Tomato GDSL1 Is Required for Cutin Deposition in the Fruit Cuticle

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The plant cuticle consists of cutin, a polyester of glycerol, hydroxyl, and epoxy fatty acids, covered and filled with waxes. While the biosynthesis of cutin building blocks is well documented, the mechanisms underlining their extracellular deposition remain unknown. Among the proteins extracted from dewaxed tomato (Solanum lycopersicum) peels, we identified GDSL1, a member of the GDSL esterase/acylhydrolase family of plant proteins. GDSL1 is strongly expressed in the epidermis of growing fruit. In GDSL1-silenced tomato lines, we observed a significant reduction in fruit cuticle thickness and a decrease in cutin monomer content proportional to the level of GDSL1 silencing. A significant decrease of wax load was observed only for cuticles of the severely silenced transgenic line. Fourier transform infrared (FTIR) analysis of isolated cutins revealed a reduction in cutin density in silenced lines. Indeed, FTIR-attenuated total reflectance spectroscopy and atomic force microscopy imaging showed that drastic GDSL1 silencing leads to a reduction in ester bond cross-links and to the appearance of nanopores in tomato cutins. Furthermore, immunolabeling experiments attested that GDSL1 is essentially entrapped in the cuticle proper and cuticle layer. These results suggest that GDSL1 is specifically involved in the extracellular deposition of the cutin polyester in the tomato fruit cuticle.

INTRODUCTION

Cuticle is a complex assembly of a hydrophobic biopolymer (i.e., cutin, coated and filled with waxes). Waxes are mixtures of alliphatic molecules with very long hydrocarbon chains, including alkanes, fatty alcohols, aldehydes, acids, and esters as well as secondary metabolites (i.e., cyclic triterpenoids, phenylpropa- noids, and phenolics) (Waltson, 1990; Schnurr et al., 2004). Cutin, the skeleton of cuticle, is a polyester of noids, and phenolics) (Waltson, 1990; Schnurr et al., 2004). Cutin plays a major role as the primary physical barrier in plant cuticles experiencing biotic and abiotic stress (Reina-Pinto and Yephremov, 2009). Cutin could also play a major role in plant morphogenesis. Actually, cutin regulates cell adhesion during plant development by preventing organ fusion as observed in Arabidopsis thaliana cutin-deficient mutants (Sieber et al., 2000; Nawrath, 2006; Shi et al., 2011) or by regulating hull adhesion in cereal grains (Taketa et al., 2008). Taken together, these observations underline the importance of determining cutin structure and biosynthesis to delineate the biological function of plant cuticles.

Considerable progress in the understanding of cutin formation has been gained by the screening of Arabidopsis organ fusion mutants (Wellesen et al., 2001; Schnurr et al., 2004; Xiao et al., 2004; Bessire et al., 2007; Kannangara et al., 2007) and the discovery of Wax inducer/Shine 1 (WIN1/SHN1) protein, a transcription factor that regulates wax and cutin biosynthesis (Aharoni et al., 2004; Kannangara et al., 2007; Shi et al., 2011). The formation of the extracellular cutin polymer involves three major steps: (1) synthesis of the cutin precursors, (2) their translocation and diffusion in the apoplast, and (3) their polymerization. Cutin monomers (i.e., hydroxy- and epoxy-fatty acids) are synthesized in epidermal cells. The major pathway involves ω-hydroxylases, such as HOTHEAD oxidase (HTH) or cytochrome P450 of the CYP86A subfamily (Wellesen et al., 2001; Kudryukov et al., 2006a), long-chain acyl-CoA synthases (Schnurr et al., 2004; Lü et al., 2009), and glycerol-3-phosphate acyltransferases (Beisson et al., 2007; Li et al., 2007b; Yang et al., 2010). Midchain hydroxylation of ω-hydroxypalmitate to form the dihydroxyypalmitate is catalyzed by a specific cytochrome P450, CYP77A6 (Li-Beisson et al., 2009). Another pathway, the lipoxigenase-peroxygenase pathway, could be involved in the midchain epoxidation of C18 unsaturated fatty acids (Blee and Schuber, 1993). Glycerol-3-phosphate

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acyltransferases catalyze the transfer of acyl-CoA to glycerol-3-phosphate to form lysophosphatidic acid, a precursor of acylglycerols and especially of monoaoylglycerol (Pollard et al., 2008). An elemental building block of the cutin polymer (Graça et al., 2002). In Arabidopsis, GLYCERAL-3-PHOSPHATE ACYLTRANSFERASE4 (GPAT4) and GPAT6 are involved in cutin biosynthesis and are unique glycerol-3-phosphate acyltransferases containing a regio-selective acyltransferase and a phosphatase leading to the production of 2-monoglycerides (Yang et al., 2010). The apoplastic secretion of sn-2 monoglycerides observed upon the ectopic overexpression of the suberin-associated and sn-2 regioselective GPAT5 strengthened their role as synthons (i.e., biosynthetic monomers) for extracellular cutin polymerization (Li et al., 2007a; Yang et al., 2010). Finally, a cytosolic acyltransferase of the BAHD family of acyltransferase with in vitro diacylglycerol-acyltransferase activity was involved in the incorporation of dihydroxymalminate into the cutin of Arabidopsis flowers. It was suggested that this diacylglycerol-acyltransferase could be involved in the formation of other cutin precursors, cutin oligomers, or triacylglycerols (Rani et al., 2010; Panikashvili et al., 2011), whereas members of the BAHD family of acyltransferase usually catalyze the acylation of secondary metabolites (D’Auria, 2006).

The transport of hydrophobic cutin precursors and their polymerization in the aqueous cell wall–filled apoplast are less documented. ATP binding cassette (ABC) transporters located in the plasmalemma of epidermal cells are required for both cutin and wax deposition (Bessire et al., 2007; Panikashvili et al., 2007, 2011; Bird, 2008; Chen et al., 2011) and could be involved in the apoplastic translocation of the cutin precursors. Owing to their extracellular localization, generally close to cuticle surfaces, and their ability to bind lipids in a hydrophobic cavity, lipid transfer proteins often have been proposed to fulfill this function (Douliez et al., 2000; Blein et al., 2002). However, no functional genomics study has confirmed this transport function or the involvement of these proteins in cuticle assembly.

Although the polyesterification of monomers through a chemical process could not be excluded (Heredia-Guerrero et al., 2009), most studies since the pioneering work of Croteau and Kolattukudy (1973, 1974) have focused on an enzyme-catalyzed mechanism of polymerization. The α/β hydrolase BODYGUARD (BDG) has been identified as a candidate protein to fulfill this role in Arabidopsis. However, this protein does not fit perfectly with its proposed function in cutin polymerization because mutation of this protein induces other pleiotropic effects besides cuticle modification (Kurdyukov et al., 2006b).

Recently, the proteins of the Gly-Asp-Ser-Leu family of esterases/acylhydrolases, commonly called GDSL-lipases, which, like BDG, belong to the α/β hydrolase family, have triggered considerable interest. Indeed, GDSL-lipases are expressed in epidermal cells of the peel, close to the cuticular membranes (Lemaire-Chamley et al., 2005; Reina et al., 2007; Mintz-Oron et al., 2008; Matas et al., 2010; Yeats et al., 2010), and the expression of an Arabidopsis GDSL-lipase is upregulated by WIN1/SHN1, a transcription factor that regulates cutin deposition (Kannangara et al., 2007; Shi et al., 2011). In addition, GDSL-lipases are synthesized as preproteins containing signal peptides predicted to facilitate extracellular exportation. This extracellular location was confirmed for most characterized GDSL-lipases in latex (Abdelkafi et al., 2009), in nectar (Kram et al., 2008), in the secretome of plant cells (Oh et al., 2005; Naranjo et al., 2006; Hong et al., 2008; Kusumawati et al., 2008), and in sporoellenin, a cutin-like polymer of the pollen coat (Updegrave et al., 2009). However, to date, most of the studies have highlighted the role of GDSL-lipases in the tolerance of plants to biotic and abiotic stress (Oh et al., 2005; Naranjo et al., 2006; Hong et al., 2008). Nevertheless, through the cuticular defects induced by the ectopic overexpression of an Arabidopsis pollen GDSL-lipase (CUTICLE DESTRUCTING FACTOR1 [CDEF1]), Takahashi et al. (2010) recently suggested that CDEF1 could be a plant cutinase.

Tomato (Solanum lycopersicum) has recently become an attractive plant model because of the development of functional genomic tools and the recent completion of the tomato genome sequence. In addition, the cuticle of tomato fruits can be isolated (López-Casado et al., 2007), and its cutin polymer was found to be dominated by a single monomer (i.e., 9,16 hydroxy-palmitic acid) (Graça et al., 2002). Furthermore, the tomato fruit is astomatous, providing a cuticle with an intact surface. In this study, we characterize tomato GDSL1, the main GDSL-lipase protein expressed in the epidermis of developing tomato fruit. Silencing of GDSL1 expression by the RNA interference (RNAi) strategy alters cutin load and cutin structure of the fruit. GDSL1 localized to both the cuticle proper and cuticle layer. We therefore conclude that the extracellular and cutin-embedded GDSL-lipase is specifically required for the formation of the cutin polymer.

RESULTS

Proteome Analysis of Tomato Fruit Cutinized Exocarp Highlights an Epidermis GDSL-Lipase

One of the most abundant proteins identified from tomato cutin, with 54% sequence coverage, was a GDSL-lipase (accession number SGN-585129) (Figure 1A). Although GDSL-lipases form a multigenic family in tomato, this protein, hereafter named GDSL1, was the sole GDSL-lipase identified. A proteome analysis performed on material released from tomato fruits immersed in methanol/chloroform identified two additional GDSL-lipases (SGN-U583101 and SGN-U579520) and a peptide of GDSL1 (Yeats et al., 2010). A large number of cell wall–modifying proteins or cellular proteins were also identified. Real-time RT-PCR analysis of GDSL1 expression in tomato fruit tissues indicated that GDSL1 transcripts accumulate specifically in the exocarp (Figure 1B). The highest transcript accumulation was reached at 20 d postanthesis (DPA) in the ‘Ailsa Craig’ tomato fruit (see Supplemental Figure 1 online) (i.e., during the cell expansion phase) (Mounet et al., 2009).

The full-length GDSL1 cdNA sequence (1042 bp) revealed an open reading frame specifying a 362–amino acid putative protein with a predicted N-terminal signal peptide of 19 residues (see Supplemental Figure 2 online). Like 99 of the 107 Arabidopsis GDSL-lipases (Ling, 2008), tomato GDSL1 is predicted to be located extracellularly. Although the overall sequence of the members of this large multigenic family is barely conserved, five sequence blocks are highly conserved and were also found in
tomato GDSL1 (see Supplemental Figure 2 online). The three amino acids of the catalytic triad are present in block I (Ser-32) and block V (His-326), whereas, as previously suggested, the Asp residue is either localized to block III (Asp-166) (Upton and Buckley, 1995) or block V (Asp-323), immediately upstream of His (Akoh et al., 2004).

The P35S-SlGDSL1 RNAi Transgenic Lines Display Major Changes in Fruit Cuticle

To delineate the function of GDSL1 in tomato, we generated GDSL1 RNAi-silenced tomato transgenic lines (P35S-SlGDSL1 RNAi lines), hereafter named L-10, L-4, L-3, and L-17, which showed moderate to severe downregulation of GDSL1. GDSL1 transcript levels were highest in 12-DPA fruit and decreased thereafter during fruit development. At 12 DPA, silencing appeared less effective in L-10 and L-4, whereas L-3 and L-17 presented a 70 to 95% reduction in GDSL1 transcript abundance (Figure 2A). From 20 DPA on, all of the transgenic lines showed a reduction in the expression of GDSL1, ranging from 78% (L-4) to 98% (L-17) compared with the wild type. Immunoblotting revealed a decrease in GDSL1 content in all transgenic lines, especially in line V (Asp-323), immediately upstream of His (Akoh et al., 2004).

In fruits from L-10 and L-4, the cuticular layer surrounded all the fruit pavement epidermal cells as in wild-type fruit. By contrast, cuticularization of anticlinal walls and subepidermal deposition of cuticular material was significantly reduced in L-3 and almost disappeared in L-17 (Figure 4A). The most striking change in transgenic lines was the impressive reduction in cuticle thickness above the pavement epidermal
cells, especially in L-3 and L-17, where cuticle was reduced to a thin film at the surface of the fruit (Figure 4C). Because mutation in the epidermal-expressed gene BDG, proposed to control cuticle formation, also affects epidermis proliferation/differentiation status in Arabidopsis (Kurdyukov et al., 2006b), we examined the collenchyma cell layers underneath the epidermis. However, no visible alteration of epidermal cells was observed in fruit of the RNAi lines, even in L-17 (Figure 4E). Altogether, these results suggest that silencing of GDSL1, which induces major changes in the cuticle, has no other apparent pleiotropic effect on plant and fruit development.

**GDSL1 Is Embedded in the Cutinized Matrix of Tomato Cuticle**

To gain insight into the relationship between GDSL1 and cuticle biosynthesis, the localization of GDSL1 in the cuticle was monitored (Figure 5; see Supplemental Figure 3 online). According to the expression profile of GDSL1 (Figure 2), immunolocalization of the corresponding protein was performed in the exocarp of wild-type and L-17 and L-3 plants harvested at 20 DPA. In wild-type fruits, the cuticle forms an electron dense layer of ~6 μm (see Supplemental Figure 3D online) deposited over a less dense layer corresponding to cell walls. Cell wall cutinization of wild-type fruits occurs between epidermal cells, thus forming anticlinal pegs (Figure 4). In the L-17 line, anticlinal pegs are almost absent and the cuticular layer is very thin but nevertheless regularly deposited (~1.5 μm) on the surface of the epidermal cells (Figures 4A and 5C).

Both immunogold and fluorescence labeling revealed that GDSL1 was located in the cuticular layer and to a lesser extent in the cytoplasm of the underlying epidermal cells (Figure 5). Intense labeling was observed in the anticlinal pegs and in the cutin region between epidermal cells, which begin to undergo cutinization at this stage of fruit development (Figure 5J). Auromine staining of 20-DPA fruits allowed the distinction between the cuticle proper and cuticle layer (see Supplemental Figure 3 online). GDSL1 labeling was observed in the both cuticle layer and cuticle proper, thus confirming that this protein is associated to cutin. Finally, no labeling was observed in the noncutinized cell wall layer of the exocarp (see Supplemental Figure 3 online).

In agreement with GDSL1 expression, the level of detected protein was greater in the wild type and in L-3 than in L-17 in both experimental conditions (Figure 5).

**GDSL1 Silencing Specifically Impacts Cutin Monomer Deposition**

The effect of GDSL1 silencing on cutin and wax biosynthesis was further characterized in red ripe fruits (Table 1). Consistent with the reduction in cuticle thickness (Figure 4), the level of cutin monomers per surface unit decreased proportionally to the reduction in GDSL1 expression (Table 1). Indeed, the total amount of cutin monomers represented 95.2% (L-10), 52% (L-3), and only 5% (L-17) of the level in wild-type fruits. In all lines, dihydroxyhexadecanoic acid was the most abundant monomer. The relative proportion of the different cutin constituents was generally well conserved within the various transgenic lines, except for 16-hydroxyhexadecanoic acid, which gradually decreased from 3% in the wild type (and L-10) to 0.7% in L-17, while the hexadecanoic acid increased from 0.5% (the wild type) to 2.4% (L-17).

We further investigated the effect of GDSL1 silencing on wax deposition in fruit. About half of the total waxes quantified were alkanes, 24% were fatty acids, and 23% were plant triterpenoids and sterols. Waxes were mostly composed of C31 alkanes (26% of total) and δ, β, or α amyrins (19.5%), in agreement with previous reports (Leide et al., 2007; Saladié et al., 2007; Isaacson et al., 2009). No significant difference in wax content and composition were detected when transgenic lines L-10, L-4, and L-3 are compared with the wild type (Table 1). Wax load was significantly affected only in the most severely affected L-17 line where the wax content dropped 10-fold when compared with the wild type and displayed a significant reduction in the proportion of sterol and terpenoid compounds. Thus, the decrease in wax content per surface unit did not follow the decrease in the corresponding cutin monomer content.

No accumulation of cutin monomers or monoglycerides (i.e., the building blocks of cutin) (Pollard et al., 2008) was detected in the fruit wax fractions or in the exocarp lipid extracts of the RNAi
Accordingly, no significant alteration in osmium-stained lipids was detected by transmission electron microscopy (TEM) in 20-DPA exocarps from L-17 compared with the wild type (see Supplemental Figure 4 online). In particular, we do not detect any increase in plastoglobules, as observed in cuticular Arabidopsis ltpg1 mutants (Lee et al., 2009b), or the presence of lipidic inclusions, as reported for ABC transporter Arabidopsis mutants impaired in the transport of cutin precursors (Bird et al., 2007; Bessire et al., 2011).

Finally, we analyzed the expression of some tomato candidate genes known to be involved in the Arabidopsis cutin biosynthesis pathway. Real-time RT-PCR analysis was performed in the exocarp of 20-DPA fruits from the most severely altered lines L-3 and L-17. The results were compared with those of the wild type and with the mildly affected L-10 line, which displays a fruit cutin composition roughly similar to that of wild-type plants (see Supplemental Figure 5 and Supplemental Table 1 online). A significant downregulation of CYP86A7, GPAT4, and GPAT6 and of three HTH-like genes as well as one lipid transfer protein gene (LTPG2) was observed in L-3 and L-17, in which GDSL1 is strongly silenced. These results suggest a possible feedback regulation of the cutin biosynthesis pathway.

![Figure 3. Analysis of Fruit Brightness, Fruit Size, Postharvest Water Loss, and Permeability in P35S-SigGDSL1 RNAi Plants Compared with the Wild Type.](image)

(A) Fruit brightness was measured using an L*a*b* colorimeter, where L corresponds to luminance in red ripe fruits at breaker + 7. Fruit size was represented using mean fruit surface value (extrapolated from diameter measurements). Comparisons were made between wild-type and transgenic lines (L-10, L-4, L-3, and L-17). Vertical bars represent s.e. (n = 13 to 19). Asterisks indicate a significant difference with fruits of the wild type (t test, P < 0.05). AU, arbitrary units; WT, the wild type.

(B) Postharvest water loss of red ripe fruits from wild-type plants and L10, L-4, L-3, and L-17 transgenic lines was measured over a 38-d period. Values are means (±s.e) of six samples from three biological replicates stored at 30°C.

(C) TB (1% [v/v] solution) coloration of 20-DPA fruits from wild-type and transgenic lines.

[See online article for color version of this figure.]
pathway, in agreement with the absence of cutin monomer accumulation and the absence of alteration in epidermal cell ultrastructure in the SlGDSL1-silenced plants.

**GDSL1 Silencing Affects the Structure of the Cutin Polyester**

Attenuated total reflectance (ATR)-Fourier transform infrared (FTIR) analysis was performed on cutin isolated from red ripe fruits (breaker + 7 d). The spectra of dewaxed cutinized fragments are dominated by aliphatic methylene (CH$_2$) asymmetric and symmetric stretching vibrations of its aliphatic monomers at 2919 and 2850 cm$^{-1}$ and the carbonyl (CO) stretching vibration of ester bonds at 1729 cm$^{-1}$ (see Supplemental Figure 6A online). These bands are specific to cutin polyester, since CH$_2$ and CO vibrations were either weak or absent, respectively, in the polysaccharide residues obtained after cutin depolymerization (see Supplemental Figure 6B online). When focusing on these stretching vibrations and compared with the wild type, a gradual decrease of absorbance of both CH$_2$ and CO bands in transformant lines consistent with the severity of GDSL1 silencing was observed (Figure 6C). The penetration depth in our experimental conditions (i.e., incident light angle of 45° and a diamond refractive index of 2.4) was estimated to be 0.7 µm at 3000 cm$^{-1}$ and 1.1 µm at 1000 cm$^{-1}$ for a refractive index of cutin estimated at 1.5 (da Luz, 2006). Considering the thickness measurements (Figure 4), this means that, in wild-type and all transformant lines, the infrared signal of methylene and carbonyl groups, which is specific to the cutin polyester, arose mainly from the cutin deposited on the cutinized surface of epidermal cells (from 8 to 1.5 µm for the wild type and L-17, respectively; Figure 4). Therefore, the gradual decrease in absorbance is not mainly related to the decrease in cutin thickness but to the decrease in cutin density. After normalization of the FTIR spectra, no significant differences were observed for methylene bands, indicating that the conformation of aliphatic chains was not significantly affected by GDSL1 silencing (Figure 6A). Conversely, significant modification in the carbonyl vibration, which is associated with a reduction in GDSL1 expression (Figure 6A). The carbonyl stretching vibration centered at 1729 cm$^{-1}$ displayed a shoulder at 1712 cm$^{-1}$. Similar lower frequency carbonyl bands have been observed in the gel state of phospholipids with iso- and anteiso-branched fatty acids (Mantsch et al., 1987) and in polyurethane polymers (Queiroz et al., 2003). Such splitting of the carbonyl band in two components is a common feature, attributed to hydrogen bonding (Blume et al., 1998; Queiroz et al., 2003). The relative increase in the intensity of the 1712 cm$^{-1}$ shoulder suggests that the ester carbonyl groups are more involved in hydrogen bonding following the decrease in GDSL1 expression. This could either originate from a higher interaction with the OH groups of the cell
wall polysaccharides or from a modification in the extent of cutin polymerization due to an increase in nonesterified hydroxyl groups in the cutin polymer. By integrating methylene stretching and carbonyl bands specific to the alkyl chain and ester groups of cutin, respectively, it was possible to determine a ratio that can be considered as an index of the extent of polymerization (Figure 6C). When compared with wild-type cutin, a significant decrease in polymerization was observed in L-17, in which GDSL1 is efficiently silenced.

Since tomato cutin is mainly composed of dihydroxyhexadecanoic acid, it contains more hydroxyl groups than carbonyl groups. Consequently, a lower polymerization index does not necessarily induce a corresponding increase in the carboxylate band. Indeed, the carboxylate vibration at 1590 cm$^{-1}$ was not observed in both wild-type and L-17 cutin. Besides, it was recently demonstrated that the structure of wild-type tomato cutin is characterized by a high rate of branched linkage, where the midchain hydroxyl groups are more involved in ester bonds than the omega-hydroxyl groups (Graça and Lamosa, 2010). Therefore, the polymerization defect in L-17 could mainly affect midchain branching of the cutin polymer.

At both 20-DPA and the red ripe stages, atomic force microscopy (AFM) highlighted differences in cutin surface morphology between wild-type and RNAi lines, in particular in L-3 and L-17 (Figure 7; see Supplemental Figure 7 online). For the 20-DPA and red ripe fruits, AFM images showed a continuous cutin displaying the imprint of the underlying epidermal cells. In addition, the comparison of cutin AFM images from 20-DPA and red ripe stages shows that, during fruit development, a higher deposition of cutin occurs in the intercellular region than on the surface of epidermal cells. This result is in good agreement with the intense GDSL1 immunolabelling observed in the cutin region between epidermal cells in 20-DPA wild-type exocarp (Figure 5J).

Figure 5. Immunolocalization of GDSL1 in the Exocarp of 20-DPA Tomato Fruits from Wild-Type and Transgenic Lines L-3 and L-17.

Tomato exocarp from wild-type ([A], [D], [G], and [J]) and L-3 ([B], [E], [H], and [K]) and L-17 ([C], [F], [I], and [L]) transgenic lines were embedded in LR white resin and labeled with polyclonal anti-GDSL1 antibodies coupled either with gold-conjugated antibodies ([A] to [I]) or fluorescent dyes ([J] to [L], bright field in inset). Black dots represent gold labeling. CL, cuticular layer; CW, cell wall; Cyt, cytoplasm, SCD, subepidermal cutin deposition; V, vacuole; Wt, the wild type. For immunofluorescence labeling ([J] to [L]), bars = 20 µm and the corresponding light micrographs are presented in the inset.
Higher magnification AFM images of cutin isolated from 20-DPA fruits show an amorphous smooth surface with regular structure, including some nodules-structure from 20 to 80 nm as previously described (Round et al., 2000; Benitez et al., 2004) (Figure 7). The calculated root mean squared roughness parameter ranged from 7 to 12 nm at 20 DPA. This parameter appears to be approximately invariant for all of the RNAI lines. Nevertheless, nanopores with a mean diameter of ∼20 nm were observed on the cutin surfaces of some 20-DPA fruits of L-17. These irregularly distributed nanopores indicate a defect in the continuity of the cutin structure, as highlighted by the TB test, and are probably related to modifications of the polymerization process, as suggested by ATR-FTIR data (Figure 6C).

**DISCUSSION**

**GDSL1 Is an Extracellular Protein Primarily Involved in Cutin Deposition**

From the synthesis of cutin monomers (i.e., hydroxy-fatty acids) and cutin building blocks (either monoglycerides or oligomers) to the translocation in the apoplast compartment through ABC
transporters (Pollard et al., 2008; Yang et al., 2010), much progress has recently been made in delineating the intricate pathway leading to cutin deposition. In this research area, the black box remains the mechanisms of extracellular polymerization of cutin. The three-dimensional structure of the cutin polyester is still unknown, and solid state NMR techniques have provided only limited information on the dynamics (Zlotnik-Mazori and Stark, 1988; Round et al., 2000) and cross-linking of cutin polyesters (Fang et al., 2001; Deshmukh et al., 2003). Genetic approaches based on the identification of mutant plants exhibiting cuticular defects or on reverse genetics analysis of candidate genes are promising but, to date, have provided only limited characterization of the extracellular assembly of cutin polyesters.

In this article, we demonstrate that an extracellular protein of the ubiquitous plant GDSL-lipase family (i.e., S. lycopersicum GDSL1) affects cutin deposition. Several lines of evidence reported here support this role. First, in the tomato fruit, GDSL1 is expressed in the exocarp of developing fruits (Figure 1). Second, proteomic analysis and immunolabeling showed that GDSL1 is embedded in the extracellular cutinized matrix of fruit cuticles, while it is absent from the noncutinized cell wall (Figure 5). Third,
we observed that the extent of the alterations of cutin deposition (i.e., cuticle thickness, cutin monomer contents, cutin monomer composition, and cutin density, considered here as the cutin monomer to cutin-associated cell wall ratio) is closely related to the severity of GDSL1 silencing. In addition, GDSL1 silencing did not lead to pleiotropic effects and the cuticle appears to be the primary affected target. However, the wax load of transgenic fruit cuticles was significantly impacted only when the expression of GDSL1 was severely reduced and this likely represents a secondary effect.

A biological role of GDSL-lipases in cutin deposition is in agreement with previous data showing that these proteins are localized to the apoplastic space of seedlings (Takahashi et al., 2010; Teutschbein et al., 2010) or to the epidermis of leaves and fruits (Reina et al., 2007; Matas et al., 2010; Yeats et al., 2010). Although the structure and composition of cutin were not characterized, the cosilencing of two Arabidopsis GDSL-lipases led to severe floral organ fusion and major changes in the architecture of epidermis cells (Shi et al., 2011). The cuticular localization of GDSL1 also fits perfectly with the role of these proteins in mediating the plant’s tolerance to biotic and abiotic stress (Oh et al., 2005; Naranjo et al., 2006; Hong et al., 2008). Indeed, cutin is recognized as a physical barrier as well as a source of signaling molecules that regulate the dialog between plants and their environment (Kolattukudy, 2001; Reina-Pinto and Yephremov, 2009).

It has been shown (Park et al., 2010) that the rice (Oryza sativa) T-DNA insertional wdl1 mutant, which exhibits increased water loss, was affected in the expression of a GDSL-lipase-like gene. However, WDL1 is only distantly related to the canonical plant GDSL-lipases and shows poor conservation of the invariant blocks I, II, and V. In particular, the GDSL motif, localized in block I, is replaced by glycine-serine-serine-isoleucine. Furthermore, this motif, which is involved in the catalytic triad of the GDSL-lipase family (Ling et al., 2006), is predicted to be localized to the putative signal peptide (SignalP and TargetP software). Finally, in contrast with GDSL1, WDL1 deficiency does not impact the cutin monomer content but leads to irregular cuticle deposition as well as to cell wall modifications.

It should be emphasized here that GDSL-lipases form a large multigenic family of plant proteins that are divided into three subfamilies (Volokita et al., 2011). The extremely conserved GDSL-motif and the highly conserved domains are not sufficient criteria for an ab initio prediction of the function of the protein. In support of such an assertion, one can mention that the maize (Zea mays) acetylcholine esterase, Brassica napus sinapine esterase, and the chlorogenate:gluconate caffeoyltransferase of tomato seedlings share the same GDSL motif and the five conserved domains with GDSL1 (Sagane et al., 2005; Clauss et al., 2008; Teutschbein et al., 2010).

**The GDSL1-Silenced Lines Do Not Display Pleiotropic Phenotypic Alterations**

Many Arabidopsis cutin mutants, such as bdg, fdh, lcr, and dcr (Pruitt et al., 2000; Wellesen et al., 2001; Kurdyukov et al., 2006b; Bird et al., 2007; Panikashvili et al., 2009), exhibit pleiotropic phenotypes, including organ fusion, abnormal development, or deformation of epidermal cells accompanied by distorted deposition of cutin polymer. Remarkably, the drastic (98%) reduction in GDSL1 expression and the corresponding decrease in cuticle thickness do not compromise fruit growth and development and do not induce any visible modification of the epidermal cell morphology.

Furthermore, in contrast with Arabidopsis mutants affected in the expression of cutin-specific ABC transporters (Bird et al., 2007; Bessire et al., 2011), we did not observe any accumulation of lipid inclusions and lamellar bodies in epidermis cells. This agrees with our analysis of the expression of some candidate genes of the cutin biosynthesis pathway. Indeed, the significant downregulation of oxidase genes (i.e., HOTHEAD-like and CYP86A7-like genes) and GPAT4 and GPAT6-like genes indicates a possible feedback regulation that could prevent epidermis cells from accumulating potentially toxic cutin monomers and precursors.

Only changes in cuticle permeability to hydrophilic molecules (TB) and increased postharvest water loss were observed in P35S-SigGDSL1 RNAi lines. The permeance properties of tomato cuticles were mostly affected in line L-17, where AFM images and ATR-FTIR analysis highlighted defects in cutin assembly. However, these defects are probably not sufficient to lead to major developmental abnormalities, like those observed in the Arabidopsis cutin mutants. This suggests that a 2% residual expression of GDSL1 provides a sufficient level of enzyme to sustain fruit development. However, at this stage, we cannot exclude the possibility that a more stringent GDSL1 silencing or a null mutation might trigger pleiotropic alterations and impair fruit development. Nevertheless, these results also indicate that tomato can afford a huge reduction in the thickness of fruit cuticle, as long as the cutin is homogeneously deposited. Such a conclusion is in full agreement with the characterized tomato cutin deficiency mutants cd1, cd2, and cd3, in which a 95%
Potential Mechanisms Relating GDSL1 to Extracellular Cutin Deposition

The impact of GDSL1 deficiency on cutin deposition raises the question of how this extracellular enzyme could operate in planta. Plant GDSL-lipases are generally depicted as acyl-hydrolases (Abdelkafi et al., 2009; Updegraff et al., 2009). Their hydrolytic activity has been widely associated with seed germination (Clauss et al., 2008), pollen hydration (Updegraff et al., 2009), pathogen defense (Oh et al., 2005; Kwon et al., 2009; Lee et al., 2009a), and the abiotic stress response (Hong et al., 2008; Zhou et al., 2009). In the P3SS-SIGDSL1 RNAi plants, both AFM analyses (showing the presence of nanopores) and FTIR spectra (indicating a lower polymerization index) support a potential polymerase activity of GDSL1. The acyl-hydrolase activity of plant GDSL-lipases is based on in vitro assays performed with recombinant proteins in diluted aqueous solutions, far from their genuine biological context. Actually, the localization of GDSL1 in the cuticle matrix and not in the cell walls shows that the protein operates in a peculiar hydrophobic environment. It is known that lipases are active in water-depleted organic solvents (Klibanov, 2001) and can catalyze the polyesteri

cation of hydroxy-fatty acids in such conditions (Mahapatro et al., 2004; Ebata et al., 2007). Moreover, the extent of polymerization is improved when the lipase is immobilized on an acrylic resin (Ebata et al., 2007). The ester precursors of the transesterification process could be the monoglycerides produced by specific intracellular GPATs (Yang et al., 2010) or oligomers (Panikashvili et al., 2011). The monoglycerides are secreted in the apoplastic compartment (Li et al., 2007a) and small lipid molecules, monoglycerides, and oligomers are compatible with the involvement of ABC transporters in cutin deposition (Bird, 2008). These putative substrates of GDSL-lipase also fit well with previous data indicating that esters are more convenient substrates than free fatty acids for the acyl-transfer activity in a hydrophobic context (Ebata et al., 2007). Therefore, the immobilization of GDSL1 in the cutin matrix, a water-depleted hydrophobic environment, can favor the reverse reaction of this lipase (i.e., transesterification activity of ester precursors). It was recently established, in vitro, that CD1 protein, identical to GDSL1, is endowed with acyltransferase activity and that 2-mono-(10,16-dihydroxyhexadecanoyl)-glycerol is a substrate for this enzyme (Yeats et al., 2012). This acyltransferase activity supports our major conclusion that GDSL1 plays an essential role in the process of cutin deposition during fruit cuticle development.

However, in planta, we also have to consider that GDSL1 can exhibit both hydrolase and transesterase activities. Indeed, a plant GDSL-lipase involved in pyrethrin biosynthesis harbors transesterase activity in vivo and esterase activity in vitro (Kikuta et al., 2003; Rose et al., 2002). These catalytic specificities are related to subtle structural modifications (Eklöf and Brumer, 2010). Different XTHs are expressed during organ development or upon environmental stress, processes that involve cell wall remodeling (Rose et al., 2002). In this regard, it was recently shown that the ectopic overexpression of a pollen GDSL-lipase CDEF1 in the vegetative organs of Arabidopsis disrupts the cuticle (Takahashi et al., 2010) in a manner similar to cute mutants expressing a fungal cutinase (Sieber et al., 2000). This apparent contradiction between GDSL1 and CDEF1 activities strengthens our hypothesis that GDSL-lipases could have hydrolase or transesterase activities, depending on whether they are present in the cell wall or aqueous apoplast, as observed for CDEF1, or in the hydrophobic cuticle, as shown here for GDSL1.

All our experimental data converge toward a key role of GDSL-lipase in the extracellular polymerization and/or cross-linking of the cutin polyester. However, cutin is a complex polyester that includes lipids, glycerol, and aromatics and is anchored to cell wall polysaccharides. Therefore, we cannot exclude the possibility that GDSL1 is involved in the cross-linking between cutin and the cell wall. In this regard, it was reported that TBL (for Trichome birefregence-like) genes containing a GDSL motif are involved in the esterification of pectic polymers within cellulose in Arabidopsis trichomes (Bischoff et al., 2010). In addition, a GDSL-lipase was involved in the esterification of phenolic compounds, which are minor components of the cuticle, in the apoplastic compartment of tomato seedlings (Teutschbein et al., 2010). To delineate this sophisticated enzymatic process, including the identification of substrates and potential protein partners, in vitro and biomimetic devices have to be designed. Conceivably, different extracellular acyltransferases may be required to catalyze the multiple cross-links present in the cuticular matrix. Moreover, it has been shown that different GDSL-lipases are expressed in the epidemis during the growth of tomato fruits (Yeats et al., 2010). GDSL1 is undoubtedly an element of a complex network required for the establishment of the plant cuticle. In this regard, the GDSL1-silenced plants provide a powerful tool to further investigate the genetic and metabolic networks underlying plant cuticle formation.

METHODS

Plant Material and Growth Conditions

Transcript analyses of fruit tissues and proteome experiments were performed on Solanum lycopersicum ‘Ailsa Craig’ grown as previously described (Mounet et al., 2009). A cherry tomato variety was used to generate transgenic plants (S. lycopersicum ‘West Virginia 106’). In vitro culture and growth of transgenic plants were performed as previously indicated (Alhagdow et al., 2007).

Plant Transformation

The GDSL1 cDNA fragment was obtained by reverse transcription (SuperScript II reverse transcriptase; Invitrogen, Life Technology) of 2 μg of total RNA from tomato fruit. A fragment of 509 bp, located in the 5′-region of GDSL1, was amplified by PCR using ExTa DNA polymerase and the gene-specific primers 5′-AAAAAGCAAGGCTTTTTGCTAATTTTGGCCTA-3′ and 5′-AGAAAGCTGGTCAACATTACCCACACCTCCACCTAAGACCACTC-3′, where the underlined sequence corresponds to partial attB extensions.
After a second PCR using full attB primers (attB1, 5'-GGGGACAA-GTTTGATACAAAAACAGGCT-3'; attB2, 5'-GGGGACACTTTGTA-CAAGAAGCTGGGT-3'), the purified DNA fragment was introduced in the Gateway system entry vector (pDONR 201) and then transferred as an inverted repeat under the control of the 3SS promoter in the RNAi destination vector (pK7GWIWG2), which confers kanamycin resistance to transformed plants (Karimi et al., 2007). After sequence checking, constructions were used to transform cherry tomato plants (‘West Virginia 106’) via Agrobacterium tumefaciens strain GV3101 (Hamza and Chupeau, 1993). Homozygous T2 plants were used for all of the analyses.

**Isolation of Tomato Fruit Cuticles**

Cuticles were isolated from the fruits of wild-type and transgenic lines according to a previously described protocol (Schönner and Riederer, 1986). Cutin was obtained after subsequent dewaxing by immersion in methylene chloride and repeated three times. Purified cutin slices were scanned and their surface was assessed using Image J software (http://rsb.info.nih.gov/ij/).

**Phenotypic Characterization of Fruit from Wild-Type and Transgenic Lines**

Red ripe fruits (breaker + 7 d) from wild-type and P35S-SigDSL1 RNAi transgenic lines were harvested and submitted to L*a*b* analyses (Tanaka et al., 2004). Mean values for the L*, which correspond to luminance character, were obtained by three measurements in the equatorial zone of 16 fruits for each line. A desiccation tolerance test, through weight loss measurement, was performed by incubating the fruits at 30°C for 21 d. Finally, a TB assay was performed to identify defects in the permeability of fruit cuticles (Tanaka et al., 2004; Hovav et al., 2007).

For cuticle thickness measurements, the fruit pericarp (including cuticle) was obtained from three independent fruits (breaker + 7) of wild-type and P35S-SigDSL1 RNAi transgenic lines. Samples were fixed and embedded in paraffin as previously described (Bereterbide et al., 2002). Ten-micrometer slices of pericarp were stained using saturated and fixed Sudan Red solution in ethanol. Mean cutin thickness was assessed from 60 measurements.

For auramine O staining, paraffin-embedded excocarp of 20-DPA wild-type fruits were stained according to Buda et al. (2009) and were observed through an A1 Nikon confocal laser scanning microscope with a ×40 water immersion objective. Excitation was conducted by a light-emitting diode at 485 nm, and emission was collected between 400 and 718 nm. The resulting images were acquired, stored, and visualized with a Nikon NIS-Elements software program.

**Proteomic Analysis of Tomato Fruit Cutin**

Cutin powder was suspended in 5 volumes of 0.5 M NaCl and 1 mM phenylmethylsulfonyl fluoride overnight at room temperature and sonicated (24 W, 15 min). After dialysis (cutoff of 3500 D), the protein extract was freeze-dried and analyzed by one-dimensional SDS-PAGE. Protein bands were excised and subjected to trypsin digestion before liquid chromatography. Urea was removed by extensive dialysis against a 50 mM Tris–HCl and 200 mM NaCl, pH 7.5, buffer. This led to the aggregation of the purified protein. After centrifugation at 10,000g, the protein pellet was suspended in Freund’s adjuvant for immunization. Antibody production was conducted according to Dubreil et al. (2002). The specificity of polyclonal antibodies was tested by immunoblotting and indirect capture ELISA. For immunoblot analysis, total protein was extracted from 50 mg fruit pericarps ground in liquid nitrogen and then washed twice with a methylene chloride/methanol (2:1) solution. Proteins were extracted with 50 mM Tris–HCl buffer, pH 8.0, 2% SDS, and 1% mercaptoethanol. After electroblotting, SigDSL1 was revealed using the Immuno-Star AP kit (Bio-Rad).

**Immunocytochemical Localization of GDSL1**

Fruit excocarp (including cuticle) was collected from three independent 20-DPA fruits from wild-type and P35S-SigDSL1 RNAi L-3 and L-17 lines using a single reflection accessory fitted with a thermostated diamond crystal with a 45° angle of light incidence. Two spectra were acquired on four different parts of the fruit cutin, and four fruits per line were analyzed. Surface calculations were conducted from the non-normalized spectra after baseline correction, which was established in the same conditions through Galactic software (Thermo Scientific). The surfaces of CH2 (2978 to 2838 cm⁻¹) and CO (1750 to 1695 cm⁻¹) bands were measured and used to calculate the ratio R2838/1750.

AFM height, phase, and error-signal images of isolated cutins were acquired in air using an Autoprobe CP Park Scientific Instrument. AFM images were recorded in the tapping mode using conventional pyramidal silicon nitride cantilevers obtained from Digital Instruments. All the tapping mode images were acquired at the lowest possible stable scanning force (<10 nN). Different surface areas were scanned for each sample, from 80 μm × 80 μm to 2 μm × 2 μm. At least three fruits from each wild-type and P35S-SigDSL1 RNAi transgenic line were used for the 80 × 80-μm scans, and the 2 × 2-μm AFM image was taken at two different locations for each sample. The mean surface roughness, which was calculated from the root mean square roughness, was deduced from the 2 × 2-μm² height images.

**Wax and Cutin Monomer Analyses**

Cuticular waxes were extracted from isolated cuticles of three tomato fruits in 6 mL of methylene chloride and analyzed as previously described (Kurdyukov et al., 2006b). Overnight cutin depolymerization was performed with 14% BF3 in methanol (Sigma-Aldrich), and 10 μg heptadecanoc acid was used as the internal standard (Osman et al., 1989). After extraction with methylene chloride, methylated cutin monomers were silylated with bis-(trimethylsilyl) trifluoroacetamide containing 1% trimethylchlorosilane (Sigma-Aldrich). Exocarp lipids were extracted by hexane/isopropanol (3:2) and were either directly silylated or transmethylated in methanol/BF3 before silylation. Lipids were analyzed by gas chromatography–mass spectrometry (Thermo DSOIL; 70 eV, mass-to-charge ratio of 50 to 700) and gas chromatography–flame ionization detection (Hewlett Packard 5890) using identical columns (DB5ms; 30 m × 0.25 mm, 0.1 μm [J&W]) and temperature gradients.

**Production of Recombinant GDSL1 for Generation of Polyclonal Antibodies**

Recombinant GDSL1 was produced in Escherichia coli (Origami) as previously described (Elmorjani et al., 2004; de Zélicourt et al., 2007) after amplification using specific primers (GDSL3’, 5’-TAAAGCTTATGCATG-TGAAT-3’; and GDSL5’, 5’-ATCCATGGGGAAGTGAAGCTTCCGGC-ATT-3’) and insertion into expression vectors. Recombinant GDSL1 was extracted in a buffer containing 8 M urea and further purified by Ni2⁺ affinity chromatography. Urea was removed by extensive dialysis against a 50 mM Tris–HCl and 200 mM NaCl, pH 7.5, buffer. This led to the aggregation of the purified protein. After centrifugation at 10,000g, the protein pellet was suspended in Freund’s adjuvant for immunization. Antibody production was conducted according to Dubreil et al. (2002). The specificity of polyclonal antibodies was tested by immunoblotting and indirect capture ELISA. For immunoblot analysis, total protein was extracted from 50 mg fruit pericarps ground in liquid nitrogen and then washed twice with a methylene chloride/methanol (2:1) solution. Proteins were extracted with 50 mM Tris–HCl buffer, pH 8.0, 2% SDS, and 1% mercaptoethanol. After electroblotting, SigDSL1 was revealed using the Immuno-Star AP kit (Bio-Rad).

**Immunoblotting and AFM**

FTIR spectra (200 scans) were recorded at a resolution of 2 cm⁻¹ on a Nicolet Magna IR 550 spectrometer equipped with a liquid nitrogen-cooled mercury–cadmium–telluride detector. The instrument was continuously purged with dry air. Spectra of cutin films were obtained by ATR
and fixed as previously described (Guillon et al., 2011). Thin ultramicro-
tome sections (Microm RMC MT 700) of either 1 μm for confocal laser
scanning microscopy or 100 nm for electron microscopy were obtained.
For nanogold immunolabelling, sections of tomato exocarp from wild-
type, P3SS-SiGDSL1 L-3, and L-17 fruits were incubated in 3% (w/v) BSA in
10 mM PBS (pH 7.2) and incubated for 1 h with the polyclonal antibody
diluted in 10 mM PBS supplemented with 1% BSA and 0.05% Tween 20.
After extensive washing, the section was incubated for 1 h with secondary
antibodies conjugated with goat anti-rabbit 1-nm colloidal gold complexes
(Auron). Labeling was intensified through the use of a silver enhancement
kit (Auron) according to the manufacturer’s instructions. After washing,
the grids were stained with 2% uranyl acetate. Electrogrooms were taken with
a Jeol 100S TEM electron microscope. In control experiments, preimmune
grids were stained with 2% uranyl acetate. Electrographs were taken with
the Nikon NIS-Elements software program.
For fluorescence immunolabeling, 1-μm exocarp sections were sat-
urated with 0.01 M Na-PBS, pH 7.2, containing 4% fat-free milk powder
(PBS milk) during the 30 min prior to incubation for 1 h with polyclonal
antibodies (1:100). After extensive washing in PBS, the sections were
incubated for 1 h with the goat anti-rabbit IgG coupled to Alexa Fluor-546
(1:100; Molecular Probes, Invitrogen). Immunostained sections were then
thoroughly washed in PBS and deionized water and mounted in deionized
water to be visualized by fluorescence. The immunofluorescence sections
were analyzed by an A1 Nikon confocal laser scanning microscope with a
×40 water immersion objective. Excitation was conducted by a light-
emitting diode at 561 nm, and emission was collected between 570 and
620 nm. The resulting images were acquired, stored, and visualized with
the Nikon NIS-Elements software program.

Real-Time RT-PCR
For measurement of GDSL1 transcript level in fruit tissues, the seed,
locomer tissue, columella, mesocarp, and exocarp were separated from
20-DPA ‘Ailsa Craig’ fruit, ground in liquid nitrogen, and stored at −80°C
until RNA extraction, as described previously (Mounet et al., 2009). The
exocarp sample represents the entire tomato peel, comprising the cuticle,
epidermal cells, and a few layers of collenchyma cells. For each wild-type
and P3SS-SiGDSL1 RNAi line (Wv 106 ‘cultivar), measurements of
GDSL1 transcript levels were performed on pools of fruit. Total RNA was
isolated, treated with DNase, and reverse transcribed as previously de-
scribed (Mounet et al., 2009).
PCR primers were designed in the 3’ untranslated region and are
available in Supplemental Table 1 online. Real-time PCR was performed on
a Bio-Rad CFX96 real-time system with the following parameters:
Denaturation at 95°C for 90 s, 40 amplification cycles with a denaturation
step at 95°C for 30 s, and a hybridization/synthesis step at 60°C for
30 s. Data acquisition and analysis were done using the Bio-Rad CFX
Manager software (version 1.1.308.1111). E/EFT (BT013166), β-tubulin
(DQ205342), and actin (SGN-U213132), based on de Jong et al., (2009),
were used as housekeeping genes to calculate relative transcript ex-
pression using gene expression analysis for the iCycler IQ real-time PCR
machine. Data were analyzed using the iCycler IQ real-time PCR
detection system. A Student’s t test was performed using the mean value
and SD of four replicates.

Accession Numbers
Sequence data from this article can be found in the SOL Genomics
Network (http://www.sgn.cornell.edu/) under the following accession
number: GDSL1, SGN-U585129.

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Figure 1. Expression Pattern of Si GDSL1 in Ailsa Craig
Fruit Tissues.

Supplemental Figure 2. Nucleotide and Deduced Protein Sequences of
Si GDSL1.

Supplemental Figure 3. Localization of Si GDSL1 in 20-DPA Tomato
Fruit Cuticle.

Supplemental Figure 4. Ultrastructural Feature of Epidermal Cells of
Cutinized Exocarps.

Supplemental Figure 5. Transcript Levels of Cutin-Associated Genes
in Wild-Type and P3SS-SiGDSL1 RNAi Transgenic Lines.

Supplemental Figure 6. Total ATR-FTIR Spectra.

Supplemental Figure 7. AFM Images of Tomato Cutin Surfaces of
Fruits from Wild-Type and P3SS-SiGDSL1 RNAi Plants at 20 DPA and
Breaker + 7 d Stages.

Supplemental Figure 8. Immunolabeling of Tomato Exocarp with
Preimmune Sera.

Supplemental Table 1. Accession and Sequence Information Con-
cerning Real-Time RT PCR Primers.

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AUTHOR CONTRIBUTIONS
M.L.-C., C.R., D.M., and B.B. designed the research. A.-L.G., F.M., C.G.,
B.B. analyzed the data. M.L.-C., K.E., C.R., D.M., and B.B. wrote the
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