

CHS silencing suggests a negative cross-talk between wax and flavonoid pathways in tomato fruit cuticle

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Tomato fruits (*Solanum lycopersicum* L.) accumulate flavonoids in their cuticle and epidermal cells during ripening. These flavonoids come from *de novo* biosynthesis due to a significant increase in chalcone synthase (CHS) activity during ripening. Virus-induced gene silencing (VIGS) of tomato fruits have been used to down-regulate *SICHS* expression during ripening and analyze the effects at the epidermal and cuticle level. Besides the expected change in fruit color due to a lack of flavonoids incorporated to the cuticle, several other modifications such as a decrease in the amount of cutin and polysaccharides were observed. These indicate a role for either flavonoids or *CHS* in the alteration of the expression levels of some genes involved in cuticle biosynthesis. Moreover, a negative interaction between the 2 cuticle components, flavonoids and waxes, suggests a relationship between these 2 metabolic pathways.

The plant cuticle is an extracellular membrane that covers the outer epidermal cell wall of aerial organs such as leaves, flowers and fruits.¹ It is chiefly composed of a lipid matrix of interesterified polyhydroxy fatty acids named cutin that is intertwined with cell wall polysaccharides. Other components are waxes and phenolics. In tomato, phenolics accumulated in the fruit cuticle vary with the developmental stage, being the main compounds coumaric and benzoic acid during fruit growth and flavonoids such as naringenin chalcone and naringenin flavanone during ripening.² Incorporation of flavonoids was postulated to modify the cuticle's mechanical properties by increasing its resistance

to deformation.³ Thus, during the period of organ expansion, the cuticle can be deformed with little stress applied.² CHALCONE SYNTHASE (CHS) is the enzyme responsible for the synthesis of chalconaringenin, the flavonoid mainly accumulated in tomato fruit cuticle and responsible for its orange-yellow color. Flavonoids are being shown to interact with several metabolic pathways sometimes by altering hormone levels. In this sense, constitutive *SICHS* suppression in tomato led to parthenocarpy, probably as a response to the observed pollen growth impairment.⁴

In a recent publication we analyzed how flavonoid accumulation during ripening affected epidermal and cuticle properties. In this work, we studied the results of down-regulating the expression of *SICHS* during ripening by means of virus-induced gene silencing (VIGS) in different tomato genotypes. Firstly, we analyzed the epidermal expression level of the 2 *CHS* genes (*SICHS1* Solyc09g091510.2 and *SICHS2* Solyc05g053550) known to be expressed in the fruit during ripening. Both *CHS* showed a very low expression at mature green but peaked at breaker, with *SICHS2* showing a higher expression level than *SICHS1*. Silencing of flavonoid synthesis during ripening rendered tomatoes with differentially colored regions: red (non-silenced) and pink (silenced) which allowed their physical isolation and ulterior analysis. Silenced regions showed an alteration in the final step of cell expansion since the epidermal cells of pink sectors were similar in size and shape to those of mature green fruits. Thus, cells were more rounded due to a decreased in

Keywords: cutin, flavonoids, plant cuticle, tomato, waxes

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Submitted: 01/05/2015

Revised: 02/11/2015

Accepted: 02/12/2015

<http://dx.doi.org/10.1080/15592324.2015.1019979>

Addendum to: España L, Heredia-Guerrero JA, Reina-Pinto JJ, Fernández-Muñoz R, Heredia A, Domínguez E. Transient silencing of CHALCONE SYNTHASE during fruit ripening modifies tomato epidermal cells and cuticle properties. *Plant Physiol* 2014; 166: 1371-1386

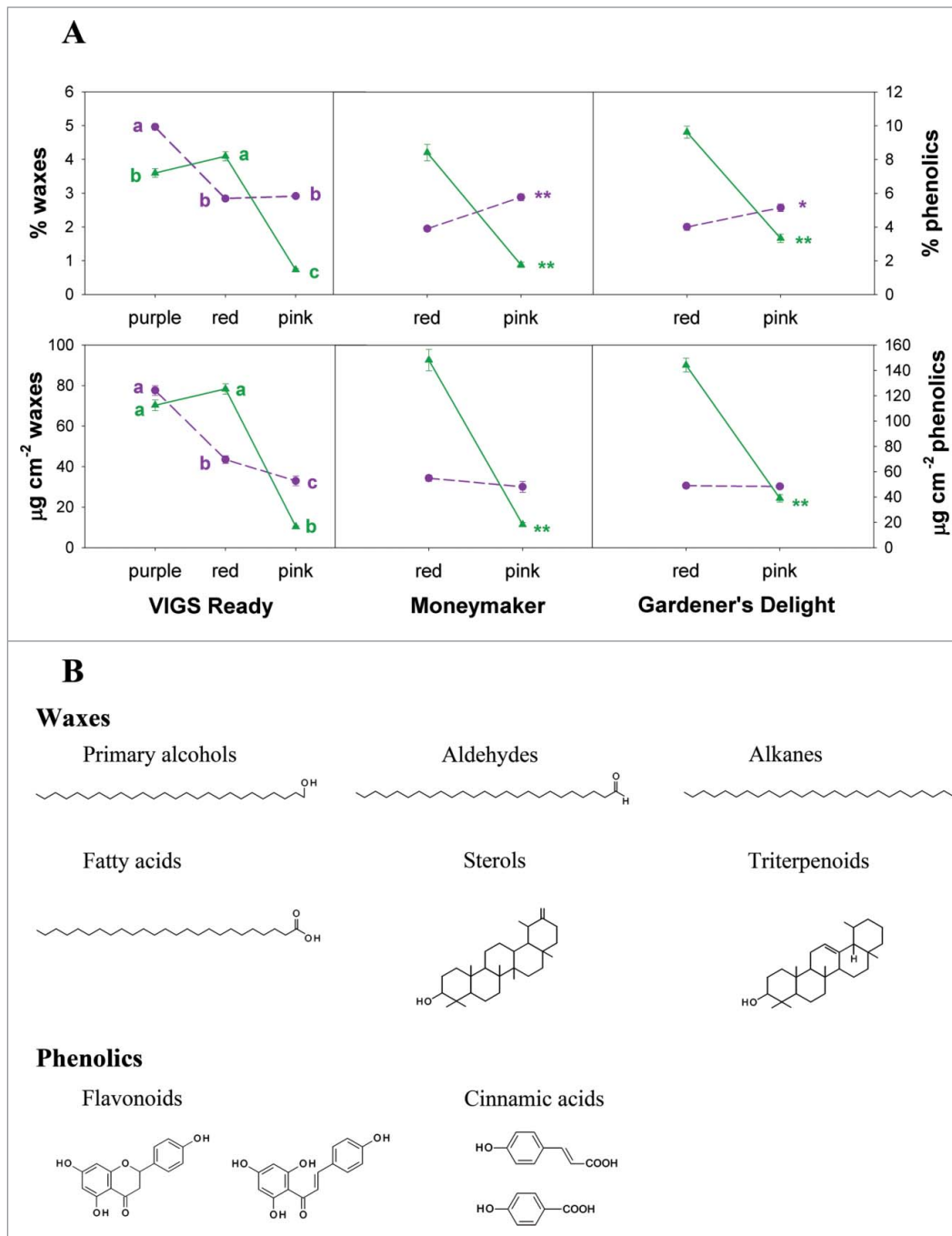


Figure 1. (A) Changes in the percentages and amount (expressed as $\mu\text{g cm}^{-2}$) of phenolics (solid green lines) and waxes (dashed purple lines) in the cuticles isolated from the different sectors obtained after fruits were agroinoculated to silence *SICH5*. Letters indicate significant differences according to one-way ANOVA with $P < 0.05$ or with asterisks according to t-tests with $P < 0.01$ (**). (B) Main chemical components of the wax and phenolic fractions of tomato fruit cuticles.

tangential width. These results clearly support a role of flavonoids in modifying cell expansion possibly mediated by auxins. Flavonols, such as quercetin, have been shown to modify auxin transport thus altering tissue growth.⁵ More recently, naringenin has also been involved in

retarding cotton cell development.⁶ Despite the results observed in the epidermal cells suggested that mature silenced regions were similar to the mature green stage, this was not the case for the cuticle. Cuticles of the pink regions were significantly different from those of the red

sectors but also from the mature green stage already reported in the literature.^{2,4} In tomato, flavonoids are synthesized during fruit development^{7,8} and most of them are assumed to accumulate in the vacuole.^{9,10} Inhibition of flavonoid synthesis during ripening clearly indicated

Table 1. Resume of changes observed in the epidermis and cuticle of tomato fruits agroinoculated to silence *SICH5*. Comparisons were established between the control red sectors and the *SICH5*-silenced pink sectors

Traits affected by <i>SICH5</i> silencing	
Amount ($\mu\text{g cm}^{-2}$)	
cuticle	decreased
cutin	decreased
polysaccharides	decreased
phenolics	decreased
Cuticle biomechanical properties	
stiffness	lower
deformation	increased
Cuticle water permeability	lower
Cuticle invagination	decreased
Cutin esterification index	higher
Epidermal cell shape	more rounded

that flavonoids incorporated to the cuticle come from *de novo* synthesis and not from vacuole stored flavonoids. This lack of flavonoids decreased the amount of cuticle accumulated mostly due to a reduction of its main components cutin and polysaccharides (Table 1). Although a decrease in cuticle polysaccharides during fruit ripening has been reported,¹¹ this association of flavonoid biosynthesis and cuticle deposition has not been described before. Ripening has been shown to cause a reduction in the number of ester bonds in the cutin matrix.² This reduction was shown to be associated with flavonoid incorporation to the cuticle and not ripening since the pink sectors ripened but had a higher number of ester bonds than the red sectors. Clearly, flavonoids seem to affect the expression of several genes involved in the last stages of cuticle deposition and/or remodeling.

Waxes, on the other hand, did not seem to be affected by the inhibition of *SICH5* expression since their amount did not change between the silenced and non-silenced sectors. Hence, their relative contribution to the overall cuticle (% of waxes) was increased in the pink sectors due to the decrease in the amount of cuticle (Fig. 1A). Results obtained from 2 of the genotypes studied supported this explanation. However, results from the VIGS-Ready genotype did not support it. VIGS-Ready is a tomato that accumulates anthocyanins in the pericarp due to the ripening specific expression of 2 *Antirrhinum majus* transcription factors: *ROSEA1*

and *DELILA*.¹² VIGS silencing of these transcription factors rendered ripe tomato with purple (non-silenced) and red (silenced) sectors. In both cases, cuticles were very similar except for a significant increase in the amount of waxes present in the purple sectors. Thus, another explanation for the results would be a negative cross-talk between the flavonoid and wax pathways. Indeed, comparison of the percentages of waxes and phenolics showed an inverse relationship between these 2 minor cuticle components (Fig. 1A) which seems controlled by the phenolic compounds. Waxes and flavonoids are minor compounds present in the cuticle of the tomato fruit (Fig. 1B). As such, they have been shown to modify the physical properties of the cuticle, especially the biomechanics.^{2,13} Waxes are complex mixtures of very long chain aliphatic molecules (aldehydes, alcohols, fatty acids, alkanes) and cyclic compounds (sterols, triterpenoids)¹⁴ which are the product of different metabolic pathways.¹⁵ Our results indicate a negative interaction between the flavonoid and wax pathways; however, it remains to be determined which type or types of waxes are modified. This change in the relative contribution of waxes associated with flavonoids could also be responsible for the decreased water permeability observed in the cuticles of the silenced regions (Table 1). In tomato, cuticle water permeability has been negatively correlated with the amount of fruit waxes, mostly with the very-long-chain aliphatic fraction.¹⁶

It remains an unanswered question whether the 2 *SICH5* genes play a redundant role or a differential one. Both shared the same epidermal expression profile during ripening, with *SICH52* showing the highest expression level. Nuclear localization of *CHS* and flavonoids has been found in some species and clearly suggests a key function in the control of transcription of specific genes.^{17,18} Considering the significant number of effects found to be regulated by *CHS* at the epidermal level, both *SICH5* might be playing different roles with one more related to the regulation of gene expression and the other one to flavonoid biosynthesis. This is however speculative since the high degree of similarity of both genes

prevents an efficient down regulation of one of them without affecting the expression level of the other.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Funding

This work was supported by grant AGL2012-32613 from the Plan Nacional de I+D, Ministry of Science and Innovation (MICINN), Spain, co-funded by EU. FEDER. José A. Heredia-Guerrero is supported by a Marie Curie Intra-European Fellowship (BIOPROTO project), financed by the EU's 7th Framework Program for Research (FP7).

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