Transient Silencing of CHALCONE SYNTHASE during Fruit Ripening Modifies Tomato Epidermal Cells and Cuticle Properties¹[C][W]

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Tomato (*Solanum lycopersicum*) fruit ripening is accompanied by an increase in CHALCONE SYNTHASE (CHS) activity and flavonoid biosynthesis. Flavonoids accumulate in the cuticle, giving its characteristic orange color that contributes to the eventual red color of the ripe fruit. Using virus-induced gene silencing in fruits, we have down-regulated the expression of SI*CHS* during ripening and compared the cuticles derived from silenced and nonsilenced regions. Silenced regions showed a pink color due to the lack of flavonoids incorporated to the cuticle. This change in color was accompanied by several other changes in the cuticle and epidermis. The epidermal cells displayed a decreased tangential cell width; a decrease in the amount of cuticle and its main components, cutin and polysaccharides, was also observed. Flavonoids dramatically altered the cuticle biomechanical properties by stiffening the elastic and viscoelastic phase and by reducing the ability of the cuticle to deform.

There seemed to be a negative relation between SI*CHS* expression and wax accumulation during ripening that could be related to the decreased cuticle permeability to water observed in the regions silencing SI*CHS*. A reduction in the overall number of ester linkages present in the cutin matrix was also dependent on the presence of flavonoids.

**Fruit ripening involves a shift in primary and secondary metabolism that renders the fruit palatable.** Tomato (*Solanum lycopersicum*) has become a model of fleshy fruit development and ripening (Seymour et al., 2013) because of its economic importance and impact on the human diet. During ripening, tomato fruit changes its texture due to cell wall softening, modifies pigment biosynthesis, produces volatile compounds, and accumulates sugars and acids (Giovannoni, 2004). The major changes in pigment biosynthesis that occur in tomato are chlorophyll degradation and carotenoid and flavonoid accumulation. This flavonoid increase is tissue specific, since the highest concentration is present in the epidermal peel with little in the flesh. Nar-ingenin chalcone and the flavonol glycoside rutin are the main flavonoid compounds accumulated in ripe tomatoes (Muir et al., 2001).

Flavonoids represent a large group of plant secondary metabolites that include flavonols, anthocyanins, condensed tannins, and isoflavonoids. There are more than 9,000 flavonoids already described (Williams and Grayer, 2004). Flavonoids are likely present in every plant organ and serve several functions. They provide color to flowers, fruits, and seeds, protect from UV light, are essential for male fertility, regulate reactive oxygen species, and participate in plant defense and molecule signaling, among other roles (Koes et al., 1994). On the other hand, flavonoids can exert their effects directly or indirectly, modulating plant hormones or controlling gene transcription. Thus, they can affect an important number of biological processes. In this sense, flavonoids have been reported to modulate auxin movement (Peer and Murphy, 2007) and hence could be involved in regulating plant growth (Buer and Djordjevic, 2009). Plant flavonoids are usually stored in the vacuole or secreted to the environment, although some accumulate on the surface of different tissues. They are known as surface or lipophilic flavonoids (Onyilagha and Grotewold, 2004). These surface flavonoids are predominantly not glycosylated and mainly locate in glandular trichomes, laticifers, or the cuticle (Wink, 2010). Their biological significance in the physiological survival of the plant is largely based on their location as a first line of defense against pests, herbivores,
or harmful environmental conditions (e.g. UV radiation; Harborne and Williams, 2000; Osnylagha and Grotewold, 2004). However, other roles cannot be ruled out.

The cuticle is a membrane layer that covers the outer epidermal cell walls of the aerial parts of higher plants (Heredia, 2003). It is mainly composed of an insoluble, amorphous, and high-M₆ matrix of esterified fatty acids named cutin in which other compounds such as waxes and phenolics are deposited. Since the cutin matrix is deposited and intertwined with the outer epidermal cell wall, polysaccharides from the cell wall are also an integral part of the cuticle (Domínguez et al., 2011a). In recent years, tomato fruit has become a model for cuticle analyses mostly because its cuticle can be isolated, the availability of an extensive collection of genetic resources and mutants, and the recent publication of its complete genome sequence. Tomato fruit cutin is mainly composed of C₁₆ dihydroxy and hydroxy fatty acids (Heredia, 2003), while the main wax compounds are long-chain alkanes and cyclic compounds such as triterpenoids (Vogg et al., 2004). These waxes can be deposited on the surface (epicuticular) or be embedded in the cutin matrix (intracuticular). The phenolic fraction of tomato fruit cuticle changes during development. It is mainly composed of hydroxycinnamic acid derivatives such as p-coumaric and p-hydroxybenzoic acids during growth, whereas the flavonoid naringenin chalcone becomes the major compound during ripening (Hunt and Baker, 1980; Baker et al., 1982; Luque et al., 1995; España et al., 2014). As a layer located at the boundary with the external environment, the plant cuticle plays several roles related to plant protection. The main function is to prevent water loss while regulating gas exchange. At the same time, it protects from mechanical injury caused by the environment or pests, attenuates UV light, acts as a deterrent against insects, but also allows the generation of an environment suitable for the growth of certain microorganisms (Riederer, 2006a). More recently, an important additional role related to fruit physiology and quality was suggested for the tomato fruit cuticle. A significant increase in cuticle mechanical stiffness was observed during ripening that overlapped with cell wall softening, suggesting that the loss of pericarp strength is compensated with a mechanical reinforcement of the cuticle (España et al., 2014). Although several changes occur in the tomato fruit cuticle during ripening (Domínguez et al., 2008), it was postulated that flavonoids could be responsible for this increase in mechanical strength (Bargel et al., 2006; Domínguez et al., 2009, 2011b).

CHALCONE SYNTHASE (CHS) is the enzyme responsible for the first committed step of the multi-branched flavonoid pathway. It involves the sequential condensation of one p-coumaroyl and three malonyl-CoA molecules to form naringenin chalcone. Schijlen et al. (2007) constitutively suppressed CHS expression in tomato via RNA interference and obtained pink tomatoes due to a reduction of cuticle flavonoids. However, the alteration of plant reproduction due to pollen growth impairment present in these plants led to the development of parthenocarpic fruits, which are known to have altered hormone levels as well as several genes involved in cell wall, sugars, and lipid metabolism (Martinelli et al., 2009).

In this work, we used virus-induced gene silencing (VIGS) to silence CHS in tomato fruits during ripening and study the effect of the absence of flavonoids on the final stages of cuticle development, thus avoiding any indirect effect derived from flavonoid depletion in other tissues or developmental stages. Three different genotypes were analyzed: VIGS-Ready, a tomato genetically engineered to accumulate anthocyanins during ripening due to the expression of two *Antirrhinum majus* transcription factors, *DELILA* (*AmDEL*) and *ROSEA1* (*AmROS1*; Butelli et al., 2008; Orzáez et al., 2009), and two tomato cultivars, cv Moneymaker and cv Gardener’s Delight. We studied the changes in the cuticle and epidermis of ripe fruits associated with the inhibition of the flavonoid pathway. Absence of flavonoids in the cuticle led to dramatic changes in the cuticle biochemical and biophysical properties.

**RESULTS**

**CHS Expression during Ripening and VIGS**

Two *CHS* genes are known to be expressed in tomato fruit during ripening, *SICH51* (Solycc09g091510.2) and *SICH52* (Solycc05g053550; Ballester et al., 2010). The *SICH51* fragment selected to silence both genes corresponded to a 320-bp region of the second exon (between nucleotides 431 and 751 of the annotated complementary DNA [cDNA] sequence) sharing 86% identity with *SICH52*. A nucleotide BLAST search (at http://solgenomics.net) of this fragment did not render high similarity with any other gene in the tomato genome other than both *CHSs*.

In order to test if VIGS agroinjection at mature green would be a suitable strategy to obtain ripe tomatoes with colorless cuticles due to *CHS* silencing, we first measured the accumulation of *SICH51* and *SICH52* in fruit epicarp during ripening (Fig. 1). The results showed differences in *CHS* expression among different genotypes. In general, *SICH52* was more expressed than *SICH51* throughout the whole ripening period in all genotypes. Maximum accumulation of *SICH51* and *SICH52* mRNA was detected at the breaker stage, where *SICH52* showed an 8-fold increase compared with *SICH51* in VIGS-Ready and cv Gardener’s Delight and a 20-fold increase in cv Moneymaker. *SICH51* was barely detectable at mature green or red ripe in the three genotypes studied. On the other hand, *SICH52* was clearly expressed at mature green, although low, and had a considerable expression at red ripe. An exception was cv Gardener’s Delight, where *SICH52* expression at red ripe was notably lower than in cv Moneymaker and VIGS-Ready.

Ripe tomato fruits with colorless cuticles are pink in color. However, red and pink fruits are not always easy to discriminate, especially if a mosaic pattern of red and pink regions with transition zones in between is expected. Thus, *CHS* silencing was first carried out...
in the VIGS-Ready genotype using the pTRV2_DR_CHS construct, which carried DNA fragments for combined silencing of DEL/ROS1/CHS. Since anthocyanin accumulation in these tomatoes occurs during ripening due to DEL/ROS1 expression under the control of the E8 fruit-ripening promoter, it can be used as a visual marker of silencing (Orzáez et al., 2009). Hence, two easily distinguishable colored regions could be expected: a purple one where anthocyanin accumulation was not silenced and a pink one due to the silencing of DEL/ROS1 plus CHS. However, DEL/ROS1/CHS silencing in the VIGS-Ready genotype at mature green rendered a mosaic pattern at the ripe stage where three colored sectors could be observed (Fig. 2): a purple region where no silencing had occurred, a red one that corresponded to DEL/ROS1 silencing with low, if any, CHS silencing, and a pink one where full silencing of DEL/ROS1/CHS would be expected.

Since CHS is still expressed at red ripe in the VIGS-Ready genotype (Fig. 1), we measured the expression of SICH1, SICH2, and AmDEL at the ripe stage in the epicarp of the three different sectors (Fig. 3). A marked decrease in both SICH1 and SICH2 expression was observed in the red and pink regions compared with the purple ones (Fig. 3, A and B). Nevertheless, the expression of both SICH1 and SICH2 was significantly lower in the pink sectors compared with the red ones. This was not the case for AmDEL, which showed a marked but similar decrease in the red and pink regions compared with the purple ones (Fig. 3C). Comparison of the accumulation of SICH1, AmROS1, and AmDEL transcripts at breaker in control VIGS-Ready tomatoes indicated that SICH2 was 10 times more expressed at this stage than AmROS1 or AmDEL (Fig. 3D). Despite the significant decrease in CHS expression in the red sector, physical isolation of the purple and red sectors followed by their cuticle extraction showed a similar orange color in both of them due to flavonoid accumulation (Fig. 4).

Since the VIGS methodology was shown to be a good strategy to silence CHS during ripening, and considering that the VIGS-Ready tomatoes already have the flavonoid pathway modified, we decided to agroinoculate two other nongenetically modified genotypes, cv Moneymaker and cv Gardener’s Delight, with the pTRV2_CHS construct for CHS silencing. The results of these new agroinoculations showed tomatoes with a mosaic pattern of red and pink regions too, although sometimes they were not easy to distinguish, especially in cv Gardener’s Delight. Figure 5 shows isolated red ripe cuticles corresponding to control (not agroinoculated) and CHS-silenced tomatoes of VIGS-Ready, cv Moneymaker, and cv Gardener’s Delight. The colorless regions of the cuticle corresponded to the pink sectors. While in VIGS-Ready and cv Moneymaker, almost colorless cuticle regions can be observed, in cv Gardener’s Delight, only a decrease in cuticle color was obtained.

### CHS Silencing Affects Cuticle Deposition

Table I shows the amount of cuticle and its components present in the different sectors of VIGS-Ready, cv Moneymaker, and cv Gardener’s Delight silenced fruits. First, no differences in the amount of cuticle and its major component cutin were observed for the purple and red regions of VIGS-Ready. Similarly, the amount of phenolics was the same in these two regions, which agreed with the color already analyzed (Fig. 4). However, a small but significant decrease in polysaccharides and, interestingly, an almost 2-fold decrease in the amount of cutin was observed in the pink sectors.
In order to test if the differences observed in the amount of waxes and polysaccharides between the cuticles of the purple and red areas were side effects of DEL/ROS1 expression or of CHS expression (compare Fig. 4 with Supplemental Fig. S1). Combination of the above results indicated that in VIGS-Ready and cv Moneymaker, the loss of cuticle cutinization was mostly due to a decrease in cuticle density, which in cv Moneymaker was also accompanied by a decrease in epidermal cell wall cutinization, whereas in cv Gardener’s Delight it could mostly be attributed to a decrease in the invagination index and the thickness of such invaginations.

Epidermal Cell Expansion during Ripping Is Dependent on CHS Expression

Histological sectioning of the different sectors of fruit epicarp showed other interesting differences (Fig. 6). Epidermal cells showed a different shape in the pink areas compared with that of the red ones. This different behavior could be attributable to the observed indirect effect of DEL/ROS1 expression at the wax level.
sectors compared with the red ones. Differences in shape and size were sometimes also observed in the hypodermal and some parenchyma cells of the pink sectors. This may be a side effect of the strong mosaic pattern characteristic of the VIGS methodology in tomato. Hence, only the changes consistently displayed in all the cross sections analyzed were measured. However, it is possible that CHS silencing would also modify the size and shape of parenchyma fruit cells.

Table III shows the differences in epidermal cell size and shape observed in the different sectors of the three genotypes studied. Comparison of pTRV2_DR_CHS purple and red sectors of VIGS-Ready epicarp revealed a significant increase in epidermal cell area and perimeter in the red sector compared with the purple one. In the purple area, radial cell width was smaller, while tangential cell width did not change significantly. These changes were also observed in the purple and red sectors of the tomatoes agroinoculated with pTRV2_DR (Supplemental Table S1C), suggesting that the differences are due to an effect of the genes DEL/ROS1 and not to the CHS knockdown present in the red sector of the pTRV2_DR_CHS tomatoes (Fig. 4). Epidermal cells from the VIGS-Ready pink sector were similar to those from the red sector except for a significant increase in radial cell width. Similarly, changes in tangential and radial cell widths were also observed in the pink sectors of cv Moneymaker and cv Gardener’s Delight. In both cases, the cells from the pink sectors were significantly shorter and wider than their red counterparts. In cv Gardener’s Delight, significant decreases in epidermal cell area and perimeter were also observed in the pink sector.

The aspect ratio is a dimensionless shape factor that describes the geometry of any particle independently of its size (Wojnar and Kurzydlowski, 2000). It varies from close to 0 (very elongated) to 1 (equiaxial). In our case, it describes how elongated or rounded cells are. Epidermal cells from the pink sectors were significantly more rounded than those from the red sectors in the three genotypes analyzed. Moreover, differences in the shape of epidermal cells were observed among the different genotypes, with cv Gardener’s Delight having more rounded epidermal cells than VIGS-Ready...
and cv Moneymaker, mostly due to their higher radial cell width (Table III).

**CHS-Mediated Changes in the Biophysics and Molecular Arrangement of the Cuticle**

Table IV shows the biomechanical parameters analyzed for the isolated cuticles of the different sectors in the three genotypes studied here. Comparison of the cuticles from the purple and red areas of VIGS-Ready showed no differences in any of the analyzed parameters. However, cuticles from the pink sectors had a significant decrease in the Young’s modulus and an increase in maximum strain. This general behavior was confirmed in the two other genotypes, cv Moneymaker and cv Gardener’s Delight, where the cuticles from the pink areas were less stiff and more deformable at the breaking point than the corresponding cuticles from the red sectors. Also, a decrease in the breaking stress was observed in the cuticles of the pink regions for the three genotypes, although it was only significant in cv Moneymaker. Figure 7 shows an example of a stress-strain curve for the cuticles of each sector in the three genotypes studied. The typical presence of two phases, elastic at low stresses and viscoelastic at higher stresses, could be observed in most cases (Fig. 7). The slope of the elastic phase was always higher than that of the viscoelastic phase. No differences were observed between the cuticles of the purple and red sectors regarding the lengths of the elastic and viscoelastic phases, since they both reached the yield point (change from elastic to viscoelastic behavior) at similar stresses and strains (Table IV).

Analysis of the cuticles from the pink sectors showed that in VIGS-Ready and cv Gardener’s Delight, the yield stress (stress needed to enter viscoelasticity) was almost similar to the cuticles of the red regions (Table IV; Fig. 7, arrows). These results were similar to those observed for the breaking stress. However, cuticles of the pink areas of cv Moneymaker did not show any viscoelastic behavior, and they broke under elastic deformation. The amount of elastic versus viscoelastic deformation showed differences between the cuticles of the pink and red regions. In all the genotypes, an increase in the elastic strain was observed in the pink regions except for cv Moneymaker, where only elastic deformation was present. Also, a decrease in the slope of the viscoelastic phase could be observed in the cuticles of the pink sectors for VIGS-Ready and cv Gardener’s Delight (Fig. 7).

The effect of CHS silencing on the cuticle biomechanics varied among the different genotypes. Thus, cv Moneymaker showed a more severe effect in stiffness (6-fold) and breaking stress (2.6-fold) decrease than the

![Figure 5](image-url) Photograph of enzymatically isolated cuticles from not agro-injected control and agroinoculated ripe tomatoes. Regions without color can be observed in the agroinoculated cuticles. A, VIGS-Ready. B, cv Moneymaker. C, cv Gardener’s Delight.

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Table 1. *Amounts of tomato fruit cuticle and its components*

Significant differences are indicated by different letters according to one-way ANOVA with \( P < 0.05 \) or with asterisks according to Student’s *t* tests with \( P < 0.01 (**).\)

<table>
<thead>
<tr>
<th>Plant</th>
<th>Cuticle</th>
<th>Cutin</th>
<th>Polysaccharides</th>
<th>Waxes</th>
<th>Phenolics</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>µg cm(^{-2})</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VIGS-Ready</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Purple</td>
<td>1,563 ± 47 a</td>
<td>1,011 ± 30 a</td>
<td>475 ± 14 a</td>
<td>77 ± 2 a</td>
<td>112 ± 4 a</td>
</tr>
<tr>
<td>Red</td>
<td>1,531 ± 66 a</td>
<td>1,084 ± 47 a</td>
<td>404 ± 17 b</td>
<td>43 ± 2 b</td>
<td>125 ± 4 a</td>
</tr>
<tr>
<td>Pink</td>
<td>1,132 ± 83 b</td>
<td>767 ± 56 b</td>
<td>332 ± 24 c</td>
<td>33 ± 2 c</td>
<td>16 ± 1 b</td>
</tr>
<tr>
<td>cv Moneymaker</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red</td>
<td>1,763 ± 76</td>
<td>1,342 ± 58</td>
<td>387 ± 17</td>
<td>34 ± 1</td>
<td>148 ± 8</td>
</tr>
<tr>
<td>Pink</td>
<td>1,043 ± 93**</td>
<td>740 ± 66**</td>
<td>273 ± 24**</td>
<td>30 ± 3</td>
<td>18 ± 2**</td>
</tr>
<tr>
<td>cv Gardener’s Delight</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red</td>
<td>1,498 ± 58</td>
<td>1,063 ± 41</td>
<td>404 ± 15</td>
<td>31 ± 1</td>
<td>144 ± 5</td>
</tr>
<tr>
<td>Pink</td>
<td>1,171 ± 45**</td>
<td>819 ± 31**</td>
<td>322 ± 12**</td>
<td>30 ± 1</td>
<td>39 ± 3**</td>
</tr>
</tbody>
</table>

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other two genotypes. These differences in stress were not correlated with differences in strain, since a similar increase in the maximum strain was observed in the cuticles of the pink sectors of the three genotypes. These differences cannot be attributed to a higher reduction in the amount of phenolics, since the cuticles of the pink areas of cv Moneymaker and VIGS-Ready showed similar decreases in phenolics.

Transition regions between the colored and noncolored sectors of the cuticle showed small mosaic patterns of colored and noncolored regions, attributable to the presence/absence of flavonoids (Supplemental Fig. S2, A and B). The biomechanics of these cuticle regions with clusters of flavonoids in a noncolored matrix (i.e. without flavonoids) showed a gradation in the Young modulus, from values similar to the red regions to others closer to the pink ones (Supplemental Fig. S2C). This decrease was related to the general amount of coloration in the sample. However, no similar gradual behavior was observed in the maximum strain, probably because the mosaic colored regions might have acted as fillers within the sample itself and prevented further deformation.

The water barrier properties of the cuticles isolated from the different sectors were also studied. Figure 8 shows the differences in water permeability for the three genotypes analyzed. In VIGS-Ready, cuticles from the purple regions displayed a significantly lower permeability to water than their red counterparts, whereas cuticles from the pink regions showed the lowest permeability to water. Similarly, cuticles from the pink sectors of cv Moneymaker and cv Gardener’s Delight had significant 3- and 2-fold decreases, respectively, in permeability in comparison with those from the control red regions. Cuticles from the pink regions of cv Gardener’s Delight showed the highest permeability compared with the pink regions of the two other genotypes (VIGS-Ready and cv Moneymaker).

Cutin chemical characterization was carried out by attenuated total reflectance (ATR)-Fourier transform infrared (FTIR) spectroscopy (Supplemental Fig. S3). Spectra were mainly characterized by infrared absorptions of hydroxyl (approximately 3,270 cm\(^{-1}\)), methylene (2,925, 2,853, 1,463, and 724 cm\(^{-1}\)), and ester (1,729 cm\(^{-1}\)) functional groups (Villena et al., 2000; Mazurek et al., 2013; Heredia-Guerrero et al., 2014). No differences in the infrared spectral pattern were observed between the cutin isolated from VIGS-Ready purple and red regions (Supplemental Fig. S3). However, some interesting differences were detected between the cutin of the red and pink sectors for the three genotypes studied (Table V). These are ascribed to the presence of absorption bands related to phenolic compounds (1,650–1,550 cm\(^{-1}\) region and 836 cm\(^{-1}\)) and to the changes in the bands associated with ester and free carboxylic acids (1,729 and approximately 1,705 cm\(^{-1}\), respectively). The ɣ-band (836 cm\(^{-1}\)), which has been associated with the presence of phenolic compounds (España et al., 2014), showed a significant decrease in the cutin isolated from the pink sectors compared with the red ones. Similarly, a significant increase in the esterification index and in the ratio between ester and carboxylic acids (\(I_{\text{COOR}}/I_{\text{COOH}}\)) was observed in the cutin isolated from the pink sectors compared with the red ones. The esterification index is an indicator of how esterified a cutin matrix is, whereas the \(I_{\text{COOR}}/I_{\text{COOH}}\) ratio shows how many carbonyl groups are present as esters versus carboxylic acids. These results indicated a shift in the chemical bonding of the carbonyl groups present in the cutin matrix in relation to the incorporation of phenolics: a reduction in ester and a consequent increase in carboxylic acids in the cutin isolated from the red regions and the opposite behavior (more ester and less carboxylic acids) in the pink sectors.

**DISCUSSION**

**DEL/ROS1 Expression Affects Several Cuticle Traits**

VIGS methodology has been proven to be a fast and efficient tool to study gene function without having to resort to the development of stable transgenic lines. Fruit VIGS offers several advantages over whole-plant VIGS: it is a shortcut to the study of fruit-specific processes and also avoids the potential side effect of
gene silencing at the vegetative tissue or fruit development (Orzáez et al., 2006). However, VIGS partial penetration allows the screening of visually identifiable phenotypes but needs the addition of an internal visual reference to identify the silenced sectors. Transgenic tomato plants expressing genes involved in anthocyanin synthesis during ripening (Butelli et al., 2008) have been shown to be useful for this purpose, since the inhibition of anthocyanin accumulation in ripe tomatoes can be used as a visual marker for VIGS (Orzáez et al., 2009). Ballester et al. (2010) reported the presence of only pink and purple sectors in VIGS-Ready tomatoes that silenced SlMYB12, a transcription factor involved in the flavonoid pathway, in combination with DEL/ROS1. However, in our case, CHS silencing in combination with DEL/ROS1 also rendered red sectors. The red sectors resulted from DEL/ROS1 down-regulation, but with insufficient CHS down-regulation to affect naringenin chalcone accumulation in the cuticle. The differences in the expression of SlCHS2, AmDEL, and AmROS1 during ripening (Fig. 4) could explain this gradient of CHS gene silencing. These results indicate that significant differences in expression between the reporter gene and the gene of interest can be a drawback, since they would render different levels of silencing of the gene of interest with the same visual silencing of the reporter.

Figure 6. Micrographs of unstained fruit epicarp sections from ripe tomatoes agroinoculated to silence CHS. A and B, cv Moneymaker red (A) and pink (B) sectors. C and D, cv Gardener’s Delight red (C) and pink (D) sectors. E to G, VIGS-Ready purple (E), red (F), and pink (G) sectors.
gene. This uncoupling in the silencing of both genes could be avoided by checking in advance the expression levels of both the reporter and the gene of interest for the desired tissue and stage of development. The development of other reporter genes, including some highly expressed during fruit ripening, would be of interest, since it would allow choosing the reporter gene more suitable to a given experiment.

Heterologous expression in tomato fruit of AmDEL and AmROS1 activates several genes of the flavonoid pathway during ripening, including PHENYLALANINE AMMONIA LYASE (SIPAL) and CHALCONE ISOMERASE (SICHI; Butelli et al., 2008). Although CHS expression was not modiﬁed by DEL/ROS1 expression (Supplemental Fig. S1), the activation of other genes in the pathway could alter the flavonoids deposited in the cuticle. Muir et al. (2001) reported a signiﬁcant depletion of naringenin chalcone in the peel of ripe tomato fruit overexpressing petunia (Petunia hybrida) CHI despite the low increase of CHI transcripts. These results were accompanied by a reduction in tomato peel color that could have been the result of a lower amount of naringenin chalcone present in the cuticle or the incorporation of a noncolored flavonoid to the cuticle. Nevertheless, the transient increase in SICHI transcript caused by DEL/ROS1 activity did not affect cuticle color, since the cuticles of the purple and red sectors had the same color and displayed the same amount of flavonoids (Fig. 4; Table I). Yet, naringenin chalcone content was decreased in the peel of purple tomatoes, and flavonol derivatives increased (Butelli et al., 2008). Comparison of the red sectors of control pTRV2_DR and pTRV2_DR_CHS agroinoculated tomatoes could provide an explanation. Whereas SICHS1 and SICHS2 expression in pTRV2_DR red sectors were similar to the purple ones, in pTRV2_DR_CHS, a significant decrease in both transcripts was detected. However, this reduction was not enough to affect flavonoid incorporation to the cuticle. Thus, it could be that in pTRV2_DR red sectors, the SICHI expression was not high enough to prevent naringenin chalcone incorporation to the cuticle. Also, SIPAL activation in VIGS-Ready tomatoes would increase the amount of substrate for CHS.

The changes in the flavonoid profile present in the peel of the purple tomatoes (Butelli et al., 2008) have an effect on some of the cuticle traits analyzed. Thus, DEL/ROS1 expression clearly modiﬁed the amount of waxes accumulated in the cuticle. An almost 2-fold increase was observed in the cuticles from the purple sectors compared with the red ones in silenced tomatoes. Waxes have been known to modify the mechanical properties of the cuticle conferring stiffness (Petracek and Bukovac, 1995) and also play a signiﬁcant role as a transpiration barrier (Schönherr and Lendzian, 1981). However, according to the literature, while wax extraction decreased cuticle stiffness, a decrease in the amount of waxes did not have a clear effect (España et al., 2014). This is in agreement with our results, since the mechanical properties of the cuticles from the purple and red sectors were similar. Waxes are a complex mixture of compounds; hence, it is possible that the composition and speciﬁc localization of the wax mixture, more than the wax load, were more important in the mechanical properties. In this sense, cuticular water permeability was found to increase with the reduction of the very-long-chain aliphatic fraction of tomato fruit waxes (Vogg et al., 2004) but did not correlate with the total amount of waxes (Riederer and Schreiber, 2001). Recently, Zhang et al. (2013) reported an extended shelf life in purple tomatoes expressing DEL/ROS1 genes. Fruit shelf life is determined by a combination of factors, such as degree of softening, shriveling, and rotting. This long shelf life present in the purple tomatoes can be explained, at least in part, by the significant decrease in water permeability of the cuticle, which would lead to reduced fruit shriveling. In turn, this lower permeability could be attributed to the signiﬁcant increase in cuticle waxes present in the purple sectors.

Similarly, the increase in cuticle polysaccharides of the purple sectors could be explained by the reported reduction of cell wall degradation observed in the purple tomatoes (Zhang et al., 2013). This reduction in fruit softening present in the purple tomatoes could also explain the higher invagination index and the differences in epidermal cell shape in comparison with the red sectors.

Table III. Analysis of epidermal cell size and shape of ripe fruits

<table>
<thead>
<tr>
<th>Plant</th>
<th>Area</th>
<th>Perimeter</th>
<th>Tangential Width</th>
<th>Radial Width</th>
<th>Aspect Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \mu^2 )</td>
<td>( \mu m )</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VIGS-Ready</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Purple</td>
<td>330.8 ± 14.2 b</td>
<td>72.5 ± 1.6 b</td>
<td>29.9 ± 0.7</td>
<td>14.4 ± 0.3 c</td>
<td>0.49 ± 0.01 b</td>
</tr>
<tr>
<td>Red</td>
<td>376.0 ± 15.3 a</td>
<td>77.6 ± 1.5 a</td>
<td>32.1 ± 0.7</td>
<td>15.4 ± 0.3 b</td>
<td>0.48 ± 0.01 b</td>
</tr>
<tr>
<td>Pink</td>
<td>379.7 ± 10.4 a</td>
<td>76.9 ± 1.3 b</td>
<td>31.0 ± 0.6</td>
<td>16.5 ± 0.2 a</td>
<td>0.55 ± 0.01 a</td>
</tr>
<tr>
<td>cv Moneymaker</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red</td>
<td>387.4 ± 14.2</td>
<td>80.1 ± 1.6</td>
<td>34.1 ± 0.8</td>
<td>14.9 ± 0.3</td>
<td>0.44 ± 0.01</td>
</tr>
<tr>
<td>Pink</td>
<td>389.0 ± 12.2</td>
<td>76.2 ± 1.2</td>
<td>29.5 ± 0.6**</td>
<td>17.0 ± 0.3**</td>
<td>0.59 ± 0.01**</td>
</tr>
<tr>
<td>cv Gardener’s Delight</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red</td>
<td>610.3 ± 17.3</td>
<td>94.7 ± 1.5</td>
<td>36.2 ± 0.7</td>
<td>21.9 ± 0.3</td>
<td>0.61 ± 0.01</td>
</tr>
<tr>
<td>Pink</td>
<td>550.6 ± 15.8*</td>
<td>87.1 ± 1.3**</td>
<td>31.2 ± 0.5**</td>
<td>23.2 ± 0.4*</td>
<td>0.75 ± 0.01**</td>
</tr>
</tbody>
</table>
De Novo Flavonoid Biosynthesis Is Responsible for Flavonoid Incorporation to the Cuticle

Tomato fruits synthesize and accumulate flavonoids in their peel (epidermal and hypodermal cells) throughout development (Slimestad and Verheul, 2009; Meléndez-Martínez et al., 2010). This synthesis is accompanied by a significant expression of CHS during most stages of fruit development (Schijlen et al., 2004), despite naringenin chalcone accumulation not being detected until the beginning of ripening (Muir et al., 2001; Meléndez-Martínez et al., 2010). However, flavonoids are not present in the cuticles of immature or mature green tomatoes but are only incorporated during ripening (Domínguez et al., 2009; España et al., 2014). Of the several flavonoids known to be present in tomato peel (Slimestad and Verheul, 2009), only naringenin is transported and incorporated to the outer cuticle matrix (Hunt and Baker, 1980). Flavonoids synthesized during fruit development are either stored in the vacuole, from where they can be remobilized under certain conditions, or directly transported to their cell targets (Zhao and Dixon, 2010; Petruzza et al., 2013). The fact that CHS disruption during ripening impeded flavonoid accumulation in the cuticle indicates that the naringenin chalcone incorporated comes from de novo synthesis and not from remobilized vacuole-stored flavonoids. This flavonoid specificity of the cuticle might be related to the activation of its transport until the cuticle only during ripening, when SICHI is not expressed (Muir et al., 2001) and only naringenin chalcone is synthetized, or even to a putative specificity of the transporter for this compound. Although a trafficking pathway of phenolic compounds to the cell wall has been shown in some species (Lin et al., 2003), this topic needs further research. Moreover, the analysis of tomato plants with altered flavonoid pathways during ripening could provide insights into the specificity of the transport to the cuticle or the affinity for a certain flavonoid molecule.

**CHS-Related Changes in Epidermal Cell Shape**

During tomato fruit ripening, a significant thickening of the cuticular pegs and cutinization of the epidermal and some hypodermal cell walls occur (Domínguez et al., 2008). Also, a final increase in fruit size, mostly due to cell expansion, is detected between mature green and red ripe (Domínguez et al., 2008, 2012), which leads to an increase in tangential width and a decrease in radial width of the epidermal cells (Domínguez et al., 2008; España et al., 2014). These final changes in the epidermis modified its shape, with cells from red ripe fruits being significantly more elongated than their mature green counterparts. These modifications during ripening are clearly affected by CHS silencing, since the epidermal cells from the pink sectors shared several traits common in the cells of mature green fruits: more rounded cells due to a lower tangential width and higher radial width and with thinner pegs and less cuticle invagination. A reduction in radial cell width and an increase in tangential width associated with growth also have been reported for other species, suggesting a physical stretching of the epidermis (Hammami and Rapoport, 2012). Considine and Brown (1981) showed that cell geometry is modified with the increase of internal fruit forces as a consequence of the orientation of mechanical stresses along the fruit surface. An increase in internal pressure has been observed during tomato fruit ripening (Almeida and Huber, 2001), which could act as the driving force for these changes.

**CHS silencing and/or changes in the flavonoid profile have been shown to modify several parameters related to shoot and root phenotype in Arabidopsis (Arabidopsis thaliana); Buer and Djordjevic, 2009; Buer et al., 2013). In a few cases, these changes in size were related to differences in cell size or cell expansion (Ringli et al., 2008; Laffont et al., 2010). Flavonoids have been shown to negatively regulate auxin polar transport in vivo (Jacobs and Rubery, 1988; Buer and Muday, 2004), one of the hormones that control cell anisotropy (Hamant and Traas, 2010). A decrease in free auxin concentration has been reported prior to the onset of tomato fruit ripening; this decrease was accompanied by an increase in conjugated auxin (Buta and Spaulding, 1994; Böttcher et al., 2010). In grape (Vitis vinifera), treatment with synthetic auxin delayed

### Table IV. Biomechanical parameters of the isolated fruit cuticles

<table>
<thead>
<tr>
<th>Plant</th>
<th>Young’s Modulus</th>
<th>Breaking Stress</th>
<th>Maximum Strain</th>
<th>Yield Stress</th>
<th>Yield Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MPa</td>
<td>MPa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VIGS-Ready</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Purple</td>
<td>649 ± 70 a</td>
<td>58 ± 6 a</td>
<td>14 ± 1 b</td>
<td>32 ± 3 a</td>
<td>5 ± 0 b</td>
</tr>
<tr>
<td>Red</td>
<td>735 ± 80 a</td>
<td>57 ± 9 a</td>
<td>13 ± 2 b</td>
<td>32 ± 3 a</td>
<td>4 ± 0 b</td>
</tr>
<tr>
<td>Pink</td>
<td>242 ± 19 b</td>
<td>42 ± 3 a</td>
<td>21 ± 2 a</td>
<td>29 ± 0 a</td>
<td>12 ± 1 a</td>
</tr>
<tr>
<td>cv Moneymaker</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red</td>
<td>573 ± 46</td>
<td>60 ± 3</td>
<td>16 ± 1</td>
<td>36 ± 1</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>Pink</td>
<td>91 ± 6**</td>
<td>23 ± 3**</td>
<td>23 ± 2*</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>cv Gardener’s Delight</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red</td>
<td>755 ± 49</td>
<td>59 ± 6</td>
<td>12 ± 1</td>
<td>38 ± 4</td>
<td>5 ± 1</td>
</tr>
<tr>
<td>Pink</td>
<td>293 ± 78**</td>
<td>46 ± 2</td>
<td>26 ± 2**</td>
<td>30 ± 5</td>
<td>12 ± 2**</td>
</tr>
</tbody>
</table>

Significant differences are indicated by different letters according to one-way ANOVA with P < 0.05 (*) or P < 0.01 (**). –, Data not available.
the increase of CHS expression associated with fruit ripening (Davies et al., 1997). Recently, Medina-Puche et al. (2014) showed that \( \text{FaMYB10} \) expression, a transcription factor involved in flavonoid synthesis, was repressed by auxin in strawberry (\( \text{Fragaria x ananassa} \)). \( \text{TOMATO AGAMOUS-LIKE1 (TAGL1)} \) is involved in fruit expansion at early stages of development but also in ripening (Vrebalov et al., 2009). Ripening fruits that silence \( \text{TAGL1} \) have shown changes in the phenylpropanoid pathway (Giménez et al., 2010), although no characterization of the cuticle or CHS expression levels have been carried out. Changes in epidermal cell shape associated with CHS silencing reported in this work could respond to the above-mentioned relationship between flavonoids and auxins. However, a link of flavonoids and epidermal cell size and shape via auxin levels (or more generally hormone levels) and fruit ripening still needs to be addressed.

### Flavonoids Are Involved in Cuticle Wax Deposition, Water Permeability, and Mechanical Resistance

Several transcription factors involved in tomato ripening have been found to impact cuticle development (for review, see Hen-Avivi et al., 2014), including in many cases the regulation of CHS expression. Thus, \( \text{SIMYB12} \) inhibition down-regulated \( \text{SICH1} \) and \( \text{SICH2} \) expression (Adato et al., 2009; Ballester et al., 2010), \( \text{RIPENING INHIBITOR (RIN)} \) positively regulated CHS expression (Fujisawa et al., 2012), while inhibition of \( \text{FRUITFULL1/2} \) up-regulated \( \text{SICH1} \) expression (Bemer et al., 2012). Cuticle isolation and thorough analysis have been limited to a few transcription factors, such as \( \text{colorless fruit epidermis (y)} \) and the naturally occurring ripening-delayed mutants \( \text{rin} \) and \( \text{nonripening (nor)} \). In these instances, cuticles did not accumulate flavonoids despite the changes in fruit color during ripening (Bargel and Neinhuis, 2004; Adato et al., 2009).

Changes in tomato fruit cuticle during ripening have been described previously (Domínguez et al., 2008, 2009). These included a loss of polysaccharides due to cell wall degradation and, in cherry tomatoes, a decrease in cuticle thickness. However, the observed differences in the cuticles of the red and pink sectors cannot be explained only by a cuticle that retained some characteristics of the mature green cuticles. In this case, a decrease in the amounts of cutin and polysaccharides and a comparative increase in waxes were

**Figure 7.** Mean stress-strain curves of isolated cuticles from the different sectors of fruits agroinoculated to silence CHS. Arrows indicate the yield point (transition from elastic to viscoelastic behavior). The pink sector of cv Moneymaker did not show viscoelastic behavior. \( n = 5 \) to 7 biological replicates. [See online article for color version of this figure.]

**Figure 8.** Water permeability of the cuticles isolated from the different sectors of fruits agroinoculated to silence CHS. Data are presented as means of seven to 10 biological replicates \( \pm \) se. Letters indicate significant differences according to one-way ANOVA with \( P < 0.05 \), and asterisks indicate significant differences according to Student’s \( t \) test with \( P < 0.01 \) (**). [See online article for color version of this figure.]
observed. These novel results indicate that flavonoids modify the cuticle during ripening. Saladié et al. (2007) reported an increase in cuticle waxes of the delayed fruit deterioration (ddf) mutant compared with cv Ailsa Craig. This mutant is characterized by a colorless cuticle at the ripe stage. Adato et al. (2009) studied the cuticle of the tomato y mutant, which is characterized by a lack of flavonoids together with several other changes at the fruit level. Comparison of the control and mutant cuticles showed some similarities with those from the red and pink sectors: a decrease in the amounts of cuticle and cutin in the y mutant cuticle, while the amount of waxes remained unchanged (Adato et al., 2009). The decrease in polysaccharides present in the cuticles of the pink sectors could be related to a higher cell wall degradation and possible fruit softening.

Comparison of the mechanical behavior of cuticles from different red and pink tomato genotypes showed significant changes in stiffness (Dominguez et al., 2009). However, other effects associated with the different genetic backgrounds studied prevented a more in-depth analysis. In this study, CHS silencing during ripening allowed us to further study the sole involvement of flavonoids in cuticle biomechanics. Thus, flavonoids modified not only the Young's modulus but also the slope of the viscoelastic phase, hence reducing the deformation capacity of the cuticle matrix. Moreover, flavonoids also modified the elastic and viscoelastic deformation of the cuticle. Cuticle polysaccharides play a similar mechanical role, conferring stiffness and reducing strain (López-Casado et al., 2007). However, contrary to polysaccharides, which also reduced the breaking stress, flavonoids did not affect the breaking stress, which indicated that their absence did not reduce the stress able to be sustained by the cuticle. A similar mechanical behavior was reported for the ddf mutant compared with cv Ailsa Craig: a decrease in stiffness and an increase in strain without affecting the breaking stress (Saladié et al., 2007). On the other hand, Bargel and Neinhuis (2004) observed a significant decrease in the breaking stress, as well as Young's modulus and strain, for the tomato mutant nor, whereas the cuticles of the y mutant only showed a decrease in the yield stress, the force needed to enter viscoelastic behavior (Adato et al., 2009).

Waxes are known to modify the mechanical properties of the cuticle (Petracek and Bukovac, 1995). Wax removal in species with a heavy wax load, such as apple (Malus domestica), pear (Pyrus communis), persimmon (Diospyros kaki), and others, led to decreases in Young's modulus and breaking stress as well as an increase in cuticle deformation (Khanal et al., 2013; Tsubaki et al., 2013). In tomato, however, only the cuticular stiffness was reduced (Khanal et al., 2013). This suggests that the relative increase in waxes observed in the pink sectors was not enough to compensate the mechanical role of the flavonoids.

A negative correlation between flavonoid incorporation during ripening and changes in the cutin esterification index was reported recently (España et al., 2014). However, our work here shows that changes in the esterification of the cutin matrix are only related to CHS expression and not to general fruit ripening, since the pink sectors ripened but their cuticles had less carboxylic acids than those from the red ripe sectors. Thus, a hydrolysis of the ester groups of the cutin induced or regulated by the presence of phenolic compounds should be posited. Hence, in the absence of flavonoids, the cutin matrix did not modify its degree of esterification during ripening.

Luque et al. (1995) reported a decrease in the water permeability of tomato fruit cuticle during ripening. They suggested an important role for flavonoids in the control of water transport through the cuticle. Our results, however, indicate that the absence of flavonoids led to a series of changes in the cuticle of ripe fruits: polymer cross-linking degree, density and wax accumulation, and water flux through the cuticle. Hence, biophysical changes of the cuticle matrix could take part in water permeability in addition to the already mentioned principal function of waxes. In this sense, Knoche et al. (2001) suggested that the strain associated with fruit expansion increased permeability in cherry (Prunus avium). The fact that altering the flavonoid pathway via either DEL/ROS1 expression during ripening or CHS silencing has similar

Table V. Infrared parameters calculated from ATR-FTIR cutin spectra

<table>
<thead>
<tr>
<th>Plant</th>
<th>γ-Band Area</th>
<th>Esterification Index</th>
<th>υ_{COOH}−υ_{COOH}</th>
<th>a.u.</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIGS-Ready</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Purple</td>
<td>1.74 ± 0.10 a</td>
<td>0.96 ± 0.01 b</td>
<td>1.12 ± 0.02 b</td>
<td></td>
</tr>
<tr>
<td>Red</td>
<td>1.50 ± 0.09 a</td>
<td>0.97 ± 0.00 b</td>
<td>1.14 ± 0.03 b</td>
<td></td>
</tr>
<tr>
<td>Pink</td>
<td>0.27 ± 0.07 b</td>
<td>1.14 ± 0.02 a</td>
<td>1.48 ± 0.01 a</td>
<td></td>
</tr>
<tr>
<td>cv Moneymaker</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red</td>
<td>2.00 ± 0.11</td>
<td>0.90 ± 0.01</td>
<td>1.05 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Pink</td>
<td>0.55 ± 0.06**</td>
<td>1.12 ± 0.01**</td>
<td>1.39 ± 0.02**</td>
<td></td>
</tr>
<tr>
<td>cv Gardener’s Delight</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red</td>
<td>2.08 ± 0.08**</td>
<td>0.78 ± 0.01**</td>
<td>0.83 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>Pink</td>
<td>0.53 ± 0.07</td>
<td>1.07 ± 0.01</td>
<td>1.29 ± 0.03**</td>
<td></td>
</tr>
</tbody>
</table>

Significant differences are indicated by different letters according to one-way ANOVA with $P < 0.05$ or with asterisks according to Student’s $t$ tests with $P < 0.01 (**). a.u., Arbitrary units.
effects on water permeability implies that a specific type of flavonoid(s) is responsible for the changes leading to decreased cuticle permeability.

CONCLUSION

SICH1 and SICH2 down-regulation by means of VIGS allowed the study of cuticle-related changes. Silencing experiments showed that flavonoids incorporated to the cuticle come from de novo synthesis. Moreover, flavonoid accumulation during ripening seems to modulate wax synthesis and deposition and cuticle water transpiration. They also act as modulators of the cuticle mechanical properties, conferring stiffness and reducing the cutin matrix deformation. Several ripening-related changes of the epidermal cells and cuticle seem to be controlled by CHS during fruit ripening. These include a reduction in the physical stretching of the epidermal cells due to changes in their shape, together with the decrease of the amount of cuticle and a change in the molecular arrangement of the cutin matrix.

MATERIALS AND METHODS

Plant Material

Three tomato (Solanum lycopersicum) genotypes, cv Moneymaker, cv Gardener’s Delight, and VIGS-Ready, were used in this study. The cv Gardener’s Delight is a cherry genotype, whereas cv Moneymaker is a medium-sized tomato. VIGS-Ready is a stable line derived from the cross between cv Micro-Tom transformed with DEL and ROS1 GUSAs from Asteranthum majus under the control of the ripening-specific promoter E3 (Butelli et al., 2008) and cv Moneymaker (Orzáez et al., 2009). Plants were grown in a glasshouse at the Estación Experimental La Mayora, Consejo Superior de Investigaciones Científicas, in the southeast of Spain. Tomato seedlings were transplanted to 16-L pots at the four true-leaf growth stage. Plants were watered when necessary, fertilized with the nutrient solution recommended by Cánovas (1995), supported by strings, and pruned to a single stem.

Cloning Procedures and Agroinoculation

pTRV-based vectors (pTRV1 and pTRV2) were kindly provided by Dr. Diego Orzáez (Orzáez et al., 2009). Two modified pTRV2 vectors suitable for Gateway cloning were employed. pTRV2-GATEWAY (pTRV2_GW) was used for silencing cv Moneymaker and cv Gardener’s Delight, whereas pTRV2_DEL/ROS1-GATEWAY (pTRV2_DELDR_GW), containing fragments of the genes DEL and ROS1, was used for transformation of the VIGS-Ready genotype. Primers CHS1L (5’-AGCGGCTGAAAGGCAACCGT-3’) and CHS1R (5’-TCCCTAGAGGTTGGAAGCTG-3’), CHS2L (5’-GCGGCGGATCTTAGATCA-3’), CHS2R (5’-TTTCGGCTTTTCTGCTATTG-3’; Ballester et al., 2010), DELL (5’-GGGACATAGTGGGAAATTTGG-3’), DELR (5’-AGTATGGCTGTGGAACGCTAC-3’), ROS1L (5’-ATCGATGTTCCACACACAACGGG-3’), ROS1R (5’-CAAGAATAATGACCACCACTCAG-CC-3’; Orzáez et al., 2009); CACL (5’-CTCCCCGTGTGATGTAACTGGA-3’), and CACR (5’-ATCTGGCAAGAATATACATCTAG-3’; Espósito-Rodríguez et al., 2008). Three biological replicates corresponding to pools of epicarp pieces from different tomatoes and plants were analyzed. For each biological replicate, three technical replicates were performed. PCR efficiency was calculated for each PCR using the LinRegPCR 11.0 program (Ramakers et al., 2003). Different pairs of primers showed similar efficiencies (differences in efficiency below 2%).

Tissue Sectioning and Staining

Small pericarp pieces from three fruits of different plants were collected per genotype and agroinoculated and then fixed in a formaldehyde, acetic acid, and ethanol solution (1:1:18). Later, they were dehydrated in an ethanol dilution series (70%–95%) and embedded in a commercial resin (Leica Historesin Embedding Kit). Cross sections were cut in 8-μm thick sections using a Leica microtome (RM2125) and stained with Sudan IV (Jensen, 1962) to visualize the cuticle. Cuticle thickness was estimated from a minimum of 30 measurements from the cross-sectioned samples of the three fruits using the image-capture analysis program Visilog 6.3 (Noesis). Epidermal surface area was calculated using the same program and a minimum of 10 measurements per genotype and stage from 10 different biological samples (different fruits and plants). Cuticle density was calculated following the protocol established by España et al. (2014). Briefly, the cuticle area of 20 cross-sectioned samples from three different fruits was measured and referred to the weight of the cuticular surface area already estimated. Epidermal cell measurements were performed with Visilog using stained epicarp cross sections. A minimum of 100 cells from three different fruits was measured. The aspect ratio was calculated as the function of the minimum and maximum diameter (in this case, cell radial/tangential width).

The degree of cuticle invagination was estimated from a minimum of 50 microscopic fields of the stained cross sections corresponding to three different biological replicates (three fruits from three different plants). An arbitrary index was employed to account for the cutinization of the anticlinal and inner periclinal cell walls of the epidermis. 0, no cutinization of the anticlinal cell wall (i.e. absence of pectic); 0.25, cutinization of the upper half of the anticlinal cell wall; 0.5, cutinization of the whole anticlinal cell wall; 0.75, cutinization of the anticlinal and half of the inner periclinal cell wall; 1, the epidermal cell is completely surrounded by cutinized cell walls.

Cuticle Isolation

Cuticles were enzymatically isolated from a minimum of 30 tomato fruits from 10 different plants following the protocol of Petracek and Bukovac (1995) using an aqueous solution of a mixture of fungal cellulase (0.2%, w/v; Sigma), pectinase (2%, w/v; Sigma), and 1 mM NaN3 to prevent microbial growth in sodium carbonate buffer (50 mM, pH 7.7). A vacuum was used to facilitate enzyme penetration, and fruit samples were incubated with continuous agitation at 35°C for at least 14 d. The cuticle was then separated from the epidermis, rinsed in distilled water, and stored under dry conditions.

Cuticle Components

Cuticular waxes were removed by heating with Rnase-free DNase. First-strand cDNA synthesis was carried out with the SuperScript III First Strand synthesis kit according to the manufacturer’s instructions (Invitrogen). Relative transcript amounts of SICH1 and SICH2 were measured by quantitative reverse transcription-PCR using SYBR Permix Ex Taq (Takara). Each sample was normalized using SlCAC (Solyclg000960) as an internal control. Relative normalized expression was calculated using the Delta Delta quantification method. Primers used were CHS1L (5’-AGCGGCTGAAAGGCAACCGT-3’) and CHS1R (5’-TCCCTAGAGGTTGGAAGCTG-3’), CHS2L (5’-GCGGCGGATCTTAGATCA-3’), CHS2R (5’-TTTCGGCTTTTCTGCTATTG-3’; Ballester et al., 2010), DELL (5’-GGGACATAGTGGGAAATTTGG-3’), DELR (5’-AGTATGGCTGTGGAACGCTAC-3’), ROS1L (5’-ATCGATGTTCCACACACAACGGG-3’), ROS1R (5’-CAAGAATAATGACCACCACTCAG-CC-3’; Orzáez et al., 2009); CACL (5’-CTCCCCGTGTGATGTAACTGGA-3’), and CACR (5’-ATCTGGCAAGAATATACATCTAG-3’; Espósito-Rodríguez et al., 2008). Three biological replicates corresponding to pools of epicarp pieces from different tomatoes and plants were analyzed. For each biological replicate, three technical replicates were performed. PCR efficiency was calculated for each PCR using the LinRegPCR 11.0 program (Ramakers et al., 2003). Different pairs of primers showed similar efficiencies (differences in efficiency below 2%).

Gene Expression Analysis

Trizol reagent (Life Technologies) was used to isolate RNA from tomato fruit peels at different stages of ripening or from different-colored sectors. Genomic DNA was removed by treating with RNase-free DNase. First-strand cDNA synthesis was carried out with the SuperScript III First Strand synthesis kit according to the manufacturer’s instructions (Invitrogen). Relative transcript amounts of SICH1 and SICH2 were measured by quantitative reverse transcription-PCR using SYBR Permix Ex Taq (Takara). Each sample was normalized using SlCAC (Solyclg000960) as an internal control. Relative normalized expression was calculated using the Delta Delta quantification method. Primers used were CHS1L (5’-AGCGGCTGAAAGGCAACCGT-3’) and CHS1R (5’-TCCCTAGAGGTTGGAAGCTG-3’), CHS2L (5’-GCGGCGGATCTTAGATCA-3’), CHS2R (5’-TTTCGGCTTTTCTGCTATTG-3’; Ballester et al., 2010), DELL (5’-GGGACATAGTGGGAAATTTGG-3’), DELR (5’-AGTATGGCTGTGGAACGCTAC-3’), ROS1L (5’-ATCGATGTTCCACACACAACGGG-3’), ROS1R (5’-CAAGAATAATGACCACCACTCAG-CC-3’; Orzáez et al., 2009); CACL (5’-CTCCCCGTGTGATGTAACTGGA-3’), and CACR (5’-ATCTGGCAAGAATATACATCTAG-3’; Espósito-Rodríguez et al., 2008). Three biological replicates corresponding to pools of epicarp pieces from different tomatoes and plants were analyzed. For each biological replicate, three technical replicates were performed. PCR efficiency was calculated for each PCR using the LinRegPCR 11.0 program (Ramakers et al., 2003). Different pairs of primers showed similar efficiencies (differences in efficiency below 2%).
cuticle pieces from different fruits and plants. Cuticle color was objectively measured with a colorimeter (Minolta) using the International Commission on Illumination L’C’*H’* color space, where L’ represents lightness, C’ chroma or saturation, and H’ hue (CIE, 1978). Ten samples per sector, corresponding to cuticle pieces from 10 different fruits and plants, were analyzed.

**ATR-FTIR Spectroscopy**

Infrared spectra of samples were obtained with an ATR accessory (MBraun ATR; Pike Technologies) coupled to an FTIR spectrometer (FT/IR-4100; JASCO). All spectra were recorded in the range from 3,800 to 600 cm\(^{-1}\) with 4-cm\(^{-1}\) resolution and accumulating 50 scans. Samples were gently colocated on the spot of the ATR accessory and slowly pressed. Three biological replicates of cutin per genotype and sector were analyzed. The degree of cutin esterification (esterification index) was estimated from the ratio between absorbances of the C=O stretching vibrations of the ester (1,729 cm\(^{-1}\)) and the asymmetric ones of the methylene (2,925 cm\(^{-1}\)) functional groups. \(\text{UCOO-COO}^\circ\) was measured as the ratio between the intensities of the C=O stretching vibrations of the ester (1,729 cm\(^{-1}\)) and the carboxylic acid (1,705 cm\(^{-1}\)) functional groups. Deconvolution was applied to the C=O stretching region (1,770–1,650 cm\(^{-1}\)) using the PeakFit version 4.11 software.

**Mechanical Tests**

The mechanical properties of the cuticle were measured with an extensometer equipped with a linear displacement transducer (Mitutoyo) and customized to work with cuticular membrane samples (resolution of ± 1 µm) following the protocol already described by López-Casado et al. (2007). Rectangular uniform segments (3 mm × 9 mm) of isolated cuticles were sectioned using a metal block and inspected microscopically to confirm the absence of small cracks before mechanical testing. Mechanical tests were performed as a transient creep test maintaining samples in uniaxial tension, under a constant load for 1,200 s, with the longitudinal extension being also determined for each sample. A minimum of five to seven biological samples (cuticle pieces from different fruits and plants) per genotype and sector were analyzed at 25°C and 40% relative humidity. The yield point was determined from the creep tests as the stress and strain needed to enter viscoelastic (time-dependent) deformation (España et al., 2014). To obtain the corresponding stress-strain curves and elastic modulus, the applied stress was plotted against the total change in length (%) for every tensile force. Breaking stress and maximum strain at the breaking stress were also determined for each sample.

**Cuticle Permeability Measurements**

Cuticle permeability was determined by water loss through the outer surface according to Riederer (2006b). Cuticles were mounted with the outer surface facing the atmosphere in custom-made transpiration chambers following the design reported by Schönherz and Lensdian (1981). The transpiration chambers were filled with distilled water and deposited upside down in desiccators with dry silica gel at 25°C. Water loss was calculated gravimetrically by measuring the chambers at regular time intervals. The transpiration rate at maximum driving force (\(J\)) was calculated from the slope of the regression line of weight loss versus time and divided by the exposed area. Cuticle permeability (\(P\)) was then calculated following:

\[
P = \frac{1}{\rho(\delta_{\text{chamber}} - \delta_{\text{air}})}
\]

where \(\rho\) is the density of water vapor at saturation in the air and \(\delta\) is the water activity in each compartment. Seven to 10 biological replicates per genotype and sector were carried out. Each replicate corresponded to a cuticle piece from a different fruit and plant.

**Statistical Data Analysis**

Data are expressed as means ± SE. One-way ANOVA or Student’s t tests were used to compare means between sectors for each genotype (SPSS, 2013). Letters indicate significant differences at \(P < 0.05\), while asterisks indicate significant differences at \(P < 0.05\) (*) and \(P < 0.01\) (**).

**Supplemental Data**

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

**Supplemental Figure S1.** Relative normalized expression of SICH51, SICH52, and AmDEl genes in the purple and red epicarp sectors of VIGS-Ready fruits agroinoculated with pTRV2-DR.

**Supplemental Figure S2.** Transition regions between the VIGS-Ready red and pink sectors.

**Supplemental Figure S3.** ATR-FTIR spectra of cutin isolated from the cuticles of the sectors obtained after agroinoculation of VIGS-Ready fruits with pTRV2_DR_CHS.

**Supplemental Table S1.** Cuticle and epidermal cell traits of the sectors obtained after agroinoculation with pTRV2_DR.

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**LITERATURE CITED**


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SPSS (2013) IBM SPSS Statistics for Windows, Version 22.0. IBM, Armonk, NY