturer's instructions. The gel was fixed in 40% methanol/10% acetic acid and stained overnight with SYPRO Ruby (Invitrogen) according to the manufacturer's instructions. Gels were visualized with UV illumination and individual bands were excised (see Fig. 2A) and frozen at -80°C .

Isolation of broad slabs from 1D polyacrylamide gels

Proteins were separated as above, except MES running buffer (Invitrogen) was used according to the manufacturer's instructions.

Slabs were excised (see Fig. 2B), cut into small pieces, and frozen at -80°C .

Gel-free in-solution trypsin digest

Precipitated proteins were resuspended in 100 μl of 50 mM ammonium bicarbonate, 6 M guanidinium chloride. To this, 5 μl of dithiothreitol (DTT) stock solution (200 mM DTT in 50 mM ammonium bicarbonate) was added and the mixture boiled for 10 min. Proteins were alkylated by addition of 4 μl of 1 M iodoacetamide in 50 mM ammonium bicarbonate, followed by a 1 h room temperature incubation in the dark. To this, 40 μl of DTT stock was added and incubation was continued for an additional hour. The sample was then diluted by addition of 846 μl of 50 mM ammonium bicarbonate and digested by the addition of 5 μl of 200 $\text{ng } \mu\text{l}^{-1}$ solution of sequencing grade trypsin (Promega, Madison, WI, USA). The reaction was incubated overnight at 37°C and then terminated by the addition of concentrated acetic acid to lower the pH below 6.0.

Analysis of the gel-free extract was conducted by online liquid chromatography–electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS), essentially as described by Yang *et al.* (2007). The sample was pre-fractionated by strong cation-exchange chromatography, eluting bound peptides in five fractions with a step gradient of 25, 50, 100, 200, and 500 mM KCl. Each fraction was then analysed by LC-ESI-MS/MS as previously described. For the two gel-fractionated samples, in-gel trypsin digestion was performed as previously described (Shevchenko *et al.*, 1996), with modifications as described by Yang *et al.* (2007), and tryptic peptides were recovered with C18 ZipTips (Millipore, Bedford, MA, USA), according to the manufacturer's directions. Peptides from each fraction were separated and analysed by offline LC-MALDI-TOF/TOF (liquid chromatography–matrix-assisted laser desorption ionization time of flight tandem mass spectrometry) analysis (Yang *et al.*, 2007).

Peak lists from the mass spectrometers were searched against the longest six-frame translation of the Sol Genomics Network (SGN) *Lycopersicon* Combined unigene build from May 2009 (www.solgenomics.net) using MASCOT (Perkins *et al.*, 1999). For all experiments, the database was searched allowing for one missed cleavage, cysteine carboxyamidomethylation, and variable methionine oxidation, requiring peptide scores corresponding to $\geq 95\%$ confidence. For MALDI-TOF/TOF experiments, a peptide mass tolerance of 10 ppm and fragment tolerance of 0.025 Da was used. For ESI-MS/MS experiments, these tolerances were set to 1.5 ppm and 0.6 Da, respectively. To limit the number of false-positive results, the results were filtered by requiring that each identified protein be represented by at least two unique peptides in the same or multiple analyses.

Laser-capture microdissection, RNA amplification, and cDNA synthesis

Tissue fixation and microdissection were performed based on the protocol of Nakazono *et al.* (2003). Pericarp tissue from 10 DPA immature green tomato fruits was manually dissected into 2 mm cubes using a razor and fixed by vacuum infiltration with 75% ethanol, 25% acetic acid. The ethanol/acetic acid was replaced with a fresh aliquot and the sample was left overnight at 4°C . The fixative was decanted and replaced twice with a solution of 10% (w/v) sucrose in 100 mM phosphate-buffered saline (PBS). Upon penetration of the solution into the tissue, as indicated by the tissue sinking, the solution was replaced twice more with a solution of 20% (w/v) sucrose in 100 mM PBS. The tissue was then embedded in TissueTek OCT medium (Sakura Finetek USA, Torrance, CA, USA), frozen in a beaker submerged in a liquid nitrogen bath, and the resulting cryoblocks stored at -80°C until sectioning.

A Microm HM550 cryostat (ThermoFisher Scientific, Waltham, MA, USA) was used to prepare 10 μm and 16 μm pericarp

sections and the CryoJane tape-transfer system (Instrumedics, St Louis, MO, USA) was used to transfer sections to 0.5× adhesive-coated slides, where they were adhered by UV cross-linking. Slides were stored at -80°C until later use. Immediately prior to laser-capture microdissection, slides were thawed and dehydrated as follows (all solvents at -20°C): 1 min, 50% ethanol; 30 s, 95% ethanol; 1 min, 100% ethanol; 2 min, xylene; 2 min, fresh xylene. After air drying, cells were harvested into PALM adhesive cap tubes (Carl Zeiss, Oberkochen, Germany) using a PALM MicroBeam System (Carl Zeiss). Epidermal cells were captured from the 10 μm sections, while the larger, more vacuole-rich collenchyma cells were captured from the 16 μm sections. Total RNA was isolated from the harvested cells using an RNeasy Micro Kit (Qiagen, Valencia, CA, USA) and the mRNA amplified using the TargetAmp 2-Round aRNA Amplification Kit 2.0 (Epicentre Biotechnologies, Madison, WI, USA), according to the manufacturers' instructions. A 1.5 μg aliquot of amplified RNA was used for cDNA synthesis using SuperScript III reverse transcriptase and random hexamer primers (Invitrogen), according to the manufacturer's instructions.

RNA isolation and cDNA synthesis for developmental time course

RNA was isolated from frozen tissue (Schneiderbauer *et al.*, 1991) and 1.5 μg of total, DNase-treated RNA was used for cDNA synthesis using SuperScript II reverse transcriptase and oligo(dT) primers (Invitrogen), according to the manufacturer's instructions.

Quantitative PCR

Quantitative PCR experiments were performed using an iQ5 system (BioRad, Hercules, CA, USA). The cDNA samples were diluted 5-fold with water and 0.5 μl or 1 μl was used as a template for each 25 μl quantitative PCR, prepared using HotStart-IT SYBR Green qPCR Master Mix (Affymetrix, Santa Clara, CA, USA). For each gene, qPCRs were performed in technical triplicates. The sequences of oligonucleotide primers are given in Supplementary Table S1 available at *JXB* online. Specificity of the products was determined by gel electrophoresis, product sequencing, and high-resolution melt curve analysis. For tissue specificity, quantification was performed using REST 2008 software (Pfaffl *et al.*, 2002) with *RPL2* serving as a constitutive control, assuming

PCR efficiency of 1.0 for all genes. For time course experiments, expression ratios for each gene and time point were calculated relative to *RPL2* expression. For each gene, expression was linearly normalized, with a value of 0.0 assigned to the stage with lowest expression and 1.0 to the stage showing the highest expression.

Bioinformatics and software

The area-proportional Venn diagram was constructed using BioVenn (Hulsen *et al.*, 2008; <http://www.cmbi.ru.nl/cdd/biovenn/>). Normalized gene expression profile data were converted into a heat map using Cluster 3.0 (bonsai.ims.u-tokyo.ac.jp/~mdehoon/software/cluster/software.htm) and Java TreeView (Saldanha, 2004). Alignment of protein sequences was performed with Clustal W (Thompson *et al.*, 1994), and a Neighbor-Joining tree was constructed using MEGA4 (Tamura *et al.*, 2007). The alignment parameters and the settings for the phylogenetic reconstruction were the defaults of the MEGA4 package.

Results

Protein isolation and identification of candidate genes

As illustrated in Fig. 1A–C, the fluorescently stained cuticle (Fig. 1A) covers the surface of the tomato fruit but is separated from the epidermal cells by a subcuticular polysaccharide cell wall (Fig. 1B). Previous studies have indicated that wax, rather than cutin, is the major barrier to the diffusion of polar molecules, including water (Leide *et al.*, 2007; Isaacson *et al.*, 2009), and presumably proteins, across the cuticle. It was reasoned that, despite the relatively low abundance of wax in the tomato fruit cuticle, which is of the order of 5 $\mu\text{g cm}^{-2}$ compared with 1 mg cm^{-2} for the cutin polymer (Baker *et al.*, 1982), a brief immersion of the fruits in an organic solvent would allow the isolation of proteins directly associated with cuticular wax, as well as those localized within the subcuticular epidermal cell wall

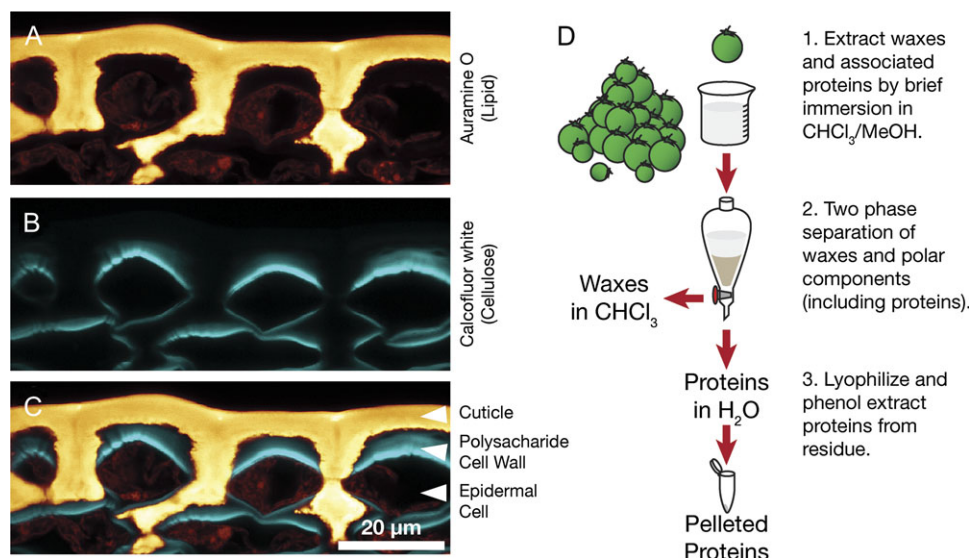


Fig. 1. Epidermis structure and experimental design. Confocal microscopy of cryosectioned tomato breaker stage fruit epidermis, co-stained with the fluorescent lipid stain Auramine O (A) and the cellulose stain Calcofluor white M2R (B). The merged image (C) illustrates the cuticle and epidermal cell wall in the context of the epidermal cell layer. (D) Schematic representation of the extraction protocol used to isolate proteins from the cuticle and epidermal cell wall.

and possibly epidermal intracellular proteins, depending on the degree to which the cells were compromised. A standard protocol was therefore used to remove waxes by immersion of intact plant organs in an organic solvent to obtain extracts for profiling of the fruit surface proteome, as was previously attempted on a smaller scale with *Brassica oleracea* leaves (Pyee *et al.*, 1994).

In order to target proteins that might be associated with cuticle biosynthesis more specifically, young, rapidly expanding tomato fruits were used in this study, since this represents the phase of most rapid cuticle deposition (Baker *et al.*, 1982; Mintz-Oron *et al.*, 2008). After extraction of cuticular waxes and other co-extracted components, the wax was separated from the more polar proteins by partitioning of polar constituents into an aqueous phase and wax into chloroform. The aqueous phase was then lyophilized and proteins were further purified from the residue by phenol extraction and precipitation (Fig. 1D).

In the initial analysis, the protein extract was separated by denaturing polyacrylamide gel electrophoresis (PAGE) and the 16 most distinct bands (Fig. 2A) were excised and subjected to in-gel tryptic digestion, followed by offline LC-MALDI-TOF/TOF analysis. This use of reverse phase liquid chromatography to separate tryptic peptides and robotic mixing of chromatographic fractions with a MALDI matrix (Bodnar *et al.*, 2003) combines the capacity for analysing complex mixtures offered by online LC-ESI-MS/MS analysis with the increased precision and reduced sensitivity to ion suppression that is offered by MALDI-TOF/TOF analysis (Yang *et al.*, 2007). Using this approach,

a total of 44 different proteins were identified from the 16 bands following MASCOT searching of the mass spectra against a database of translated tomato unigene sequences (Supplementary Table S2 at *JXB* online). Since an initial analysis using the spectra obtained from each band separately revealed some redundancy in the proteins identified in each band, as well as the presence of many proteins in each band (data not shown), the spectra from all bands were combined for this search.

Using a second experimental strategy and a new protein isolate, proteins were pre-fractionated by PAGE, but, rather than cutting distinct bands, 10 contiguous gel slabs were excised and subjected to in-gel trypsin digestions (Fig. 2B). It was noted that the banding pattern did not closely resemble that seen in the first analysis (Fig. 2A). This probably reflects the fact that a different buffer system was used (MES), which favours the resolution of smaller proteins at the expense of larger proteins, or that the proteins may be subjected to varying degrees of post-extraction proteolysis. When spectra from these 10 slabs were combined and a MASCOT search of the tomato predicted protein database was performed, a total of 25 proteins were identified (Supplementary Table S3 at *JXB* online).

A third protein extract was prepared as before, but, rather than fractionating the sample by denaturing PAGE, the entire protein extract was subjected to in-solution tryptic digestion. The resulting solution of tryptic peptides was then pre-fractionated by step elution of a strong cation exchange solid phase extraction cartridge and each fraction was subjected to LC-ESI-MS/MS. A database search using MASCOT with the spectra from this analysis identified 192 unique proteins (Supplementary Table S4 at *JXB* online). In addition to identifying more proteins, in the cases where a protein was identified by both a gel-based and gel-free approach, the latter strategy generally resulted in greater percentage protein coverage and total ion scores.

In summary, proteins corresponding to 202 distinct tomato unigenes were identified. The three analyses showed a substantial amount of overlap, as shown by the relatively high degree of redundancy between the sets of proteins identified in each analysis (Fig. 3). Notably, only 5% of the proteins were identified only by the gel-based analysis and not by the gel-free approach. Given that so little is known about extracellular cuticle assembly and restructuring, the subset of proteins that are potentially secreted to the cell wall were of particular interest. Of the 202 proteins identified, 78 (39%) had secretory signal peptides (SPs) as predicted by SignalP 3.0 (Bendtsen *et al.*, 2004), and these were sorted into 40 putative functional families based on BLAST annotations (Table 1). Several of the putative secreted protein families had lipid-related domains, or similarity to proteins that have previously been implicated in cuticle biology. For example, five LTPs, and an MD-2-related lipid recognition domain-containing (ML) protein that is predicted to bind lipids, were identified. Also of interest were two GDSL-motif lipase/hydrolase family proteins. In addition to the proteins with putative roles in

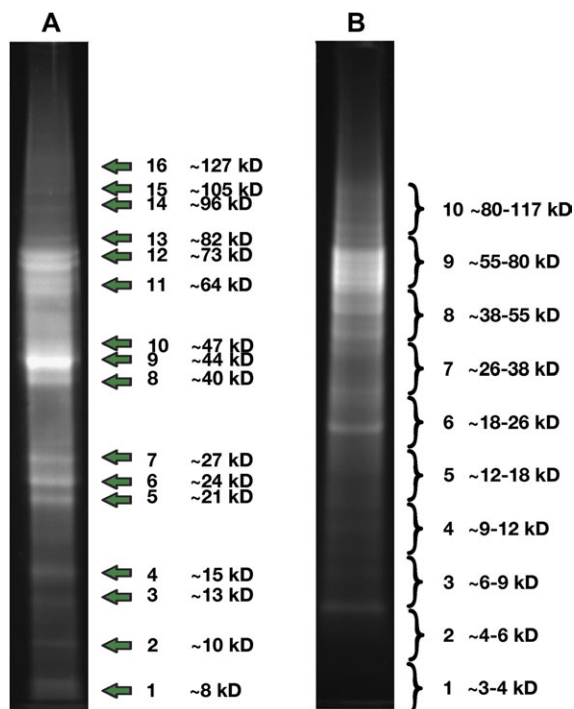


Fig. 2. Denaturing polyacrylamide gel electrophoresis of protein extracts. Proteins were separated and distinct bands (A), or broad slabs covering the indicated ranges (B), were isolated.

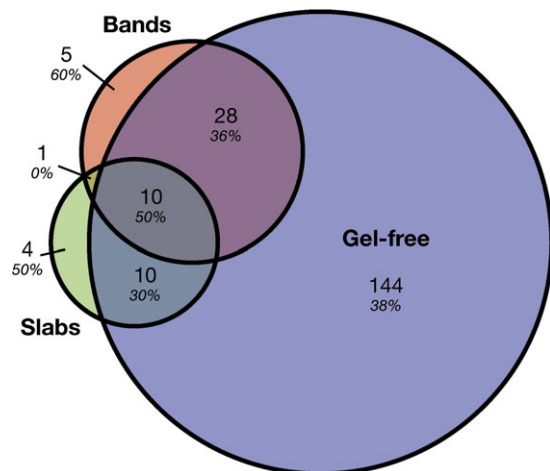


Fig. 3. Venn diagram of proteins found in the three proteomic analyses and signal peptide prediction. The total number in each unique or overlapping set is shown, with the percentage of each set with a predicted signal peptide (SignalP 3.0) indicated in italics.

lipid metabolism, many defence-related proteins were also identified, including several PR-1 proteins, protease inhibitors, chitinases, and endo- β -1,3-glucanases. A large number of proteins belonging to the category of cell wall-modifying and structural proteins, such as expansin, xyloglucan endotransglucosylase-hydrolase, and extensin, were also identified.

Several compelling candidates with homology to previously reported cuticle-related proteins were found in the set of proteins that were identified by only a single peptide and thus did not meet the stringent filtering criteria (Supplementary Tables S1, S3 at *JXB* online). While it was decided not to include these in the list of proteins that were confidently identified, their homology to previously identified cuticle-related proteins warranted further investigation. Thus, a glucose-methanol-choline (GMC) oxidoreductase family protein (SGN-U570812) with high similarity to the *Arabidopsis* protein HOTHEAD (HTH) (57% amino acid identity), which is involved in cuticle biosynthesis (Krolkowski *et al.*, 2003; Kurdyukov *et al.*, 2006b), and three additional GDSL-motif lipase/hydrolase family proteins (SGN-U577181, SGN-U583107, and SGN-U585129) were included in the expression and phylogenetic studies below.

Gene expression analysis of identified proteins

Since the initial aim of this study was to identify surface proteins with a possible role in cuticle formation, the further characterization of candidates with a previously reported cuticle association, or those with lipid-related domains, was of particular interest. Previous studies have shown that many genes encoding cuticle biosynthetic enzymes are specifically expressed in the epidermis (Suh *et al.*, 2005; Mintz-Oron *et al.*, 2008) and so the cell type-specific expression of several candidate genes was investigated. Epidermis and collenchyma cells from immature green fruits were harvested using laser-capture microdissection (Fig. 4A),

RNA was isolated, amplified, and qRT-PCR was performed. The expression of the epidermis-specific (Mintz-Oron *et al.*, 2008) cuticle biosynthesis gene *LeCer6* was used as a positive control, and the expression of transcripts encoding four defence-related proteins, a class that represented a substantial portion of the identified proteins, was also monitored. One of these, xyloglucan-specific endoglucanase inhibitor protein (*XEGIP*), was only identified by a single peptide (Supplementary Fig. S2 at *JXB* online), but its well characterized expression and biological activity warranted its inclusion as a positive control for defence-related transcripts (Qin *et al.*, 2003). Of the 10 genes selected for further characterization, five showed much greater expression in the epidermis relative to the collenchyma (90- to 1700-fold), three showed more modest epidermal enrichment (4- to 8-fold), and two showed low expression ratios (2- and 0.03-fold), suggesting that their transcripts were not epidermis specific (Fig. 4B). The positive control *LeCer6* and the six cuticle-related candidate genes all showed epidermal enrichment of >4-fold, while the four defence-related transcripts showed mixed epidermal specificity: the *XEGIP* and PR-1 transcripts were more highly expressed in the epidermis while the defensin and chitinase both showed low expression ratios, indicating weak epidermal specificity and collenchyma-specific expression, respectively.

Since deposition of wax and cutin follows a specific temporal pattern during fruit development, typified by maximal accumulation during fruit growth followed by a second phase of cuticle deposition during ripening (Baker *et al.*, 1982; Bauer *et al.*, 2004), the expression of the eight epidermis up-regulated genes during fruit growth and ripening was further characterized using qRT-PCR (Fig. 5). *LeCer6* expression was again used as a positive control, as it encodes a part of the fatty acid elongation complex required for aliphatic wax biosynthesis (Vogg *et al.*, 2004) and so its expression would be expected to correlate with wax deposition. The expression pattern of *LeCer6* was most similar to that of the GDSL-motif lipase/hydrolase family gene SGN-U585129 and GMC oxidoreductase, as all three were maximally expressed during the most rapid phase of fruit expansion, peaking at 15–20 DPA (Fig. 5). The two defence-related transcripts, *XEGIP* and the PR-1 SGN-U579545, as well as the LTP SGN-U579687, showed related expression patterns with broad peaks of expression spanning the late phases of fruit growth and early ripening. It was noted that the expression pattern of *XEGIP* corresponded well with a previously reported northern blot analysis of its expression (Qin *et al.*, 2003). Finally, the genes encoding the ML protein, the LTP SGN-U581465, and the GDSL-motif lipase/hydrolase family protein SGN-U583101 all showed similar expression patterns, with high levels of transcript in very young fruit and a substantial reduction by 15 DPA.

Phylogenetic analysis of the GDSL-motif lipase/hydrolase family proteins

Two of the confidently identified proteins and three proteins identified by a single peptide belong to the GDSL-motif

Table 1. Proteins identified by MASCOT with predicted signal peptides

Annotation/gene family ^a	SGN unigene	Identified in analysis ^b	Best hit	
			Total ion score	Percentage coverage
Lipid and putative cuticle related				
GDSL-motif lipase/hydrolase family protein	SGN-U583101	A	37	7.5
	SGN-U579520	ABC	173	22
Inducible plastid lipid-associated protein	SGN-U577010	C	76	15.9
Lipid transfer protein (LTP)	SGN-U577838	C	43	11.7
	SGN-U579033	C	149	46.7
	SGN-U579687	C	252	55.7
	SGN-U580659	C	69	43.5
	SGN-U581465 ^c	C	171	33.1
MD-2-related lipid recognition domain-containing (ML) protein	SGN-U577903	ABC	93	8.6
Defence related				
Allergen V5/Tpx-1-related family protein	SGN-U578890	C	105	13.8
Bet v I allergen family protein	SGN-U577856	AC	67	13.6
Chitinase (GH family 18 and 19) ^d	SGN-U580366	BC	245	23.3
	SGN-U579068	C	244	27.3
	SGN-U579551	C	219	19.8
	SGN-U579696	C	72	8.7
	SGN-U581507	C	91	14.4
Chitin-binding lectin	SGN-U562887	C	46	7.1
Defensin	SGN-U577872	BC	341	47.4
	SGN-U591780	C	62	17.5
Endo β -1,3 glucanase (GH family 17)	SGN-U590837	C	102	7.8
Hevein-like protein	SGN-U567805	C	103	12.3
	SGN-U579235	C	863	68.2
Osmotin-like protein	SGN-U574403	AC	473	40.7
	SGN-U579414	C	505	31
	SGN-U581103	C	574	30.6
Peroxidase	SGN-U581155	AC	588	21.9
	SGN-U583085	BC	550	23.5
	SGN-U564185	C	49	7.4
	SGN-U566251	C	200	25.1
	SGN-U571844	C	102	8.5
	SGN-U575184	C	243	36.8
	SGN-U578562	C	149	6.8
	SGN-U580369	C	199	17.3
	SGN-U580709	C	211	34.3
	SGN-U583086	C	461	23.8
Peroxiredoxin	SGN-U579538	C	74	7.4
Polygalacturonase inhibitor protein	SGN-U579059	AC	44	11.6
PR-1	SGN-U578279	C	180	48.7
	SGN-U579345	C	93	12.2
	SGN-U579426	C	276	34.8
	SGN-U579545	C	771	52.8
	SGN-U579883	C	160	25
Protease	SGN-U578421	AC	71	6.2
	SGN-U582837	AC	134	8.3
	SGN-U578351	C	76	4.7
	SGN-U578475	C	102	7.4
	SGN-U579972	C	159	6.1
Protease inhibitor protein	SGN-U573941	ABC	194	18.3
	SGN-U574346	AC	83	7.3
	SGN-U577283	C	194	14.8
	SGN-U578389	C	163	20
	SGN-U578863	C	62	9.6
	SGN-U585465	C	134	15.5

Table 1. Continued

Annotation/gene family ^a	SGN unigene	Identified in analysis ^b	Best hit	
			Total ion score	Percentage coverage
Snakin-like protein	SGN-U578258	C	168	9
Carbohydrate cell wall metabolism related				
α-Galactosidase (GH family 27)	SGN-U571081	C	102	5.9
β-Glucosidase (GH family 1)	SGN-U580766	A	49	3.9
Expansin	SGN-U577727	C	124	11.6
Ole e 1 allergen/extensin like	SGN-U563658	C	85	13.8
Other				
ADP/ATP translocator like	SGN-U577960	C	91	5.1
Ascorbate peroxidase	SGN-U578449	C	98	14
Enolase	SGN-U579393	C	581	28.9
Formate dehydrogenase	SGN-U579280	C	65	7.3
Fructokinase	SGN-U586194	AC	250	9.2
Fructose-bisphosphate aldolase	SGN-U578572	AC	209	17.6
Glyceraldehyde 3-phosphate dehydrogenase	SGN-U580213	ABC	438	38.2
Glycine-rich RNA-binding protein	SGN-U578513	B	37	19.4
Histone H2B	SGN-U579310	C	57	19.4
Leucine-rich repeat transmembrane protein kinase	SGN-U579197	C	122	25.2
Malate dehydrogenase	SGN-U565569	C	399	25.8
Protein disulphide isomerase-like (PDIL) protein	SGN-U575297	C	61	6.5
	SGN-U577569	C	108	8.8
Ribulose bisphosphate carboxylase large chain	SGN-U565452	ABC	50	4.8
SOUL haem-binding protein	SGN-U584870	A	48	9.2
Strictosidine synthase family protein	SGN-U583542	AC	175	12.4
Transketolase	SGN-U577918	C	109	4.2
Unknown	SGN-U593950	B	23	11.3
	SGN-U565851	C	41	10.7
	SGN-U566943	C	52	18.2

^a Gene family groupings and annotation based on BLAST search of the NCBI non-redundant database.

^b Analyses [(A) is gel band-based analysis, (B) is gel slab-based analysis, (C) is gel-free analysis] from which members of the protein family were identified. The analysis that yielded the highest protein total ion score is shown in bold.

^c The longest-six frame translation of SGN-U581465, corresponding to CAJ19705, has an incorrect start codon that was manually adjusted before SignalP analysis

^d Glycosyl hydrolase (GH) families, www.cazy.org. The SGN annotation refers to the unigene identifier in the Sol Genomics Network database (www.solgenomics.net).

lipase/hydrolase family, which is widely distributed in both eukaryotes and prokaryotes (Akoh *et al.*, 2004). Plant GDSL-motif lipase/hydrolases comprise large gene families; for example, there are 113 predicted members in *Arabidopsis*, although few have a known function. Several lines of circumstantial evidence suggest a role for these enzymes in cutin metabolism. First, biochemically characterized isozymes have been shown to have acyl hydrolase activity, and the presence of an SP suggests that many are secreted (Akoh *et al.*, 2004). Secondly, microarray analysis of *Arabidopsis* stem peels revealed that a subset of 18 members of the gene family is preferentially expressed in this cuticle-synthesizing tissue (Suh *et al.*, 2005). Furthermore, one of these, At2g04570, is highly induced by expression of the cuticle-associated transcription factor WIN1/SHN1 (Kannangara *et al.*, 2007). Microarrays with RNA from isolated tomato peel also identified three tomato GDSL-motif lipase/hydrolase family proteins that are preferentially expressed in the epidermis (Mintz-Oron *et al.*, 2008). Finally, the transcripts corresponding to a GDSL-motif

lipase/hydrolase family protein, AgaSGNH, from *Agave americana* were shown to be highly abundant in the epidermis during leaf elongation, when cutin is being rapidly synthesized (Reina *et al.*, 2007).

Phylogenetic analysis of the five GDSL-motif lipase/hydrolase family proteins described in this present study, as well as the 18 epidermis-specific *Arabidopsis* sequences, AgaSGNH, and the three tomato sequences previously identified by Mintz-Oron *et al.* (2008) indicated that the candidate cuticle-related GDSL-motif lipase/hydrolase family proteins can be grouped into four clades (Fig. 6). The sequences identified in this study align within Clades I, III, and IV. Co-expression analysis of the *Arabidopsis* members of Clades I and III using CressExpress (Srinivasasainendra *et al.*, 2008; www.cressexpress.org) showed high levels of co-expression with nine cutin biosynthesis-related genes (*ATT1*, *LACS2*, *LCR*, *GPAT4*, *GPAT8*, *GPAT6*, *CYP86A4*, *CYP86A7*, and *CYP77A6*), which were used as bait (Supplementary Table S5 at JXB online). While co-expression is less pronounced in Clade IV, its smaller size

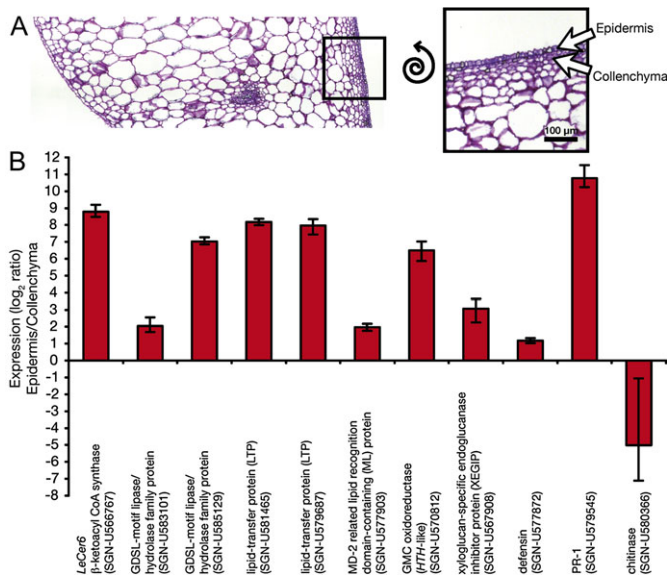


Fig. 4. Tissue-specific expression of selected genes by qRT-PCR of RNA from microdissected cells. Epidermal cells and collenchyma cells were harvested from immature green tomato fruits by laser-capture microdissection as illustrated (A), and extracted, amplified RNA was used for qRT-PCR expression analysis of selected genes (B). The error bars are the standard error as determined by REST 2008 using three technical replicates.

and higher degree of conservation make it an attractive source of candidate cuticle-related GDSL-motif lipase/hydrolase family proteins, particularly in light of the expression patterns of *AgasGNH* and SGN-U585129 that coincide with cutin deposition.

Discussion

In this study, the use of modern mass spectrometry-based proteomic techniques and a diverse set of protein fractionation strategies resulted in a large set of proteins putatively associated with the cuticle of the developing tomato fruit. During the first step of surface protein extraction, care was taken to minimize the time the fruits were submerged in the solvent, in order to reduce cell lysis and increase the proportion of secreted proteins. Bioinformatic analysis suggested that 39% of the cognate genes are predicted to encode N-terminal SPs that would direct their secretion, and this represents a substantial enrichment. For comparison, when the *Arabidopsis* predicted proteome (TAIR release 8, www.arabidopsis.org) is subjected to the same analysis, only 19% of proteins are predicted to have an SP (data not shown). Moreover, it is likely that the N-termini containing the SPs of some of the identified proteins are absent from the sequence databases, since a full genome sequence is not yet available for tomato. However, the presence of known intracellular proteins can be taken to indicate the lysis of some epidermal cells. In addition, it is emphasized that a subset of extracellular proteins will not be extracted or successfully fractionated with the protocols

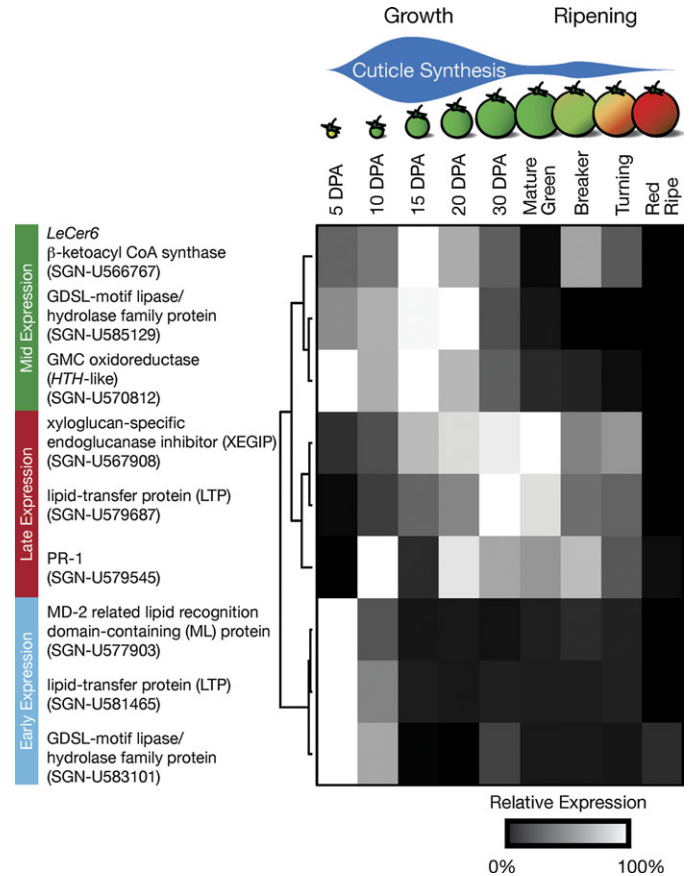


Fig. 5. Time course expression of selected genes during fruit growth and ripening. Gene expression was determined by qRT-PCR relative to the constitutive control *RPL2* and normalized as described in the Materials and methods. The two phases of cuticle deposition are indicated above the fruit development stages considered.

used here due to the recalcitrant nature of the cell wall proteome (reviewed in Lee *et al.*, 2004; Isaacson and Rose, 2006). Moreover, computational tools for predicting SPs are imperfect, and so the presence, or absence, of a predicted SP is not a *de facto* indication of protein extracellular or intracellular localization, respectively. Nonetheless, the enrichment observed suggests that the protein extracts will provide a valuable starting point for researchers interested in cuticle assembly and restructuring.

The three fractionation strategies employed were generally complementary and helped to confirm findings in other analyses, as indicated by the significant overlap between the sets of proteins found in each analysis (Fig. 3). However, the gel-free approach has clear advantages in terms of the number of proteins that were identified and the higher identification confidence scores, as indicated by MASCOT total ion score. Conversely, the gel slab-based analysis (Fig. 2B) yielded the fewest identified proteins (Fig. 3). Since the initial goal was to identify candidate proteins that might be involved in cuticle metabolism, several proteins attracted attention because they had lipid-associated domains, or shared sequence similarity with proteins that are known, or proposed to have, roles in cuticle biogenesis.

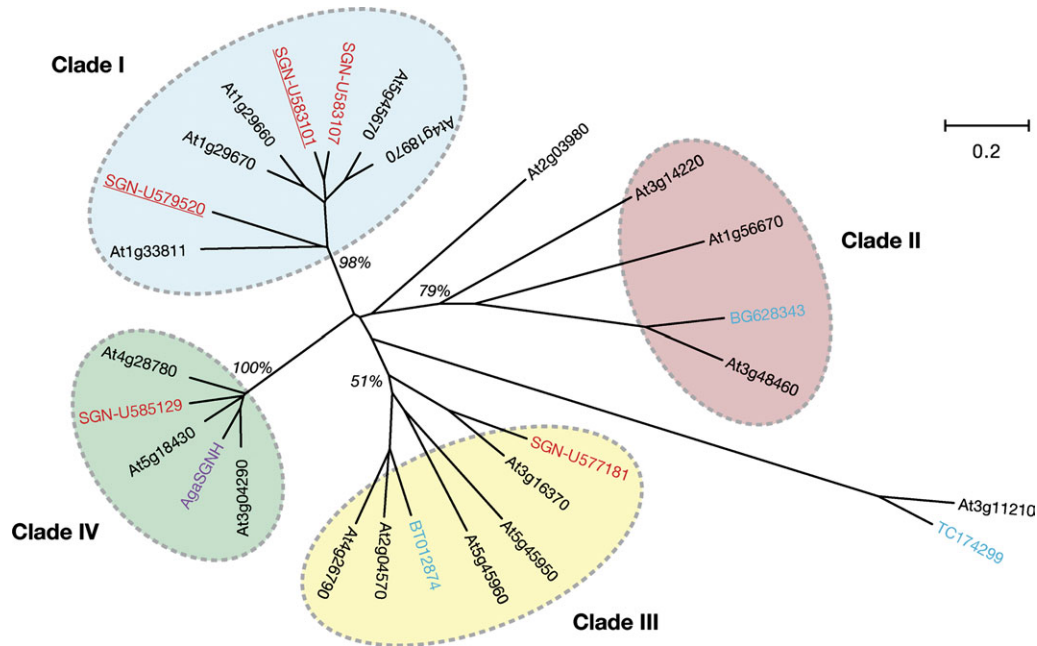


Fig. 6. Phylogenetic analysis of GDSL-motif lipase/hydrolase family proteins. The *Arabidopsis* genes (AGI numbers, www.arabidopsis.org, in black) are those showing >2-fold enrichment in epidermal peels relative to whole stem tissue (Suh *et al.*, 2005). The blue TIGR plant transcript assembly numbers (plantta.jcvi.org) and NCBI EST accessions were identified as tomato transcripts enriched in the peel relative to tomato flesh (Mintz-Oron *et al.*, 2008). AgaSGNH (purple) was identified as an epidermal-specific transcript in *Agave americana* (Reina *et al.*, 2007). SGN unigenes (www.solgenomics.net) described in the current study are shown in red and are underlined if they were identified with confidence (spectra matching two or more peptides, see text). Bootstrap support of the four arbitrarily numbered clades is indicated in italics (500 replications). The bootstrap support of Clade III increases to 92% if the outlying At3g11210 and TC174299 sequences are discarded. Branch lengths are proportional to distance, as indicated by the scale legend.

Putative lipid-binding proteins

Of the five LTPs that were identified, four belong to family 1 of LTPs, and one to family 2 (SGN-U577838) (Yeats and Rose, 2008). The cDNA le16, corresponding to SGN-U579033, was previously identified as being up-regulated by drought and abscisic acid (ABA) (Plant *et al.*, 1991), and the same gene, as well as the gene corresponding to SGN-U581465, was later shown to encode the tomato Lyc e 3 allergen (Le *et al.*, 2006). In a microarray analysis of tomato peel transcripts, SGN-U579687 was seen to be more highly expressed in the exocarp than in the inner pericarp (Mintz-Oron *et al.*, 2008), a result that supports the finding that this transcript is more highly expressed in the epidermis than in the underlying collenchyma.

Aside from LTPs, SGN-U577903, which encodes an ML protein, and has predicted extracellular localization and lipid-binding activity, is also a candidate for contributing to cuticle biogenesis. The ML domain is shared by proteins from diverse eukaryotic species and takes its name from MD-2, a soluble extracellular protein in humans that binds lipopolysaccharide (LPS) in the first step of a signalling cascade that triggers the innate immune response (Jerala, 2007). Other members of this family include the human cholesterol-binding-protein NPC2 (Friedland *et al.*, 2003) and the dust mite allergen Der f 2, which was recently also shown to bind LPS (Ichikawa *et al.*, 2009). Structurally, the domain is composed of two β -sheets that enclose a deep

lipid-binding pocket (Ohto *et al.*, 2007), although no ligand is known or function proposed for the protein family in plants. The transcript abundance of the ML protein was \sim 4-fold greater in the epidermis than in the underlying collenchyma cells (Fig. 4B), and its expression, like that of the LTP SGN-U581465, was highest at the earliest stage of fruit development before rapidly declining (Fig. 5). This precedes the extensive cutin and wax deposition that occurs during the phase of greatest fruit expansion, from 10 DPA to 30 DPA. However, this does not necessarily lead to rejection of the LTP or ML proteins as candidates for wax or cutin transporters: both proteins have extremely stable folds that may result in the protein remaining functional far longer than steady-state mRNA levels are maintained. While no structural or biochemical characterization of any plant-derived ML proteins has been reported, it is suggested that the large lipid-binding cavity of this domain may accommodate the pentacyclic triterpenoids that are abundant in tomato cuticular wax. In contrast, the family 1 LTPs that have been previously proposed as lipid-binding proteins that transport wax across the cell wall are unable to bind planar sterols that are structurally analogous to triterpenoids (Cheng *et al.*, 2004).

Putative HTH orthologue

The GMC oxidoreductase gene that was tentatively identified and shown to have epidermis-specific expression,

SGN-U570812, has 57% amino acid identity with the *Arabidopsis* gene *HTH*. The *hth* mutant has a fused floral organ phenotype that is attributed to a defective cuticle (Krolikowski *et al.*, 2003), and a biochemical activity for HTH has been proposed based on *hth* cutin polymer composition (Kurdyukov *et al.*, 2006b). Mutant plants accumulate increased amounts of ω -hydroxy fatty acids and lower levels of α,ω -dicarboxylic fatty acids that predominate in *Arabidopsis* cutin. Thus, the authors propose that HTH oxidizes ω -hydroxy fatty acids to ω -oxo fatty acids prior to formation of α,ω -dicarboxylic fatty acids.

In tomato, α,ω -dicarboxylic fatty acids comprise only ~1% by weight of cutin monomer composition (Leide *et al.*, 2007; Isaacson *et al.*, 2009). Nevertheless, this monomer may play an important structural role in determining the degree of cross-linking between either cutin chains and the polysaccharide cell wall, or other cutin chains. Expression analysis of the *HTH*-like SGN-U570812 indicated that it is highly expressed (~90-fold) in the epidermis relative to the collenchyma (Fig. 4) and that its expression during fruit development coincides with the rapid expansion and cuticle deposition that occurs 10–20 DPA (Fig. 5).

GDSL-motif lipase/hydrolase family proteins

The genes encoding two confidently identified and three tentatively identified GDSL-motif lipase/hydrolase family proteins were of particular interest, and qRT-PCR characterization of the expression patterns of the five genes was attempted. Despite repeated attempts, this was only successful for SGN-U583101 and SGN-U585129. Both were shown to be more highly expressed in the epidermis than in the underlying collenchyma, although the ratio for SGN-U585129 was much greater (Fig. 4B). The time course of their expression during fruit development was also distinct as SGN-U583101 was highly expressed only very early in fruit development, while SGN-U585129 was expressed throughout fruit expansion (Fig. 5).

GDSL-motif lipase/hydrolase family proteins have previously been proposed as cutin synthases (Reina *et al.*, 2007), or enzymes involved in modification or recycling of the cutin polymer (Pollard *et al.*, 2008). Thus, in the absence of genetic or biochemical evidence for their activity, three specific biochemical activities that may be required for cutin metabolism can be imagined. As cutin synthases, they may incorporate either cutin monomers or oligomers. Secondly, controlled hydrolysis of cutin during organ expansion may be required, so they may act as cutin hydrolases. A third hypothetical enzyme activity is that of a cutin transacylase, wherein the cutin polymer could be loosened by simultaneous cleavage and religation (transacylation) of ester bonds, allowing for organ expansion during growth. This activity, combined with synthesis of cutin oligomers by intracellular enzymes such as GPATs and BAHF family acyltransferases, could be sufficient for cutin polymer synthesis, allowing the oligomers to be ‘stitched’ into the growing cutin polymer matrix only by exchange of existing ester bonds.

In conclusion, using a proteomic approach, a diverse collection of proteins with putative roles in lipid metabolism was identified. Several of these have gene expression patterns that correlate with cuticle biosynthesis; that is they are specifically expressed in the epidermis and their expression coincides with or precedes the deposition of wax and cutin. The results further suggest that there are discrete phases of cuticular lipid metabolism and/or trafficking, which are associated with different gene classes and, even more interestingly, distinct members of the same gene family (e.g. GDSL-motif lipase/hydrolase family proteins and LTPs). However these remain candidates for cuticle biogenesis, and reverse-genetic experiments are currently underway for functional confirmation. Analysis of the phenotypes of these plants coupled with *in vitro* demonstration of their proposed biochemical activities will advance the goal of better understanding of cuticle biosynthesis.

Supplementary data

Supplementary data are available at *JXB* online.

Table S1. PCR primers use for gene expression analysis.

Table S2. Complete MASCOT results for gel band analysis.

Table S3. Complete MASCOT results for gel slab analysis.

Table S4. Complete MASCOT results for gel-free analysis.

Table S5. Co-expression results of *Arabidopsis* GDSL-motif lipase/hydrolase family proteins and known cutin-biosynthesis genes.

Acknowledgements

The authors gratefully acknowledge Dr Mike Scanlon for generously providing access to the PALM MicroBeam System. This work was supported by NSF Plant Genome Research Program grant (DBI-0606595) and THY was supported in part by an NIH chemistry/biology interface training grant (grant no. T32 GM008500). LC-ESI-MS/MS analysis was performed by Dr Sheng Zhang and the Cornell Proteomics and Mass Spectrometry Core Facility.

References

- Akoh CC, Lee GC, Liaw YC, Huang TH, Shaw JF.** 2004. GDSL family of serine esterases/lipases. *Progress in Lipid Research* **43**, 534–552.
- Baker EA, Bukovac MJ, Hunt GM.** 1982. Composition of tomato fruit cuticle as related to fruit growth and development. In: Cutler DF, Alvin KL, Price CE, eds. *The plant cuticle*. London: Academic Press, 33–44.
- Bauer S, Schulte E, Thier H.** 2004. Composition of the surface wax from tomatoes: II. Quantification of the components at the ripe red stage and during ripening. *European Food Research and Technology* **219**, 487–491.

- Beisson F, Li Y, Bonaventure G, Pollard M, Ohlrogge JB.** 2007. The acyltransferase GPAT5 is required for the synthesis of suberin in seed coat and root of *Arabidopsis*. *The Plant Cell* **19**, 351–368.
- Bendtsen JD, Nielsen H, von Heijne G, Brunak S.** 2004. Improved prediction of signal peptides: SignalP 3.0. *Journal of Molecular Biology* **340**, 783–795.
- Bird D, Beisson F, Brigham A, Shin J, Greer S, Jetter R, Kunst L, Wu X, Yephremov A, Samuels L.** 2007. Characterization of *Arabidopsis* ABCG11/WBC11, an ATP binding cassette (ABC) transporter that is required for cuticular lipid secretion. *The Plant Journal* **52**, 485–498.
- Bodnar WM, Blackburn RK, Krise JM, Moseley MA.** 2003. Exploiting the complementary nature of LC/MALDI/MS/MS and LC/ESI/MS/MS for increased proteome coverage. *Journal of the American Society of Mass Spectrometry* **14**, 971–979.
- Bonaventure G, Beisson F, Ohlrogge J, Pollard M.** 2004. Analysis of the aliphatic monomer composition of polyesters associated with *Arabidopsis* epidermis: occurrence of octadecacis-6, cis-9-diene-1,18-dioate as the major component. *The Plant Journal* **40**, 920–930.
- Buda GJ, Isaacson T, Matas AJ, Paolillo DJ, Rose JKC.** 2009. Three-dimensional imaging of plant cuticle architecture using confocal scanning laser microscopy. *The Plant Journal* **60**, 378–385.
- Cheng CS, Samuel D, Liu YJ, Shyu JC, Lai SM, Lin KF, Lyu PC.** 2004. Binding mechanism of nonspecific lipid transfer proteins and their role in plant defense. *Biochemistry* **43**, 13628–13636.
- Cruz MAL, Gomes VM, Fernandes KVS, Machado OLT, Xavier J.** 2002. Identification and partial characterization of a chitinase and a beta-1,3-glucanase from *Copernicia cerifera* wax. *Plant Physiology and Biochemistry* **40**, 11–16.
- Debono A, Yeats TH, Rose JKC, Bird D, Jetter R, Kunst L, Samuels L.** 2009. *Arabidopsis* LTPG is a glycosylphosphatidylinositol-anchored lipid transfer protein required for export of lipids to the plant surface. *The Plant Cell* **21**, 1230–1238.
- Franke R, Briesen I, Wojciechowski T, Faust A, Yephremov A, Nawrath C, Schreiber L.** 2005. Apoplastic polyesters in *Arabidopsis* surface tissues—a typical suberin and a particular cutin. *Phytochemistry* **66**, 2643–2658.
- Friedland N, Liou HL, Lobel P, Stock AM.** 2003. Structure of a cholesterol-binding protein deficient in Niemann–Pick type C2 disease. *Proceedings of the National Academy of Sciences, USA* **100**, 2512–2517.
- Gidda SK, Shockey JM, Rothstein SJ, Dyer JM, Mullen RT.** 2009. *Arabidopsis thaliana* GPAT8 and GPAT9 are localized to the ER and possess distinct ER retrieval signals: functional divergence of the dilysine ER retrieval motif in plant cells. *Plant Physiology and Biochemistry* **47**, 867–879.
- Gonzalez-Bosch C, Brummell DA, Bennett AB.** 1996. Differential expression of two endo-1,4-beta-glucanase genes in pericarp and locules of wild-type and mutant tomato fruit. *Plant Physiology* **111**, 1313–1319.
- Graca J, Schreiber L, Rodrigues J, Pereira H.** 2002. Glycerol and glyceryl esters of omega-hydroxyacids in cutins. *Phytochemistry* **61**, 205–215.
- Hulsen T, de Vlieg J, Alkema W.** 2008. BioVenn—a web application for the comparison and visualization of biological lists using area-proportional Venn diagrams. *BMC Genomics* **9**, 488.
- Ichikawa S, Takai T, Yashiki T, Takahashi S, Okumura K, Ogawa H, Kohda D, Hatanaka H.** 2009. Lipopolysaccharide binding of the mite allergen Der f 2. *Genes to Cells* **14**, 1055–1065.
- Isaacson T, Damasceno CM, Saravanan RS, He Y, Catalá C, Saladié M, Rose JKC.** 2006. Sample extraction techniques for enhanced proteomic analysis of plant tissues. *Nature Protocols* **1**, 769–774.
- Isaacson T, Kosma DK, Matas AJ, et al.** 2009. Cutin deficiency in the tomato fruit cuticle consistently affects resistance to microbial infection and biomechanical properties, but not transpirational water loss. *The Plant Journal* **60**, 363–377.
- Isaacson T, Rose JKC.** 2006. The plant cell wall proteome, or secretome. In: Finnie C, ed. *Plant proteomics*. Oxford: Blackwell, 175–209.
- Jerala R.** 2007. Structural biology of the LPS recognition. *International Journal of Medical Microbiology* **297**, 353–363.
- Jetter R, Kunst L, Samuels AL.** 2006. Composition of plant cuticular waxes. In: Riederer, M, Müller C, eds. *Biology of the plant cuticle*. Oxford: Blackwell, 145–181.
- Kannangara R, Branigan C, Liu Y, Penfield T, Rao V, Mouille G, Hofte H, Pauly M, Riechmann JL, Broun P.** 2007. The transcription factor WIN1/SHN1 regulates cutin biosynthesis in *Arabidopsis thaliana*. *The Plant Cell* **19**, 1278–1294.
- Krolikowski KA, Victor JL, Wagler TN, Lolle SJ, Pruitt RE.** 2003. Isolation and characterization of the *Arabidopsis* organ fusion gene *HOTHEAD*. *The Plant Journal* **35**, 501–511.
- Kurdyukov S, Faust A, Nawrath C, et al.** 2006a. The epidermis-specific extracellular BODYGUARD controls cuticle development and morphogenesis in *Arabidopsis*. *The Plant Cell* **18**, 321–339.
- Kurdyukov S, Faust A, Trenkamp S, Bar S, Franke R, Efremova N, Tietjen K, Schreiber L, Saedler H, Yephremov A.** 2006b. Genetic and biochemical evidence for involvement of *HOTHEAD* in the biosynthesis of long-chain α -, ω -dicarboxylic fatty acids and formation of extracellular matrix. *Planta* **224**, 315–329.
- Le LQ, Lorenz Y, Scheurer S, Fötisch K, Enrique E, Bartra J, Biemelt S, Vieths S, Sonnewald U.** 2006. Design of tomato fruits with reduced allergenicity by dsRNAi-mediated inhibition of ns-LTP (Lyc e 3) expression. *Plant Biotechnology Journal* **4**, 231–242.
- Lee SJ, Saravanan RS, Damasceno CMB, Yamane H, Kim BD, Rose JKC.** 2004. Digging deeper into the plant cell wall proteome. *Plant Physiology and Biochemistry* **42**, 979–988.
- Leide J, Hildebrandt U, Reussing K, Riederer M, Vogg G.** 2007. The developmental pattern of tomato fruit wax accumulation and its impact on cuticular transpiration barrier properties: effects of a deficiency in a beta-ketoacyl-coenzyme a synthase (LeCER6). *Plant Physiology* **144**, 1667–1679.
- Li Y, Beisson F, Koo AJ, Molina I, Pollard M, Ohlrogge J.** 2007. Identification of acyltransferases required for cutin biosynthesis and production of cutin with suberin-like monomers. *Proceedings of the National Academy of Sciences, USA* **104**, 18339–18344.

- Li-Beisson Y, Pollard M, Sauveplane V, Pinot F, Ohlrogge J, Beisson F.** 2009. Nanoridges that characterize the surface morphology of flowers require the synthesis of cutin polyester. *Proceedings of the National Academy of Science, USA* **106**, 22008–22013.
- Martin JT, Juniper BE.** 1970. *The cuticles of plants*. London: Edward Arnold, 147.
- Mintz-Oron S, Mandel T, Rogachev I, et al.** 2008. Gene expression and metabolism in tomato fruit surface tissues. *Plant Physiology* **147**, 823–851.
- Mueller LA, Solow TH, Taylor N, et al.** 2005. The SOL Genomics Network: a comparative resource for Solanaceae biology and beyond. *Plant Physiology* **138**, 1310–1317.
- Nakazono M, Qiu F, Borsuk LA, Schnable PS.** 2003. Laser-capture microdissection, a tool for the global analysis of gene expression in specific plant cell types: identification of genes expressed differentially in epidermal cells or vascular tissues of maize. *The Plant Cell* **15**, 583–596.
- Ohto U, Fukase K, Miyake K, Satow Y.** 2007. Crystal structures of human MD-2 and its complex with antiendotoxic lipid IVa. *Science* **316**, 1632–1634.
- Panikashvili D, Shi JX, Schreiber L, Aharoni A.** 2009. The Arabidopsis DCR encoding a soluble BAHD acyltransferase is required for cutin polyester formation and seed hydration properties. *Plant Physiology* **151**, 1773–1789.
- Perkins DN, Pappin DJ, Creasy DM, Cottrell JS.** 1999. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* **20**, 3551–3567.
- Pfaffl MW, Horgan GW, Dempfle L.** 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Research* **30**, e36.
- Pighin JA, Zheng H, Balakshin LJ, Goodman IP, Western TL, Jetter R, Kunst L, Samuels AL.** 2004. Plant cuticular lipid export requires an ABC transporter. *Science* **306**, 702–704.
- Plant AL, Cohen A, Moses MS, Bray EA.** 1991. Nucleotide sequence and spatial expression pattern of a drought- and abscisic acid-induced gene of tomato. *Plant Physiology* **97**, 900–906.
- Pollard M, Beisson F, Li Y, Ohlrogge JB.** 2008. Building lipid barriers: biosynthesis of cutin and suberin. *Trends in Plant Science* **13**, 236–246.
- Pyee J, Yu H, Kolattukudy PE.** 1994. Identification of a lipid transfer protein as the major protein in the surface wax of broccoli (*Brassica oleracea*) leaves. *Archives of Biochemistry and Biophysics* **311**, 460–468.
- Qin Q, Bergmann CW, Rose JKC, Saladié M, Kolli VS, Albersheim P, Darvill AG, York WS.** 2003. Characterization of a tomato protein that inhibits a xyloglucan-specific endoglucanase. *The Plant Journal* **34**, 327–338.
- Riederer M.** 2006. Introduction: biology of the plant cuticle. In: Riederer M, Müller C, eds. *Biology of the plant cuticle*. Oxford: Blackwell, 1–10.
- Reina JJ, Guerrero C, Heredia A.** 2007. Isolation, characterization, and localization of AgaSGNH cDNA: a new SGNH-motif plant hydrolase specific to *Agave americana* L. leaf epidermis. *Journal of Experimental Botany* **58**, 2717–2731.
- Ruzin SE.** 1999. *Plant microtechnique and microscopy*. Oxford: Oxford University Press.
- Saldanha AJ.** 2004. Java Treeview—extensible visualization of microarray data. *Bioinformatics* **20**, 3246–3248.
- Samuels L, Kunst L, Jetter R.** 2008. Sealing plant surfaces: cuticular wax formation by epidermal cells. *Annual Review of Plant Biology* **59**, 683–707.
- Schneiderbauer A, Sandermann HJ, Ernst D.** 1991. Isolation of functional RNA from plant tissues rich in phenolic compounds. *Analytical Biochemistry* **197**, 91–95.
- Shepherd RW, Wagner GJ.** 2007. Phyloplane proteins: emerging defenses at the aerial frontline? *Trends in Plant Science* **12**, 51–56.
- Shevchenko A, Wilm M, Vorm O, Mann M.** 1996. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Analytical Chemistry* **68**, 850–858.
- Srinivasasainagendra V, Page GP, Mehta T, Coulibaly I, Loraine AE.** 2008. CressExpress: a tool for large-scale mining of expression data from *Arabidopsis*. *Plant Physiology* **147**, 1004–1016.
- Suh MC, Samuels AL, Jetter R, Kunst L, Pollard M, Ohlrogge J, Beisson F.** 2005. Cuticular lipid composition, surface structure, and gene expression in *Arabidopsis* stem epidermis. *Plant Physiology* **139**, 1649–1665.
- Tamura K, Dudley J, Nei M, Kumar S.** 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**, 1596–1599.
- Thompson JD, Higgins DG, Gibson TJ.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**, 4673–4680.
- Vogg G, Fischer S, Leide J, Emmanuel E, Jetter R, Levy AA, Riederer M.** 2004. Tomato fruit cuticular waxes and their effects on transpiration barrier properties: functional characterization of a mutant deficient in a very-long-chain fatty acid β -ketoacyl-CoA synthase. *Journal of Experimental Botany* **55**, 1401–1410.
- Yang Y, Zhang S, Howe K, Wilson DB, Moser F, Irwin D, Thannhauser TW.** 2007. A comparison of nLC-ESI-MS/MS and nLC-MALDI-MS/MS for GeLC-based protein identification and iTRAQ-based shotgun quantitative proteomics. *Journal of Biomolecular Techniques* **18**, 226–237.
- Yeats TH, Rose JKC.** 2008. The biochemistry and biology of extracellular plant lipid-transfer proteins (LTPs). *Protein Science* **17**, 191–198.