Cutinsomes and CUTIN SYNTHASE1 Function Sequentially in Tomato Fruit Cutin Deposition

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The aerial parts of plants, including the leaves, fruits and non-lignified stems, are covered with a protective cuticle, largely composed of the polyester cutin. Two mechanisms of cutin deposition have been identified in tomato (Solanum lycopersicum) fruit. The contribution of each mechanism to cutin synthesis and deposition has shown a temporal and coordinated sequence that correlates with the two periods of organ growth, cell division and cell expansion. Cutinsomes, self-assembled particles composed of esterified cutin monomers, are involved in the synthesis of the procuticle during cell division and provide a template for further cutin deposition. CUTIN SYNTHASE1 (CUS1), an acyl transferase enzyme that links cutin monomers, contributes to massive cuticle deposition during the early stages of the cell expansion period by incorporating additional cutin to the procuticle template. However, cutin deposition and polymerization appear to be part of a more complex biological scenario, which is yet not fully understood. CUS1 is also associated with the coordinated growth of the cutinized and non-cutinized domains of the outer epidermal wall, and affects cell size. A dynamic and complex interplay linking cutin synthesis with cell wall development and epidermal cell size has been identified.

The cuticle that covers aerial parts of leaves, fruits, and nonlignified stems of land plants has a chemically heterogeneous nature, with lipids representing between 60% and 80% of the cuticle, depending on the plant organ and species (Heredia, 2003). These lipids can be polymerized, represented by cutin, and non-polymerized, i.e. waxes located either inside or on the cuticle. Cutin is the main framework of the cuticle; it is a polyester formed after the condensation of polyhydroxy fatty acids synthesized in the epidermal cells (Yeats and Rose, 2013; Domínguez et al., 2015). Additionally, cell wall polysaccharides are present in the cuticle (López-Casado et al., 2007) as well as phenolics (España et al., 2014a). Hence, the cuticle is an extreme modification of the outer epidermal cell wall (OEW) and, as such, it can be envisioned as a cutinized cell wall of variable extension depending of the species, organ considered, and stage of development (Domínguez et al., 2011; Fernández et al., 2016).

Over the years, many attempts to identify the mechanism of cutin synthesis and monomer transport to the outside have been carried out (Domínguez et al., 2015). A few enzymes have been shown to be involved in cutin synthesis and/or polymerization. DEFECTIVE IN CUTICULAR RIDGES (DCR) is an epidermis-specific member of the BAHD acyltransferase family (name based on the first four characterized enzymes of this family) that was postulated to carry out cytoplasmic cutin oligomerization in Arabidopsis (Arabidopsis thaliana; Panikashvili et al., 2009), in a similar fashion to what it had been reported for fava bean (Vicia faba; Croteau and Kolattukudy, 1974). Silencing the DCR tomato (Solanum lycopersicum) ortholog caused a dramatic decrease of the main cutin monomer as well as a reduction in cuticle thickness and the presence of lipid bodies in the cytoplasm of the tomato fruit epicarp cells (Lashbrooke et al., 2016). This accumulation of lipid material would be in agreement with its previously identified cytoplasmic location (Panikashvili et al., 2009). Lipases/hydrolases with a GDSL motif have also been associated with cutin synthesis. Identification

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P.S. performed the gene expression analyses, optical and electron tissue sectioning, immunolocalization, and sample visualization; J.A.H.-G. performed the x-ray analyses and interpreted the data; A.H. and E.D. designed the research plan, interpreted data, and wrote the article.

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of a GDSL lipase in the epidermis of expanding Agave americana leaves, and its immunolocalization to the epidermal cell and cuticle pointed to its involvement in cutin polymerization outside the cell (Reina et al., 2007). Indeed, the tomato putative ortholog of this protein, CUTIN SYNTHASE1 (CUS1), was found to be the gene responsible for the cutin deficient1 (cd1) mutation, which causes a dramatic reduction in cuticle deposition (Isaacson et al., 2009; Yeats et al., 2012). Protein activity analysis of CUS1 suggested that it acts as an acyltransferase enzyme linking activated cutin monomers (Girard et al., 2012; Yeats et al., 2012). This enzyme has been characterized as an extracellular protein and located by means of antibodies to the cutinized region of the OEW (Girard et al., 2012; Yeats et al., 2012). Putative orthologs of CUS1 have been identified in other species, suggesting a conserved cutin formation mechanism among several land plant groups (Yeats et al., 2014). Moreover, four CUS1 paralogs have been identified in tomato. One of them, CUS2, has been shown to participate in the maintenance of sepal cuticle ridges in Arabidopsis (Hong et al., 2017); however, their involvement in tomato cutin synthesis is yet to be determined (Yeats et al., 2014). BODYGUARD (BDG) is a gene family that belongs to the α/β hydrolase superfamily that has been characterized in Arabidopsis (Kurdyukov et al., 2006). Analysis of bdg1 showed that this gene is epidermis specific and displays an extracellular location, consistent with the suggested role as a transacylase involved in C18 cutin monomer polymerization (Kurdyukov et al., 2006; Jakobson et al., 2016). Another member of this family, BDG3, participates in maintaining the petal nanoridge structure in Arabidopsis (Shi et al., 2011), thus emphasizing the link of these proteins to cutin synthesis. On the other hand, cutin has been postulated to be the final result of an emergent molecular self-assembling process (Heredia-Guerrero et al., 2008, 2010; Heredia et al., 2009). This hypothesis delves into the ability of cutin monomers to form cutinsomes, that is, esterified lipid nanoparticles formed by spontaneous self-assembly of polyhydroxy fatty acids (Heredia-Guerrero et al., 2008). These particles could be passively or actively transported to the outermost part of the OEW, where they would accumulate and polymerize to form the cuticle (Heredia-Guerrero et al., 2008). Immunocyto localization of cutinsomes to the epidermis and nascent cuticle of tomato immature fruit, Ornithogalum umbellatum ovary, and Arabidopsis embryo cotyledons reinforces their possible role in early stages of cuticle development (Domínguez et al., 2010; Kwiatkowska et al., 2014; Stepniński et al., 2017).

Detailed analyses on tomato cuticle changes throughout growth and ripening have allowed the identification of several important features. One of them is that cuticle deposition did not cease during growth or ripening, since fruit size increased until the red ripe stage, and the amount of cuticle was either maintained or increased during the whole period (Domínguez et al., 2008, 2012). Additionally, an important shift in the pattern of cuticle deposition was detected between the early stages of development corresponding to the cell division phase and the later cell expansion period (España et al., 2014a; Segado et al., 2016). In this study, we sought to analyze the contribution of both mechanisms to cuticle deposition during development and their potential interaction. Using immunocyto localization and TEM, we have established that both mechanisms are part of a more complex temporal sequence.

RESULTS

Expression of Genes Involved in Tomato Cutin Synthesis

CUS1 protein was identified as a member of a gene family comprising five members in tomato (Yeats et al., 2014). Amino acid sequence comparison among the five members of the CUS family showed a 50.7% identity (Supplemental Fig. S1). Although CUS2 and CUS3 shared the highest protein identity with CUS1 (76% and 74%, respectively), the number of residues of CUS4 and CUS5 identical to CUS1 was still very high (69% and 67%, respectively). The expression profiles throughout development of several genes postulated to participate in cutin synthesis were analyzed by reverse transcription quantitative PCR (RT-qPCR; Fig. 1). The evolution of the amount of cuticle per fruit, a parameter that combines fruit growth and cuticle deposition per surface area, is also presented in Figure 1. As it has already been reported, tomato fruit cuticle cannot be isolated at the earliest stages of development, which correspond to the cell division period (Domínguez et al., 2008; Segado et al., 2016). Two slopes can be observed for the deposition of cuticle per fruit during the cell expansion period, a first one between 10 and 20 d after anthesis (daa) corresponding to a substantial deposition of cuticle in a very short period of time and a second one between 20 and 45 daa with a 2-fold increase in cuticle. Expression levels of CUS1 displayed very little expression in the ovary at anthesis and 5 daa, the earliest stages of development, studied, with a maximum at 10 followed by a substantial drop at 25 daa and almost no expression from 30 daa until ripening (Fig. 1). CUS1 expression was mainly associated with the first period of massive cuticle accumulation. CUS4 showed expression from anthesis until the onset of ripening with a peak detected between 10 and 20 daa. CUS5 was only expressed between 10 until 40 daa, the onset of ripening. On the other hand, CUS2 and CUS3 were not expressed in fruit epicarp in any of the stages studied.

Expression of the tomato DCR, BDG1, and BDG3 orthologs was also analyzed in fruit epidermis during growth and ripening (Fig. 1). The acyl-transferase DCR displayed an expression profile somehow similar to CUS1 with little expression before 10 daa, a period of maximum expression that remained until 25 daa, followed by a decrease around mature green and little expression at ripening. Both BDG genes were expressed throughout the whole period of fruit growth but with a
Figure 1. Relative quantity (RQ) throughout epicarp development of ‘Cascada’ tomato fruits of CUS genes and other genes postulated to be involved in cutin synthesis. Gray bars represent the RT-qPCR analysis of the expression levels of CUS1, CUS4, CUS5, DCR, BDG1, BDG3, SHN2, and SHN3. Data are presented as mean ± se of three biological samples per developmental stage. Evolution of the amount of cuticle per fruit, modified from Segado et al. (2016), is shown in all graphs for comparison purposes (lines).
clear decrease during ripening. Interestingly, BDG1 displayed two transient increases, one at 10 daa, followed by a decrease during most of fruit growth, and a second one at 40 daa, coinciding with the onset of ripening and a final increase in cuticle deposition between 40 and 45 daa (Fig. 1). This second expression peak was also observed for BDG3. The Arabidopsis WAX INDUCER/SYMPHONY (WIN/SYMPHONY) is a clade of three transcription factors that have been reported to regulate wax and cutin biosynthesis and also activate the BDG3 promoter (Shi et al., 2011). Therefore, we investigated their expression in tomato fruit epicarp throughout development. SHN2 showed an expression profile similar to that of CUS1, very little expression prior to 10 daa, with a maximum at this stage followed by a sharp decrease and little to no expression after 25 daa. SHN3 however, showed a different profile; it was expressed in the epicarp throughout the whole growth period, including ripening. On the other hand, SHN1 did not show any expression on fruit epicarp. In general, with the exception of BDG3 and SHN3, the rest of the studied genes showed little to no expression prior to 10 daa, followed by an important increase during the period between 10 and 20 to 25 daa.

Electron Microscopy Immunolocalization of CUS and Cutinsomes

Following the description of tomato fruit epidermis and cuticle carried out in Segado et al. (2016), the OEW is divided into the noncutinized OEW, i.e. the polysaccharide region of the wall and the cuticle, or cutinized OEW, the outermost part of the wall rich in lipid material. Cellular localization of CUS1 and cutinsomes was performed by transmission electron microscopy (TEM) with specific antibodies. Table 1 shows changes in CUS1 and cutinsome labeling density throughout fruit development. CUS1 and cutinsomes were solely located to the epidermal cells and OEW, with no labeling present in the cell layers underneath. CUS1 was detected during the first 25 d of fruit development and was absent from 30 daa until ripening. This temporal pattern mirrored that of gene expression. Likewise, cutinsomes were also identified during early stages of development, from anthesis until 10 daa, while their presence was scarce or undetected for the rest of fruit growth and development. In both cases, a maximum in labeling density was identified, earlier from cutinsomes around 5 to 9 daa and later for CUS1 10 to 15 daa.

Antibody immunolocalization of CUS1 in the epidermis suffered considerable changes throughout development. At early stages of fruit growth, from anthesis until 6 daa, CUS1 labeling was only found inside the epidermal cell and on the noncutinized OEW (Fig. 2, A–D). At 7 daa, a few gold particles were already present in the cuticle, although the majority of the protein was still located in the cytoplasm of the epidermal cells or the noncutinized OEW (Fig. 2E). Later on, during the 8 to 9 daa period, CUS1 was not found again inside the epidermal cells, but only in the cutinized and noncutinized OEW (Fig. 2, F and G). Finally, from 10 until 25 daa, the protein was always located in the cuticle without any additional labeling to the noncutinized OEW (Fig. 2H). Protein distribution, based on gold particle labeling, showed a nonuniform pattern. Inside the epidermal cells, CUS1 was located to the upper part of the cell, more or less in close proximity to the plasma membrane and the OEW (Fig. 2, A, D, and E). On the other hand, CUS1 was found throughout the whole cuticle, with no preference for its inner or outer side (Fig. 2H), although at 7 to 9 daa, the gold particles were mostly found closer to the inner side of the cuticle (Fig. 2, E–G). The procuticle is a uniform and electron-dense thin layer present during the early stages of development, mainly during the cell division period, from fruit set until 10 daa (Segado et al., 2016). This procuticle showed a nonhomogeneous inner side due to the presence of electron-dense globules of variable size and shape in the interface between the noncutinized OEW and the procuticle (Fig. 2, C–G). CUS1 localization did not coincide with these osmiophilic droplets, although in a few cases the gold particles were in close proximity to these globules (Fig. 2D). At 10 daa, this irregular morphology of the inner side disappeared, while CUS1 changed its distribution and was only present in the cuticle (Fig. 2H).

Contrary to what was observed with CUS1, cutinsome immunolabeling did not show any main changes in location during development. From anthesis until 10 daa, they were found in the upper part of the epidermal cells, specifically in the cytoplasmic region close to the plasma membrane and throughout the noncutinized region of the OEW (Fig. 3, A–D). Gold particles were identified in close proximity to the inner side of the procuticle (Fig. 3D), indicating a pattern of labeling close or within the electron-dense regions of the noncutinized OEW. High-magnification pictures of the region between the inner side of the procuticle and the cell wall showed an amorphous structure of tightly

| Stage  | CUS1 Density | Cutinsomes Density |
|--------|--------------|--------------------|
| Ovary  | 1.31 ± 0.09  | 1.17 ± 0.11        |
| 2 daa  | 1.45 ± 0.16  | 0.99 ± 0.07        |
| 5 daa  | 1.60 ± 0.13  | 1.78 ± 0.10        |
| 7 daa  | 1.83 ± 0.15  | 1.76 ± 0.07        |
| 9 daa  | 1.96 ± 0.11  | 1.95 ± 0.11        |
| 10 daa | 1.78 ± 0.37  | 0.26 ± 0.04        |
| 15 daa | 7.02 ± 0.29  | –                  |
| 20 daa | 1.36 ± 0.09  | –                  |
| 25 daa | 1.64 ± 0.18  | –                  |
| 35 daa | –             | –                  |
| 45 daa | –             | –                  |
| 55 daa | –             | –                  |

Cutin Synthesis and Deposition
Figure 2. Immunolocalization of CUS1 in the epicarp of cv Cascada fruits at different stages of development. Ovary (A); 2 (B); 5 (C); 6 (D); 7 (E); 8 (F); 9 (G); and 10 (H) daa. Gold particles are encircled in red.
Figure 3. Immunolocalization of cutinsomes in epicarp cross-sections of cv Cascada fruits. Ovary (A); 2 (B); 7 (C); 9 (D); and 5 (E–H) daa. Gold particles are encircled in red.
appressed coalescent electron-dense globules that are attached to or in close vicinity to the procuticle (Fig. 3, E–G). Cutinsome labeling was present in these regions of the OEW with localized electron-dense material (Fig. 3, E–G). Figure 3H shows a high-magnification picture of cutinsome labeling inside the epidermal cell and outside on the inner side of the noncutinized OEW.

Structural Changes in the Cutin Matrix throughout Development

Structural characterization of the cuticle and cutin matrix during growth and development was carried out by X-ray diffraction (XRD). Diffractograms of both cuticle and cutin at 15 and 50 daa showed a pattern with a broad diffuse halo centered at ~20°, which is characteristic of amorphous polymers (Fig. 4A). This halo is associated with the average molecular spacing between aliphatic chains of cutin matrices, as it was previously reported by Luque et al. (1995). No differences were observed in the XRD diffractograms between the cuticle and the cutin matrix, indicating that the features identified were attributable to cutin, and the diffraction pattern of the other cuticle components (i.e. polysaccharides, waxes, and phenolics) are most likely masked by cutin. Despite the fact that no major structural differences were detected throughout development, there were some interesting changes. The basal spacing distance was ~4.60 Å between 10 and 35 daa but decreased to ~4.35 Å during ripening (Fig. 4B). This indicates that the average distance among molecular chains remained constant throughout most of fruit growth but decreased with ripening, revealing that the material became more compact. The full width at half maximum (FWHM) of each halo is indicative of the qualitative distribution of average distances between macromolecular chains in amorphous polymers (Halasa et al., 1991). Thus, high FWHM values in amorphous systems are indicative of a high disorder degree. The FWHM increased from ~0.7 Å at 10 and 15 daa to ~1.0 Å at 20 daa and remained unchanged until red ripe (Fig. 4C).

Microscopic Characterization of the cus1-a Mutant Epidermis

cus1-a is a cv Microtom mutant line that has a 96% reduction of CUS1 expression and no detectable protein in the epicarp (Petit et al., 2014). Optical cross sections of cv Microtom and cus1-a pericarp showed irregularly oriented cells with variable sizes and shapes during the first 10 d of development (Fig. 5, A–F). The surface was irregular, with some cells protruding. The clear cell layer arrangement of an epidermis, several layers of collenchyma, followed by parenchyma cells was not clearly established until 15 daa in both lines (Fig. 5, G and H). Although some cellular differences can be observed between the mutant and the wild type prior to 15 daa, given the extent of cellular variability within and among replicates, they were not considered. However, image analysis of epidermal cells at two stages of development showed that cus1-a cells were significantly different in size and shape from cv Microtom at 15 daa (Table 2). In the mutant, epidermal cells were bigger and more rounded due to a significant increase in radial width, whereas the tangential width was not altered and remained similar (Table 2). The differences in cell shape remained until red ripe, although in this case the mutant showed more elongated cells. Additionally, a more gradual increase in parenchyma cell size was observed in the mutant compared to cv Microtom (Fig. 5, G and H).

CUS1 immunolocalization in the epicarp of cv Microtom showed a similar subcellular profile to that of cv Cascada (Supplemental Fig. S2). Prior to 10 daa, the protein was mainly located inside the epidermal cell or...
Figure 5. Light microscopy images of cv Microtom (A, C, E, and G) and cus1-a (B, D, F, and H) pericarp. Ovary (A and B); 5 (C and D); 10 (E and F); and 15 (G and H) daa. Bars = 20 μm. Sections stained with epoxy tissue stain.
at the noncutinized OEW. At 10 daa, there was a clear switch and most of the protein was located to the cutinized OEW with only a few gold particles still in the cytoplasm of the epidermal cell. The number of gold particles increased at 15 daa, and all of them were located to the cutinized OEW (Supplemental Fig. S2). Figure 6 shows the OEW of cv Microtom and cus1-a during the early stages of development. No differences in cuticle deposition or inner surface contour were detected between cv Microtom and cus1-a from ovary at anthesis to 10 daa (Fig. 6, A–F). Indeed, cuticle thickness was similar between the wild type and the mutant during this period, as it can be observed in Table 3, which shows the evolution of cutinized and noncutinized wall thickness of cv Microtom and cus1-a during development. However, at 15 daa, a sharp increase in cuticle deposition was observed in the wild type, while in cus1-a the procuticle was still clearly visible (Fig. 6, G and H). In cv Microtom, cuticle thickness displayed a 19-fold increase between 10 and 15 daa and then remained constant until red ripe (Table 3). This dramatic increase in cutin deposition at 15 daa coincided with CUS1 sole location to the cutinized OEW (Supplemental Fig. S2). In the cus1-a mutant, the cuticle displayed a small yet significant increase between 10 daa and red ripe (Table 3). A close look at the cus1-a cuticle at red ripe allowed us to identify two regions, an outermost and more electron-dense region and an underneath, less electron-dense and with a globular appearance one (Supplemental Fig. S3, A and B). These two regions were first visible at 15 daa and remained distinguishable until red ripe (Supplemental Fig. S3C). The uppermost region corresponded to the procuticle, since its thickness (0.25 ± 0.01, 0.26 ± 0.01 for 15 daa and red ripe, respectively) was similar to that observed at 10 daa (Table 3) and did not change during development. Therefore, the increase in cuticle thickness detected in cus1-a during the cell expansion period was due to changes in this inner region. It is interesting to note that the noncutinized OEW mirrored most of the changes detected in the cuticle (Table 3). Thus, from anthesis until 10 daa, the thickness of the noncutinized OEW increased similarly in cv Microtom and cus1-a. However, at 15 daa, the increase in the noncutinized section of the OEW was significantly lower in cus1-a compared to cv Microtom. In the mutant, unlike the control, the noncutinized OEW continued increasing its thickness during the rest of the growing period, thus reaching in both genotypes a similar thickness at red ripe (Table 3). Cutinsome immunolocalization during the first 15 d of development was not affected by CUS1 knockdown, showing the expected location inside the epidermal cell and in the OEW (Fig. 7). Indeed, a higher number of cutinsomes seemed to be identified in cus1-a mutant compared to cv Microtom throughout this period (Fig. 7).

### DISCUSSION

#### Involvement of the Acyl Transferase CUS1 in Cutin Formation

CUS1 is a GDSL lipase/hydrolase protein located to the cutinized OEW of tomato fruit that has been postulated to play an important role in cutin synthesis (Girard et al., 2012; Yeats et al., 2012). Although its mechanism of action and substrate recognition is still undetermined, in vitro analysis of CUS1 activity has delivered linear oligomers with up to six fatty acid units (Yeats et al., 2014; San Segundo et al., 2019). Results presented herein displayed a good correlation between CUS1 immunolocalization and gene expression. Of the other CUS family members, only CUS4 and CUS5 were expressed in tomato epicarp. Although the possibility of epitope cross recognition due to the high sequence similarity of CUS proteins exists, it is unlikely because no protein was detected at the mature green stage (35 daa) despite CUS4 and CUS5 being expressed until the onset of ripening. The peak in CUS1 expression observed between 10 and 20 daa coincided with the location of the protein to its natural molecular scenario, the growing cuticle. These results agreed with the pattern of cuticle accumulation observed during this period (Domínguez et al., 2008). CUS1 immunolocalization did not show any specific distribution, since it could be detected throughout the whole cuticle. It should be noted that CUS1 was fully located within the cuticle at the beginning of the epicarp cell expansion period (Segado et al., 2016), suggesting that CUS1 is mainly involved in cutin deposition during this period, but not before. Moreover, the fact that the cus1-a mutant did not show any differences in cuticle thickness prior to cell expansion (15 daa) supports this idea. Yeats et al. (2014) reported a pH 5 as the optimum for CUS1 activity. Yet, the presence of acid pectins was recently detected in the

#### Table 2. Epidermal cell size and shape

Aspect ratio is dimensionless. Data presented as means ± s. Asterisks indicate statistically significant differences compared with the wild type of the same developmental stage by t test (*P < 0.05, **P < 0.01, and ***P < 0.001).

| Parameters        | cv Microtom  | cus1-a     | Red Ripe  | cus1-a     |
|-------------------|--------------|------------|-----------|------------|
| Area (µm²)        | 163.97 ± 13.80 | 229.39 ± 12.39* | 293.93 ± 11.47 | 337.38 ± 18.03 |
| Perimeter (µm)    | 47.74 ± 1.64  | 55.65 ± 1.54*  | 66.30 ± 1.37 | 78.17 ± 2.88*  |
| Radial width (µm) | 11.26 ± 0.88  | 16.39 ± 0.37** | 14.97 ± 0.31 | 16.42 ± 0.95   |
| Tangential width (µm) | 15.42 ± 0.69 | 14.22 ± 0.78   | 24.66 ± 0.64 | 31.24 ± 0.75***|
| Aspect ratio      | 0.74 ± 0.03   | 1.20 ± 0.07**  | 0.61 ± 0.02  | 0.47 ± 0.04**  |
Figure 6. Transmission electron micrographs. High-magnification pictures of the epicarp of cv Microtom (A, C, E, and G) and cus1-a (B, D, F, and H) epicarp.
OEW from anthesis until 7 daa (Segado et al., 2016), coinciding the location of CUS1 to the growing cuticle with a clear reduction in acid pectins. How this decrease in unesterified pectins affect the apoplastic pH or, more importantly, the pH of the growing cuticle is a matter that needs to be elucidated. CUS1 expression was clearly reduced at 25 daa and almost not detected at 30 daa. Tomato fruit growth continues until red ripe (Domínguez et al., 2012), and its amount of cuticle per surface unit does not decrease during the later stages of development (Domínguez et al., 2008). There is an approximate 31% to 35% fruit surface expansion (Segado et al., 2016), and concomitantly cuticle deposition, occurring between 30 and 35 daa and red ripe in cv Cascada, that must therefore be accompanied by additional lipid deposition and polymerization. Hence, additional enzyme/s or chemical mechanisms have to be responsible for cutin deposition during later stages of development. This is in agreement with gradual increase in cuticle thickness displayed by the cus1-a mutant from 15 daa until red ripe. It is possible that the two other CUS members expressed in tomato epicarp (CUS4 and CUS5) could be participating in cutin polymerization during these later stages of fruit growth, although their enzymatic activities have not been discerned yet. Although CUS2 has been reported to participate in Arabidopsis cuticle deposition (Hong et al., 2017), its role is probably restricted to sepals or vegetative tissues, since it was not detected in the epidermis of tomato fruit.

### Early Stages of Cuticle Development: The Procuticle

Studies of OEW cutinization in numerous species have consistently described the presence at early stages...
of development of electron-dense globules of variable size that seem to migrate through the OEW to a nascent procuticle, which had an irregular inner contour. These globular structures were proposed as cutin precursors, that is, sites of cutin synthesis or assembly (Frey-Wyssling and Mühlenthal, 1965; Hallam, 1970; Heide-Jørgensen, 1978, 1991; Jeffree, 2006; Segado et al., 2016). Similar osmiophilic globules were found at the interface between the plasma membrane and OEW in the epidermis of young tissues after auxin treatment to induce growth (Kutschera et al., 1987). Cutinsomes, lipid particles formed in vitro by self-assembly and partial polymerization of cutin monomers, have been identified in close proximity to such globular structures in several species (Domínguez et al., 2010; Kwiatkowska et al., 2014; Štepiński et al., 2017) and proposed to play a role in cuticle deposition of olive (Olea europaea) fruit (D’Angeli et al., 2013, 2016). These particles have also been suggested to participate in lipid adsorption to the cell wall-air interphase (Bakan and Marion, 2017). However, the significance of these particles to the overall cutin synthesis and deposition had not been addressed.

Localization of cutinsomes to the epidermal cells and OEW showed a specific time frame that corresponded with the cell division period, from ovary until 10 daa. The fact that they were already present in close contact to the procuticle of the ovary can be considered an indication of their active participation in its procuticle development. In the epidermal cells of O. umbellatum ovary, cutinsomes were identified in specific cytoplasmic domains named lipotubuloids, where lipid synthesis occurred (Kwiatkowska, 2004, 2014). These lipotubuloid structures have been postulated to act as a functionally and structurally integrated metabolon for lipid synthesis (Kwiatkowska et al., 2015). An acid environment has been shown to favor cutinsome formation (Heredia-Guerrero et al., 2008), since it could facilitate its in vivo chemical stabilization, induce aggregation in branched chains, and favor condensation between cutin monomers, as it has been found to occur in vitro (Guzmán-Puyol et al., 2015). Hence, cutinsome identification during the cell division stage, a period that has been characterized to have an OEW rich in acid pectins (Segado et al., 2016), supports their involvement in the deposition of the procuticle. However, the pH of the cytoplasmic location where their formation takes place is a topic that should be further studied.

It should be emphasized that cutinsomes provide both a hypothesis for procuticle formation as well as a vesicle-like transport between the epidermal cell and the outermost region of the OEW during this early stage of development. Transport to the cell surface of partially synthesized cell wall macromolecules and cuticle waxes via extracellular vesicles have been previously described (Casadevall et al., 2009; Toyooka et al., 2009; McFarlane et al., 2014). On the other hand, how CUS1 proteins are transported from the epidermal cell to the cutinized OEW remains unknown. The putative involvement of lipid transfer proteins or even a vesicular transport has been suggested (Domínguez et al., 2015). Cutinsomes could act as delivery vehicles for the transport of proteins to the cuticle, as it has been suggested for GPAT6 and DGAT2 (Štepiński et al., 2016); however, no colocalization between CUS1 and cutinsomes were observed. Thus, cutinsomes seem to be involved in the deposition of the procuticle, whereas CUS1 participates in cutin deposition during the period of early cell expansion.

The procuticle remained distinguishable in the cus1-a mutant; thus, the additional cuticle material displayed an aggregated and globular appearance, which reminded a partially polymerized cutin or oligomeric cutin domains. This indicates that, despite some additional cutin material can be incorporated to the cuticle in the absence of CUS1, this material could not be fully polymerized or integrated with the existing procuticle. Although this additional cutin could be the result of some residual CUS1 activity present in the mutant, the fact that it is accumulated until the later stages of development, when CUS1 is not detected, suggests otherwise.

The intimate relationship between the cuticle and the epidermal wall

The OEW has several features different from the rest of the cell walls. In addition to changes in sugar content (Bret-Harte and Talbott, 1993), it is much thicker and less extensible than the rest of the cell walls (Kutschera, 2008). Recently, analysis of the polysaccharide domain of the tomato fruit cuticle has also shown chemical differences with the pericarp walls (Philippe et al., 2020). The clear reduction observed in the cus1-a mutant of both the cutinized and noncutinized fractions of the OEW at the onset of the expansion period indicates a relationship between CUS1 activity and cell wall development. This decrease, more pronounced in the cutinized region of the OEW, hampered the growth of the OEW from 15 daa until ripe. The gradual increase in thickness of the noncutinized OEW during the later stages of development showed that polysaccharide material continued its deposition in the cell wall of the mutant, even after the control had reduced or stopped this process. These changes in the noncutinized OEW detected in the cus1-a mutant probably also affected its chemical composition, since it has recently been uncovered that the polysaccharide fraction of cus1-a cuticle displayed differences in pectin content (Philippe et al., 2020). These alterations in polysaccharide composition and OEW thickness could also explain the increase in epidermal cell size and altered shape of the cus1-a mutant, since a thicker OEW would be a limiting factor for growth and would sustain higher tensile stresses (Hamant and Traas, 2010). Therefore, a reduction in the OEW thickness, even in the absence of chemical changes, could favor cell expansion.

If the cuticle and the epidermal cell wall development were two independent processes, it would be expected...
that a decrease in cuticle deposition would increase the fraction of noncutinized OEW in order to maintain the overall OEW thickness. A reduction of the overall OEW thickness due to cuticle deposition impairment was detected in mature tomato fruits silencing SHN3 and in the cd mutants (Isaacson et al., 2009; Shi et al., 2013). Nevertheless, whereas red ripe cd mutant fruits exhibited a similar noncutinized OEW as the control, red ripe SHN3-silenced fruits showed a thicker, noncutinized OEW, although not enough to compensate for the decrease in cuticle. Thus, a reduction in cutin deposition seems to have a negative effect on the development of the OEW throughout development, pointing to a coordinate growth and deposition of cutin and polysaccharide cell wall material. This reinforces the current view of the cuticle as a special modification of the cell wall and, moreover, clearly indicates that cutin deposition, and hence cuticle development, fosters the OEW development.

The cuticle has been reported to contribute to the establishment and maintenance of the epidermal identity (Javelle et al., 2011). The altered epidermal size and shape found in the cusi1-a mutant from the beginning of the cell expansion period could also be the consequence of the substantial significant reduction in cuticle deposition. Changes in epidermal cell patterning were detected in Arabidopsis after silencing DCR or BDG1, the two other genes putatively involved in cutin synthesis (Kurdyukov et al., 2006; Panikashvili et al., 2009; Jakobson et al., 2016). A relationship between changes in cuticle deposition and epidermal cell size and shape has also been reported for tomato fruits silencing CHALCONE SYNTHASE (CHS) and either silencing or constitutively expressing TOMATO AGAMOUS-LIKE1 (TAGL1; España et al., 2014b; Giménez et al., 2015).

Cutin Synthesis and Deposition Is a Complex Scenario

Cuticle synthesis and deposition is a complex polygenic trait not only due to its modulation by the environment and hormonal cues but also to its heterogeneous and composite nature. Any of the three genes postulated to be involved in cutin polymerization, CUS1, DCR, and BDG1, caused a significant reduction in cutin deposition when they were silenced individually (Girard et al., 2012; Yeats et al., 2012; Jakobson et al., 2016; Lashbrooke et al., 2016), yet a cuticle was still synthetized. In this sense, bdg1 showed a significant reduction in Arabidopsis leaf cutin at early stages of development that was later compensated, probably by other enzymes (Kurdyukov et al., 2006; Jakobson et al., 2016). The substantial increase observed in CUS1, DCR, and BDG1 expression at the onset of cell expansion is an indication of their involvement in the massive cutin deposition occurring during this period. Nevertheless, it remains to be elucidated whether DCR and BDG1 participate, in combination with cutinsomes, to the synthesis of the procuticle. Interestingly, no major structural changes were detected in the transition between the procuticle and the beginning of cuticle deposition, only an increase in disorder that could be indicative of a reorganization of cutin polymeric domains, as additional components are being incorporated or modifications of cutin cross linking as secondary hydroxyls are being esterified.

An alternative enzymatic mechanism for DCR has been proposed that involves oligomerization of phenolics to cutin monomers (Rani et al., 2010). However, the DCR expression profile herein identified is not in accordance with phenolic accumulation in the tomato fruit cuticle (España et al., 2014a) but with a role in cutin polymerization. BDG1 has been suggested to polymerize C18 unsaturated cutin precursors in Arabidopsis (Jakobson et al., 2016). In tomato fruit cutin, C18 monomers are minor components, hence, BDG1 would either be responsible for the incorporation of minor compounds to the cutin matrix or perform a different role in tomato fruit cuticle. The increase of BDG1 and BDG3 expression at 40 daa coincided with a final accumulation of cuticle at the onset of ripening and the transition to a more compact cutin matrix. The transcription factor SHN3 has been shown to be involved in tomato fruit cuticle formation and to regulate the expression of DCR and CUS1 (Shi et al., 2013). Comparison of SHN2 and SHN3 expression profiles showed an overlap during the early stages of cell expansion, suggesting that both transcription factors participate in the regulation of epidermal growth and cutinisation during this period, whereas cuticle accumulation during the later stages of fruit growth and ripening would be regulated by SHN3. Given the coexpression observed between CUS1 and SHN2, it would be interesting to study the role of SHN2 as well as its possible regulation of CUS1 and the other genes involved in cutin deposition. SHN1 has been shown to be induced after drought conditions in tomato and rice (Wang et al., 2012; Abdallat et al., 2014), and that could explain the absence of SHN1 expression in tomato fruit epidermis under the experimental conditions employed.

Tomato fruit cutin is a highly cross linked polymer with primary as well as secondary hydroxyls heavily involved in polymerization (Domínguez et al., 2015). In vitro CUS1 analysis has been shown to deliver a linear polymer with only head-to-tail esterification of fatty acids (Yeats et al., 2014), although recently it has been suggested that in planta CUS1 could be implicated in cross linking the cutin matrix (Philippe et al., 2016). These discrepancies have been attributed to differences in the environment and raise a very interesting point regarding cutin synthesis and deposition. Initiation of cutin polymerization would be expected to occur in a more hydrophilic primary wall environment but, as cutin is being deposited, this environment would become more hydrophobic. CUS1 is located to well-developed cutin regions when the phenotypic differences become apparent in the cusi1-a mutant. It could be then hypothesized that cytoplasmic cutin oligomers, synthetized by DCR, could be transported to the OEW, where they would provide a hydrophobic environment.
for CUS1 further cutin polymerization. Alternatively or in combination with this, BDG1 or BDG3 could participate together with CUS1 in cutin synthesis.

CONCLUSION

There is a clear differentiation in cuticle synthesis during organ growth between the initial cell division and the later cell expansion period. Cutisomes are involved in cutin deposition during the cell division providing a primary protective procuticle. Later on, CUS1 participates in cutin synthesis from the onset of the cell expansion until approximately mature green. Furthermore, CUS1 is also involved in the coordinated development of the cutinized and noncutinized regions of the OEW and affects the epidermal cell size and shape. These mechanisms postulated for cutin synthesis only partially address cuticle development and are part of a more complex scenario. Considering the critical role that the cuticle plays in plant survival and how it responds to changes in the environment, it is of great importance to identify the rest of the players involved as well as their regulatory processes.

MATERIALS AND METHODS

Plant Material

Three randomly distributed blocks of 10 Solanum lycopersicum ‘Cascada’ plants each were grown in a polyethylene greenhouse during spring at the Estación Experimental La Mayora, Consejo Superior de Investigaciones Científicas, Spain. Tomato seedlings were grown in an insect-proof glasshouse and transplanted to soil in a plastic house at the three true-leaf growth stage. Plants were watered with nutrient solution when needed, supported by string, and pruned to a single stem. Flowers were labeled at anthesis and fruits collected at selected time points, from anthesis to red ripe.

Tissue Sectioning and Staining for Light Microscopy

Small pericarp pieces from three fruits of each block were dissected and fixed in 4% (w/v) paraformaldehyde in 0.1 M of phosphate buffer (pH 7.4). Later on, they were dehydrated in an ethanol dilution series (50% to 100%) and embedded in LR White resin. Alternatively, samples were embedded in Unicryl resin without prior OsO4 postfixing. Ultrathin sections (around 50 nm thick) were cut with an EM UC7 ultramicrotome (Leica Microsystems) and contrasted with Epoxy tissue stain (Electron Microscopy Sciences). Epidermal cell size was measured using ImageJ software (Schindelin et al., 2012). A minimum of 50 cells of one fruit per developmental stage and block were measured. The aspect ratio was calculated as the function of the cell case radial/tangential width.

Tissue Sectioning and Immunolabeling for TEM

Three fruits from each block were harvested at the corresponding developmental stages, and small pericarp pieces of each fruit were cut and fixed overnight with 4% (w/v) paraformaldehyde in 0.1 M of phosphate buffer (pH 7.4), rinsed in the same buffer, and postfixed in 1% (w/v) OsO4 in distilled water for 1 h, following the same postfixing procedure described in Yeats et al. (2012). After dehydration in a graded ethanol-water series (50% to 100%), samples were embedded in LR White resin. Alternatively, samples were embedded in Unicryl resin without prior OsO4 postfixing. Ultrathin sections (around 50 nm thick) were cut with an EM UC7 ultramicrotome (Leica Microsystems) using a diamond knife. Sections were placed onto nickel-Formvar-coated grids and examined in a JEOL JEM-1400 transmission electron microscope at 80 kV acceleration voltage.

Sections embedded in LR White were treated with 10% (v/v) hydrogen peroxide in distilled water for 15 min to remove osmium and washed in distilled water and in phosphate-buffered saline (PBS) buffer (0.01 M, pH 7.4; Kvitkowska et al., 2013). Samples were then blocked with small drops of PBS buffer containing 3% (w/v) bovine serum albumin for 1 h at room temperature and later washed three times in PBS buffer. Rabbit polyclonal antibodies against GDSL2 (CUS1) protein (for details, see Girard et al., 2012) and mouse polyclonal antibodies against cutisomes (for details, see Domínguez et al., 2010) were employed. Sections were incubated in a 500-fold dilution of CUS1 or CUS2 antibodies in PBS buffer for 1.5 h. After three washes in PBS buffer, grids were incubated in a 500-fold dilution in PBS buffer of goat anti-rabbit immunoglobulin G coupled to 10-nm diameter colloidal gold particles for CUS1 detection or goat anti-mouse immunoglobulin G coupled to 25-nm diameter colloidal gold particles (Aurion) and incubated at room temperature for 1 h. After thorough wash in distilled water, sections were stained with a 4% (w/v) uranyl acetate in distilled water for 15 min and later on carefully washed with distilled water. Controls without primary and/or secondary antibodies were carried out. No differences in antibody location were observed between samples embedded in both resins.

Labeling density, i.e. number of gold grains per unit area (micrometers squared), was estimated using ImageJ and measuring ten micrographs corresponding to one fruit per stage of development and block. Cuticle and OEW thickness were estimated from the cross sectioned sample from a minimum of ten measurements of one fruit per developmental stage and block using the same software.

X-ray Diffraclograms

XRD diffraction patterns were obtained with an X’Pert Pro (Malvern PANalytical) automated diffractometer using Ge(111)-monochromated CuKα radiation and an X'celerator detector. Diffractionograms were recorded between 5° and 45° (2θ) in 0.017° steps at 45 kV and 35 mA for 30 min. Isolated cuticle and cutin samples were placed on an aluminum support adapting them to the goniometer in a 2θ to 2θ configuration. The position and the FWHM of peaks were determined after fitting using the software PeakFit 4.11 (Systat Software). The basal spacing d, the average separation between polymer chains in the cuticles, was calculated from the rearrangement of Bragg’s diffraction equation:

\[ d = \frac{\lambda}{2\sin(\theta)} \]

where \( \lambda \) is the wavelength of radiation (~1.54 Å) and \( \theta \) is the diffraction maximum angle. The FWHM parameter was calculated by difference of the basal spacings for the strong maximum at half-height intensity.

Gene Expression Analyses

RNAeasy plant kit (Qiagen) was employed to isolate RNA from tomato fruit peels at different stages of development. Genomic DNA was removed by treating with RNase-free DNase and RNA was cleaned with Nucleospin RNA cleanup kit (Macherey-Nagel). First-strand cDNA synthesis was carried out with the Super Script III first-strand synthesis super mix for RT-qPCR according to the manufacturer’s instructions (Invitrogen). Relative transcript-quantity of the different genes was measured by RT-qPCR using SsoAdvanced SYBR green supermix (Bio-Rad) following the modified ∆ Ct method (Vandesompele et al., 2002) with three endogenous genes recommended by Exposito-Rodríguez et al. (2008) for tomato fruit expression analyses (SICAC Solyc08g006960, SIEXPRESSED Solyc07g025390, and SISAND Solyc03g115810). Three biological samples per developmental stage were analyzed, each corresponding to a pool of epicarp pieces from different tomatoes and plants. For each biological sample three technical replicates were performed. Primers used for amplification and their corresponding efficiencies are shown in Supplemental Table S1.

Protein alignment of the CUS family members was carried out with ClustalX (Larkin et al., 2007). Pairwise comparisons of protein sequences were performed using BLAST 2 Seq (http://www.ncbi.nlm.nih.gov/). The cuta-a mutant (IBM SPSS Statistics for Windows, 2017). Asterisks indicate significant differences at *P < 0.05, **P < 0.01, and ***P < 0.001.

Statistical analysis

Data are expressed as means ± se. Student’s t-test analyses were employed to compare means between cv Microtom and the cuta-a mutant (IBM SPSS Statistics for Windows, 2017). Asterisks indicate significant differences at *P < 0.05, **P < 0.01, and ***P < 0.001.
Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Protein alignment of the CUTIN SYNTHASE (CUS) family members.

Supplemental Figure S2. Immunolocalization of CUTIN SYNTHASE1 (CUS1) in the epicarp of cv Microtom.

Supplemental Figure S3. Transmission electron micrographs of cus1-a cuticle.

Supplemental Table S1. List of primers used for quantitative PCR.

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