Tesis Doctoral

Bases Genéticas del Desarrollo y Composición de la Cutícula del Fruto de Tomate

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Para mi Padre .. إلى أبي
Para mi Madre .. إلى أمي

GENETICS OF TOMATO FRUIT CUTICLE DEVELOPMENT AND COMPOSITION

The more you know, the less you fear.

Portada: Varias fotos de cutículas extraídas de la variedad de tomate cultivado (*Solanum lycopersicum* L.) 'Moneymaker' (MM), de una entrada (TO-937) de la especie silvestre (*Solanum pimpinellifolium* L.), de varias líneas de introgresión SP_1-2, SP_3-3, SP_4-2, SP_7-3, SP_8-1, SP_12-4 y de dos sublíneas a228 y a239, ambas derivadas del cruce SP_7-2 x MM. Esas fotos están realizadas por R. BARRAJ BARRAJ mediante una cámara NIKON. Cuanto al diseño de la portada, esta realizado por R. BARRAJ BARRAJ y Francisco José DÍAZ COBOS – MayPrint, mediante Adobe Spark y Adobe Photoshop.

UNIVERSIDAD DE MÁLAGA

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Rida BARRAJ BARRAJ

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إلى أمي، **هدى البرّاج**
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جئتُ لا أعلم من أين و لكني أتيت، و لقد أبصرت قدامي طريقاً فمشيت. و سأبقى ماشياً إن شئتُ أم أبيت. كيف جئت ؟ كيف أبصرتُ طريقي ؟ لستُ أدري.

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SUMMARIES

SUMMARY

The plant cuticle, as the interface between the plant and the environment, plays an important role in plant performance, fruit quality and postharvest. Plant cuticles show compositional and structural variability among and within species and also between organs of the same species. It is composed by a cutin polymer matrix with waxes that can be accumulated on the surface (epicuticular waxes) or embedded in the matrix (intracuticular waxes), phenolics and polysaccharides from the cell wall. In tomato fruit cuticle, the phenolic fraction is a characteristic component and is the main modulator of the cuticle’s stiffness, deformation and strength. This phenolic domain is composed of cinnamic acids derivatives present during fruit growth and increases significantly during ripening with the incorporation of the flavonoid naringenin chalcone, responsible for the yellow-orange color of the cuticle in red ripe tomatoes.

Understanding the genetic basis of cuticle composition and color is needed for breeding in crop species. Little is known about the range and the genetic bases of naturally occurring variation for cuticle composition and structure in tomato. The general objective of this work is to dissect the genetic architecture underlying these variations and to exploit available natural genetic diversity in wild species to transfer the beneficial alleles affecting cuticle-associated traits to commercial varieties.

In this work, we have explored for the first time the cuticle variability of red-fruited species in two mapping populations derived from the interspecific cross between the domesticated tomato (*Solanum lycopersicum* L.) and its closest wild relative *Solanum pimpinellifolium* L., a Recombinant Inbred Line population and an Introgression Line population. Quantitative trait locus (QTL) analyses were conducted to investigate tomato fruit cuticle components and color traits. A total of 60 QTLs were identified, indicating that all the cuticle traits analyzed have a complex polygenic nature. A combination of additive and epistatic interactions was observed for all the traits, with positive contribution of either both parental lines to most of them. Colocalization of QTLs for various traits uncovered novel genomic regions producing extensive changes in the cuticle composition and color. Two genomic regions, located in chromosomes 1 and 12, were found to be responsible for the negative relationship between cuticle waxes and phenolics percentage contents. Several candidate genes, including transcription factors and structural genes, are postulated and their expression analyzed throughout development. We also pyramided several of the phenolic content-related QTLs from the *S. pimpinellifolium* accession into the genetic background of the cultivated tomato with the aid of single nucleotide polymorphisms (SNP) molecular markers, polymorphic between both parental lines. Our pyramiding strategy resulted in 23 new introgression lines, combining more than one QTL for cuticle phenolics in the genetic background of the cultivated tomato to produce new genetic materials with higher or lesser cuticle flavonoid content. The interactions between the combined QTLs were also dissected and the interactions, either additive or epistatic, of each created combination was identified. Accumulation of phenolics QTLs was only partially effective in reducing phenolics in the cuticle since no QTL-combined line showed phenolic content comparable to that of TO-937. Results of pyramided QTLs emphasized that the genetic control of phenolics-related traits was very complex, and epistatic interactions changed the expected outcome of several of the QTLs depending on the specific alleles of the combined QTLs.

RESUMEN

La cutícula de la planta, como interfaz entre la planta y el medio ambiente, juega un papel importante en el rendimiento de la planta, la calidad del fruto y la postcosecha. Las cutículas de las plantas muestran variabilidad composicional y estructural entre y dentro de las especies y también entre órganos de la misma especie. La cutícula está compuesta de una matriz polimérica de cutina con ceras que pueden acumularse en la superficie (ceras epicuticulares) o incrustadas en la matriz (ceras intracuticulares), compuestos fenólicos y polisacáridos de la pared celular. En la cutícula del fruto del tomate, la fracción fenólica es el componente característico y el principal modulador de la rigidez, deformación y resistencia de la cutícula. Esta fracción fenólica está compuesta de derivados de ácidos cinámicos presentes durante el crecimiento del fruto y aumenta significativamente durante la maduración con la incorporación del flavonoide chalconaringenina, responsable del color amarillo naranjado de la cutícula en tomates rojos maduros.

Es necesario entender las bases genéticas de la composición y del color de la cutícula para la mejora genética de los cultivos. Se sabe poco sobre el rango y las bases genéticas de la variación natural de la composición y estructura de la cutícula en el tomate. El objetivo general de este trabajo es analizar la arquitectura genética responsable de estas variaciones y aprovechar la diversidad genética natural disponible en las especies silvestres para transferir los alelos beneficiosos que afectan los caracteres agronómicos a las variedades comerciales.

En este trabajo, hemos explorado por primera vez la variabilidad de la cutícula de especies de frutos rojos en dos poblaciones de cartografiado derivadas del cruce interespecífico entre el tomate cultivado (*Solanum lycopersicum* L.) y su pariente silvestre más cercano *Solanum pimpinellifolium* L., una población de Líneas Recombinantes Consanguíneas (RIL) y una población de Líneas de Introgresión (IL). Se realizaron análisis de loci de caracteres cuantitativos (QTL, quantitative trait locus) para localizar regiones genómicas implicadas en la composición y color de la

cutícula del fruto de tomate. Se identificaron un total de 60 QTLs, lo que indica que todos los caracteres de la cutícula analizados tienen una naturaleza compleja y poligénica. Se observó una combinación de interacciones aditivas y epistáticas para todos los caracteres, con una contribución positiva de ambas líneas parentales a la mayoría de ellos. La colocalización de los QTLs para varios caracteres reveló nuevas regiones genómicas que producen grandes cambios en la cutícula. Se identificaron dos regiones genómicas, ubicadas en los cromosomas 1 y 12, responsables de una relación negativa entre los porcentajes de ceras y de compuestos fenólicos. Se analizó la expresión de varios genes candidatos a lo largo del desarrollo, entre ellos, factores de transcripción y genes estructurales. También hemos combinado varios QTLs implicados en el contenido fenólico de la accesión de *S. pimpinellifolium* en el fondo genético de la variedad de tomate cultivado mediante el empleo de marcadores moleculares tipo SNP (single nucleotide polymorphism/polimorfismo de nucleótido único), polimórficos entre ambas líneas parentales. Con esta estrategia se obtuvieron 23 nuevas líneas de introgresión que combinan más de un QTL asociados a contenido en compuestos fenólicos y color de la cutícula del fruto y así producir nuevos materiales genéticos con aún mayores o menores contenidos de fenoles en la cutícula. Con estos materiales se analizaron las interacciones entre los QTLs combinados y se identificaron los efectos aditivos acumulados y las interacciones epistáticas implicadas en el control genético de estos caracteres. La acumulación de QTLs sólo fue parcialmente eficaz para reducir el contenido fenólico en la cutícula, ya que ninguna línea con QTL combinados mostró un contenido fenólico comparable al de TO-937. Los resultados de la piramidación de QTLs mostraron que el control genético del contenido en fenoles y caracteres relacionados es muy complejo, y las interacciones epistáticas encontradas alteraron los resultados esperados para varios de los QTLs dependiendo de los alelos específicos de los QTL combinados en cada línea.

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ABBREVIATIONS

BC	backcross
Br	breaker
C*	chroma
CER	*ECERIFERUM*
CHI	*CHALCONE ISOMERASE*
CHS	*CHALCONE SYNTHASE*
cm	QTL amount of cuticle
Cr	chromosome
cu	QTL cutin
FDH	*FIDDLEHEAD*
H°	hue
IG	immature green
IL	introgression line
KCS	*β-KETO ACYL-CoA SYNTHASE*
L*	lightness
LACS	*LONG CHAIN ACYL-CoA SYNTHETASE*
lyc	*S. lycopersicum* ‘Moneymaker’ allele
MAS	marker-assisted selection
MG	mature green
MM	Moneymaker
nSP	newly created SP line
ph	QTL cuticle phenolics
pim	*S. pimpinellifolium* ‘TO-937’ allele
ps	QTL cuticle polysaccharides
QTL	quantitative trait locus
RIL	recombinant inbred line
RR	red ripe
SNP	single nucleotide polymorphism
SP	*S. pimpinellifolium* introgression line
wt	wild type
wx	QTL cuticle waxes
y	*colorless fruit epidermis*

GENERAL INTRODUCTION

1 THE TOMATO CROP: TAXONOMY, ECONOMIC IMPORTANCE AND GENETIC IMPROVEMENT

1.1 Origin, Domestication and Genetic Diversity

The tomato crop species (*Solanum lycopersicum* L.) belongs to the large and diverse family of Solanaceae (division Magnoliophyta, class Magnoliopsida, subclass Asteridae and order Solanales), also called nightshades. This family consists of around 90 genera (D'Arcy, 1979) and assembles a wide range of flowering plants. Plants from Solanaceae family show remarkable morphological and ecological variability. They can range from herbaceous perennials to trees; among them there are major crops in terms of food production (eggplant, tomato, pepper, potato), ornamental (petunia) and medicinal plants (datura, henbane and deadly nightshade). As for the habitat, they can occupy a broad range of ecosystems, from rainforests to deserts and from highlands to coasts (Knapp *et al.,* 2004). They are found on all continents especially in Central and South America, except Antarctica.

In the first edition of *Species Plantarum*, Linnaeus initiated the binominal nomenclature system that consists of defining plants by a genus and a species name (Linnaeus, 1753). Before that, plants were given polynomial Latin names that described them and distinguished them from others. Tomato has been subjected to numerous name changes by botanists. Linnaeus, in *Species Plantarum* classified the tomato plant in the genus *Solanum* and named it: *S. lycopersicum* (Linnaeus, 1753). However, the traditional nomenclature till 2005 was the one by Miller, who utilized the generic name *Lycopersicon*, meaning 'wolf peach' to include cultivated tomato, eggplant and potato. He justified his taxonomic classification based on fruit characters (Miller, 1731). Many twentieth century authors as Müller, (1940); Rick and Holle, (1990); Hunziker, (2001) did the same. They distinguished and considered *Solanum* and *Lycopersicon* two different genera.

A more accurate taxonomic classification in tomato was revealed in 2005 ending a controversial debate in the botanical society. Based on biogeographic, morphological and molecular analyses, today *Lycopersicon* is considered a section within the genus *Solanum*, usually referred as 'the tomato clade'*.* Molecular analyses included molecular markers, such as restriction fragment length polymorphism (RFLP), chloroplast deoxyribonucleic acid (DNA) marker (Spooner *et al.,* 1993), amplified fragment length polymorphism (AFLP) (Spooner *et al.,* 2005) and granule-bound starch synthase gene sequencing (Peralta and Spooner, 2001). As for the morphological analyses, characters of the corolla, anthers, stigma, leaf etc., were used to differentiate taxa (Peralta and Spooner, 2005). As a result, the *Lycopersicon* section includes the domesticated tomato, *S. lycopersicum*, and its 12 closest wild relatives *Solanum arcanum* Peralta, *Solanum chmielewskii* (C.M. Rick, Kesicki, Fobes & M. Holle) D.M. Spooner, G.J. Anderson & R.K. Jansen, *Solanum neorickii* D.M. Spooner, G.J. Anderson & R.K. Jansen, *Solanum pennellii* Correll, *Solanum habrochaites* S. Knapp & D.M Spooner, *Solanum chilense* (Dunal) Reiche, *Solanum huaylasense* Peralta, *Solanum peruvianum* L., *Solanum corneliomuelleri* J.F. Macbr., *Solanum cheesmaniae* (L. Riley) Fosberg, *Solanum galapagense* S.C. Darwin & Peralta*, and Solanum pimpinellifolium* L. (Peralta and Spooner, 2005). Tomato wild species are endemic to the Andean region that encompasses parts of Chile, Bolivia, Ecuador, Colombia and Peru (Atherton and Rudich, 1986) with two species (*S. galapagense* and *S. cheesmaniae*) native to the Galapagos Islands.

Although the cultivated tomato is originated from the new world, the time, the site and the early events of domestication are undetermined (Peralta and Spooner, 2007). This uncertainty about domestication resulted into developing two competing hypotheses. The first assumption proposed Peru as the center of origin and domestication. De Candolle suggested, based on linguistic (Roxburgh, 1832), historical (Hernandez, 1651) and botanical (Bauhin, 1623; Ruiz and Pavon, 1797) proofs, a Peruvian origin of tomato domestication. His conclusions were based on the fact that: i) 'mala peruviana' and 'pomi del Peru' were used to mention the tomato; ii) the cultivated tomato was believed to emerge from the wild cherry tomato (*S. lycopersicum* var. *cerasiforme*), which was acknowledged to be centered in the area from coastal Peru through Mexico to the southwestern USA (California) (de Candolle, 1882). However, de Candolle's hypothesis is not supported by strong archaeological evidence (McMeekin, 1992). The second hypothesis pointed at Mexico as the primarily site of domestication (Jenkins, 1948). The lack of evidence of pre-Colombian tomato cultivation in South America in comparison to sufficient proof in Mexico was Jenkins´s argument to defend his hypothesis. Based on the etymology, Jenkins also argued that the name 'tomato' derives from the Mexican Nahuatl word 'tomatl' which refers to 'plants bearing globous and juicy fruit' (Bauchet and Causse, 2012). Both hypotheses on the origin of tomato domestication are uncertain. This could indicate that domestication took place independently in both regions as proposed by Rick and Holle (1990).

More recently, understanding the history of tomato domestication has made significant advancements (Blanca *et al.,* 2012). Extensive genetic characterization of 272 accessions using Tomato Illumina Bead Chips (Illumina, San Diego, California, USA) created by the SolCAP project (Hamilton *et al.,* 2012; Sim *et al.,* 2012), containing 7414 single-nucleotide polymorphism markers led to the hypothesis that tomato was probably domesticated in two waves: from *S. pimpinellifolium* to *S. lycopersicum* var. *cerasiforme* in Ecuador and Northern Peru; and later from *S. lycopersicum* var. *cerasiforme* to *S. lycopersicum* in Central America (Blanca *et al.,* 2012). Consequently *S. pimpinellifolium* is considered the closest wild ancestor to cultivated tomato (Bretó *et al.,* 1993; Blanca *et al.,* 2012; The Tomato Genome Consortium, 2012). Razifard *et al.* (2020) postulated that the origin of *S. lycopersicum* var. *cerasiforme* preceded domestication. Moreover, the latter authors suggested that many attributes of the cultivated tomatoes emerged in South American *S. lycopersicum* var. *cerasiforme*, but were lost or diminished once these partially domesticated forms expanded towards the north. And before its spread all over the world, these characteristics were probably reselected in Mexico in a convergent way in the common cultivated tomato.

The worldwide distribution of cultivated tomato was achieved starting from the sixteenth century after the discovery of the New World by Spanish conquistadors (Mann, 2011). Prior to the Spanish conquest of Mexico in 1523, tomato plants were already domesticated (Rick, 1978). Cortes, the Spanish conquistador, might be the first to bring the small yellow tomato to Spain (McCue, 1952). From Spain, the tomato arrived in Italy through Naples, which was part of the Spanish kingdom at that time. The tomato in Europe was first documented in the descriptions published in 1544 by Italian botanist Pietro Andrea Mattioli who described it as 'green at first and when ripe of a golden color that is eaten in the same manner' (Mattioli, 1544). However, it was in a cookbook in 1692 where the tomato appeared for the first time, almost two hundred years after Columbus headed for the new world. Even then and because of the speculations regarding its poisonous nature, tomato didn't gain acceptance rapidly except for ornamental or medicinal purposes (Rick, 1978). Its domestication as consumable fruit began during the 19th century.

Domestication of some crop species including the cultivated tomato began 10,000 years ago (Tanksley and McCouch, 1997). Through domestication, tomato has undergone severe natural and artificial selections while the crop was carried out from the Andean region to Central America, and from there to Europe (Blanca *et al.,* 2012). Many new desirable agronomic traits such as increased fruit size, compact growth habitat, non-shattering seeds and loss of germination inhibition were then obtained by early agriculturists (Harlan, 1975). During this process, a selective propagation of lines holding these beneficial mutations has led to a progressive narrowing of the genetic variation of the cultivated tomato (Tanksley and McCouch, 1997). It can be calculated that the genome of tomato cultivars contains <5 percent (%) of the genetic diversity of their wild relatives (Miller and Tanksley, 1990). However, for morphological traits like fruit size and shape, tomato cultivars show far larger phenotypic variation than their wild progenitors (Stevens and Rick, 1986; Tanksley, 2004). Indeed, tomato domestication has led to a wide diversity of fruit sizes and shapes. Molecular genetic investigations resulted into the identification of at least six morphological key *loci* controlling fruit shape and size: *Fruit Weight2.2* (*FW2.2*) and *FW3.2* leading to increased fruit size; *OVATE* and

SUN leading to fruit elongation; *FASCIATED* controlling locule number and leading to flat or oxheart shape; *LOCULE NUMBER* controlling locule number and meristem size (Tanksley, 2004; Rodriguez *et al.,* 2011; Monforte, 2014; van der Knaap, 2014).

After domestication, modern plant breeding has played a key role in reducing the genetic variation in crop plants. The genetic basis of breeding populations narrows through intracrossing, selection and fixation of specific alleles. Consequently, heritability declines and limits breeding progress. Moreover, many beneficial agricultural attributes do not exist in modern cultivars. Thus, and to maintain genetic diversity, breeding strategies relied on molecular genetic methodologies to introduce new traits into cultivated plants from wild species, which are a rich reservoir of favorable genes and poorly exploited. As mentioned before, the growing environment of the wild tomato species is very wide (Knapp *et al.,* 2004), contributing thus to their diversity at molecular, sexual, morphological and physiological levels (Peralta and Spooner, 2005). The mating system contributes to species diversification through outcrossing. In the plant kingdom, genetically controlled self-incompatibility is a usual mechanism. It is gametophytic in tomato clade and varies from allogamous self-incompatible, to facultative allogamous, to autogamous and self-compatible (Grandillo *et al.,* 2011). Self-incompatibility highly correlates with allelic diversity, the degree of outcrossing, floral display and it is favored by the degree of stigma exertion in wild tomatoes (Peralta *et al.,* 2008). Indeed, a recessed stigma below the anthers will develop self-fertilization whereas an exserted stigma above the anthers will promote outcrossing by buzz pollination, in facultative or completely self-compatible species (Chen and Tanksley, 2004). By exploring flower characteristics and the bases of self-compatibility/incompatibility, Rick (1982a) concluded that the mating system in the tomato clade progressed from self-incompatible, as the ancestral condition, to self-compatible. Alterations from self-incompatibility to self-compatibility are events expected to occur independently and infrequently. For example, in the case of *S. pennellii* and *S. habrochaites*, both self-incompatible and self-compatible populations have been characterized (Rick, 1982a). The self-incompatible populations are more diverse and are characterized by exserted stigma and larger flower parts whereas the self-compatible populations have reduced genetic variation, little or no stigma exertion and smaller flower parts. Furthermore, Miller and Tanksley (1990) found that genetic diversity is higher within a single accession of the self-incompatible species than in all accessions of any of the self-compatible species. Thus, in tomato clade, gametophytic incompatibility and flower stigma exertion lead to greater genetic variation and outcrossing. The species with the highest variability are the self-incompatible species: *S. chilense, S. huaylasense, S. habrochaites, S. pennellii, S. peruvianum, S. corneliomuelleri,* except *S. arcanum* (Grandillo *et al.,* 2011). Species such as *S. arcanum, S. chmielewskii and S. pimpinellifolium* have intermediate variability, whereas the least variable species are, besides *S. lycopersicum*, *S. neorickii, S. cheesmaniae, S. galapagense* (Grandillo *et al.,* 2011). As for the size and shape, wild species are all small and round (approximately 1–2 g) (Peralta and Spooner, 2005) whereas for fruit color, it varies among the wild relative species. Nearly all of them have green fruits, except for the two species from the Galapagos Islands (with orange and yellow fruits) and *S. pimpinellifolium*, which is the only wild relative species that carries red fruits.

Wild relative species of tomato play a key role as donors of many economically important traits. However, most of these agronomic traits are complex. They are regulated by polygenes or quantitative trait loci (QTL) (Labate *et al.,* 2007). Therefore, several linkage maps have been created from more than 20 mapping populations derived from interspecific crosses between cultivated tomato and its wild relatives to identify these QTLs. Almost all wild species of tomato have been used for gene and/or QTL mapping. *S. pimpinellifolium*, *S. habrochaites* and *S. pennellii* have been employed extensively (Eshed and Zamir 1995; Bernacchi and Tanksley 1997; Monforte and Tanksley, 2000; Monforte *et al.,* 2001; Lippman *et al.,* 2007; Di Matteo, 2010a; Di Matteo 2010b; Yeats *et al.,* 2012a; Haggard, 2014). By contrast, the wild species *S. peruvianum* and *S. chilense* have been used scarcely and mostly for mapping a few major involved in disease resistance mechanisms (Foolad, 2007). According to Alseekh *et al.* (2013), more than 3,000 QTLs are reported in the *S. pennellii* library of introgression lines created by Eshed and Zamir (1995). Among them, nearly 2,000 QTLs are related to fruit quality traits including volatile aromas (Tadmor *et al.,* 2002), color (Liu *et al.,* 2003), antioxidant compounds (Rousseaux *et al.,* 2005), fruit weight and composition in sugars and acids (Causse *et al.,* 2004), , various metabolites (Schauer *et al.,* 2006) and firmness (Chapman *et al.,* 2012).

As for *S. pimpinellifolium*, it is not only regarded as a source of resistance against pathogens as cited in many studies (Martin *et al.,* 1993a,b; Fernández-Muñoz, 2000; Merk *et al.,* 2012), but also as a donor for fruit quality traits to both processing and fresh markets including yield, fruit weight, flavor, color, shape, nutritional value etc. It is noteworthy to mention that natural introgression into the cultivated tomato has been identified only in *S. pimpinellifolium* (Rick, 1982b). Additionally, plant breeding has contributed to the introduction of many genetic introgressions from this species into the cultivated tomato during the last several decades (Rick, 1982b; Warnock, 1988). Cryptic introgressions from the green-fruited wild species have also been acquired through plant breeding (Labate and Robertson, 2012). Accessions within *S. pimpinellifolium* are bidirectionally cross compatible and highly self-compatible with the cultivated tomato. The close phylogenetic relationship between the two species has facilitated considerably initial crosses and the subsequent generations of prebreeding and breeding activities. Moreover, *S. pimpinellifolium* is the only wild species characterized by its red ripe fruits, a basic feature for fruit quality traits (Chen and Foolad, 1999). Many accessions of *S. pimpinellifolium* are known for high fruit soluble solids and lycopene contents (Grandillo and Tanksley, 1996), both of which are extremely important traits to the tomato industry. Thus, the exploitation of genetic variation within *S. pimpinellifolium* is more advantageous *a priori* than the use of variation with more distantly related, green-fruited wild species such as *S. pennellii* or *S. habrochaites*. *S. pimpinellifolium* has numerous desirable horticultural and agronomic traits and significantly fewer undesirable ones in comparison to other wild relatives. Many genetic linkage maps have been created and numerous QTL analyses were conducted using different mapping populations derived from the cross *S. lycopersicum* × *S. pimpinellifolium*. Thus, several QTLs/genes were mapped and identified for pest and disease resistance (Martin *et al.,* 1991; Danesh *et al.,* 1994; Zamir *et al.,* 1994; Ganal *et al.,* 1995; Chague *et al.,* 1997; Chunwongse *et al.,* 2002; Alba *et al.,* 2009; Ashrafi *et al.,* 2009; Sharma *et al.,* 2009; Lopez *et al.,* 2011; Salinas *et al.,* 2013), for abiotic stress tolerance (Foolad, 2004; Foolad *et al.,* 2005) and for fruit quality (Kinzer *et al.,* 1990; Grandillo and Tanksley, 1996; Tanksley *et al.,* 1996; Chen *et al.,* 1999; Chen and Foolad, 1999; Lippman and Tanksley, 2001; Doganlar *et al.,* 2002a; Fulton *et al.,* 2002; van der Knaap *et al.,* 2002, 2003; Frary *et al.,* 2004; Stevens *et al.,* 2007; Xiao *et al.,* 2008; Powell *et al.,* 2012; Capel *et al.,* 2015; Barrantes *et al.,* 2016; Capel *et al.,* 2017a,b; Rambla *et al.,* 2017).

1.2 Economic and Nutritional Value

The fruit of the cultivated tomato is extensively grown and consumed worldwide. It ranked first among the leading world fresh vegetables in 2019 in terms of value of production (FAOSTAT – February (Feb) 2021; http://faostat.fao-org) and constitutes a major agricultural industry. The total harvested area worldwide reached 5,030,545 hectare (ha) with a total production of 180,766,329 tonnes (t) in 2019 (FAOSTAT - Feb. 2021; http://faostat.fao-org). These data show the huge importance of tomato at the economic level. The top 10 tomato producers between 2009 and 2019 were, in descending order, China, India, USA, Turkey, Egypt, Italy, Iran, Spain, Brazil and Mexico (FAOSTAT – Feb. 2021; http://faostat.fao-org). Spain is considered one of the leading countries in tomato production for the fresh market and for the processing industry. Tomato production in Spain has increased remarkably over the last decade (Fig. G.I-1) and has reached 5,000,560 t with 56,940 ha of the total harvested area in 2019 (FAOSTAT – Feb. 2021; http://faostat.fao-org).

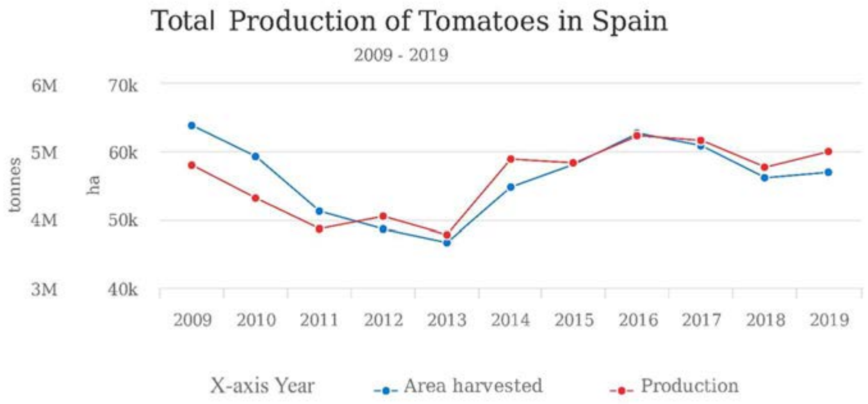


Fig. G.I-1: **The evolution of tomato production (t) and area harvested (ha) in Spain over the last decade**. Adapted from source: FAOSTAT (Feb. 2021).

Tomato industry can be separated into two distinct markets. The fresh market, where tomato products are added to other food items or consumed directly as raw vegetables and the processing food industry that includes tomato processed products such as, ketchup, canned tomatoes, chili sauce, powder, juice, salad dressings, soups, sauce and paste. Tomatoes, fresh or processed, play a vital role in maintaining a diverse and balanced human diet. The nutritional value of the tomato fruit is not high. One medium fresh tomato (135 g) provides 25 Kcal and 47% and 22% recommended dietary allowances of vitamin C and A respectively. However, inverse correlations exist between age-related macular degeneration, cardiovascular diseases and the risk of certain types of cancer on one hand and tomato consumption on the other hand (Dorais *et al.,* 2008). Tomatoes contribute largely to nutrition globally and is a source of various antioxidant molecules, including vitamins such as tocopherols (vitamin E) and ascorbic acid (vitamin C), phenolic compounds such as carotenoids, mainly lycopene but also beta-carotene, a precursor of vitamin A, and flavonoids (Moco *et al.,* 2006; Borguini and Torres, 2009; Kotkov *et al.,* 2009; Vallverdu-Queral *et al.,* 2011). These molecules have inhibited to a large extent the generation of reactive oxygen species (ROS) involved in many human diseases' development (Raiola *et al.,* 2014). They are vital for immune cell development, iron assimilation and cardiovascular function (Heber and Lu, 2002; Zou *et al.,* 2006). As cardio-protectors, flavonoids are potent inhibitors of low-density lipoprotein oxidation, a mechanism that is considered key in the development of atherosclerosis. Because flavonoids are antioxidants, they may provide protection from neurodegenerative and age-related diseases such as Alzheimer and Parkinson (Hussain *et al.,* 2018). Additionally, *β*-carotene is the most important precursor of vitamin A and an increased lycopene intake with the diet correlates with reduced rates of heart attack and has been considered a promising cancer chemo preventive, particularly for prostate cancer (Miller *et al.,* 2002). Trace amount of minerals such as zinc, manganese and copper can also be found in tomato. They are vital co-factors for several antioxidant enzymes such as catalase, and superoxide dismutase (Lavelli *et al.,* 2000; Tyssandier *et al.,* 2004).

Currently, consumers are paying great attention to health and nutritional value of horticultural products such as antioxidants, mineral elements and vitamin content (Causse *et al.,* 2010). Consequently, fruit quality in terms of both organoleptic and nutritional contents has become a paramount objective for most innovative and conventional tomato breeding with particular attention being given to fruit antioxidant molecules. For this reason, there is a rising focus on the generation of tomato cultivars with optimized levels and composition of antioxidant compounds that are associated with health benefits. This has resulted into extensive investigations highlighting the antioxidant content of cultivars and the molecular mechanisms underlying their synthesis and accumulations in plants. The isolation and characterization of many regulatory and structural genes implicated in the biosynthetic pathways of several antioxidant molecules in different species were both achieved using specific mutants characterized by visible phenotype. These genes have been employed to control these metabolic pathways in both crop and model plant species, not only from a basic research perspective, but also to change and influence, for example their nutritional value. Many studies have investigated the improvement of vitamin content in strawberry (Agius *et al.,* 2003), tomato (Davuluri *et al.,* 2005) and rice (Paine *et al.,* 2005). Many efforts have been made to increase the content of carotenoids and flavonoids, either by genetic manipulation or traditional breeding. The strategies applied have focused on metabolic engineering of genes involved in carotenoids and flavonoid biosynthesis, transgenic modulation of transcription factors affecting these metabolic pathways or the employment of single-point mutations (spontaneous or induced), and/or QTL with pronounced effects. A good example of the molecular alternative are the tomato *high pigment* (*hp*) mutations. *hp* mutations have been introduced into elite tomato germplasm due to their effect on fruit lycopene content (Levin *et al.,* 2003). When one of these mutations, *hp-2*^{dg}*,* was introgressed into elite processing cultivars, fruit lycopene content increased to about 3.5-fold (from 80–90 µg/g to 280 µg/g fruit weight) (Levin *et al.,* 2003). Moreover, these mutations also stimulate the production of higher levels of other health promoting metabolites including ascorbic acid, tocopherol and flavonoids (Yen *et al.,* 1997; Liu *et al.,* 2004). Functional characterization of *DE-ETIOLATED1* (*DET1*), the gene responsible for the *hp2* mutation, confirmed these results (Davuluri *et al.,* 2004, 2005). Additionally, genetic modification of the carotenoid biosynthetic pathway in tomato has been achieved with fruit-specific expression of several bacterial genes (Romer *et al.,* 2000; Fraser *et al.,* 2002; Enfissi *et al.,* 2005). Transgenic strategies to increase the content of flavonoids in tomato have also been successful. The transformation of tomato plants with the *CHALCONE ISOMERASE* (*CHI*) gene from *Petunia* x *hybrida* hort. ex E. Vilm. under the control of the 35S promoter resulted in a dramatic 78-fold increase of flavonoids, mainly rutin (a quercetin glucoside), in the red ripe peel (Muir *et al.,* 2001). In a different study, a significant production of rutin, up to 130 times higher than the content of wild tomato peel, was achieved by ectopic expression of *CHI* structural gene from onion (*Allium cepa* L.) (Lim and Li, 2017). To augment flavonols production in the tomato skin, the ectopic expression of *CHI* is sufficient. However, this increase in quercetin glucoside was not observed in leaves or in the flesh of red ripe tomato tissues despite the detection of relatively high amounts of *CHI* transcripts (Willits *et al.,* 2005). This shows that the biosynthesis of flavonoids is regulated in a tissue specific manner in tomato. Also, it suggests that a different perspective is needed to significantly augment flavonol content in the tomato flesh (Willits *et al.,* 2005). Indeed, flavonoid accumulation in tomato flesh was achieved with the fruit specific expression of two maize (*Zea mays* L.) transcription factors, *LEAF COLOR (Lc) and COLORED ALEURONE1 (C1)*, reaching a 60-fold increase of kaempferol glycosides in tomato flesh tissue and 20-fold increase in total fruit flavonols (Bovy *et al.,* 2002). In an alternative approach, genes encoding four key biosynthetic enzymes from *P.* × *hybrida* hort. ex E. Vilm. leading to flavonols *CHALCONE SYNTHASE* (*CHS*), CHI, *FLAVANONE-3-HYDROXYLASE* (*F3H*), and *FLAVONOL SYNTHASE* (*FLS*) were ectopically and simultaneously expressed in tomato plants. About 75% of the primary transformants containing all four transgenes accumulated very high levels of quercetin glycosides in the peel and a more modest, but significant, increased levels of kaempferol- and naringenin-glycosides were observed in the columella (Verhoeyen *et al.,* 2002).

Many QTL mapping studies have identified useful genetic material for breeding purposes seeking to increase tomato's nutritional value. More than 20 QTLs for increased °Brix were identified using the library of introgression lines (ILs) from *S. pennellii* created by Eshed and Zamir (1995). One of them, °*Bx9-2-5*, improves Brix by more than 20% and was cloned by map-based cloning (Fridman *et al.,* 2004). A total of 20 QTLs for fruit antioxidants, among them nine QTLs for phenolic content (ph) and 6 QTLs for ascorbic acid (AsA), were identified in a library of ILs from *S. pennellii* (Rousseaux *et al.,* 2005). Lines harboring some of these QTLs, *AsA12-4*, *ph6-2* and *ph7-4*, display increased phenolic and acid ascorbic contents when compared to the parental line *S. lycopersicum* (Rousseaux *et al.,* 2005). Two *S. pennellii* introgression lines (IL_7-3 and IL_12-4) characterized by accumulating higher soluble solids and antioxidant fruit contents in comparison with *S. lycopersicum* \`M82´. For high AsA content, one QTL was detected in the introgressed region 12-4 (Di Matteo *et al.,* 2010a). As for high ascorbic acid, phenols and °Brix contents, another QTL was identified in the introgressed region 7-3 (Di Matteo *et al.,* 2010b). Another study done by the same group provided novel genetic tools by pyramiding QTLs involved in high fruit antioxidant and °Brix contents into the genetic background of M82 (Sacco *et al.,* 2012). Similar studies using *S. pimpinellifolium* accession `TO-937´ and *S. lycopersicum* `Moneymaker´ as parental lines identified many QTLs involved in several fruit quality traits including carotenoids, vitamins and °Bx contents (Capel *et al.,* 2015; Barrantes *et al.,* 2016), trace elements and mineral contents (Capel *et al.,* 2017a) and volatile aromas (Rambla *et al.*, 2017). The colocalization of a fruit weight QTL, *FW7.1*, with QTLs implicated in glucose, soluble solid and vitamin C contents, dry weight/fresh weight, and most importantly with the *Sucrose Phosphate Synthase* gene, proposes that polymorphism in this gene could have an impact on the genetic variation of many fruit quality attributes (Capel *et al.,* 2015). On the other hand, Barrantes *et al.* (2016) identified two QTLs located on chromosomes 2 and 9 that increased solid soluble content without affecting fruit weight. These two *loci* might be regarded as good candidates to be introgressed into modern cultivars (Barrantes *et al.,* 2016). As for flavonoids, there are not many studies published on their inheritance in tomato, yet a few genomic regions controlling flavonoid content of the peel have been reported by studying ILs from *S. chmielewskii* (Ballester *et al.,* 2016).

1.3 The Tomato Today: A Model Species for Scientists

Tomato is one of the crops that have been studied the most in both agronomical and basic research. It has many interesting features such as fleshy fruit covered with an astomatous cuticle and its plant has a sympodial shoot and compound leaves, which other model plants such as rice (*Oryza sativa* L.) and *Arabidopsis thaliana* L. lack (Schmitz and Theres, 1999; Sinha, 1999; Franke *et al.,* 2005). Due to certain characteristics, such as self-pollination, high multiplication rate, short life cycle and ease of mechanical crossing and culture under a wide range of environments (Gillaspy *et al.,* 1993; Tanksley, 2004) the tomato crop has become the model species for fleshy fruits. The common feature of fleshy fruits is the fine balance between organic acids and sugars, and the accumulation of vitamins, aromatic compounds, pigments, minerals and water that confer upon the fruit its attractiveness and juiciness. All these compounds control fruit quality traits that are economically important and cannot be studied using other model plant systems. Moreover, it is a model for fruit development studies, including endoreplication studies (van der Knaap *et al.,* 2014; Azzi *et al.,* 2015). Over several decades, numerous varieties and mutants of tomato have been collected and almost one thousand monogenic stocks have been characterized so far (Emmanuel and Levy, 2002). The availability of a broad spectrum of mutants (Menda *et al.,* 2004; Watanabe *et al.,* 2007; Just *et al.,* 2013) has facilitated the screening with strong genetic and genomic materials to study mechanisms involved into the tomato fruit development and ripening including the cuticle lipids biosynthesis (Mintz-Oron *et al.,* 2008; Kosma *et al.,* 2010; Wang *et al.,* 2011). Among these tomato mutants, several are well described and exhibit alterations in all aspects of fruit growth and ripening, and for which many of the underlying genes have been cloned (Giovannoni, 2007; van der Knaap *et al.,* 2014; Azzi *et al.,* 2015). Also, the availability of high molecular weight insert genomic libraries, including yeast artificial chromosomes (YAC) (Bonnema *et al.,* 1999) and bacterial artificial chromosome (BAC) (Hamilton *et al.,* 1999; Budiman *et al.,* 2000) has made positional cloning and map-based less complicated. All these libraries are easily accessible and available for purchase. Functional analyses of candidate genes are regularly carried out in tomato since it is considered a common and highly effective tool for transformation via *Agrobacterium tumefaciens (Smith & Townsend) Conn*, and susceptible to transient gene expression via agroinjection (Orzaez *et al.,* 2006). In addition, the repression of target genes can be achieved by a genome-editing strategy using the RNA-guided DNA endonuclease system called the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated9 (Cas9) (Brooks *et al.,* 2014) or by virus-induced gene silencing (VIGS) (Orzaez *et al.,* 2009).

Different types of molecular markers have been developed and mapped onto the twelve tomato chromosomes including single-nucleotide polymorphism (SNPs), amplified fragment length polymorphism (AFLPs), restriction fragment length polymorphism (RFLPs), cleaved amplified polymorphic (CAPs), expressed sequence tag (ESTs), simple sequence repeat (SSRs), resistance gene analog (RGAs) and conserved orthologue set (COS) (Broun and Tanksley 1996; Saliba-Colombani *et al.,* 2000; Suliman-

Pollatschek *et al.,* 2002; Frary *et al.,* 2005). These markers have been extensively employed in the construction of high-density genetic maps and in the dissection of quantitative traits into discrete QTLs. They have also been used sometimes for marker-assisted breeding, fingerprinting and germplasm screening. Moreover, the sequencing and the publishing of the full tomato genome in 2012 (Tomato Genome Consortium, 2012) represented an exceptional breakthrough for mapping studies in fleshy fruits. Following this release, the identification and fine mapping of additional QTLs in tomato have increased remarkably (Rodriguez *et al.,* 2013). In the past decade, a large number of SNP markers, emerging from the resequencing of quite diverse accessions of *S. lycopersicum* and its wild relatives, has been reported (Xu *et al.,* 2013; Aflitos *et al.,* 2014; Lin *et al.,* 2014), and therefore, this may help identify novel markers in genetic maps. The highly favorable mating system between domesticated and wild species has been broadly employed to create segregating populations such as F_2, recombinant inbred lines (RILs) and ILs. F_2 populations are easily obtainable and are frequently employed in most crop species. However, their resolution for map-based cloning is low and cannot be used to analyze experiments in different environmental conditions. These issues are overcome with RIL and IL populations representing a mosaic of the two parental genomes. RILs are created from a F_1 hybrid by repeated selfing towards advanced homozygous lines. Likewise, ILs are developed by repeatedly backcrossing F_1 hybrids with the recurrent parent and ultimately fixing by selfing and selection of homozygous introgressions. ILs are identical to the recurrent parent except for the small homozygous introgressed genomic fragment from the other parent. Both RIL and IL populations are immortal and permanent. In tomato, RIL populations have been employed to map QTLs for several traits; including fruit weight and composition (Goldman *et al.,* 1995; Capel *et al.,* 2015; Capel *el al.,* 2017a) several morphological traits (Paran *et al.,* 1997; Capel *et al.,* 2015) abiotic stress tolerance (Foolad *et al.,* 2004) seed weight (Goldman et *al.,* 1997), disease and pest resistance (Diwan *et al.,* 1999; Alba *et al.,* 2009). As for IL populations, they have been used to facilitate the introgression of genetic material from wild species (Eshed and Zamir, 1995; Tanksley *et al.,* 1996). In tomato, IL populations have been developed from many wild relatives including *S. habrochaites* (Monforte and Tanksley, 2000; Francis *et al.,* 2001; Finkers *et al.,* 2007), *S. pimpinellifolium* (Tanksley *et al.,* 1996; Bernacchi *et al.,* 1998; Kinkade and Foolad, 2013; Barrantes *et al.,* 2014) and *S. pennellii* (Eshed and Zamir, 1994). IL populations were proved to be extremely useful and powerful for QTLs identification and localization (Eshed and Zamir, 1995; Rousseaux *et al.,* 2005; Ben Chaim *et al.,* 2006), QTL effects verification and validation (Tanksley *et al.,* 1996), studying QTL x genetic background, QTL x QTL and QTL x environmental interactions (Monforte *et al.,* 2001), QTL fine mapping (Eshed and Zamir, 1996; Ku *et al.,* 2000; Monforte and Tanksley, 2000b; Ashrafi *et al.,* 2012), studying epistatic interactions between *loci* (Chapman *et al.,* 2012) and introducing new genetic material from wild species into elite germplasm (Tanksley and McCouch, 1997; Zamir, 2001; Gur and Zamir, 2004). Crosses between ILs have been made to combine more than one QTL into the same genetic background and study their effects (Gur *et al.,* 2011; Sacco *et al.,* 2013; Rigano *et al.,* 2014). Metabolomic analyses have been employed to identify QTLs (metabolite QTL) involved in the accumulation of compounds related to fruit quality such as sugars, pigments or volatiles (Bovy *et al.,* 2007; Capel *et al.,* 2017a). Combination of metabolite QTL mapping and transcriptomics to identify the key factors (Mounet *et al.,* 2009; Do *et al.,* 2010). Such research can improve the biochemical knowledge of fruit content and be an enhancer for quality breeding (Fernie and Schauer 2009; de Vos *et al.,* 2011).

As described here, extensive genomic research on tomato has led to the emergence of valuable resources that will help advance the genetic characterization of complex attributes and will provide tools for breeders and geneticists alike, not only in tomato but also in other fleshy-fruited and Solanaceae crops.

2 THE CUTICLE: BIOSYNTHESIS, STRUCTURE AND BIOLOGICAL FUNCTIONS

2.1 Key Feature of Land Plants

Up until the Mid-Ordovician era, approximately 470 million years ago, the continents of Earth were practically lifeless (Gensel, 2008). Around this time the passage of modern land plants ancestors from water to the terrestrial environment started, revealing one of the most defining evolutionary events in the history of life on earth, and contributing to profound consequences on both, atmospheric and animal evolution levels (Bateman *et al.,* 1998). The lack of water was the major challenge for the first migrating plants to land. They had to adjust to an environment where their internal water content was much higher than the relative water content of the atmosphere. To survive severe conditions such as radiation, drought and water deficiency, primitive land plants were urged to evolve and develop new strategies to adapt to the new ecological habitat. Seemingly, one of their most significant innovations was the development of lipophilic barriers covering the outermost surface of their aerial parts as well as some inner cellular layers in specialized tissues (Goodwin and Jenks, 2005; Riederer and Muller, 2006; Budke *et al.,* 2012). The plant cuticle is one of these lipophilic barriers. It opened the way towards plants expansion in terrestrial environments, permitting them to reduce their transpirational water loss and to control the mobility of solutes, gases and water (Bargel *et al.,* 2006; Riederer and Müller, 2006; Chen *et al.,* 2011). The plant cuticle is one of the key features that define and characterize land plants. In general, it is the interface between non-woody aerial plant organs and the surrounding environment (Riederer and Schreiber, 2001). It is located at the external periclinal cell wall of epidermal cells, being also projected between anticlinal walls in some species and/or organs (Javelle *et al.,* 2011). Thus, the cuticle is present on the surface of leaves, including trichomes and substomatal chambers (Osborn and Taylor, 1990; Bessire *et al.,* 2007; Fernández *et al.,* 2011; Buschhaus and Jetter, 2012; Fich *et al.,* 2016), petals (Li-Beisson *et al.,* 2009; Buschhaus *et al.,* 2015), primary stems (Xue *et al.,* 2014), seeds (de

Giorgi *et al.,* 2015) and fruits (Khanal *et al.,* 2011; Lara *et al.,* 2015, Segado *et al.,* 2016; Lara, 2018).

2.2 Cuticle Composition and Structure

The cuticle is a composite structure formed by a combination of biopolymers that serves as a matrix for chemically heterogeneous compounds embedded in it. The cuticle is composed by a cutin polymer matrix with waxes that can embedded in the matrix (intracuticular waxes) or accumulated on the surface forming crystalloids or smooth films (epicuticular waxes), a phenolics fraction and polysaccharides from the cell wall (Fig. G.I-2) (Domínguez *et al.,* 2011). Trace amount of amino acids and mineral elements can also be found (Guzmán-Delgado *et al.,* 2016).

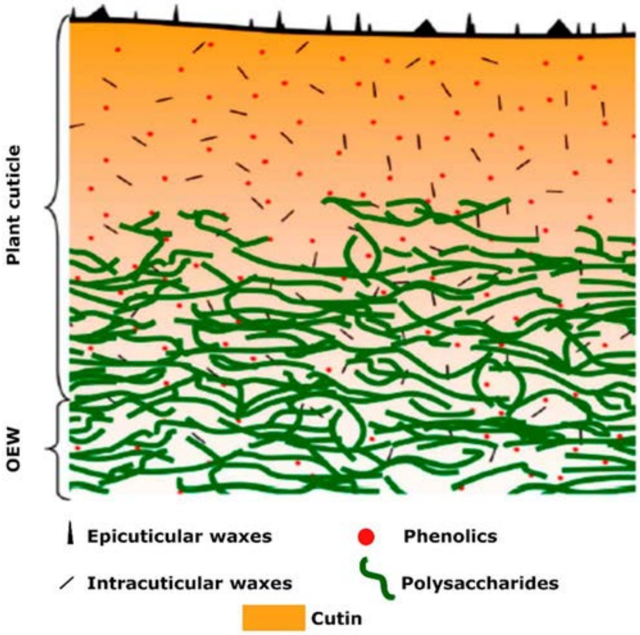


Fig. G.I-2: Scheme of a transverse section of the cuticle representing the different components and main structural features. OEW, outer epidermal wall. Adapted from Domínguez *et al.* (2011), New Phytologist Copyright ©.

Plant cuticles show compositional and structural variability among and within species and also between organs of the same species (Kolattukudy, 1980a; Jetter and Schäffer, 2001; Jeffree, 2006; Lara *et al.,* 2015;

Fernández *et al.,* 2017). For example, tomato fruit cuticles contain 2.3–5.2% of wax (Petracek and Bukovac, 1995; Domínguez *et al.,* 2009a; Khanal *et al.,* 2013a; España *et al.,* 2014b), while the content of wax in apple fruit cuticles is ~10-fold higher (Khanal *et al.,* 2013a). Moreover, differences in wax composition among varieties have been observed in *Prunus persica* L. Batsch and *Prunus avium* L. (Lara *et al.,* 2018). In tomatoes, fruits have significantly higher cutin and wax contents than leaves (Vogg *et al.,* 2004; Buxdorf *et al.,* 2014; Petit *et al.,* 2014). Alterations in cuticle thickness and composition occur frequently during organ development (Takahashi *et al.,* 2012; España *et al.,* 2014a,b; Segado *et al.,* 2016, 2020). Furthermore, changes in the structure and composition of cuticles also occur during postharvest and storage (Veraverbeke *et al.,* 2001; Bing *et al.,* 2018; Lara, 2018; Belge *et al.,* 2019). Moreover, environmental conditions have been known to influence cuticle synthesis and thickness (Domínguez *et al.,* 2012). In general, the amount of cuticle deposited can vary between 20 and >3000 $\mu g.cm^{-2}$ while its thickness ranges from <0.03 to >10 µm (Heredia, 2003).

The description of the plant cuticle has changed over time. Initially, it was understood as a lipid layer deposited on top of epidermal cell walls (von Mohl, 1847) (Fig. G.I-3A left). Later on, ultrastructural studies on some cuticles during development showed regions with different electron density that were attributed to compositional changes within the cuticle. Thus, the cuticle was postulated to be composed of an upper cutin layer (cuticle proper) and an inner one where cutin and cell wall polysaccharides intertwined (cuticle layer) (Fig. G.I-3A right) (Jeffree, 2006). However, this layered structure concept (cuticle proper and cuticle layer) has been doubted in recent years. Currently, based on polysaccharide immunolocalization within the cuticle and ultrastructural analyses, it is considered, at least in some species, as a non-layered lipidized structure, derived from a modified epidermal cell wall (Guzman *et al.,* 2014a,b; Segado *et al.,* 2016) (Fig. G.I-3B).

Cutin, the principal and major constituent of the cuticle, is an insoluble, aliphatic, and amorphous polyester (Kolattukudy, 1980b) mostly constituted

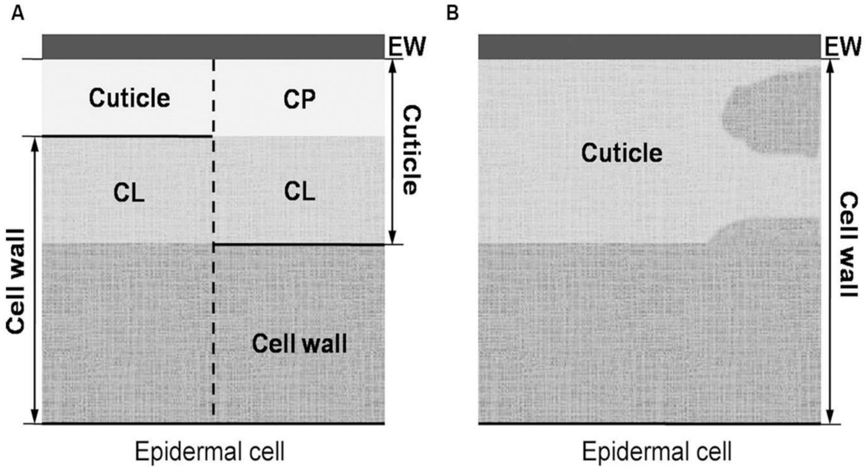


Fig. G.I-3: The evolution of the plant cuticle structural and compositional concept. (A, left side) Preliminary model of von Mohl (1847) in which the cuticle is restricted to its outermost cellulose-free region (i.e., the cuticle proper), as basis for (A, right side) the prevailing plant cuticle model in which the cuticle proper is believed to be free of polysaccharides. (B) The cuticle understood as a lipidized, chemically and structurally heterogeneous region of the epidermal cell wall. Epicuticular waxes can be considered a layer that is located over the cuticle. EW= Epicuticular wax; CP= cuticle proper; CL= cuticle layer. Adapted from Fernández *et al.* (2016), Frontiers in Plant Science Copyright ©.

of polyhydroxylated and epoxy hydroxylated C_{16} and/or C_{18} fatty acids. These esterified fatty acids are displayed in a net-like structure via numerous cross-links developed between the carboxyl and the secondary hydroxyl moieties (Kolattukudy, 1980b), and sometimes linked with glycerol (Graça *et al.,* 2002). Depending on the chain length of the predominant fatty acid, three types of cutin constituents can be identified: C_{16}, C_{18} or mixtures of C_{16} and C_{18}. The main cuticle monomers are shown in Fig. G.I-4. The 9- or 10,16-dihydroxyhexadecanoic acid and 16-hydroxyhexadecanoic acids are the major components of the C_{16} family.

The 18-hydroxy-9,10-epoxyoctadecanoic acid and 9,10,18-trihydroxyoctadecanoic acid and their monosaturated homologues are the major components of the C_{18} family (Kolattukudy, 1980b). Although the classes of cutin monomers are usually conserved within plant families, organs and developmental stages, some variations may exist (Kosma *et al.,*

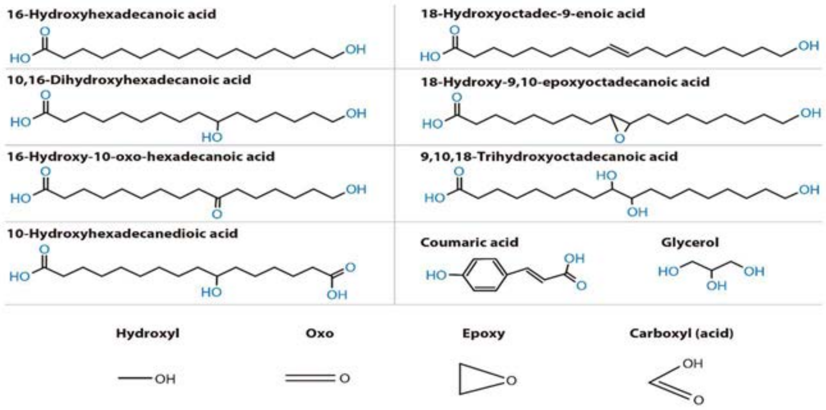


Fig. G.I-4: Examples of common types of cutin monomers, as well as typical monomer functional groups. Taken from Fich *et al.* (2016), Annual Review of Plant Biology Copyright ©.

2010; Parsons *et al.,* 2012; Belge *et al.,* 2014a,b; Petit *et al.,* 2014). As well as fatty acid derivatives, trace amounts of glycerol and phenolic compounds can also be detected in cutin (Stark and Tian, 2006). *A. thaliana* stems and leaves have an unusual cutin mainly composed of dicarboxylic acids and glycerol (Graça *et al.,* 2002; Franke *et al.,* 2005). Additionally, some cuticles such as peach fruit cuticle contain cutan, an extremely insoluble, nonhydrolyzable polymer (Boom *et al.,* 2005; Johnson *et al.,* 2007; Fernández *et al.,* 2011). Cutan is rich in C-O-C bonds; however, its chemical nature remains unclear (Tegelaar *et al.,* 1991; Deshmukh *et al.,* 2005).

Cuticular waxes are complex mixtures of very long-chain fatty acids and their derivatives including aldehydes, primary alcohols and alkanes. Additionally, pentacyclic triterpenoids have also been reported (Jetter *et al.,* 2006; Buschhaus and Jetter, 2011). Triterpenoids can be present as minor compounds of wax mixtures as in *A. thaliana* leaves (Jenks *et al.,* 1995) or dominating the mixture as in apple (Belding *et al.,* 1998), sweet cherry (Peschel *et al.,* 2007), citrus (Liu *et al.,* 2012) or persimmon fruits (Tsubaki *et al.,* 2013). It has been reported that triterpenoids are present only as intracuticular waxes (Buschhaus and Jetter, 2011). Most plant species investigated to date, have shown some differences in the chemical composition of epi and intracuticular waxes (Buschhaus and Jetter, 2011).

The cuticle and the cell wall are composed of similar polysaccharides (hemicelluloses, celluloses and pectins) (López-Casado *et al.,* 2007). These components represent the anchor by which the cuticle is attached to the outer cell wall. Cuticles related studies have traditionally focused on cutin and wax and little importance was given to polysaccharides or phenolics. However, in recent years the relevance of cuticle polysaccharides, their distribution within the cuticle, and their structural characteristics are being addressed (Phillipe *et al.,* 2020). Identification of the interconnections between polysaccharides and cutin, either physical, chemical or both, would be of great interest to understand cuticle structure, deposition and attachment to the cell wall.

The phenolic fraction principal components are flavonoids and hydroxycinnamic acid derivatives (Hunt and Baker, 1980; Pfündel *et al.,* 2006). Their presence has been widely reported in most studied species (Fernández *et al.,* 2011; España *et al.,* 2014a; Huang *et al.,* 2017; Leide *et al.,* 2018; Kong *et al.,* 2020). They may be trapped in the matrix, chemically bound to cutin or waxes or present in free form (Karabourniotis and Liakopoulos, 2005; Domínguez *et al.,* 2009b). The predominant constituents identified in the tomato fruit cuticle are ferulic, *p*-hydroxybenzoic and *p*-coumaric acids and the flavonoid naringenin chalcone (Hunt and Baker, 1980; Pfündel *et al.,* 2006; Rautengarten *et al.,* 2012; España *et al.,* 2014b). Moreover, the cuticle of the peach fruit also contains flavonoids (Fernández *et al.,* 2011). A lignin-like aromatic domain has been also observed in some gymnosperms (Reina-Pinto *et al.,* 2001).

2.3 Genetics of Cuticle Components

Fatty acids are precursors for both cutin and wax biosynthesis. They are regarded as central intermediates for all lipid classes biosynthetic pathways including first stage of cutin formation and cuticular waxes. These precursors are believed to be synthesized de novo in plastids as the result of fatty acid synthase (FAS) activity, which implicates three sequential reactions resulting in the production of fatty acyl primer (Yeats and Rose, 2013). The elongation of this fatty acyl primer by repeated condensations generates the long-chain fatty acids (Kolattukudy, 2001). Despite that de novo fatty acid biosynthesis is ubiquitous, cutin monomers precursors [palmitic (16:0), stearic (18:0), and oleic (18:1) acids] are synthesized in the plastids of the epidermal cells. Cutin monomers biosynthesis consists first of a chemical transformation in the endoplasmic reticulum and then a transport to outer cell wall where they are polymerized (Kolattukudy, 1980b; Kolattukudy, 1981; Kolattukudy, 2001).

As mentioned in the previous paragraph, Kolattukudy and co-workers have established the biosynthetic pathway leading to the production of cutin monomers using apple and bean leaves as plant materials. However, little is known about the biosynthesis and polymerization of cutin monomers including the monomer transport mechanisms, the enzymes involved in their assembly and the polymerization site itself. Several studies were conducted to elucidate the key factors underlying the latter processes. The main cutin monomers are synthesized by multiple epoxidation and hydroxylation reactions. These reactions are catalyzed by enzymes showing typical characteristics of cytochrome P450 (CYP) family (Molina *et al.,* 2008; Mizutani and Sato, 2011). The hydroxylation reactions at the terminal methyl group (ω- hydroxylases) on aliphatic fatty acid chains are carried out by members of CYP86A subfamily (Xiao *et al.,* 2004), whereas the hydroxylation reactions at midchain positions probably involve members of the CYP77A6 subfamily (Li and Beisson, 2009). Several of these CYP450-dependent monooxygenases have been cloned, including CYP86A8 (*LACERATA*; *LCR*) (Wellesen *et al.,* 2001), and CYP86A2 (*Aberrant induction of Type Three*; *ATT1*) (Xiao *et al.,* 2004). A key role in cutin biosynthesis has been proved for *LCR* (A. Yephremov, unpublished results) and *ATT1* (Xiao *et al.,* 2004). *AtATT1* mutants exihibit a 30% loss in cutin load and a much looser cuticular ultrastructure (Xiao *et al.,* 2004). Moreover, *lcr* plants display modifications in the monomer composition of residual-bound lipids (A. Yephremov, unpublished results). For cutin production *Long-chain acyl-CoA synthetases 2* (*LACS2*) are also needed, indicating that cutin monomers precursors have to be activated (CoA-bound) before the formation of the cutin network (Schnurr *et al.,* 2004; Weng *et al.,* 2010). Recombinant *LACS2* are more compatible with 16-hydroxypalmitate than with palmitate (Schnurr *et al.,* 2004). Ester bonds in monomers are formed by the ω-hydroxyl groups while the ester-type cross-links are derived from variable portion of the midchain hydroxyl groups, thus joining together and leading to the final polymeric complex (Pollard *et al.,* 2008). Acyltransferases catalyzed these ester linkage reactions. These enzymes carry the activated cutin monomers to the free hydroxyl groups present in the cutin polymer (Kolattukudy, 2001). The latter reaction might need an ATP and a cutin primer, and indeed two glycerol-3-phosphate acyltransferases, GPAT4 and GPAT8, confirmed to be essential for cutin accumulation in *A. thaliana* (Li *et al.,* 2007, Yang *et al.,* 2012). Recently, Pineau *et al.* (2017) demonstrated that *EPOXIDE HYDROLASE1* (*AtEH1*) encodes a cytosolic epoxide hydrolase implicated in the biosynthesis of poly-hydroxylated cutin monomers in *A. thaliana*. Moreover, the cutin polymerization may also require, although to a minor extent, the contribution of phenolic esters such as those of *p*-coumaric and ferulic acids (Rautengarten *et al.,* 2012).

The cutin monomers must be transported from the endoplasmic reticulum and trafficked across the plasma membrane to the cell wall to be integrated into the cutin matrix. Several mechanisms concerning monomers exportation from the endoplasmic reticulum to the plasma membrane were suggested. 'The direct way', by which cutin monomers are exported directly from the endoplasmic reticulum to the plasma membrane at sites of close contact (Samuels and McFarlane, 2012). And 'the via-way', by which the transport is achieved via exocytotic vesicles or lipid droplets (Domínguez *et al.,* 2015a), suspected to be present in the growing epidermal cell wall of some species (Jeffree, 2006). Studies in *A. thaliana* have shown that monoacylglycerols leave the cell via specific ATP binding cassette (ABC) transporters in the plasma membrane. It has been demonstrated that transporter ABCG11, ABCG13 and ABCG32 are involved in cutin deposition (Bird *et al.,* 2007; Bessire *et al.,* 2011; Panikashvili *et al.,* 2011). The passage of the hydrophobic cutin precursors across the hydrophilic cell wall to reach the polymerization site remains unknown. Lipid transfer proteins (LTPs), a family of small and soluble proteins bound to the extracellular side of the plasma membrane, may be candidates of this transfer since two glycosyl-phosphatidylinositol (GPI)-anchored LTPs: LTPG1 and LTPG2 both confirmed to be involved in cuticular wax deposition (DeBono *et al.,* 2009; Kim *et al.,* 2012).

The last and the least understood step in cutin biosynthesis is cutin polymerization, by which, cutin monomers incorporate into the polymer when arrived at the growing cuticle in the outer cell wall. Two independent studies in 2012, using distinct methodology, identified a member of Gly-Asp-Ser-Leu-pattern lipase/esterase (GDSL) superfamily functioning as cutin synthase in the epidermis of developing tomato fruits (Girard *et al.,* 2012, Yeats *et al.,* 2012b). In vitro analysis showed that GDSL1 recombinant protein catalyzes the synthetic monomer precursor: 2-mono(10,16-dihydroxyhexadecanoyl) glycerol (2-MHG) resulting in the transfer of the hydroxy acyl group from one molecule of 2-MHG to the terminus of the growing polymer chain, generating a linear head-to-tail structure (Yeats *et al.,* 2012b). This primary linear oligomeric in vitro product indicates that the elaboration of the final polymer undergoing further branching and cross-linking may require additional factors. However, GDSL1 may preferentially catalyze midchain esterification in vivo and not ω-hydroxyls as demonstrated in vitro (Fich *et al.,* 2016). The phylogenetic analysis of the first identified gene coding for cutin synthase, *Cutin Deficient 1* (*CD1*) (Isaacson *et al.,* 2009) and homologues genes indicates that *GDSLs* subfamily represented by *CD1*, is small and well conserved across divers taxa of land plants (Volokita *et al.,* 2011). The silencing of two of its orthologues in *A. thaliana* resulted in plants exhibiting floral organ fusions and changes in the petal surface morphology; such phenotypes are usually associated with cutin deficiency (Shi *et al.,* 2011). Similar localization and expression of a *CD1* orthologue in *Agave americana* L. (*A. americana*) also supports the fact that CD1-like enzymes act as cutin synthases (Reina-Pinto *et al.,* 2007). Further phylogenetic analyses done by Yeats *et al.* (2014) show that *CD1* and putative orthologues in other species belong to a gene clade that comprises an ancient family of cutin synthase-like (CUS) proteins conserved among land plants. Accordingly, *CD1* was renamed *CUS1*.

Additionally, in tomato four *CUS1* paralogs have been identified: *CUS2*, *CUS3*, *CUS4* and *CUS5*. Among the latter four *CUS* genes, only *CUS2* has been demonstrated to be involved in the maintenance of sepal cuticle ridges in *A. thaliana* (Hong *et al.,* 2017). CUS1, the only known apoplastic cutin-acting transacylase, requires a hydroxy-fatty acid-glycerol ester (2-MAG) as its acyl donor substrate. However, Xin *et al.* (2021) detected a different type of reaction in which the donor is polymeric cutin. The latter authors proposed that during organ growth, ester bonds can be transiently cleaved and then re-formed (transacylation). In other words, they detected a cutin: cutin-fatty acid endo-transacylase (CCT) activity using pea epicotyl epidermis as a model. *In-situ* growth rate correlated with CCT activity in *Hylotelephium spectabile* (Boreau) Ohba leaves and tomato fruits. This indicates that CCT activity might relax the outer epidermal wall during organ development. It is noteworthy to mention that CUS1 lacks CCT activity. Thus, Xin *et al.* (2021) provide a reference for future CCT protein identification, which can adopt their sensitive enzyme assay to screen other CUS1-related enzymes. Two additional enzymes, the BODYGUARD (BDG), a member of *α/β* hydrolase family proteins localized in the outermost portion of the epidermal cell wall (Kurdyukov *et al.,* 2006a) and the DEFECTIVE IN CUTICULAR RIDGES (DCR), an acyltransferase enzyme located in the cytosol (Panikashvili *et al.,* 2009), were suggested to contribute to cutin polymerization after phenotypic analysis of their mutants. Jakobson *et al.* (2016) demonstrated using *bdg1* plants of *A. thaliana* that *BDG1* is specific to the epidermis and showed an extracellular location. These findings are compatible with the proposed role of *BDG1* as a transacylase involved in C_{18} cutin monomer polymerization. On the other hand, silencing the *DCR* tomato ortholog resulted in a reduction in cuticle thickness as well as a drastic decrease of the main cutin monomer and the lipid bodies in the cytoplasm of the tomato fruit epicarp cells (Lashbrooke *et al.,* 2016).

All studies related to cutin biosynthesis, polymerization and deposition, have suggested processes that involve enzymes located in the epidermal cells or in the outer epidermal walls. However, Domínguez *et al.* (2010) proposed a non-enzymatic model of polymerization based on self-assembly and self-esterification of cutin monomers and transport via a novel type of nanoparticles called cutinsomes. Cutinsomes have been identified *in planta* during early stages of tomato fruit growth (Segado *et al.,* 2020).

As mentioned before, cutin and wax share the first stage of their biosynthetic pathways. However, wax biosynthesis second stage, involves the elongation of saturated C_{16} and C_{18} fatty acids in the endoplasmic reticulum into very long chain fatty acids with C_{20}-C_{34} chains. Wax biosynthesis can be summarized as follows. It begins with de novo C_{16} and C_{18} fatty acid biosynthesis in the plastid of epidermal cells. These long-chain fatty acid compounds are converted to CoA thioesters by LACS isozyme. The sequential elongation of the latter products by the action of a fatty acid elongase (FAE) complex consisting of the β-keto acyl-CoA synthase condensing enzyme (KCS) (Blacklock and Jaworski, 2006), the β-keto acyl-CoA reductase (KCR) (Beaudoin *et al.,* 2002), and the enoyl-CoA reductase (ECR) (Kohlwein *et al.,* 2001), generates the very long chain fatty acids. The *ECERIFERUM 6* (*CER6*) is the only wax specific *KCS* identified to date. *CER6* is implicated in the elongation of fatty acyl-CoAs longer than C_{24} and thus implicated in wax biosynthesis in *A. thaliana* (Fiebig *et al.,* 2000). Analysis of *cer6* mutants and transgenic sense-suppressed plants exihibit a drastic decrease in CER6 activity that almost suppressed stem wax accumulation resulting in significantly lower loads of all wax constituents and accumulation of C_{20}-C_{34} acyl group (Millar *et al.,* 1999; Fiebig *et al.,* 2000). Moreover, another enzyme, the *wax ester synthase/acyl-coenzyme A: diacylglycerol acyltransferase* (*WSD1*), located in the endoplasmic reticulum of stems in *A. thaliana*, was also identified for its involvement in wax ester synthesis (Li *et al.,* 2008). Before being transported, very long chain fatty acids undergo the final modifications into the definitive wax compounds including esters, ketones, alkanes, aldehydes, primary and secondary alcohols. To date studies indicate that the endoplasmic reticulum contains all wax biosynthesis enzymes showing that the latter process probably take place in this single intracellular structure.

By analogy to other intracellular lipid transfer mechanisms and based on inconclusive evidence, two hypothetical paths for wax molecules from the endoplasmic reticulum to the plasma membrane have been proposed: 1) Golgi-mediated vesicular transfer across the secretory pathway (Kunst and Samuels, 2003), or 2) direct molecular traffic at endoplasmic reticulum - plasma membrane contact sites (Schulz and Frommer, 2004). Wax secretion and transport through the hydrophilic outer cell walls and the lipidic cuticular layers are still unclear. Following their arrival to the plasma membrane, wax molecules need to be liberated from the lipid bilayer into the apoplastic environment. A breakthrough was made towards a more specific comprehension of wax transport when two members of the ABC family transporters, WHITE-BROWN COMPLEX 11 (WBC11) (Pighin *et al.,* 2004; Luo *et al.,* 2007; Panikashvili *et al.,* 2007) and CER5 (Bird *et al.,* 2007) necessary for wax export were discovered in *A. thaliana* stems. Analysis of *cer5* and *wbc11* plants indicate that the two ABC transporters are needed for wax transport from the plasma membrane, however, it is not clear whether these proteins use wax molecules as substrates (Samuels *et al.,* 2008). Beyond plasma membrane, the cell wall - associated LTPs are believed to facilitate the pass of the hydrophobic wax constituents to the aqueous cell wall (Bernard and Joubès, 2013; Kunst and Samuels, 2009). LTPG1 and LTPG2 were shown to be necessary for wax trafficking from the plasma membrane to the outer cell wall (DeBono *et al.,* 2009; Kim *et al.,* 2012). However, their exact function in wax export mechanism is still unclear.

Data from many studies showed that cuticle biosynthesis regulatory pathways including cutin and wax, are most often mediated by transcription factors. Most of these pathways have been identified in *A. thaliana* due to the large availability of genetic tools and mutants. The first assigned regulator of a cuticle-related metabolic pathway is *SHINE1/WAX INDUCER1* (*SHN1/WIN1*) of *A. thaliana*, belonging to a small clade of three proteins in the APETALA2/ETHYLENE-RESPONSIVE ELEMENT BINDING PROTEINS (AP2/EREBP) large superfamily (Aharoni *et al.,* 2004). *SHN1/WIN1* family are also known to regulate wax biosynthesis in *A. thaliana* (Aharoni *et al.,* 2004; Broun *et al.,* 2007), including *WAX PRODUCTION 2* (*WXP2*) (Zhang *et al.,* 2007) and its heterologous *WXP1* in *Medicago truncatula* Gaertn. (Zhang *et al.,* 2005). *SHN1/WIN1* family mode of action and biological function remains uncertain due to redundancy with the two other clade members. However, the down regulation of *SHN1* causes alteration in the petal cutin monomer composition, a reduction in the total cutin content and an increase in cuticle permeability (Kannangara *et al.,* 2007). By contrast, overexpression of these AP2/EREBP -type transcription factors drastically stimulated wax production in transgenic plants leading to a remarkably glossy leaf phenotype. Moreover, it also played a role in the induction of several wax-related genes, including *CER1*, *CER2*, and *KCS1*, and consequently enhanced alkane loads in leaves (Aharoni *et al.,* 2004; Broun *et al.,* 2007). Transgenic plants overexpressing the *WIN1*/*SHN1* paralogs *SHN2* and *SHN3* also exhibited glossy leaves and increased wax accumulation on leaves and stems (Aharoni *et al.,* 2004). Five cutin associated protein families were identified to be target genes of *SHN* transcription factors including members of CYP86A family, fatty acyl-CoA reductases, GDSL-motif lipases, BDG-like proteins (Shi *et al.,* 2011) and LACS2 proteins (Kannangara *et al.,* 2007). *WIN1/SHN1* binds directly to *LACS2* promoter (Kannangara *et al.,* 2007; Wang *et al.,* 2012). This is not the case of genes involved in wax regulation. Their induction lags behind cutin genes (Kannangara *et al.,* 2007). This delayed stimulation of wax-related genes might indicate that the control of wax biosynthesis by *WIN1/SHN1* is indirect and thus additional transcription factors acting downstream of *WIN1/SHN1* are required. Moreover, *WIN1/SHN1* may have an indirect impact on wax formation by affecting the composition or load of cutin synthesized. *A. thaliana* mutants with altered cutin, including *bdg* (Kurdyukov *et al.,* 2006a) and *lacs2* (Schnurr *et al.,* 2004; Bessire *et al.,* 2007) produce more cuticular wax than wild-type plants. Although this result can be interpreted by the fact that the two pathways have the same fatty acid precursors, it may also indicate the existence of regulatory interactions between the pathways, common biosynthetic steps, or (partially) shared mechanisms for cuticle constituents trafficking (Samuels *et al.,* 2008). It has been shown that *MYB94* and *MYB96*, two of *A. thaliana* abscisic acid responsive R2R3-type MYB transcription factors, induce genes encoding very long chain fatty acids-condensing enzymes, implicated in cuticular wax deposition via the abscisic acid pathway (Seo *et al.,* 2011; Lee *et al.,* 2016). *SHN* genes are also target of other transcription factors. The *MADS* box gene *TOMATO AGAMOUS-LIKE 1* (*TAGL1*), known for controlling tomato fruit development, has also an impact on cuticle development, structure and composition in cutin, polysaccharides, waxes and phenolic compounds (Giménez *et al.,* 2015). The latter authors demonstrated that *TAGL1* regulates tomato *SHN1* and *SHN3* genes expression. Therefore, the function of *TAGL1* in epidermis and cuticle development could be exercised through regulating the expression pattern of *SHN1* and *SHN3* genes (Giménez *et al.,* 2015). Two *MYB* transcription factors, *MYB106* and its paralog *MYB16* were found to control many aspects of cuticle and epidermis formation in *A. thaliana*, by acting upstream *SHIN1* and also by activating some cuticle biosynthetic gene (Oshima *et al.,* 2013). Zhou *et al.* (2013) demonstrated that cutin biosynthesis in rice was affected by the overexpression of ethylene response factor *WAX SYNTHESIS REGULATOR 2* (*WR2*), a homolog of *A. thaliana SHN* regulatory genes. Additional epidermis-specific homeodomain–leucine zipper (HD-ZIP) transcription factors such as tomato *CD2*, *A. thaliana ANTHOCYANINLESS 2* (ANL2) and *HOMEODOMAIN GLABROUS 1* (*HDG1*), and maize *OUTER CELL LAYER 1* (*OCL1*) regulate cuticle synthesis as well as other aspects of epidermal cell identity (Javelle *et al.,* 2010; Nadakuduti *et al.,* 2012). It is noteworthy to mention that the activity of *HDG1* is affected by an interaction with the WW-domain protein *CURLY FLAG LEAF 1* (*CFL1*), which acts as a suppressor of cuticle development (Wu *et al.,* 2011). The investigations of most of the transcription factor genes have not certainly showed whether cutin biosynthesis is directly regulated by them, or whether the cuticle alterations at both structural and compositional levels are a secondary effect of changes in the growth and morphology of the epidermal cells. Yet, some research in *A. thaliana* and rice reported that *SHN1* binds directly to the cutin biosynthesis genes promoters (Kannangara *et al.,* 2007; Wang *et al.,* 2012). Therefore, *SHN1* can thus be regarded as direct, if not necessarily specific, regulator of cutin biosynthesis.

Most of the studies related to the cuticle phenolics compounds including studies related to their biosynthesis, transport, and incorporation have been carried out in tomato fruit. Hence, biosynthesis of phenolic compounds present in the cuticle will be focused on tomato and addressed in section 3.1. Regarding their transport from the epidermis to the cuticle, flavonoids have been shown to interact with ABC transporters and suggested that they could transport flavonoids through the plasma membrane (Yazaki, 2006). In a recent study, Also, extracellular LTPs have been postulated to transport several cuticle components such as cutin monomers or waxes through the cell wall to their final destination (Yeats and Rose, 2008) and could also be involved in flavonoid transport.

2.4 General Biological Functions

The main function ascribed to the cuticle has been related to protection against water loss, together with regulation of gas exchange (Riederer and Schreiber, 2001). However, the cuticle also plays various roles in plant development, physiology, protection against biotic and abiotic stresses and modulates many traits of economic importance in agriculture (Heredia, 2003; Solovchenko and Merzlyak, 2003; Asselbergh *et al.,* 2007; Bessire *et al.,* 2007; López-Casado *et al.,* 2007; Saladié *et al.,* 2007; Taketa *et al.,* 2008; Reina-Pinto and Yephremov, 2009; Isaacson *et al.,* 2009; Popielarska-Konieczna *et al.,* 2011; Shi *et al.,* 2013, España *et al.,* 2014a,b; Segado *et al.,* 2016; Domínguez and Heredia, 2017; Vallarino *et al.,* 2017; Lara, 2018).

2.4.1 Biophysical Properties

The cuticle has many biophysical properties that derived from its composition and structural arrangement. These properties are of high physiological relevance and are strongly interconnected (Domínguez *et al.,* 2011a). These properties allow the cuticle delay fruit deterioration during postharvest and contributes to protecting organs from mechanical failure against external biotic (e.g., fungi, herbivorous animals.) and abiotic (e.g., wind, rain) stresses as well as against internal stresses (turgor pressure) (Domínguez *et al.,* 2011b).

i. Hydric Properties. It has been a common perception to assume that thicker cuticles have lower water permeabilities. However, it has been clearly demonstrated that water permeability does not correlate with cuticle thickness (Riederer and Schreiber, 2001). Analyses of the tomato cutin deficient mutants showed that water permeability correlated with wax composition and that the cutin matrix did not play an important role (Isaacson *et al.,* 2009). The *A. thaliana* double mutant of the wax-related transcription factors, *MYB94* and *MYB96*, showed an increased water loss and decreased wax load compared to the single mutants and the control (Lee *et al.,* 2016). Moreover, recent studies in grape berries, under different deficit irrigation regimes and from different vine cluster positions, provided evidence of the importance of both quantity and quality of waxes on the berry cuticle permeability along the ripening process (Zarrouk *et al.,* 2018).

Waxes, primary their composition rather than their amount, have been shown to be the principal determinant of cuticle permeability. Indeed, a direct relationship between cuticular transpiration barrier properties and cuticular wax composition has been observed, with aliphatic constituents being the main determinant of this water barrier (Vogg *et al.,* 2004; Leide *et al.,* 2007). Overexpression of some wax related genes such as *WR1* (Wang *et al.,* 2012) and *WR2* (Zhou *et al.,* 2013) in rice, *WIN1/SHN1* in *A. thaliana*, *MYB96* in *A. thaliana* and *Camelina sativa* L. Crantz (Seo *et al.,* 2011; Lee *et al.,* 2014), *WXP1* in *Medicago truncatula* Gaertn. (Zhang *et al.,* 2005) and the overexpression of *WXP1* and *WXP2* in *A. thaliana* (Zhang *et al.,* 2007) reduced water loss and enhanced drought tolerance, mainly due to the increased amount of aliphatic wax compounds. Within cuticular waxes, the intracuticular layer has been shown to play a key role in the reduction of water movement across the cuticle, with epicuticular waxes playing a minor role (Vogg *et al.,* 2004). Exceptionally, in some species such as *Citrus aurantium* L. where intracuticular waxes are rich in triterpenoids or other alicyclic compounds, the overall transpiration resistance was low and, in these cases, both the epicuticular and intracuticular waxes contributed equally to the cuticular transpiration barrier (Jetter and Riederer, 2016).

Some specialized structures in the epidermis such as trichomes also play a pivotal role in avoiding water loss and conferring drought tolerance in major crops (Antunes *et al.,* 2012; Galmes *et al.,* 2013). However, in peach, it was observed a significant increase in fruit water loss rate after wax but not trichome removal (Fernández *et al.,* 2011). These results showed that despite the importance of trichomes in water absorption, water loss depends on waxes rather than on trichomes (Fernández *et al.,* 2011).

ii. Thermal Properties. Temperature can affect the performance of polymers by inducing structural changes within their matrixes (Nicholson, 1997). Little has been done to understand the temperature-dependent alterations in the plant cuticle structure despite its implication in many developmental and postharvest processes. Both the cuticle and cutin have a significantly higher specific heat (Casado and Heredia, 2001) than other polymers. This means that to change cuticle physical state, a significant amount of heat is needed. This thermal characteristic allows the plant to maintain its physiological temperature in case of extreme temperatures exposure. Thus, the cuticle is regarded as a thermoregulator between the plant and its surrounding atmosphere. The cuticle exhibits a glass transition within physiological temperature that can be credited to the cutin matrix (Schönherr *et al.,* 1979; Matas *et al.,* 2004). The glass transition temperature is defined as the temperature at which the flexibility and fluidity of amorphous polymers increase. In other words, it is the shift point from rigid to a viscous state. Below this temperature, the cuticle is stiffer limiting the mobility of exogenous constituents across it, while above the glass temperature, the cuticular matrix seems more viscous easing exogenous compounds mobility (Domínguez *et al.,* 2011). In tomato fruit, a glass transition at 23°C was detected in the cutin matrix throughout fruit development but disappeared during ripening (Matas *et al.,* 2004). The structural changes that occur in the cuticle above the glass transition temperature have a negative effect on the mechanical and water properties since they reduce the mechanical resistance and increase water permeability (Schönherr *et al.,* 1979).

iii. Mechanical Properties. The cuticle provides structural support to plants by fulfilling a mechanical role decreasing the impact of external and internal forces on plant organs and thus preventing mechanical damage that could cause tissue breaking (Kutschera, 1989; Savaldi-Goldstein *et al.,* 2007).

Studies investigating the mechanical properties of enzymatically-isolated cuticles of fruit have significantly increased in the last two decades, to cite few: tomato (López-Casado *et al.,* 2007; Saladié *et al.,* 2007; Isaacson *et al.,* 2009; Domínguez *et al.,* 2011; España *et al.,* 2014a,b; Domínguez *et al.,* 2015b), sweet cherry (Knoche and Peschel, 2006), apple (Grotte *et al.,* 2001; Khanal *et al.,* 2013a,b), pear (Khanal *et al.,* 2013b), orange (Singh and Reddy, 2006) and persimmon (Tsubaki *et al.,* 2013) and leaves of *A. americana*, *Clivia miniata* (Lindley) Bosse or *Hedera helix* L. among others (Wiedemann and Neinhuis, 1998; Khanal *et al.,* 2013a). The cuticle has a biphasic biomechanical behavior, viscoelastic at higher stresses and elastic at low ones (Petracek and Buckovac, 1995; Wiedemann and Neinhuis, 1998; Domínguez *et al.,* 2011b). Elastic behavior implies instant deformation and recovery after stress removal, while the viscoelastic one can be described as time-dependent and irreversible (Niklas, 1992). Thus, the threshold separating both behaviors indicates the minimum stress needed to permanently deform the cuticle. Due to the composite nature of the plant cuticle, its biomechanical behavior does not only rely on the mechanical characteristics of the individual constituents by themselves but also on the sum of them (López-Casado *et al.,* 2010).

The cutin matrix is mainly responsible for the viscoelastic behavior (Domínguez *et al.,* 2011b). It is thus characterized by its deformability and low breaking stress (López-Casado *et al.,* 2007). Only intracuticular waxes have been shown to increase stiffness and reduce deformability, while the cuticle mechanical properties do not seem to be affected by epicuticular waxes (Petracek and Bukovac, 1995; Takahashi *et al.,* 2012; Khanal *et al.,* 2013a). It is postulated that intracuticular waxes reduce the mobility of cutin polymer chains since they occupy the intermolecular spaces within the cutin matrix acting as fillers (Petracek and Bukovac, 1995). It is noteworthy to note that wax triterpenoids have been shown to act as nanofillers strengthening the cuticle of persimmon fruits (Tsubaki *et al.,* 2013). Thus, it would be interesting to know if both the aliphatic and alicyclic compounds of waxes are responsible for the strengthening role attributed to intracuticular waxes. Only a few studies in tomato and persimmon fruits and in mangrove leaves have investigated the mechanical role of cuticle polysaccharides showing that they confer stiffness and reduce deformability (Lopez-Casado *et al.,* 2007, 2010; Takahashi *et al.,* 2012; Tsubaki *et al.,* 2012). As for the phenolic compounds, their role in the cuticle mechanical properties has only been studied in tomato. Unlike polysaccharides and waxes, flavonoids and phenolic acids cannot be selectively removed without depolymerizing the cuticle (Domínguez *et al.,* 2009a), and thus direct comparison of cuticles with and without phenolics is not possible. To overcome this problem, several strategies have been employed. Since flavonoids accumulate in the cuticle only during ripening, comparison of mature green and red ripe cuticles has allowed to indirectly establish the role of flavonoids in the mechanical properties (Bargel and Neinhuis, 2004; Domínguez *et al.,* 2009a). Also, analysis of red ripe cuticles of the "*y*" mutant with altered phenolic accumulation, have also shown a reduced mechanical resistance (Adato *et al.,* 2009). However, transient silencing of *CHS* in tomato allowed to prove that flavonoids, not phenolic acids also accumulated during ripening, were the main responsible for this mechanical resistance and reduced deformability (España *et al.,* 2014b). Likewise waxes, phenolics also act as fillers and, in tomato fruit cuticle, play a more important mechanical role than waxes or polysaccharides (Domínguez *et al.,* 2009a; España *et al.,* 2014a).

The mechanical properties of the cuticles respond to changes in environmental conditions such as temperature, relative humidity and light intensity (Edelmann *et al.,* 2005; Matas *et al.,* 2005; Domínguez *et al.,* 2011). Relative humidity and temperature have been shown to have a plasticizing effect by decreasing the stiffness and the stress needed to break the cuticle while at the same time increasing its deformation (Petracek and Buckovac, 1995; Edelmann *et al.,* 2005; Matas *et al.,* 2005). This plasticizing effect is attributed to the higher mobility of the polymeric chains of the cutin matrix under these environmental conditions (Stark *et al.,* 2008).

2.4.2 Protection against Pathogens and Pests' Invasion

The plant cuticle is regarded as a barrier against plant pathogens occupying the plant surface. Fungal penetration across the cuticle can be achieved by mechanical rupture and/or enzymatic degradation. During the colonization process, some phytopathogenic fungi produce cutinases to facilitate their mobility across the cuticle (Kolattukudy *et al.,* 1995). Cutinases belong to a class of lipolytic and nonspecific esterases that hydrolyze the cutin polyester and release free cutin monomers (Longhi and Cambillau, 1999). Commonly, mutants that have strongly reduced amount of cuticle have shown increased susceptibility to infection by *Botrytis cinerea* Persoon (Isaacson *et al.,* 2009). However, in other cases the opposite behavior has been observed. The *A. thaliana lacs2*, *lcr*, *att1* and *fiddlehead* (*fdh*) cuticle mutants, characterized with a reduced amount of cuticle, have displayed enhanced resistance to *B. cinerea* (Tang *et al.,* 2007), probably due to triggering secondary mechanisms involved in the induction of plant defenses (Bessire *et al.,* 2007). It has been postulated that increased cuticular permeability correlates with this unexpected phenotype that is explained by an enhanced perception of putative elicitors resulting into antifungal molecules accumulation (Bessire *et al.,* 2007; Chassot *et al.,* 2007). Strong evidence reported by Voisin *et al.* (2009) showed that compensatory defense pathways including increased wax biosynthesis and mainly, upregulation of subsets of defense genes, are stimulated by *bdg*, *lcr* and *fdh* mutations. Additionally, resistance to *B. cinerea* in these mutants can also be explained by the fact that their biosynthetic pathway lacks the production of cutin-derived lipid-type elicitor molecules, needed for pathogen invasion (Reina-Pinto and Yephremov, 2009). It is noteworthy to mention that these mutations confer general immunity against certain pathogens and not against all of them. For example, decreased resistance to the fungus *Alternaria brassicicola* Wiltshire was seen in the *gpat4/ gpat8* double mutant (Li *et al.,* 2007). As for the *lacs2* and *att1* mutants, they exihibit increased sensibility to avirulent *Pseudomonas syringae* van Hall pathovars (Xiao *et al.,* 2004; Tang *et al.,* 2007). On the other hand, epicuticular waxes can prevent insect movement or attachment on plant surfaces (Borodich *et al.,* 2010) and their reduction has been shown to have a beneficial effect on plant resistance against insects (Duetting *et al.,* 2003).

2.4.3 Role during Organogenesis

Defining organ boundaries during organogenesis is one of the plant cuticle key roles. Many *A. thaliana* cuticle mutants *lcr*, *bdg*, *abcg11*, *abcg13* and *hothead* (*hth*), displayed leaf and/or floral fusion when the growing organs touched each other during early stages of development (Wellesen *et al.,* 2001; Kurdyukov *et al.,* 2006a,b; Bird *et al.,* 2007; Luo *et al.,* 2007; Panikashvili *et al.,* 2010, 2011; Jakobson *et al.,* 2016). It is interesting to note that this fusion between organs only occurred during a very short developmental window and was affected by environmental conditions (Weng *et al.,* 2010). The more severe cases led to abnormal growth and the mechanical stress generated during growth sometimes caused tissue tearing and formation of wounds (Wellesen *et al.,* 2001; Krolikowski *et al.,* 2003; Kurdyukov *et al.,* 2006a,b; Smirnova *et al.,* 2013). *ONION* genes in rice encoding very long chain fatty acids, have been shown to be implicated in wax biosynthesis. The *oni1*, *oni2* and *oni3* mutants exihibit organ fusion and growth reduction (Ito *et al.,* 2011; Tsuda *et al.,* 2013; Akiba *et al.,* 2014). Interestingly, a few cuticle mutants such as *A. thaliana* double mutant *gpat4*/*gpat8* and *lacs2* exihibit only a limited growth reduction without displaying organ fusions (Bessire *et al.,* 2007; Li *et al.,* 2007). The differential behavior of some cuticle mutants regarding organ fusion could be specific to the developmental stage and the studied organ. For example, the *gassho* (*gso*) *gso1*/*gso2* double mutant displayed a normal cuticle in leaves, however, in embryos and seedlings, it exhibited an increased cuticle permeability and organ fusion (Tsuwamoto *et al.,* 2008). Similarly, the maize *fused leaves 1* (*fdl1*) mutant only displayed organ fusion among the coleoptile and first two leaves (La Rocca *et al.,* 2015). Changes in cuticle composition, gene expression and/or regulation throughout development could explain why organ fusion may only occur in specific organs or at specific developmental stages, depending on the mutant analyzed.

There are genes related to cuticle synthesis that are exclusively expressed in tissues derived from the L1 meristem layer. There is a well-established relationship between cuticle deposition and epidermal cell development and differentiation (Javelle *et al.,* 2011; Ingram and Nawrath, 2017; Segado *et al.,* 2020). Thus, several genes related to epidermal identity and/or differentiation have been shown to display cuticle defects. This is the case of *CD2* in tomato, *OCL1* in maize, and the *A. thaliana MERISTEM LAYER1* (*AtML1*), members of the class IV of HD-ZIP transcription factors involved in the maintenance of the epidermal identity that also affected cuticle deposition and expression of several cuticle-related genes (Isaacson *et al.,* 2009; Nadakuduti *et al.,* 2012; Javelle *et al.,* 2010; Takada *et al.,* 2013). Additionally, other genes that participate in determining the epidermal identity like the receptor-like kinases *CRINKLY4* (*CR4*) in maize and *ARABIDOPSIS CR4* (*ACR4*) or the phytocalpain *DEFECTIVE KERNEL1* (*DEK1*) showed epidermal defects associated with organ fusion and cuticle permeability (Becraft *et al.,* 1996; Jin *et al.,* 2000; Watanabe *et al.,* 2004; San-Bento *et al.,* 2014; Galletti *et al.,* 2015). To date, the exact interaction between epidermal identity and cuticle deposition has not been fully elucidated, but it seems that an established epidermal identity is needed for cuticle deposition while at the same time an intact outer cell wall and/or cuticle is needed to maintain the epidermal cell identity throughout growth and development (Javelle *et al.,* 2010).

2.4.4 Protection against Ultraviolet Radiation

Little work on the optical properties of the cuticle has been done. It is speculated that cuticles can protect to some extent the internal plant tissues from ultraviolet B (UV-B) radiation (Pfündel *et al.,* 2006; Krauss *et al.,* 1997). The damages resulting from UV-B radiation can occur at many levels: DNA, membrane lipids, and photosynthetic machinery (Rozema *et al.,* 1997). Phenolic compounds selectively absorb UV light and their accumulation in epidermal cell vacuoles is considered the main photoprotection mechanism of plants against UV light. However, phenolics are also present in the epicuticular waxes and in the cutin matrix and therefore could play a protective role. In this sense, analysis of cuticle transmittance within the UV range of various woody species showed that the cuticle can reduce UV transmission to internal tissues (Krauss *et al.,* 1997). Additionally, plant cuticles can reflect UV light to some degree, depending on the abundance and chemical characteristics of epicuticular waxes (Mulroy, 1997; Pfündel *et al.,* 2006).

2.5 Impact of Cuticle Properties on Fruit Quality

Fruit cuticles can modify many traits of economic importance. Thus, understanding the impact of cuticle properties on fruit quality, shelf life and storage potential is important for crop improvement. While the importance of cell walls is widely recognized and has been the center of attention in studies on postharvest biology and technology of fruit produce, the role of the cuticle has received much less attention.

The cuticle may influence fruits marketability by modulating their visual appearance extremely important in the consumer purchase decision. For example, the pinkish color of tomato fruits is caused by the so called "*y*" mutation in the transcription factor *MYB12* that regulates the biosynthesis of phenolics rendering a colorless cuticle when fruits are ripe (Adato *et al.,* 2009; Ballester *et al.,* 2010). Pink tomato fruits are highly appreciated in Asian countries where it is associated with high fruit quality. Alterations in the cuticle can also affect fruit brightness, from epicuticular wax deposition and composition, to epidermal cell shape or amount of cuticle (Petit *et al.,* 2014; Lashbrooke *et al.,* 2015a; Petit *et al.,* 2016). An example of glossy mutants is the "*pe*" mutant characterized by reduced cutin accumulation, altered wax deposition and epidermal cell identity (Nadakuduti *et al.,* 2012). Besides fruit appearance, the cuticle plays a role in several important traits during postharvest: water loss, physiological alterations, fruit firmness and susceptibility to infections (Lara *et al.,* 2014, 2018). Postharvest encompasses the delivery of a crop from the place of harvest to that of consumption with minimum losses and maximum efficiency (Spurgeon, 1976; Domínguez and Heredia, 2017). It includes operations at harvest, transport, cleaning, sorting, handling, storage, packing, etc. (Toivonen *et al.,* 2014). Fresh markets are no longer restricted to local markets. They opened to international competition too. This implies the necessity for prolonged shelf life while maintaining peak quality (Toivonen, 2007). Harvested fruit species are metabolically active and their cuticles are subject to compositional and structural changes and can deteriorate them. However, deterioration depends on the product itself and the conditions of storage in terms of temperature, relative humidity and atmosphere.

Fruit water loss through cuticular transpiration during postharvest is a major problem that has been studied in many fruit species including tomato (Vogg *et al.,* 2004; Leide *et al.,* 2007), pepper (Parsons *et al.,* 2012, 2013; Popovsky-Sarid *et al.,* 2017), peach (Gilbert *et al.,* 2010) and litchi (Riederer *et al.,* 2015). The fruit visual aspect and its shelf life will be significantly altered by water loss (Fig. G.I-5D-E) that can be correlated also with higher sensibility to pathogens (Fig. G.I-5F) if the integrity of the cuticle is affected (Isaacson *et al.,* 2009; Shi *et al.,* 2013). Phenotypic screening of cuticle traits associated with postharvest water loss in a pepper mapping population derived from the cross between *Capsicum annuum* L. and *Capsicum chinense* Jacquin, showed a negative correlation between the amount of triterpenoids and water loss (Parsons *et al.,* 2012). Several ripening-delayed tomato mutations, *non-ripening* (*nor*), *ripening inhibitor* (*rin*) and the alcobaça allele of *nor* (*alc*) display cuticle modifications from early stages of fruit development until ripening (Kosma *et al.,* 2010). One of them, *nor*, is employed to prolong tomato shelf-life. The *delayed fruit deterioration* (*dfd*) tomato mutant, an allelic variant of the *NOR* gene (Rose *et al.,* 2012) exihibit a decrease in fruit transpirational water loss, a significant increase in cellular turgor, delays in cell wall degradation and color changes (Saladié *et al.,* 2007). It is noteworthy to mention that NOR transcription factor plays a key role in the regulation of the fruit ripening process (Kosma *et al.,* 2010), highlighting the tight interaction that exists between fruit ripening, cuticle alterations, and postharvest shelf life.

Susceptibility to infections during postharvest and storage period is a major challenge. The cuticle plays a central role in controlling postharvest rotting, which causes important fruit losses after harvest. Postharvest fungal pathogens are generally quiescent before ripening and become necrotrophic during storage after harvest. Opportunistic invasive fungi germinate and enter the fruit through existing cuticle breaks, namely pores, cracks or wounds. Although many common postharvest pathogens are unable to directly penetrate the host cuticle and require surface injuries or natural openings (Coates and Johnson, 1997), fruits become progressively more susceptible to wounds and mechanical damages in general after long-term storage, thus opening a route for the development of infection. Relationships between infection susceptibility and cuticle thickness and structure have been suggested for apple fruit (Konarska, 2012). Lower incidence of rots and longer postharvest life in cranberries has also been attributed to a thicker cuticle and wax accumulation at the calyx end, which would retard the entry of microorganisms into the fruit during wet harvest (Ozgen *et al.,* 2002). In addition to cuticle thickness, the structure and composition of cutin, rather than those of waxes, have been proposed as good indicators of susceptibility to infections in tomato (Isaacson *et al.,* 2009; Shi *et al.,* 2013), peach (Bostock *et al.,* 1999) and grapes (Comménil *et al.,* 1997; Mlikota Gabler *et al.,* 2005). Decreased contents of the C_{16} cutin monomers and also of phenolic compounds including *p*-hydroxybenzoic and *p*-coumaric acids at early maturity phases, correlated with high sensibility to fungal postharvest infection in tomato (Shi et al., 2013).

Additionally, a reduced susceptibility to *B. cinerea* and fruit deterioration were observed in purple tomatoes, suggesting a link between an increase antioxidant capacity due to anthocyanin accumulation and extended shelf-life (Zhang *et al.,* 2013).

The collenchyma cell layers, the epidermis, and the cuticle constitute the fruit skin. Cracks can appear on this outer surface when the degree of stress applied surpass its mechanical strength. Depending on their extent, cracks can favor oxidative processes and thus tissue browning, accelerate water loss rates and create an entry for pathogens and for the uptake of minerals. Skin cracking may cause severe losses in species like tomato (Fig. G.I-5H), pepper (Fig. G.I-5I), and sweet cherry (Fig. G.I-5J). Wound healing of the cracks in fruit skin is likely associated with the origin of the formation of suberin that gives the russet appearance to tomato (Fig. G.I-5G), apple (Fig.

G.I-5K), and pear (Fig. G.I-5L) and defines the netting pattern of the fruit in melon (Fig. G.I-5M).

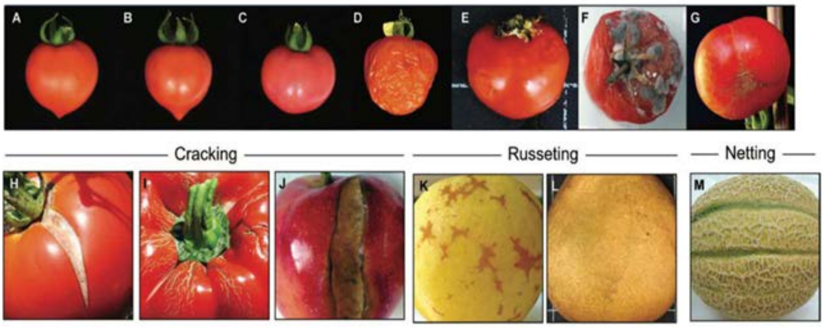


Fig. G.I-5: Cuticle-associated traits in fleshy fruits. (A–G) Cuticle alterations affecting fruit appearance in tomato (*S. lycopersicum)* EMS mutants. (A) Wild type, (B) brightness, (C) color, (D) water loss, (E) mutant fruit with extended shelf life after one year of storage at 6°C, (F) decay due to fungal pathogens, (G) russeting. (H–J) Fruit cracking in (H) tomato (*S. lycopersicum*), (I) pepper (*Capsicum annuum*) and (J) sweet cherry (*Prunus avium*). (K–L) russeting on (K) apple (*Malus x domestica*) and (L) pear (*Pyrus communis*). (M) netting on melon (*Cucumis melo*). Taken from Petit *et al.* (2017), Journal of Experimental Botany Copyright ©.

Although postharvest fruit softening is mainly affected by cell wall modifications, structural support to fruit, especially to those lacking hard internal tissues is also provided by the cuticle. Therefore, cuticle architecture and composition may participate in determining fruit firmness through its important role in controlling the skin mechanical properties (Bargel and Neinhuis, 2005; Costa, 2016). Additionally, it has been confirmed that these mechanical properties are altered by relative humidity and temperature (Edelmann *et al.,* 2005; Matas *et al.,* 2005). Thus, storage conditions will have an impact on postharvest firmness changes.

3. THE TOMATO FRUIT CUTICLE: CHARACTERISTICS AND IMPORTANCE OF FLAVONOIDS

Tomato is an excellent experimental model for the biology of fruit cuticle. The tomato fruit cuticle is particularly substantial, enabling detailed morphological analysis by light microscopy (Buda *et al.,* 2009) as well as easy isolation of a completely continuous, intact and astomatous membrane facilitating its analysis. A remarkable degree of variability in tomato fruit cuticle chemical composition, amount, thickness, biophysical properties and more, has been uncovered through the exploration of different varieties, cultivars, related wild species, naturally occurring mutants, as well as segregant and mutagenized populations (Vogg *et al.,* 2004; Saladié *et al.,* 2007; Lopez-Casado *et al.,* 2007; Domínguez *et al.,* 2009a; Kosma *et al.,* 2010; Yeats *et al.,* 2012a; Petit *et al.,* 2014; Fernández-Moreno *et al.,* 2017). Therefore, this diversity shows that the cuticle of the tomato fruit can undergo improvement of many important agronomic attributes (Petit *et al.,* 2017).

3.1 Composition and Structure

The principal cutin monomers in the cuticles of the tomato fruit are 16-hydroxy- hexadecanoic acid, 10,16-dihydroxyhexadecanoic acid, and 18-hydroxyoctadecanoic acid (Heredia, 2003). Wax composition is dominated by very long chain alkanes (predominantly n-hentriacontane, $C_{31}H_{64}$), fatty acids (C_{16}, C_{18}, and C_{24}), C_{32} n-aldehyde, and the triterpenoids (α-amyrin, β-amyrin and δ-amyrin) (Heredia, 2003; Vogg *et al.,* 2004; Leide *et al.,* 2007; Petit *et al.,* 2014). Amyrins are exclusively present in the intracuticular wax fraction (Vogg *et al.,* 2004). The phenolic fraction of tomato fruit cuticle undergoes changes during development and ripening (Domínguez *et al.,* 2008; España *et al.,* 2014a,b). It is mainly composed of hydroxycinnamic acid derivatives such as *p*-coumaric and *p*-hydroxybenzoic acids during fruit growth phase, whereas the flavonoid naringenin chalcone becomes the main constituent at the ripe stage (Hunt and Baker, 1980; Baker *et al.,* 1982; Luque *et al.,* 1995; España *et al.,* 2014a).

The cuticle composition and structure of tomato fruit varies among species and cultivars within the same species. Only a very limited number of tomato cultivars have been studied, despite the differences that have been observed in cuticle related traits including thickness, amount of cuticle and more (Bauer *et al.,* 2004b; Domínguez *et al.,* 2008; España *et al.,* 2014b). Yeats *et al.* (2012a) explored the variability among the cultivated tomato and some related wild species and observed several differences among species (Fig. G.I-6). The tomato fruit cuticle deposition is not always limited to the outer periclinal epidermal wall, but it can also be present in the anticlinal and internal periclinal walls (Domínguez and Heredia, 2017). Differences in the degree of cutinization of the anticlinal walls were observed among different tomato species with *S. lycopersicum* and *S. pimpinellifolium* displaying cuticle deposition around all epidermal walls (Yeats *et al.,* 2012a) (Fig. G.I-6). Also, significant differences in cuticle thickness were observed, being *S. pennellii* and *S. habrochaites* the thinnest (Fig. G.I-6h). Another interesting studied feature was the epidermal cell shape. Species such as, *S. lycopersicum*, *S. pimpinellifolium* and *S. cheesmaniae* (Fig. G.I-6a–c) showed a flat outer periclinal epidermal wall, whereas in the remaining studied species the surface was more or less undulated. Although there were significant differences in the amount of cuticle among the species, the main cutin components were the same. It is noteworthy to mention that *S. lycopersicum* and its closest relative *S. pimpinellifolium* showed similar cutin monomer composition. Differences in wax coverage were also observed, with *S. lycopersicum* displaying the lowest (Yeats *et al.,* 2012a). This latter work, however, is only a preliminary analysis based only in one genotype per species. Therefore, possible variation within species and cultivars of domesticated tomato needs to be addressed.

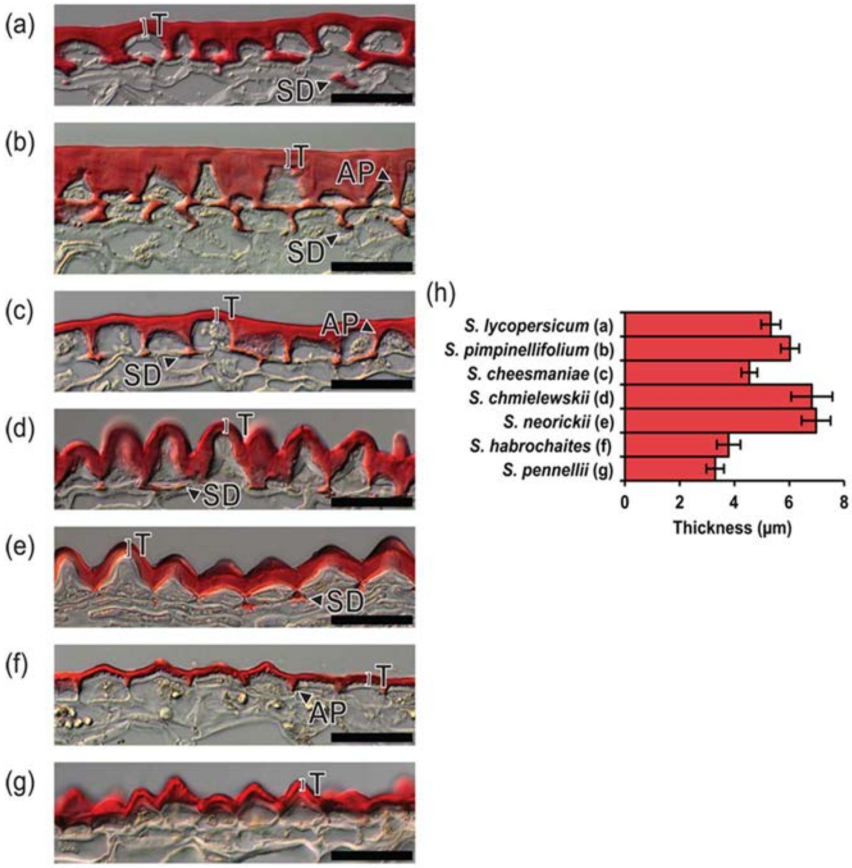


Fig. G.I-6: Two-dimensional cuticle morphology. (a–g) L Cross-sections stained with Oil Red O of fruit epidermis from different tomato species. (h) Cuticle thickness (mean ± SE) measured in the region labeled 'T' in panels a–g. T, thickness; SD, Subepidermal cuticular deposit; AP, Anticlinal peg. Taken from Yeats *et al.* (2012a), The Plant Journal Copyright ©.

3.1.1 Tomato Cuticle Changes during Development

Understanding the interaction between the deposited cuticle and the cell wall, and how each one changes during development is important to determine the stages in which significant cuticular changes happen as well as the timeframes in which genes related to cuticle synthesis would be especially active. Furthermore, it would be useful to determine the physiological stages of development on which changes in environmental factors such as light, relative humidity and temperature could affect the cuticle. Additionally, it has been speculated that changes in fruit cuticle composition and structure during development and postharvest affect shelf life and storability and could cause changes in traits such as fruit resistance to desiccation, cracking and microbial infection (Kosma *et al.,* 2010; Lara *et al.,* 2018, 2019).

Contrary to cuticle deposition in sweet cherry, red apple and some berry fruits (Peschel *et al.,* 2007; Khanal *et al.,* 2011; Dong *et al.,* 2012), two models of cuticle deposition have been identified in the tomato fruit. One observed in cherry tomatoes and characterized by fast accumulation of cuticle material at early developmental phases followed by a steady state where the rate of cuticle deposition is maintained as the fruit continues growing (Domínguez *et al.,* 2008; Segado *et al.,* 2016). In contrast, in big-sized and medium tomatoes, the cuticle amount augments continuously during fruit development and the ripening period (España *et al.,* 2014a).

From anthesis to 10-15 days post anthesis, depending on the species, the cuticle appeared as a very thin and mainly elastic procuticle layer (España *et al.,* 2014a; Segado *et al.,* 2016, 2020). This initial growth period was characterized by cell division and the cuticle represented only a minor portion of the outer epidermal cell wall (Segado *et al.,* 2016, 2020). Later on, a massive cuticle deposition was observed coinciding with the transition to the cell expansion period of growth. During this period the cuticle displayed a more viscoelastic and easily deformable mechanical behavior and the amount of cutinized cell wall increased considerably (España *et al.,* 2014a; Segado *et al.,* 2016). During ripening, significant changes were detected in the cuticle with a decrease of polysaccharides, which could be associated to cell wall degradation as the fruit ripened, and a significant increase in cuticle phenolics (Domínguez *et al.,* 2008; España *et al.,* 2014a). Although *p*-hydroxybenzoic and *p*-coumaric acids were detected in the cuticle from the earliest stages of development, its amount increased during ripening and was accompanied by the incorporation of the flavonoid naringenin chalcone (Lopez-Casado *et al.,* 2007; España *et al.,* 2014a). This phenolic incorporation marked the transition from a mechanically deformable cuticle to a more rigid and less deformable one. Additionally, changes in cutin structure, a more compact matrix with increased free carboxylic functional groups, were observed after phenolic incorporation during ripening (Segado *et al.,* 2016, 2020). Moreover, analyses of the cuticle chemical composition of three tomato fruit ripening mutants *alc*, *rin*, and *nor* showed compositional changes during ripening (Kosma *et al.,* 2010). Once the tomato fruit is detached from the vine, its cuticle is expected to keep developing and growing. For example, in ‘Ailsa Craig’ tomato cultivar, the total wax amount increases significantly during a 9 days period of postharvest (Saladié *et al.,* 2007). Unfortunately, to date, no studies on structural and compositional changes during postharvest of tomato cultivars have been conducted. However, it has been reported that some alterations in the cuticle composition or amounts occur during cold storage for some fruits such as apple (Dong *et al.,* 2012; Li *et al.,* 2019), sweet cherry (Belge *et al.,* 2014b), orange (Ding *et al.,* 2018), and peach (Belge *et al.,* 2019).

3.1.2 Naringenin Chalcone: Major Phenolic Component

As it has been mentioned earlier, tomato fruit cuticle accumulates the orange-yellow flavonoid naringenin chalcone during ripening, which contributes to the red color of ripe tomato fruits (Adato *et al.,* 2009; España *et al.,* 2014a). Flavonoids represent a large family of low molecular weight secondary metabolites that are widespread throughout the plant kingdom. They are implicated in a wide range of central functions in the biology of plants. Flavonoids provide pigmentations for seeds, fruits and flowers to attract pollinators, protect against UV radiation and phytopathogens. They also serve as signaling molecules in many processes: plant growth and development, plant fertility and pollen-tube germination (Dooner *et al.,* 1991; Koes *et al.,* 1994; Dixon and Paiva, 1995; Mol *et al.,* 1998; Winkel-Shirley, 2001; Peer and Murphy, 2007; Buer and Djordjevic, 2009).

The term flavonoid, from the Latin word ‘flavus’ meaning yellow, is normally used to describe a wide collection (over 10.000 compounds) of secondary metabolites that include a C_6-C_3-C_6 carbon framework called the flavan-nucleus (Mouradov and Spangenberg, 2014). It consists of two aromatic rings with 6 carbon atoms (ring A and B) interconnected by a hetero cycle of 3 carbon atoms (ring C) (Fig. G.I-7) (Marais *et al.,* 2006).

They can be divided into different structural classes such as chalcones, flavones, flavanones, flavanols, flavonols, isoflavones and anthocyanidins, based on the variation in the type of heterocycle involved. Within each group, there are individual variations emerging from variations in number and arrangement of the hydroxyl groups and their extent of alkylation and/or glycosylation. Enzymes like glycosyl transferases, methyl transferases and acyl transferases are mainly responsible of these structural modifications and thus of this huge diversity in the flavonoid family.

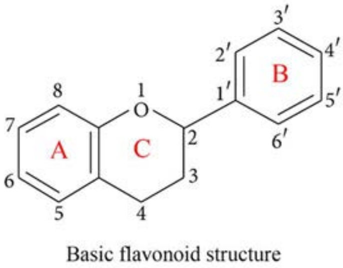


Fig. G.I-7: The flavan-nucleus, basic chemical structure of flavonoids. The different rings of basic structure are identified with red letters.

Flavonoids are synthesized via the phenylpropanoid pathway (Fig. G.I-8) that starts with the deamination of phenylalanine by the enzyme L-phenylalanine ammonia-lyase (PAL) to generate the *ρ*-coumaroyl-CoA. Together with the 3-malonyl-CoA originated from acetyl-CoA, they are precursors of flavonoids synthesis (Forkmann and Heller, 1999). The first enzymatic step of the flavonoid pathway is catalyzed by CHS, an enzyme that catalyzes the sequential decarboxylative addition of three acetate units to one molecule of *ρ*-coumaroyl-CoA followed by cyclization and resulting in the formation of the yellow colored naringenin chalcone. Subsequently, naringenin chalcone is converted into naringenin, a colorless isomer, by CHI (Muir *et al.,* 2001). Studies of different plants including *A. thaliana*, rose, tomato, maize and grape have concluded that many genes in the phenylpropanoid, flavonoid, and anthocyanin biosynthetic pathways can be regulated by the R2R3-MYB-type transcription factors (Stracke *et al.,* 2007; Adato *et al.,* 2009; Czemmel *et al.,* 2009; Ballester *et al.,* 2010; Lin-Wang

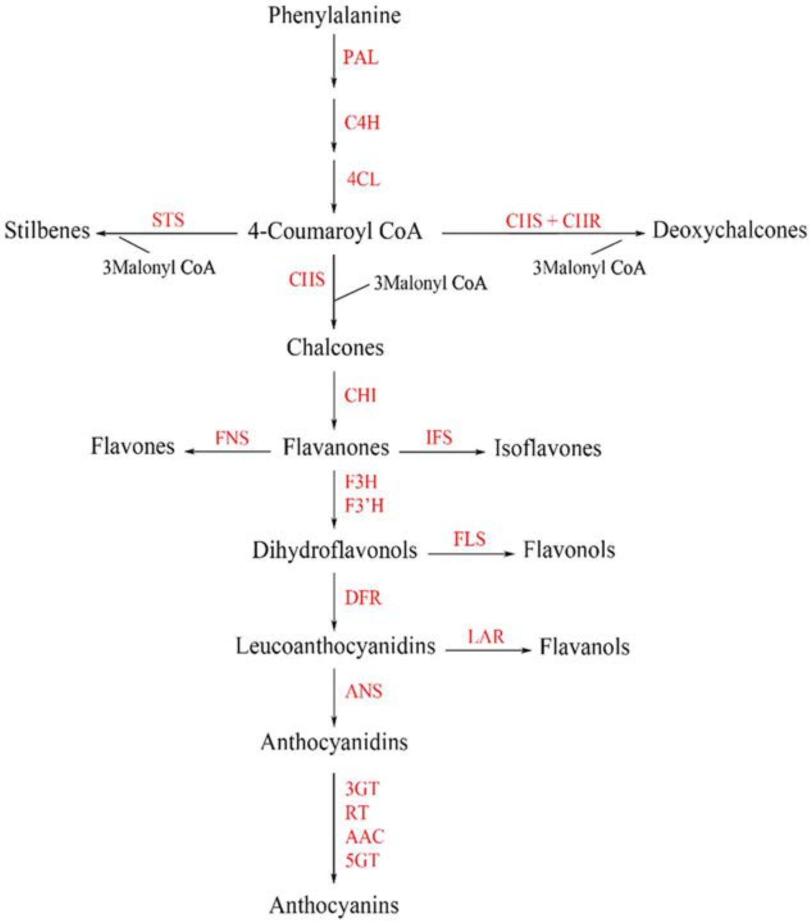


Fig. G.I-8: The biosynthetic pathways of flavonoids. The simplified scheme comprises the anthocyanidin branch and other flavonoid end products (flavonols, flavones and stilbenes). The enzymes catalyzing each step are indicated in red. PAL phenylalanine ammonia lyase; C4H cinnamate 4 hydroxylase; 4CL 4 coumarate: coenzyme A ligase; STS stilbene synthase; CHS chalcone synthase; CHI chalcone isomerase, IFS isoflavone synthase; FNS flavone synthase; F3H flavanone 3 hydroxylase; FLS flavonol synthase; DFR dihydroflavonol reductase; LAR leucoanthocyanidin reductase; ANS anthocyanidin synthase; 3GT flavonoid 3-O-glucosyltransferase; RT flavonoid 3-O-glucosyde rhamnosyl transferase; AAC anthocyanin acyl transferase; 5GT flavonoid 5 glucosyltransferase. Adapted from Domínguez *et al.* (2015), Nova Publishers Copyright ©.

et al., 2010; Hichri *et al.,* 2011; Du *et al.,* 2012; Nakatsuka *et al.,* 2012). These transcription factors are characterized by the presence of the SG7 motif (Stracke *et al.,* 2007; Czemmel *et al.,* 2009), which is located downstream of the R2R3 DNA-binding domain. MYB12 is the most broadly investigated regulator in the SG7-motif group. In *A. thaliana,* the activation of the flavonol glycoside biosynthesis is not only achieved by *MYB12* but also by two paralogs, *MYB111* and *MYB11*. These three MYB transcription factors perform additively and exhibit differential spatial activity (Stracke *et al.,* 2007, 2010). They regulate flavonol moiety production. However, in *A. thaliana* the characterization of *myb11 myb12 myb111* triple mutant, indicated that their function involves also regulating flavonol glycosylation (Stracke *et al.,* 2007, 2010). In tomato fruit, increases in caffeoylquinic acid and flavonol glycosides contents after the overexpression of *AtMYB12* were observed (Luo *et al.,* 2008; Zhang *et al.,* 2015). Moreover, overexpression of *AtMYB11* in tomato fruit resulted in a specific increase in caffeoylquinic acid content (Li *et al.,* 2015). The ortholog of *AtMYB12* in tomato is *SlMYB12* (Adato *et al.,* 2009; Ballester *et al.,* 2010) and the tomato *AtMYB111* homolog, *SlMYB111*, may have different role in tomato fruit (Adato *et al.,* 2009). Thus, *SlMYB12* appears to be the single *MYB* type regulator of flavonoid biosynthesis in tomato, in contrast to the coordinated action of *AtMYB11*, *AtMYB12*, and *AtMYB111* in *A. thaliana*.

Over 98% of the flavonoids synthetized in the tomato fruit are accumulated in the peel (Meléndez-Martínez *et al.,* 2010). Although they are synthesized throughout fruit development (Slimestad and Verheul, 2009), they are only transported and incorporated into the cuticle during ripening (España *et al.,* 2014a). During ripening, the transient gene silencing of *CHS* showed that naringenin chalcone accumulated in the cuticle came from *de novo* synthesis and did not remobilize from the vacuole-stored flavonoids synthesized at early developmental stages (España *et al.,* 2014b). Among the various flavonoids identified in the skin of ripe tomato, only naringenin chalcone is transported and incorporated to the outer cuticle matrix (Hunt and Baker, 1980; Laguna *et al.,* 1999; Slimestad and Verheul, 2009). As it has been mentioned before, pink tomatoes are well known for lacking naringenin chalcone in their cuticles (Adato *et al.,* 2009; Ballester *et al.,* 2010; España *et al.,* 2014b). This flavonoid has been located mainly within the cutin matrix where they form clusters, but it is also present, although partially, in the epicuticular wax fraction (Hunt and Baker, 1980; Laguna *et al.,* 1999; Domínguez *et al.,* 2009b; Domínguez *et al.,* 2015b). Given the importance of some of the cuticle properties modulated by flavonoids, it would be of interest to understand their interaction with the rest of cuticle components as well as to identify regulatory genes specifically involved in flavonoid synthesis and transport to the cuticle during ripening.

3.1.3 Impact of Cuticle Flavonoids in Complex Fruit Quality Traits

Many studies have demonstrated the contribution of cuticle flavonoids in several important agronomic traits that are of great interest for researchers and breeders since they have big impact on the consumer purchase decision.

i. Cracking. It is a disorder that occurs when fruit internal pressure exceeds the stress that the peel can support (Ohta *et al.,* 1997). Fruit cracking has a genetic but also an environmental component (Peet, 1992). Tomato fruits are susceptible to fruit cracking during growth and also during ripening, especially cherry tomatoes. During ripening, as the fruit softens, the internal pressure previously supported by the pericarp tissue is directly transmitted to the peel which will crack if it is not able to withstand it (Domínguez *et al.,* 2012). Cuticle cracking can be displayed in many types including concentric, radial, large, deep and in circles around the stalk or radiating from the stalk. Cracks can be oriented in all directions on the sides and the bottom of the fruit. At the economic scale cuticle cracking in tomatoes is considered as one of the physical disorders that cause significant agronomic and economic losses in tomato production for both farmers and retailers (Peet, 1992; Aloni *et al.,* 1998). Cuticle flavonoids play an important role in preventing cuticle cracking. Its accumulation is responsible for an increase in mechanical resistance of cuticles (Domínguez *et al.,* 2009a; España *et al.,* 2014a). Tomatoes carrying the "*y*" mutation phenotype, characterized by pink-colored fruit due to the colorless cuticle, have been reported to have mechanically weaker cuticles and fruits more prone to cracking (Avdeyev, 1981; Adato *et al.,* 2009). Indeed, *CHS* silencing during ripening caused an increase in tomato fruit cracking (España, 2012).

ii. Color. Together with carotenoids, flavonoids are implicated in the determination of the final color of ripe tomato fruits. The final color of the tomato fruit is the combination of lycopene and beta-carotene accumulated in the flesh plus naringenin chalcone in the cuticle. Commercial purple tomatoes have been obtained after the introgression from wild species, such as *S. chilense*, of genes related to anthocyanin accumulation in the fruit, a characteristic absent in the cultivated tomato (Bovy *et al.,* 2002; Jones *et al.,* 2003; Gonzali *et al.,* 2009). Purple tomatoes generated after overexpression of transcription factors *ROSEA* and *DELILA* (Butelli *et al.*, 2008) accumulated naringenin chalcone in the cuticle of ripe fruits similarly to normal red tomatoes (España *et al*., 2014b).

Fruit cracking and color are considered important external fruit quality traits. Together with the nutritional value of the tomato fruit, they constitute potential targets for tomato breeding programs and have big impact on the consumer purchase decision. They are complex traits and therefore, their genetic improvement and control are considered as a real challenge for tomato breeders. As described before, cuticle components were reported to be implicated directly and/or indirectly in the latter traits. However, little is known about the genetic control of cuticle flavonoids in particular, as well as the composition of the fruit cuticle in general. Research that provides additional understanding of the regulation and biosynthesis of cuticle components including flavonoids will allow improvement of many cuticle-related traits such as resistance to cracking, appearance, and texture in tomato fruits. Identifying and mapping QTLs of genes involved in the synthesis and/or the genetic control of cuticle composition and color will provide tools to breed cultivars resistant to cracking, with high nutritional value and with different colors. The challenge of identifying QTLs for cuticle traits will not only help in combining many involved genetic regions in one tomato cultivar but also in elucidating the role of gene interactions in cuticle traits.

OBJECTIVES

OBJECTIVES

Cuticle associated traits affects attributes with strong impact on tomato fruit quality such as color, cracking and shelf life. Thus, these traits can be targeted for crop improvement in breeding programs. However, little is known about the range and the genetic bases of naturally occurring variation for cuticle composition and structure in tomato. The general objective of this work is to dissect the genetic architecture underlying these variations and to exploit available natural genetic diversity in wild species to transfer the beneficial alleles affecting cuticle-associated traits to commercial varieties. To reach our main goal, four specific objectives are set:

1- Localize genomic regions underlying traits involved in cuticle composition and color through the conduction of a QTL analysis using Recombinant Inbred Line and Introgression Line mapping populations derived from a *Solanum lycopersicum* x *Solanum pimpinellifolium* cross.

2- Validate the identified QTLs through a phenotypic characterization of the Introgression Line population and a new set of subILs with shortened introgressions.

3- Identify candidate genes within the QTL genome regions using literature and bioinformatic information. And then, validate the hypothesized functional relationship between the candidate genes and the QTLs by means of gene-expression level measurements.

4- Create new introgression lines by joining more than one QTL regions involved in the percentage of cuticle phenolics to study the combined effects (epistatic or additive) of the pyramided QTLs.

GENERAL METHODOLOGY

1. EXPERIMENTAL DESIGN

1.1 Plant material

1.1.1 *Solanum lycopersicum* 'Moneymaker'

The recurrent *S. lycopersicum* parent selected for this study is the indeterminate growing, fresh market tomato cultivar 'Moneymaker' (MM) which produces medium-sized, round and red fruits. MM has been subject to extensive studies in fruit development, ripening, physiology, phytopathology that have provided a pool of accessible genetic resources such as EMS mutant collections and TILLING databases (e.g., http://www-urgv.versailles.inra.fr/tilling/tomato.htm) and collections of nearly isogenic lines containing disease-resistance genes (Laterrot, 1996), among other plant genetic resources.

1.1.2 *Solanum pimpinellifolium* 'TO-937'

The donor parent selected for this study is the wild species *S. pimpinellifolium accession* 'TO-937' (hereafter referred to as TO-937). This is a self-compatible accession obtained after at least four selfings of the accession PE-2, which was originally collected in Lambayeque, Perú (Cuartero *et al.,* 1984), and is maintained in the tomato germplasm bank of the Estación Experimental "La Mayora''. *S. pimpinellifolium* has been intensively used in breeding to detect QTLs involved in fruit quality traits (Tanksley and Nelson, 1996; Doganlar *et al.,* 2002a; Kinkade and Foolad, 2013), abiotic stress tolerance (Monforte *et al.,* 1996) and pest resistance (Salinas *et al.,* 2013). TO-937, In contrast to other *S. pimpinellifolium*, has interesting features and characteristics related to important agronomic traits. Among these traits, the resistance to *Bemisia tabacci* Gennadius, essential vector for the transmission to *tomato yellow leaf curl virus* (*TYLCV*) that causes huge economic losses in tomato production (Rodríguez-López *et al.,* 2011), as well as the resistance to the two-spotted spider mite *Tetranychus urticae* (Fernández-Muñoz *et al.,* 2000). The latter resistance is associated to acylsucrose secretion produced by type IV glandular trichomes which are present on leaves and stems, a trait controlled by a major locus modulated by minor genes (Fernández-Muñoz *et al.,* 2003; Alba *et al.,* 2009). Additional traits contributing to organoleptic and nutritional quality of the cultivated tomato fruit were also found in TO-937 including high contents of ascorbic acid (Lima-Silva *et al.,* 2012), sugar, organic acids and carotenoids (Capel *et al.,* 2011) and aroma volatile compounds (Rambla *et al.,* 2014). Therefore, TO-937 is a potential source of genetic resources used to introduce traits such as disease resistance and improved soluble solids into cultivated tomato.

1.1.3 Mapping populations

Two existing mapping populations, an F_8 population of 169 RILs derived from an interspecific cross between MM and TO-937 (Alba *et al.,* 2009) and a library of 52 introgression SP lines derived from the backcross of TO-937 into MM background (Barrantes *et al.,* 2014) were employed. The SP population have been genotyped using Solcap markers (Barrantes *et al.,* 2014). These mapping populations have been used in studies to map the *uniform ripening* (*u*) mutation (Powell *et al.,* 2012) and to detect QTLs associated with fruit weight, vitamin C, carotenoids and other antioxidant compounds, minerals, volatile aromas, and various secondary metabolites (Capel *et al.,* 2015; Barrantes *et al.,* 2016; Capel *et al.,* 2017a; Rambla *et al.,* 2017).

1.2 Greenhouse installations and growing conditions

Plant experiments were conducted at the *Estación Experimental* "*La Mayora*", belonging to the *Instituto de Hortofruticultura Subtropical y Mediterránea* (IHSM UMA-CSIC) in Algarrobo-Costa, Málaga, in the southeast of Spain.

1.2.1 Seed extraction

Seeds from all harvested ripe fruits were extracted following the fermentation method. Tomato seeds and juice of each line were extracted and kept in a marked plastic glass. After mold formation on the surface, seeds were rinsed and cleaned. Seeds were air-dried for a few days on filter paper, then packed in paper envelopes and labeled conveniently.

1.2.2 Plant growing conditions

After a pre-treatment for 30 min with 50% (v/v) bleach solution and 3-4 rinses in distilled water, tomato seeds were placed in Petri dishes on water-soaked filter paper and incubated at 25ºC in the dark. Once germinated, seedlings were transferred to plug trays of 60 mL-cells containing 85% coconut fiber substrate and 25% plant-nutrient loaded zeolite. Plantlets were grown in a polyethylene glasshouse and transplanted into soil in a multi-tunnel plastic greenhouse at the four true-leaf growth stage. Plants were watered at intervals of 3-4 days, fertilized with standard commercial nutrients through irrigation, supported by strings and pruned to a single stem. Flowers were vibrated at least twice a week to ensure fruit set. Standard phytosanitary treatments against whiteflies, aphids, spider mites, tomato pinworm and powdery mildew were applied to seedlings and plants on a weekly basis.

2. GENOTYPIC CHARACTERIZATION

2.1 DNA extraction

Genomic DNA was extracted from young leaves of each of SP_(QTL+QTL)F_1, SP_(QTL+QTL)F_2, nSPF_1 and nSPF_2 populations, following the CTAB-based extraction method developed by (Doyle and Doyle, 1987) with some modifications. For each tested genotype, 50-mg samples of young leaf tissues were collected, placed into a 1.5-mL microcentrifuge tubes and then quickly frozen in liquid nitrogen. After freezing, sample tissues were manually ground with a plastic pestle and 400 µL CTAB buffer [20 mM EDTA; 0.1 M Tris-HCl pH 8.0; 1.4 M NaCl; 2% CTAB; plus 0.4% β-mercaptoethanol; prepared before use] was added to each tube. Thereafter, samples were incubated in a 60°C bath for one hour with occasional mixing by inversion and then 400 µL of chloroform-isoamyl/alcohol (24:1) were added to the tubes and gently mixed for 1 minute. Samples were vortexed briefly and then centrifuged for 5 minutes at 14,000 rpm in a microcentrifuge. The supernatant of each sample was transferred to a new 1.5-mL microcentrifuge tube. 300 µL of ice-cold isopropanol were added to each tube, and the tubes were inverted 5 times to precipitate the nucleic acid. Samples were centrifuged at 14,000 rpm for 10 minutes in a microcentrifuge, and their supernatants were discarded. Each tube pellet was air-dried for 2 hours and then re-suspended in 100 µL of nuclease free water. The quality and the quantity of the extracted DNA of each sample were examined using NanoDrop ND-1000 spectrophotometer following the manufacturer's instructions (Nano-Drop Technologies, Wilmington, DE). After quantification, the final DNA concentration was adjusted to 50 ng/µL.

2.2 Marker Assisted Selection

To identify double homozygous introgressions in each of the SP_(QTL+QTL)F_2 and nSPF_2, 100 and 300 plants were genotyped respectively, using SNP markers from the 8K SNP SolCap Illumina Infinium tomato array (Sim *et al.,* 2012). The SNP SolCap markers used were the closest located to the QTL peaks. The primers for amplification of the SNP marker containing sequences used to differentiate between TO-937 and MM alleles for this present study were designed from the SNPs flanking sequences (obtained from the SGN database https://www.solgenomics.net/search/markers), using Primer3 web v.4.0. (https://bioinfo.ut.ee/primer3-0.4.0/) (Rozen and Skaletsky, 1999). Checking of that selected primers were specific for unique sequence regions in tomato genome was done by using In-silico PCR tool at Solgenomics webpage. SNP genotyping and precise allelic discrimination of all amplified products for all individuals of the tested populations were carried out by high-resolution melting (HRM) method. HRM consists of detecting the melting temperature (Tm) of PCR fragments amplified by each pair of primers. SNP genotyping was completed with Bio-Rad Precision Melt Analysis software v1.2. Individuals from each cross carrying more than one QTL at HC were selected. DNA amplification for HRM assays was conducted on a CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). 5 µL of Ssofast™ EvaGreen® Supermix (2x reaction buffer with dNTPs, Sso7d-fusion polymerase, $MgCl_2$, EvaGreen dye, and stabilizers; catalog number: 1725201) were mixed with 2 µL of genomic DNA (50 ng/µL), 0.3 µL of a10 µM concentration of each primer and completed with 2.4 µL of DNase-free water according to the manufacturer's protocol. The HRM PCRs were performed in Multiplate™ PCR 96-well plate, catalog number: MLL9601, provided by Bio-Rad. The PCR reaction procedure and melting analysis were performed as follows: one cycle of 2-min at 98°C for enzyme activation followed by 39 cycles of steps of denaturation for 5 sec at 98°C, annealing for 10 sec at the temperature specific to each SNP primer pair, elongation for 10 sec at 72°C and then a plate read. The amplification cycles were immediately followed by the following melting steps: 98°C for 1 min, cooling to 50°C for 3 min, and a temperature increase to 72°C for 1 min. The temperature was subsequently step-increased to 90°C at intervals of 0.2°C for 10 sec followed by a plate read. Fluorescence data provided by Bio-Rad CFX Manager software were used by Precision Melt Analysis software to precisely identify homozygous and heterozygous individuals for each SNP marker assayed.

3. PHENOTYPIC CHARACTERIZATION

3.1 Fruit Sample Collection

Fruits at four developmental stages, immature green (IG), mature green (MG), breaker (Br) and red ripe (RR) were harvested depending on the experiment. Immature green fruits were collected at 15 days post anthesis (dpa) after labeling flowers at anthesis and ensuring flower vibration the same day. Mature green, breaker and red ripe fruits were harvested after visual inspection of their color, position within the truss and number of dpa. MG corresponds to fully expanded green fruits, approximately 35 dpa. Br fruits were collected when there is a definite break in color from green to tannish-yellow, pink or red on not more than 10% of the surface, around 45 dpa. RR fruits were collected when the whole fruit surface displayed red color, around 55 dpa.

3.2 Cuticle Extraction

Cuticles were enzymatically isolated from tomato fruits following the protocol used by (Petracek and Bukovac, 1995) who applied the procedure described by (Orgell, 1955) and took into consideration the modifications of

(Yamada *et al.,* 1964). First and after visual inspection, fruits showing external lesions and damages were discarded. Cuticles from fruits at four developmental stages (IG, MG, Br, RR) were extracted from the parental lines MM and TO-937, the SP lines and the SP_(QTL+QTL) lines that holds *ph5.1* or *ph5.2*. Cuticles from red ripe fruits were isolated from the SP_(QTL+QTL) lines and nSP lines. Fruits were rinsed in distilled water, cut in halves, and incubated in an aqueous solution consisting of a sodium citrate buffer (50 mM; pH 3.7), a mixture of fungal (Aspergillus niger) cellulase (0.2% w/v; Sigma-Aldrich, EC: 3.2.1.4) and pectinase (2% w/v; Sigma-Aldrich, EC 3.2.1.15), and 1 mM of sodium azide ($NaN_{3)}$ to prevent microbial growth. Fruit samples were incubated with continuous agitation at 37ºC for at least two weeks. During these two weeks, the enzymatic solution was renewed and extracts were sonicated in an ultra-sonic bath (Selecta, Spain) to facilitate the removal of the rest of cell wall debris. Finally, isolated cuticles were rinsed with distilled water, air dried and stored under dry and dark conditions until further use.

3.2.1 Quantitative Determination of the Amount of Cuticle

The amount of cuticle per surface area was determined using cuticle pieces extracted from different tomato fruits. Small and flat cuticle sections were set on slides and fixed by cover slips made of silicate glass to hold samples in place and prevent any deformation or movement. The surface of each cuticle section was captured by LCC-RCA (SONY DFW-X700) camera and the resulting images of the surface of cuticle sections were processed by Visilog v 6.3 (Noiesis, France). Data of the surface measurements were saved, and the slides were kept in a desiccator containing silica gel. After dehydration, the weight of cuticle sections was measured using an analytical and digital precision balance from (Mettler Toledo, Spain) with weighing range up to 320g, and readability to 0.01mg. The final amount of cuticle was calculated using the formula: (weight/surface unit area) and it is expressed as the amount of cuticle per unit surface area ($\mu g \cdot cm^{-2}$). Twenty replicates for each genotype/line and forty in the case of MM were used.

3.2.2 Quantitative Determination of Cuticle Phenolics

Cuticle pieces (10 mg) from each genotype/line to be analyzed were added to 10 mL of 1% methanol/KOH (w/v) and placed for 16 h in an incubator-shaker at 60ºC for cutin depolymerization. Total phenolic components released were estimated by measuring the absorbance of the solution. A Bio Tek PowerWave XS2 plate reader equipped with Gen5™ data analysis software (Biotek Instruments Inc., Winooski, VT, USA) was used to read the absorbance of the samples at 324 nm. The percentage of phenolics (ph) in the cuticle and the amount of phenolics (a.ph) which is expressed as the quantity of phenolics per cuticle surface area (µg.cm^{-2} of cuticle) were estimated from a calibration curve of a solution of naringenin dissolved in 1% methanol/KOH (w/v). Before sample measurements, dilutions of (1:25) with methanol/KOH, used as a blank, were made for the original solutions. For each replicate, a total of 300 µL (12 of sample; 288 of Met/KOH) was added to each of the 96-well plate. The percentage of cuticle phenolics was estimated from five samples per genotype/line, except for MM where 10 samples were determined.

3.2.3 Measurement of Cuticle Color

Cuticle color was objectively measured with a colorimeter CHROMA METER CR-400 (Konica Minolta Inc., Japan) applying the International Commission on Illumination (CIE) L*C*Hº color space. The color space is three-dimensional. The L* axis represents Lightness ranging from 0, which has no L* (*i.e.,* absolute black) to 100 which is maximum L* (*i.e.,* absolute white). The C* axis represents Chroma which is a measure of color saturation that ranges from 0 which is completely unsaturated (*i.e.,* a neutral grey) to 100 or more for very high saturation reaching color purity. The Hº axis represents Hue-angle, in degrees, ranging from 0° (red) through 90° (yellow), 180° (green), 270° (blue) (CIE, 1978). For cuticle color measurements, the colorimeter was first calibrated with a white calibration plate (Minolta, Japan) and later the calibration was modified with a white paper due to the semitranslucent nature of the cuticle. Cuticles were placed with the external surface facing upwards on this same white paper to measure color. Twenty-five pieces of cuticle were measured for each SP line, SP_(QTL+QTL) line and nSP line and 50 for the parental line MM. 50 and 25 color measurements, respectively for MM and the rest of the genotypes, were done on the external side of cuticle pieces.

3.3 Statistical Analyses

For each studied trait, all statistical analyses (means comparison, variance analysis and variance homogeneity test) were performed using SPSS Statistics software package version 23.0 (IBM Corp., Armonk, NY, USA). Data are expressed as mean (±SE). Asterisks indicate significant differences * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. Pearson’s coefficient was employed to correlate different variables from the RIL population.

One-way ANOVA followed by Dunnett’s post hoc test have been used to determine statistical significance to MM. For each ANOVA analysis, trait values were used as a dependent variable and the studied genotypes were used as a fixed factor with MM as a control.

For all-possible mean comparisons among genotypes, one-way ANOVA test was followed by Tukey-b or Games Howell post hoc tests (depending on tested homogeneity or heterogeneity of variance, respectively) were used to determine statistical significance. In this case, trait values were used as the dependent variable and the studied genotypes as the fixed factor levels, with no control. Different letters on charts indicate significant mean differences between genotypes in question.

Epistatic interactions between combined QTLs in the MM genetic background for each trait were analyzed using a univariate general linear, complete 2x2 factorial model using the two tested QTLs as principal factors and their lyc/lyc and pim/pim allelic combinations as the factor levels. For this, data from MM (lyc/lyc_lyc/lyc), the two SP or nSP lines (lyc/lyc_pim/pim and pim/pim_lyc/lyc) and the pyramided, double-QTL line (pim/pim_pim/pim) were used. Percentage data were transformed with the arcsine square root prior to statistical analyses. However, for more convenient visualization of data, untransformed percentage values are shown in tables and figures.

4. NOMENCLATURE

To avoid confusion, gene symbols are italicized with all letters in uppercase. QTLs of cuticle components were named by two letters abbreviation designating the trait and italicized in lowercase. However, QTLs of cuticle color were named by the first letter of the trait name and italicized in uppercase. Mutant symbols, although very few, are also italicized between quotation marks, with all letter in lowercase.

CHAPTER I:

GENOME-WIDE QTL ANALYSIS OF TOMATO FRUIT CUTICLE COMPOSITION AND COLOR

1. INTRODUCTION

The plant cuticle, as the interface between the plant and the environment, plays a role in plant performance, fruit quality and postharvest (Petit *et al.,* 2017; Lara *et al.,* 2019). Among the functions of agronomic relevance are the protection from water loss and UV radiation, its function as a thermal regulator, and the mechanical protection against pathogens and abiotic stresses, either environmental or derived from internal tissues (Domínguez *et al.,* 2011). Thus, the cuticle participates in the protection from fungal penetration, fruit cracking, organ dehydration and fusion, and in controlling organ growth. The cuticle is composed of a lipid matrix of esterified polyhydroxy fatty acids named cutin, polysaccharides from the cell wall, phenolic compounds and waxes, a mixture of soluble lipids and triterpenoids that can be located within the cuticle matrix or deposited on the surface (Domínguez *et al.,* 2011). Waxes play a critical role in the control of water loss from internal tissues and can also confer some mechanical strength (Khanal *et al.,* 2013a; Jetter and Riederer, 2016). In tomato fruit cuticle, waxes play a small part in the mechanical resistance, being the phenolic fraction the main modulator of the cuticle's stiffness, deformation and strength (España *et al.,* 2014a,b). This phenolic domain is composed of cinnamic acids derivatives present during fruit growth and increases significantly during ripening with the incorporation of the flavonoid naringenin chalcone, responsible for the yellow-orange color of the cuticle in red ripe tomatoes (España *et al.,* 2014a).

Understanding the genetic basis of cuticle composition and color is needed for breeding in crop species. Although numerous genes related to cuticle biosynthesis have been identified, most of these have been uncovered after the analysis of mutagenized populations and/or gene silencing. However, their participation in the genetic variation present in natural populations, cultivars and wild species closely related to crops is uncertain. Natural variation is a valuable source of beneficial traits for breeding. Limited work based on variation in natural plant populations has been carried out to study the cuticle, and mainly focused on waxes (Riedel *et al.,* 2009; Würschum *et al.,* 2020). Analysis of genes involved in anatomical and biophysical traits of the cuticle, many of great importance for plant performance and fruit quality, are scarce at most. In this sense it should be mentioned the relationship found between postharvest water loss in pepper and differences in wax composition and cutin amount and the identification of QTL for cuticle thickness and invagination in tomato and cucumber (Fernández-Moreno *et al.,* 2017; Rett-Cadman *et al.,* 2019).

Tomato genetics and breeding heavily relies on exploiting natural variation from related wild species. Wild tomato species have been reported to exhibit cuticle diversity for some anatomical and chemical traits (Yeats *et al.,* 2012a), however, its genetics have only been studied in lines derived from the green-fruited *S. pennellii* Dun. (Fernández-Moreno *et al.,* 2017). In this work, we have explored for the first time the cuticle variability of red-fruited species in two populations derived from the interspecific cross between the domesticated tomato *S. lycopersicum* and its closest wild relative *S. pimpinellifolium*. A QTL analysis of the different cuticle components and color traits was performed revealing a complex polygenic nature that combined additive and epistatic interactions among the different QTLs.

2. MATERIALS AND METHODS

2.1 Plant Material and Greenhouse Experiments

The introgression SP lines derived from the backcross of TO-937 into MM were grown in summer 2013 to validate the QTLs identified and to assess the presence of additional QTLs either not detected or not significant in the RIL population. Five plants per SP line and ten of the parental lines, MM and TO-937 were grown.

To generate new SP lines with shortened introgressions for some target QTLs and/or separate QTLs present within the same SP line to study their individualized effect, three existing F_1 populations derived from the crosses SP_3-2 x MM; SP_7-2 x MM and SP_11-1 x MM were grown and self-fertilized during spring 2014. 300 plants per F_2 population were sown to identify individuals with the desired introgressions and the selected plants transplanted to a multi-tunnel polyethylene plastic house where they were vibrated daily to ensure fruit set. Eleven newly identified SP lines (nSPs) were obtained from the F_2 populations of the crosses between the corresponding SP line and MM following the procedure depicted in Fig I-1. Markers and primers employed for MAS are shown in Anx. I-1, I-2 and I-3. The nSP derived from SP_3-2 x MM were b10, b22, b40, b73 and b168, the ones derived from SP_7-2 x MM were a203, a228 and a239 and finally those derived from SP_11-1 x MM were c33, c49 and c199. These 11 nSP were sown in spring 2015 and seeds collected from each individual plant. Ten plants per nSP line together with the parental line MM were then studied in autumn 2015.

Ten plants per genotype of Ailsa Craig wt (LA2838A), the nearly isogenic Ailsa Craig *y/y* (LA3189) harboring the *colorless fruit epidermis "y"* mutation, MM and the SP lines SP_1-1, SP_8-1 and SP_12-2 were grown in spring 2016 in a multi-tunnel polyethylene plastic house. Plant of Ailsa Craig wt and *y/y* were employed to compare the amount of cuticle and percentage of each component at red ripe. The other plants were used for gene expression analyses of fruit epicarp at four stages of development.

To clean the SP_8-1 from the F_1 introgression present in chromosome 3, twenty plants were grown and MM homozygous the plants for this region were identified by MAS. Primers listed in Anx. I-1 that were located within this heterozygous region were employed. The selected plants were transplanted to the greenhouse and seeds collected. Ten plants per genotype as well as the parental MM were further studied in summer 2017.

All the SP and the 11 nSP lines, together with the parental lines MM and TO-937, were grown again in spring 2018 to validate all the cuticle traits previously studied and to obtain comparable data from the SP and nSP lines. Twelve plants per genotype, and 30 for MM and TO-937, were grown in a multi-tunnel polyethylene plastic house. A distribution of three randomly distributed blocks each with four plants per genotype and 10 for TO-937 and MM were employed. Three additional blocks of 10 MM plants each were randomly distributed.

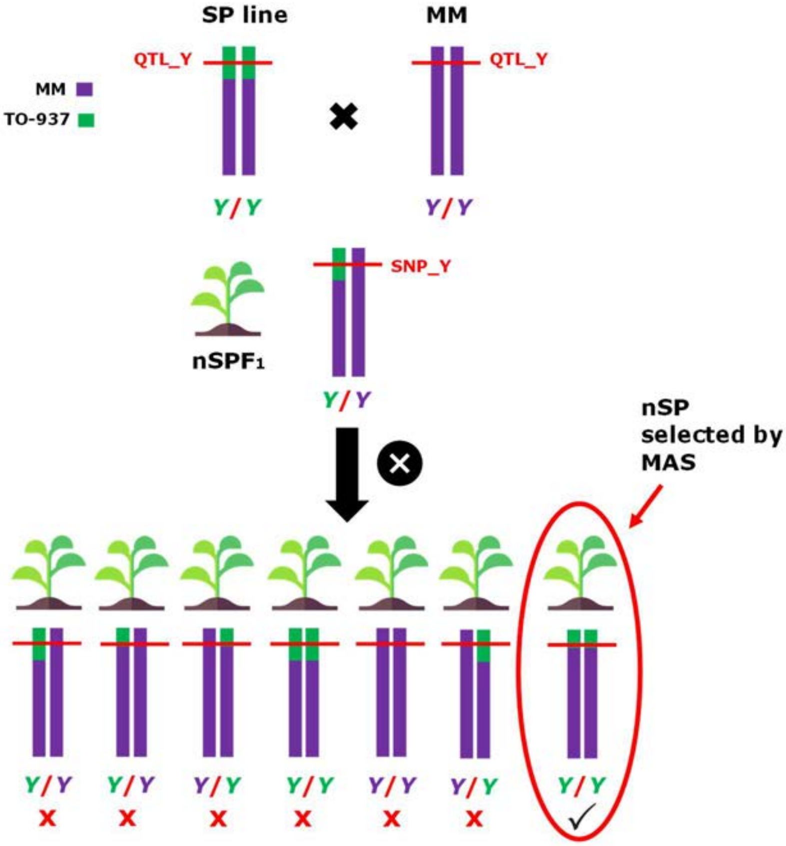


Fig. I-1: Schematic description of the generation of the new SP lines (nSP). SP lines harboring a desired QTL (QTL-Y) and MM were crossed. The F_1 plants ($nSPF_1$) were selfed to obtain a segregant F_2 population ($nSPF_2$). MAS was employed to identify individuals harboring the peak marker SNP_Y for the desired QTL-Y homozygous for the TO-937 allele. In a subsequent step, additional markers were employed to identify the length of the new introgression. MAS, marker assisted selection; QTL, quantitative trait locus; SNP, single nucleotide polymorphism.

2.2 Quantitative Determination of Waxes, Polysaccharides and Cutin

Cuticular waxes were removed by heating (50°C) 0.1 g of isolated cuticles in 100 mL of chloroform: methanol (2 : 1, v/v) for 2 h. Cutin isolates were obtained after refluxing the dewaxed cuticles in a 6 M HCl aqueous solution for 12 h. Polysaccharide material was selectively removed by immersion of the enzymatically isolated cuticles in anhydrous hydrogen fluoride in pyridine (Aldrich, Milwaukee, Wis. USA) for 4 h at 55 °C (Villena *et al.,* 1999).

2.3 QTL Analysis

The genotyping of the RIL population (Rambla *et al.,* 2017) with the 8K SNP SOLCAP Infinium chip (Sim *et al.,* 2012) was employed to construct a linkage map containing 4,885 SNP markers using JoinMap® 4.0 software (Kyazma BV, Wageningen, the Netherlands) (Van Ooijen, 2006) with the maximum likelihood algorithm and the Kosambi mapping function. QTL analysis was carried out with the software MapQTL® 5.0 (Kyazma BV, Wageningen, the Netherlands) (Van Ooijen, 2004) using interval mapping and subsequent QTL modeling by multiple QTL mapping after automatic cofactor selection. Permutation tests with 10,000 resamplings were employed to determine the probability associated to each QTL. A reduction of the map to 1,302 informative SNP markers was performed for computational purposes to carry out a composite interval mapping (Zeng *et al.,* 1994) and epistatic analysis using IciMapping (Meng *et al.,* 2015). QTL map was drawn with Mapchart 2.2 (Voorrips, 2002).

Any change in the amount of cuticle deposited also modifies the amount of cuticle components, unless their percentage distribution is modified. For this reason, the QTL analysis of the different cuticle components was carried out with the percentage and not the amount. Unless indicated otherwise, all the QTLs identified for the amount of cuticle also affected the amount of phenolics, waxes, cutin and polysaccharides.

2.4 Gene Expression Analysis

Trizol® Reagent (Life Technologies, USA) was used to isolate RNA from tomato fruit peels at four different stages of development (IG, MG, Br and RR). Genomic DNA was removed with RNase-free DNase and RNA was cleaned with the Nucleospin RNA clean-up kit (Macherey-Nagel, Germany). First-strand cDNA synthesis was carried out with the Super Script III First-Strand Synthesis Super Mix for qRT-PCR (Invitrogen, USA). Relative transcript amount of the different genes was measured by RT-qPCR using

SsoAdvanced™ SYBR® Green Supermix (Bio-Rad, USA) following the ΔCt method with three control genes: *Clathrin Adaptor Complexes* (*CAC*), *EXPRESSED* and *SAND-family protein* (SAND) previously employed for tomato peel analyses (Segado *et al.,* 2020). 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. Primers used for amplification are shown in Anx. I-4. Comparison of the mRNA sequences of MM and TO-937 genes was carried out to identify changes that would result in non-conserved amino acid substitutions.

3. RESULTS

3.1 Analysis of the Parental Lines

Cuticle analysis of the cultivated tomato MM and the wild species TO-937 showed significant differences for most of the traits studied (Fig. I-2). Thus, red ripe cuticles of TO-937 displayed a significantly lower amount of cuticle. The percentage of total waxes present in the cuticle of TO-937 was statistically higher compared to MM whereas phenolics were significantly lower. No significant differences in the percentages of the major cuticle components, cutin and polysaccharides, were detected. As for cuticle color parameters, L* and H° were significantly higher in TO-937 compared to MM whereas C* was significantly lower in TO-937 than in MM.

Cuticle characterization of the RIL population showed transgressive inheritance for most of the traits analyzed, with lines displaying values significantly higher and lower than the parental lines. This indicates that both parental lines have positive QTLs for the traits analyzed. For example, RILs with amounts of cuticle significantly lower than TO-937 (100 lines) and higher than MM (14 lines) were identified. Similarly, despite the very low percentage of cuticle phenolics detected in TO-937, 21 RILs showed values significantly higher than MM, indicating a positive contribution of TO-937 alleles. A similar behavior was observed for the percentage of waxes.

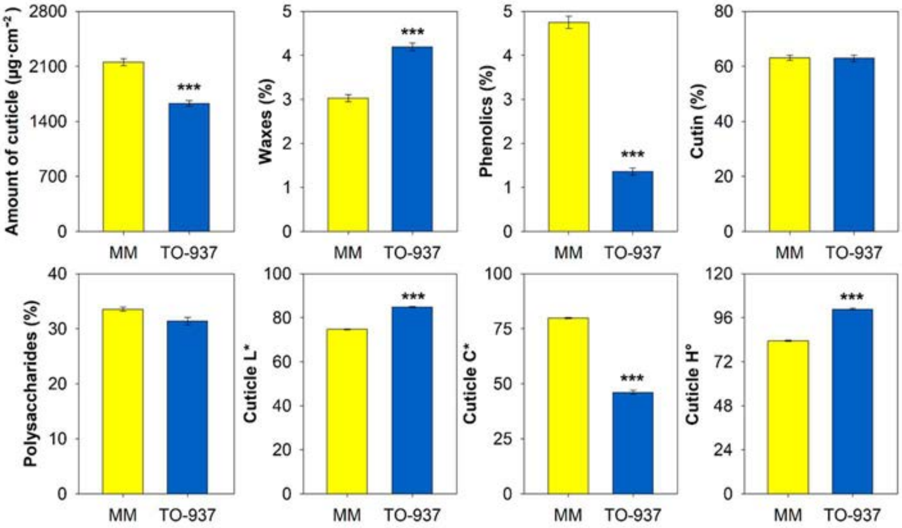


Fig. I-2: Comparison between the parental lines MM and TO-937 of the different cuticle traits studied. Red ripe fruits were analyzed. Data are presented as means ± SE. Asterisks indicate significant differences between both parental lines (****p* ≤ 0.001). L*, lightness; C*, chroma; H°, hue.

3.2 QTL Mapping of the RIL Population

To avoid rearrangement of QTL names throughout the chapter that could lead to confusion, QTLs are numbered from the beginning considering the final number of QTLs identified in both the RIL and IL populations.

Forty-two statistically significant QTLs were detected for different cuticle traits within the RIL population. Their location is presented in Fig. I-3. The peak marker for each of the identified QTLs together with the percentage of variance explained (PVE), LOD (logarithm of the odds) score and associated probability are shown in Tables I-1, I-2 and I-3. Out of the 11 QTLs identified for the amount of cuticle (Table I-1), nine had lyc effect that is, the MM allelic region increased the amount of cuticle whereas two other QTLs, *cm5.2* and *cm10.2*, had pim effect, TO-937 alleles increased the amount of cuticle. The highest additive effect was displayed by *cm3.1* and *cm11.2* with a 17 and 13,8 PVE, respectively. QTL epistatic analysis identified a significant lyc x pim interaction (triangles in Fig. I-3) between a region in chromosome 1 and c*m5.2* (LOD 5.18, 15% variance explained) that resulted in a pim effect.

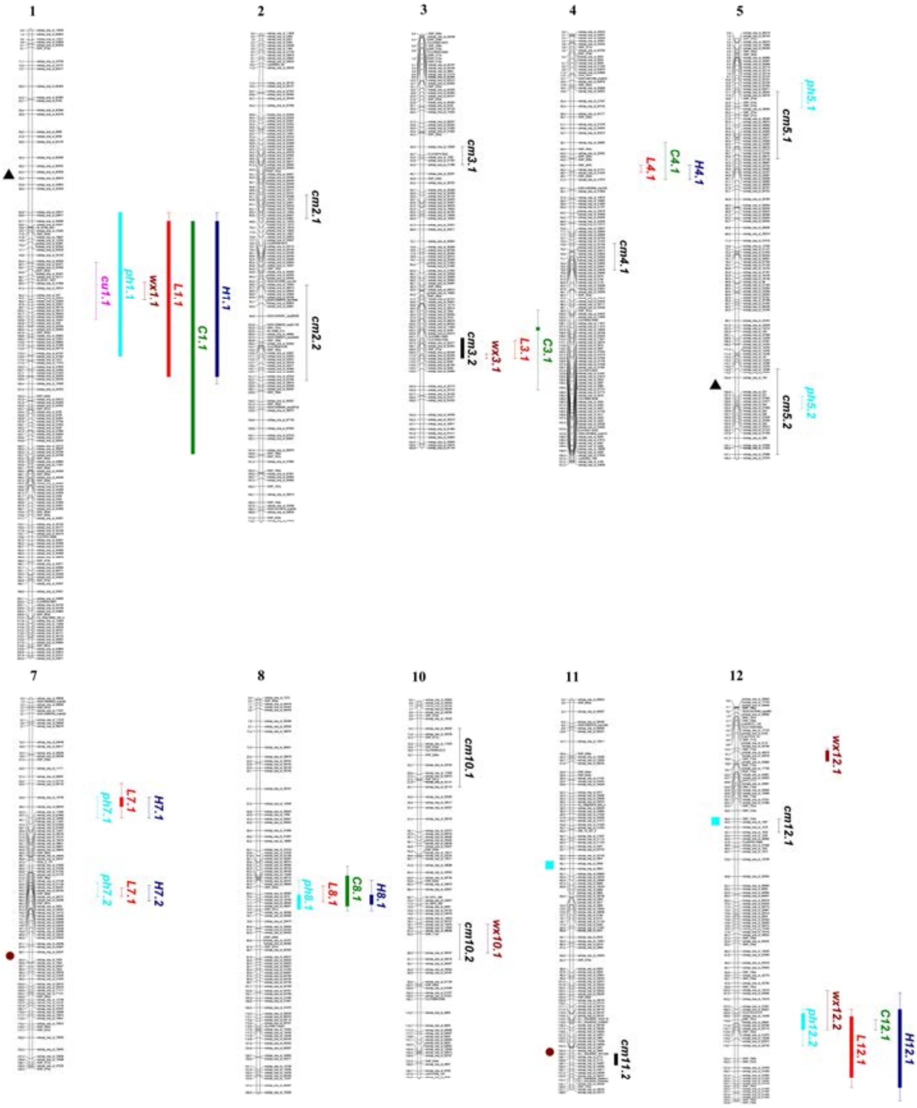


Fig. I-3: Map of the QTL regions identified in the RIL population for the different cuticle related traits. Bold lines represent QTL regions with $p < 0.001$. Symbols with the same shape and color located to the left of chromosomes denote regions with epistatic interactions. Symbol color indicates the trait. Numbers to the left of chromosomes indicate genetic distances expressed in cM (Kosambi mapping function). RIL, recombinant inbred line; *cm*, QTL amount of cuticle; *cu*, QTL percentage of cutin; *ph*, QTL percentage of phenolics; *wx*, QTL percentage of waxes; *L*, QTL cuticle lightness; *C*, QTL cuticle chroma; *H*, QTL cuticle hue.

This indicated that a lyc allelic region in chromosome 1 increases the pim effect of *cm5.2*. This region in chromosome 1 coincided with a peak with lyc effect observed in the interval mapping below the statistical level of significance p = 0.9643.

Table I-1: QTLs identified in the RIL population for the amount of cuticle. QTLs are named with a trait abbreviation followed by chromosome number and the number of the QTL within the chromosome. Effect refers to the allele (lyc/pim) that increases the trait. lyc, MM; pim, TO-937. PVE, percentage of variance explained by the QTL; *P*: probability. Marker closest to the maximum LOD (logarithm of odds) score is also shown. *cm*, QTL amount of cuticle.

QTL	Effect	LOD	PVE	*P*	Peak marker
cm2.1	lyc	2.8	6.9	0.989	Solcap_23851
cm2.2	lyc	3.67	9.8	0.997	Solcap_42471
cm3.1	lyc	4.65	11.9	1.000	Solcap_41353
cm3.2	lyc	6.85	17.0	1.000	Solcap_62180
cm4.1	lyc	3.61	7.8	0.998	Solcap_3108
cm5.1	lyc	3.04	6.8	0.992	Solcap_23797
cm5.2	pim	3.66	8.9	0.998	Solcap_37808
cm10.1	lyc	3.94	8.1	0.999	Solcap_46305
cm10.2	pim	3.08	7.3	0.995	Solcap_14879
cm11.2	lyc	6.02	13.8	1.000	Solcap_100974
cm12.1	lyc	3.84	9.4	0.998	Solcap_1497

Seven QTL regions, in chromosomes 1, 5, 7, 8 and 12, were identified for the phenolics content trait (Table I-2). Despite the very low percentage of phenolics present in TO-937 cuticle, two QTLs with pim effect were identified *ph5.1* and *ph5.2*. The QTL *ph1.1* showed the largest additive effect with 26.7 PVE. Moreover, a significant lyc x pim epistatic interaction (LOD 5.57, 10.9% variance explained) with pim effect was identified between a region in chromosome 11 and another one in chromosome 12 that coincided with *cm12.1* (squares in Fig. I-3). This indicates that a lyc allelic region in chromosome 11 increases a pim effect in chromosome 12. Again, this region coincided with a non-significant peak detected in the interval mapping (p = 0.9653).

Five QTLs were identified for the percentage of waxes, three with pim effect and two with lyc effect (Table I-2). Two of these QTLs for waxes, *wx1.1* and *wx12.2*, colocalized with the QTLs for phenolics *ph1.1* and *ph12.2* and displayed opposite effects. Whereas *wx1.1* and *wx12.*2 exhibited a pim effect, *ph1.1* and *ph12.2* had lyc effect. However, the other QTLs for phenolics were not associated with genomic regions related to wax accumulation. Also, a significant lyc x pim epistatic interaction (LOD 5.28, 15.7% variance explained) was identified between a region in chromosome 7 and another one in chromosome 11, in the same region as *cm11.2* (circles in Fig. I-3). This interaction showed a resulting lyc effect.

Table I-2: QTLs identified in the RIL population for the percentage of cuticle components. QTLs are named with a trait abbreviation followed by chromosome number and the number of the QTL within the chromosome. Effect refers to the allele (lyc/pim) that increases the trait. lyc, MM; pim, TO-937. PVE, percentage of variance explained by the QTL; *P*: probability. Marker closest to the maximum LOD (logarithm of odds) score is also shown. *cu*, QTL percentage of cutin; *ph*, QTL percentage of phenolics; *wx*, QTL percentage of waxes.

QTL	Effect	LOD	PVE	*P*	Peak marker
ph1.1	lyc	11.37	26.7	1.000	Solcap_457
wx1.1	pim	3.54	9.2	0.996	Solcap_25914
cu1.1	lyc	3.38	8.9	0.996	Solcap_25922
wx3.1	pim	3.13	7.4	0.992	Solcap_62270
ph5.1	pim	4.36	10.3	1.000	Solcap_49234
ph5.2	pim	2.90	7.2	0.991	Solcap_37562
ph7.1	lyc	3.39	9	0.998	Solcap_68044
ph7.2	lyc	3.02	7.9	0.993	Solcap_5853
ph8.1	lyc	5.64	14.3	1.000	Solcap_48550
wx10.1	lyc	3.07	8	0.992	SNP_112a
ph12.2	lyc	5.40	13.3	1.000	Solcap_55706
wx12.1	lyc	5.84	12.6	1.000	Solcap_45816
wx12.2	pim	3.48	7.2	0.997	Solcap_55712

Despite no significant differences were detected for the percentage of cutin between the parental lines, one QTLs for the percentage of cutin was identified in the RIL population, *cu1.1* with lyc effect (Table I-2). Although a peak for percentage of polysaccharides was found in chromosome 1 in the

QTL analysis, coinciding with the *cu1.1* region, it was below the statistically significant threshold.

Regarding the cuticle color traits, a total of eighteen QTLs regions were identified (Tab. I-3). For the cuticle L* trait, 7 QTLs were identified, six of them with pim effect and one with lyc effect. For the cuticle C* trait, five QTL regions were identified, four with lyc effect and one with pim effect. As for the cuticle H° trait, five QTLs with pim effect and one with lyc effect were found. The largest additive effects were detected in chromosome 1 for all cuticle color traits with *L1.1*, *C1.1* and *H1.1* having 24.5, 27.5 and 30.1 PVE,

Table I-3: QTLs identified in the RIL population for cuticle color parameters. QTLs are named with a trait abbreviation followed by chromosome number and the number of the QTL within the chromosome. Effect refers to the allele (lyc/pim) that increases the trait. lyc, MM; pim, TO-937. PVE, percentage of variance explained by the QTL; *P*: probability. Marker closest to the maximum LOD (logarithm of odds) score is also shown. *L*, QTL cuticle lightness; *C*, QTL cuticle chroma; *H*, QTL cuticle hue.

QTL	Effect	LOD	PVE	*P*	Peak marker
L1.1	pim	10.28	24.5	1.000	Solcap_457
C1.1	lyc	11.75	27.5	1.000	Solcap_457
H1.1	pim	13.13	30.1	1.000	Solcap_457
L3.1	pim	3.26	8.4	0.993	Solcap_62270
C3.1	lyc	4.57	11.7	1.000	Solcap_100243
L4.1	lyc	3.09	8.1	0.990	Solcap_51721
C4.1	pim	3.67	9.6	0.996	SNP_292a
H4.1	lyc	3.62	9.5	0.997	SNP_302a
L7.1	pim	4.32	11.2	1.000	Solcap_68044
H7.1	pim	3.65	9.5	0.998	Solcap_68044
L7.2	pim	3.08	8.1	0.994	Solcap_52557
H7.2	pim	3.62	9.4	0.998	Solcap_52557
L8.1	pim	3.25	8.5	0.995	Solcap_48592
C8.1	lyc	6.51	16.5	1.000	Solcap_48592
H8.1	pim	4.71	12.2	1.000	Solcap_48592
L12.1	pim	6.07	15.3	1.000	Solcap_62972
C12.1	lyc	4.07	10.6	0.998	SNP_752a
H12.1	pim	6.79	16.9	1.000	Solcap_62972

respectively. It should be mentioned that most of the cuticle color QTLs colocalized with those for percentage of phenolics. This agrees with the yellow-orange colored flavonoid naringenin chalcone being the main phenolic present in ripe tomato fruit cuticles (Baker and Hunt, 1980; España *et al.*, 2014 NP). Interestingly, QTLs for L*, C* and H° were detected in chromosome 4 where no significant QTL for phenolics was identified. However, a QTL for percentage of phenolics, slightly below the 0.99 threshold, was present in the RIL population that coincides with this genomic region.

3.3 Phenotyping the SP Population for the Studied Traits

The SP introgression line population and parental lines were studied for all the cuticle traits and compared with the recurrent parental line MM. Results of all the lines are presented in Anx. I-5 (amount of cuticle), Anx. I-6 (percentage of phenolics), Anx. I-7 (percentage of waxes), Anx. I-8 (percentage of cutin), Anx. I-9 (percentage of polysaccharides), Anx. I-10 (cuticle L*), Anx. I-11 (cuticle C*) and Anx. I-12 (cuticle H°). Significant changes in the amount of cuticle compared to MM were observed in several lines (Anx. I-5). All the lines harboring introgressions in chromosomes 2 and 11 showed a significant decrease in the amount of cuticle. SP_2-1, SP_2-2 and SP_2-3 carried the genomic region for *cm2.1*, SP_2-4 and SP_2-5 for *cm2.2*, whereas SP_11-2, SP_11-3 and SP_11-4 carried *cm11.2*. However, analysis of the RIL population did not detect any QTL within the region comprised in SP_11-1 that could explain its reduced amount of cuticle. SP_3-2 and SP_3-3 both carried *cm3.1+cm3.2* and showed a significantly lower amount of cuticle, the same as SP_4-2 and SP_4-3 both carrying *cm4.1*, SP_5-2, SP_5-3 and SP_5-4 which harbored *cm5.1* and SP_12-2 and SP_12-3 which contained the genomic region corresponding to *cm12.1*. The region corresponding to *cm10.1* was present in SP_10-2, and this line showed a significant decrease in cuticle. However, the pim effect of *cm10.2* could not be directly studied since the line SP_10-3 contained both *cm10.1+cm10.2* and did not show significant differences compared to MM. On the other hand, the pim effect of *cm5.2* was observed in SP_5-5, which displayed a significantly higher amount of cuticle. SP_1-1 carried the genomic region that was previously shown to have an epistatic interaction with cm5.2 and displayed a significant reduction of the amount of cuticle. This new QTL is *cm1.1*. Additionally, SP_1-4 and SP_7-3 showed a significantly lower amount of cuticle. SP_1-4 carried a genomic region where a peak below the statistical level of significance was observed in the interval mapping of the RIL population (p = 0,9329). However, none was observed in chromosome 7. This new identified QTLs are *cm1.2* and *cm7.1*.

The reduced amount of cuticle detected in SP_8-1 could be consequence of the F_1 introgression that this line has in the region of *cm3.2*, between markers Solcap_100561 and Solcap_62180. To test this hypothesis, two plants were selected from the SP_8-1 line, one carrying the chromosome 3 introgression homozygous MM and another one homozygous TO-937. Both lines were tested together with MM to analyze the amount of cuticle. Results showed that the plant homozygous MM had a similar amount of cuticle (1957 ± 50 μg·cm^{-2}) as MM (2162 ± 76 μg·cm^{-2}) whereas the line homozygous TO-937 had an amount of cuticle significantly lower than MM (1274 ± 37 μg·cm^{-2} *** $p \leq 0.001$). This indicated that the effect originally observed in SP_8-1 was due to the introgression in the *cm3.2* region, and not due to the presence of a QTL for the amount of cuticle in the chromosome 8 introgression harbored by SP_8-1.

Regarding the percentage of phenolics, significant decreases were observed in the SP_1-2 carrying *ph1.1*, SP_7-1 carrying *ph7.1*, SP_7-2 and SP_7-3 both having *ph7.1+ph7.2*, SP_8-1 harboring *ph8.1* and SP_12-4 which carried *ph12.2* (Anx. I-6). However, lines SP_5-2, SP_5-3 and SP_5-4 carrying *ph5.1* and SP_5-5 having *ph5.2* showed a percentage of phenolics similar to MM. SP_12-2 and SP_12-3, which carried the genomic region previously identified to have an epistatic interaction with chromosome 11, showed a significantly higher percentage of phenolics. This new QTL is *ph12.1*. However, no significant differences in phenolics were observed for any of the SP lines of chromosome 11. A significant increase in phenolics was also observed in SP_4-2 and SP_4-3. Again, these lines carried a genomic region where a non-significant peak (p = 0.9693) was detected in the interval mapping of the RIL population. This new QTL is *ph4.1*.

The percentage of waxes for the SP lines is presented in Anx.I-7. SP_10-2 and SP_10-3 carried the *wx10.1* and showed a significantly lower amount of waxes, the same as SP_12-1, SP_12-2 and SP_12-3, which carried *wx12.1*. SP_12-4 also showed a lower percentage of waxes, despite having the *wx12.2* region with pim effect. All the SP lines of chromosome 11 showed a significantly lower percentage of waxes. A non-significant peak (p = 0.9543) with pim effect was observed in the RIL population in chromosome 11. As it was explained earlier, a significant epistatic interaction between this region in chromosome 11 and another in chromosome 7 was detected and could explain the reduced percentage of waxes present in SP_11-2, SP_11-3 and SP_11-4. This new QTL corresponds to *wx11.2*. However, SP_11-1 did not carry this region or showed any QTL in the RIL population. Similarly, an increase in waxes was detected in SP_4-2 and SP_4-3 despite no QTLs were detected in the RIL population. This new QTL corresponds to *wx4.1*. SP_1-2 carried the QTL *wx1.1* but did not show any difference in waxes with the parental line MM. Finally, *wx3.1* was present in SP_3-2 and SP_3-3 yet, only the latter displayed a significant increase in waxes. It should be mentioned that two peaks were detected in the QTL analysis in chromosome 3 but only the first one was significant. However, the second peak was observed to improve the effect of the first one in an MQM (multiple QTL model) analysis. This second peak was located to a region present in SP_3-3 and SP_3-4. Since SP_3-4 showed a similar percentage of waxes it could be suggested that the effect of SP_3-3 is due to a combination of *wx3.1+wx3.2*. However, further analyses are needed to study this potential interaction.

Finally, a significant increase in cutin and decrease in polysaccharides was seen in SP_10-3 and SP_10-4 (Anx.I-8 and I-9) whereas SP_1-2, carrying *cu1.1*, showed values similar to MM. Both lines in chromosome 10 carried a genomic region with non-significant peaks for cutin (p = 0,9505 pim effect) and polysaccharides (p = 0,9613 lyc effect) in the RIL population. These new QTLs are *cu10.1* and *ps10.1*.

Regarding cuticle color (Anx. I-10, I-11 and I-12), a significant increase in L* and H° together with a decrease in C* was observed in the SP_1-2 carrying *L1.1*, *C1.1* and *H1.1*, SP_8-1 harboring *L8.1*, *C8.1* and *H8.1*, and SP_12-4 with *L12.1*, *C12.1* and *H12.1*. SP_3-2 and SP_3-3 harboring *L3.1* and *C3.1* displayed a significant increase in L* but also in H° without changing the cuticle's chroma. It should be mentioned that in the QTL analysis a peak with pim effect was also observed for H° in this genomic region, but it was below the statistical level of significance (p = 0.9847). This new QTL corresponds to *H3.1*. The lines SP_7-1 carry *L7.1* and *H7.1* whereas SP_7-2 and SP_7-3 carry the combination *L7.1+L7.2* and *H7.1+H7.2* and showed a significant increase in L* and H° but also had a significant decrease in C*. These new QTLs are *C7.1* and *C7.2*, and similarly to previous cases, two non-significant peaks with lyc effect were observed in the QTL analysis for C* in both genomic regions (p = 0.9956 in both cases). On the other hand, SP_4-2 and SP_4-3 carrying *L4.1*, *C4.1* and *H4.1* showed an opposite and significant behavior with decreases in L* and H° and an increase in C*. Additionally, lines SP_11-2, SP_11-3 and SP_11-4 showed a significant increase in L* and H° and a decrease in C*. These lines did not carry any QTL detected in the RIL population for color but for the amount of cuticle. These new QTLs correspond to *L11.1*, *C11.1* and *H11.1*.

3.4 Generation and Analysis of the nSPs

To study the individualized effect of *cm3.1* & *cm3.2* and *ph7.1* & *ph7.2*, nSPs were generated from SP_3-2 and SP_7-2 respectively. Additionally, to investigate the QTLs observed in the SP_11-1 for cuticle and percentage of waxes, this line was also shortened. The generated nSPs contained overlapping shortened regions (Figs. I-4, I-5 and I-6). Five nSPs were generated from SP_3-2: b10, b22, b40, b73 and b168. nSPs b10 and b73 harbored *cm3.1*, b22 and b40 *cm3.2* whereas b168 harbored both QTLs. Analysis of the amount of cuticle for these lines showed that lines with individual QTLs had a significant reduction in comparison with the parental MM whereas the combination of *cm3.1+cm3.2* displayed an even lower amount of cuticle than each individualized QTL (Fig. I-4). Also, analysis of the percentage of waxes showed that b22, b40 or b168, all of them carrying *wx3.1*, had an amount of waxes similar to MM. As for cuticle color, a significant increase in L* and H° was observed in the lines carrying QTLs for color: b20, b40 and b168. As it was previously observed in the IL population, no differences for cuticle C* were observed.

Three nSPs were generated from SP_7-2: a228, a239 and a203 harboring *ph7.1*, *ph7.2* and *ph7.1+ph7.2*, respectively. Comparison of the percentage of phenolics with MM showed that each individual QTL had a significant reduction and the line combining *ph7.1+ph7.2* displayed a stronger reduction in phenolics (Fig. I-5). Also, all the lines showed a significant increase in L* and H° while a decrease in C* compared to MM except for C* in a228.

Regarding SP_11-1 three overlapping nSPs were obtained: c33, c49 and c199. Unfortunately, a gap between Solcap_62807 and Solcap_724 was observed in all three nSPs and none of them carried this region as TO-937 (Fig. I-6). Hence c199 did not carry the same introgression as SP_11-1. Comparison of MM with these nSPs showed a similar amount of cuticle and of percentage of waxes (Fig. I-6). This indicated one of three possibilities, either the QTL previously detected in the SP_11-1 was not real or it could be located in the gap that none of the nSP lines carried as TO-937. Alternatively, the SP population was analyzed in summer but the nSP lines were tested in autumn-winter. Since cuticle deposition is affected by environmental conditions, and high temperature, irradiance and low relative humidity have been reported to increase the amount of cuticle and its components (Domínguez *et al.*, 2012), it could be possible that this QTL was significantly affected by the environment. To test any of these hypotheses, the nSP lines, MM and SP_11-1 were grown in summer 2018 and further analyzed. Results confirmed the decrease in the amount of cuticle and percentage of waxes for SP_11-1 but not for any of the nSP lines (Anx. I-13), suggesting the presence of two QTLs, *cm11.1* and *wx11.1*, in the gap. Further generation of nSPs containing this gap region would be needed to properly validate and study these QTLs.

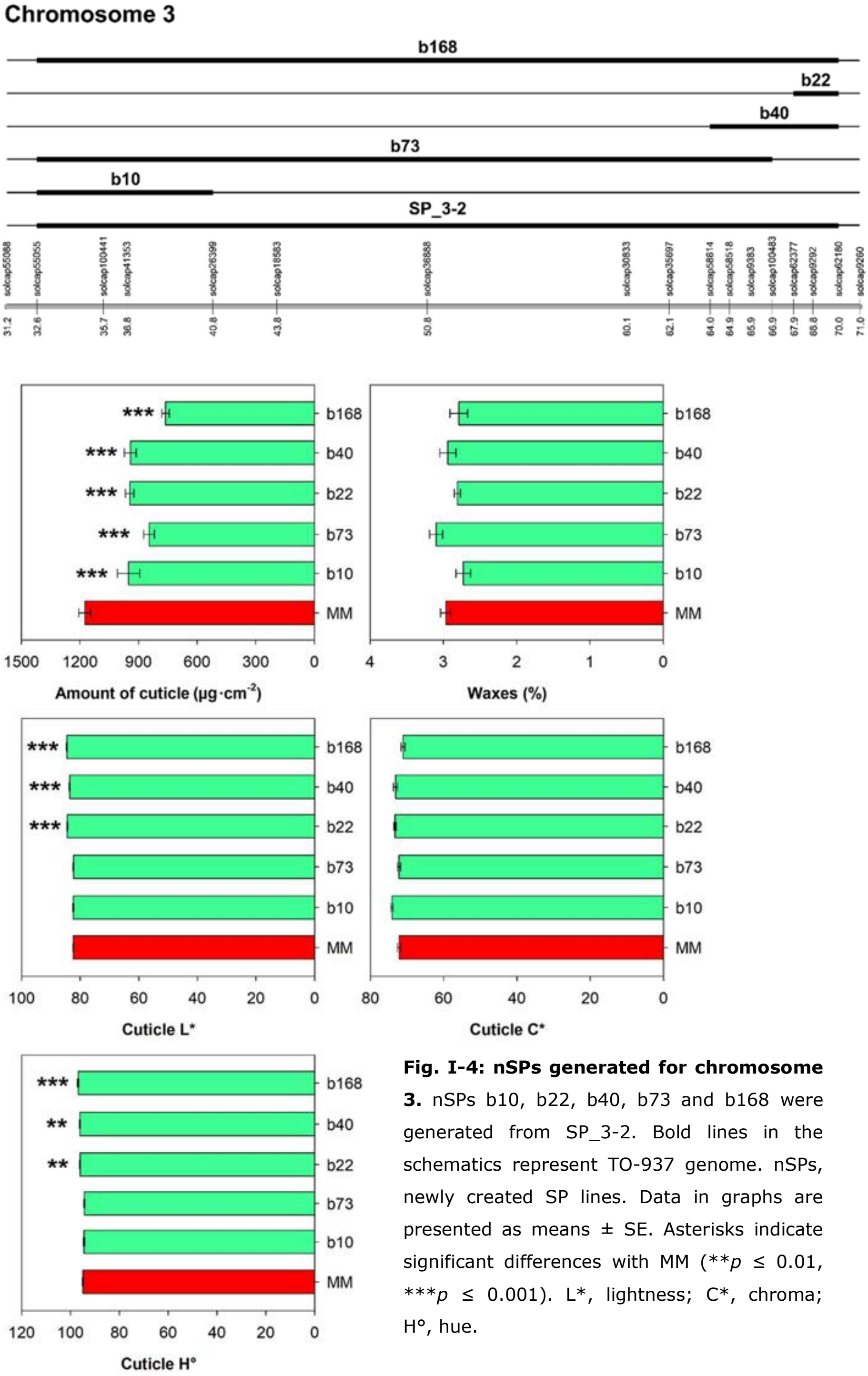


Fig. I-4: nSPs generated for chromosome 3. nSPs b10, b22, b40, b73 and b168 were generated from SP_3-2. Bold lines in the schematics represent TO-937 genome. nSPs, newly created SP lines. Data in graphs are presented as means ± SE. Asterisks indicate significant differences with MM (**$p \le 0.01$, ***$p \le 0.001$). L*, lightness; C*, chroma; H°, hue.

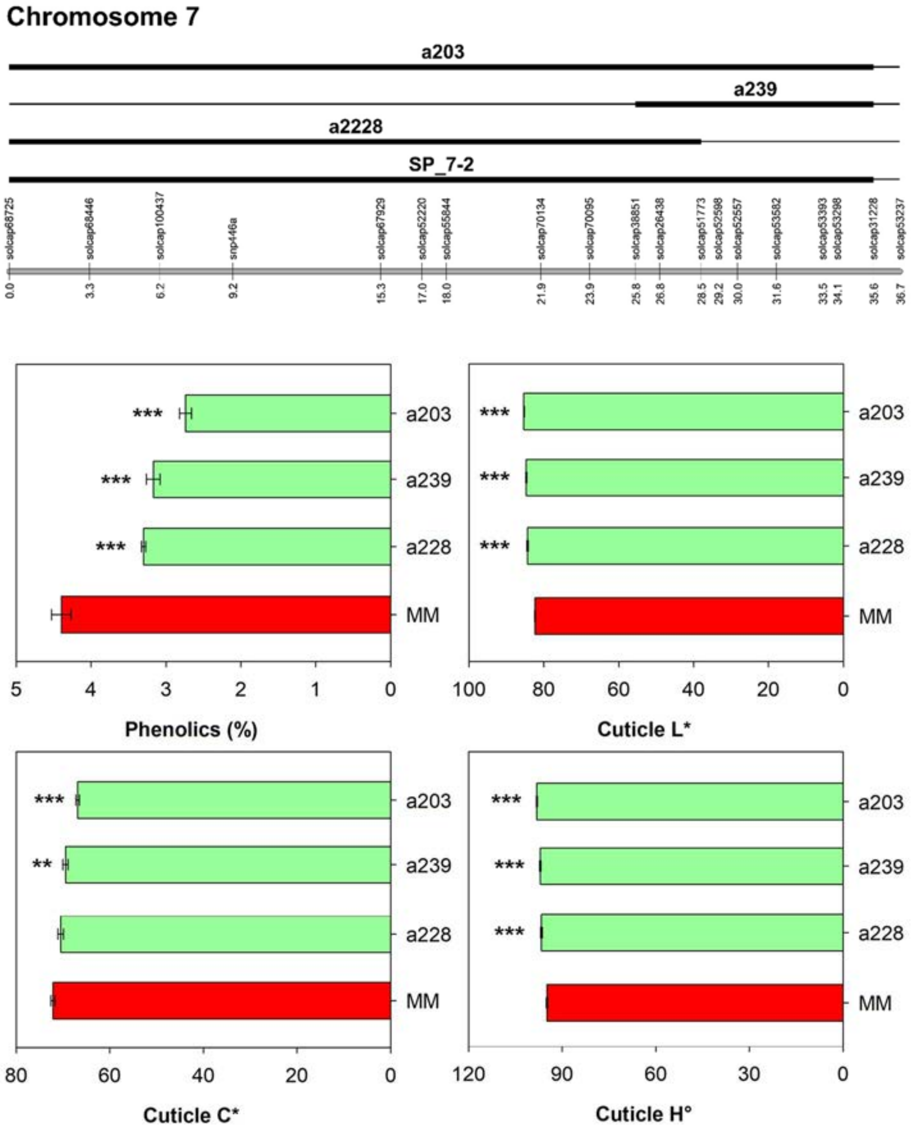


Fig. I-5: nSPs generated for chromosome 7. nSPs a203, a228 and a239 were generated from SP_7-2. Bold lines in the schematics represent TO-937 genome. nSPs, newly created SP lines. Data in graphs are presented as means ± SE. Asterisks indicate significant differences with MM (**$p \leq 0.01$; ***$p \leq 0.001$). L*, lightness; C*, chroma; H°, hue.

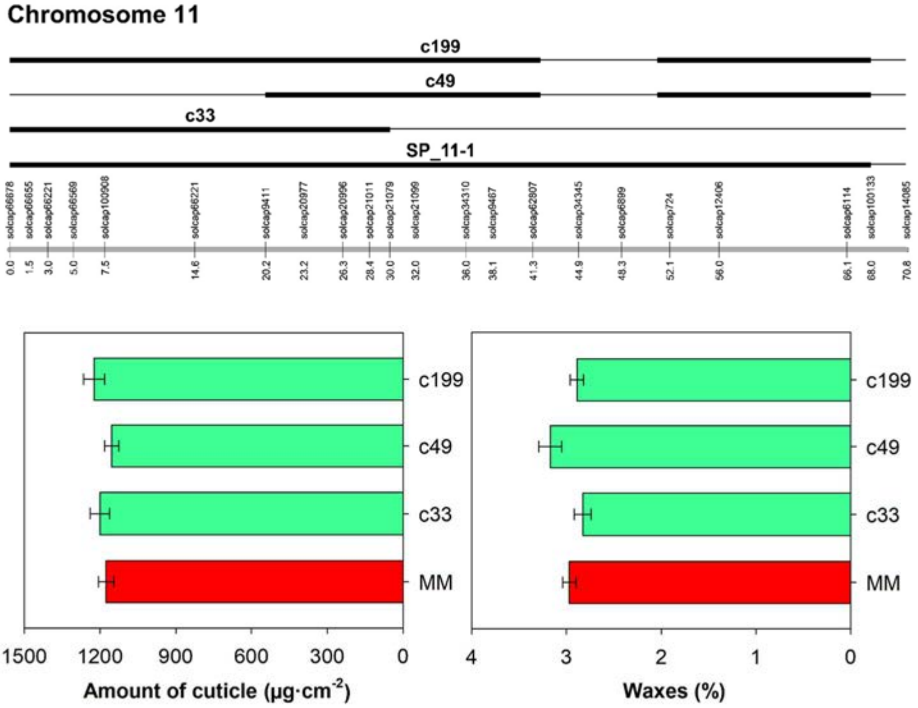


Fig. I-6: nSPs generated for chromosome 11. nSPs c33, c49 and c199 were generated from SP_11-1. Bold lines in the schematics represent TO-937 genome. nSPs, newly created SP lines. Data in graphs are presented as means ± SE.

3.5 Validation of the Identified QTLs

3.5.1 Amount of Cuticle

The highest effect on the amount of cuticle was observed in the SP line harboring *cm11.2* with a 2-fold reduction compared to MM, followed by *cm4.1* and the combination *cm3.1+cm3.2* (Fig. I-7). Each of the QTLs of chromosome 3 showed a similar reduction in cuticle, ~38%, whereas the combination showed a small additive effect, reducing 45% the amount of cuticle. On the other hand, the pim effect of *cm5.2* produced an increased 12-14% the amount of cuticle. Although *cm10.2* was not separated from *cm10.1*, comparison of *cm10.1+cm10.2* with *cm10.1* showed an increase in the amount of cuticle to levels almost similar to those of MM, indicating that the pim effect of *cm10.2* almost counteracted the lyc effect of *cm10.1*.

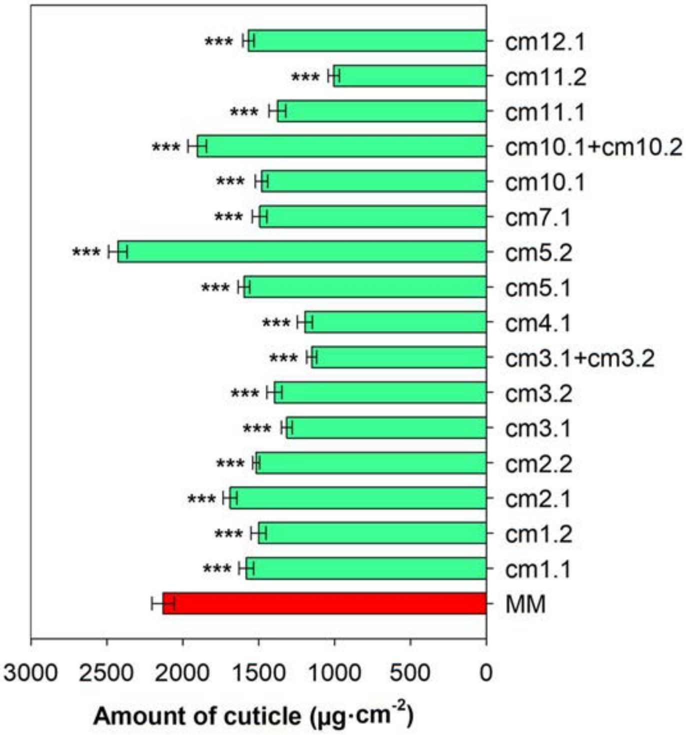


Fig. I-7: Comparison between the parental MM and the SP lines harboring QTLs implicated in the amount of cuticle. Red ripe fruits were analyzed. Data are presented as means ± SE. Asterisks indicate significant differences with MM (***$p \leq 0.001$). *cm*, QTL amount of cuticle.

3.5.2 Phenolics

Nine QTL regions, in chromosomes 1, 4, 5, 7, 8 and 12, were identified between the RIL and IL populations (Fig. I-8). Despite the very low percentage of phenolics present in TO-937 cuticle, four QTLs with pim effect were identified: *ph4.1*, *ph5.1*, *ph5.2* and *ph12.1*. The QTLs with pim effect, *ph4.1* and *ph12.1*, showed a 30 and 20% increase in phenolics, respectively, in comparison to MM. The other two, *ph5.1* and *ph5.2* did not show any difference with MM. Regarding the QTLs with lyc effect, the biggest reduction of phenolics, around 40%, was observed in *ph12.2* and the combined effect of *ph7.1+ph7.2*, whereas the remaining QTLs showed a similar reduction in the percentage of phenolics at red ripe. Surprisingly *ph1.1*, despite being the major QTL, showed an intermediate reduction in phenolics, comparable to *ph7.1*, *ph7.2* and *ph8.1*. A noticeable additive effect was observed for the QTLs located in chromosome 7, with the combination *ph7.1+ph7.2* displaying a much lower percentage of phenolics than *ph7.1* or *ph7.2*.

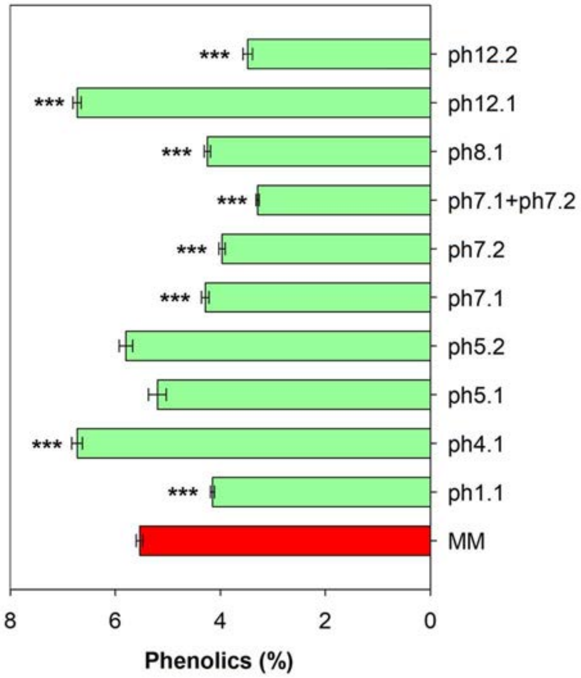


Fig. I-8: Comparison between the parental MM and the SP lines harboring QTLs implicated in the % of phenolics. Red ripe fruits were analyzed. Data are presented as means ± SE. Asterisks indicate significant differences with MM (***$p \leq 0.001$). *ph*, QTL percentage of phenolics.

3.5.3 Waxes, Cutin and Polysaccharides

Nine QTLs were identified for the percentage of waxes, six with pim effect and 3 with lyc effect. Only *wx4.1* and the combination *wx3.1+wx3.2* showed a significantly higher percentage of waxes than MM (Fig. I-9), whereas lines carrying *wx3.1* or *wx3.2* did not show any significant effect on waxes. Again, the line harboring *wx1.1* did not show any difference with MM for waxes, and the two other QTLs with pim effect, wx*11.2* and wx*12.2* showed an opposite effect. The biggest reduction in waxes was detected in the lines carrying *wx10.1*, *wx11.1* and *wx12.1*, with *wx11.2* and *wx12.2* showing quite similar reduction in waxes.

As it was previously observed, despite the identification of a QTL for percentage of cutin in chromosome 1, the corresponding SP line had values similar to MM and only the QTLs *cu10.1* and *ps10.1* showed significant differences with MM (Fig. I-9).

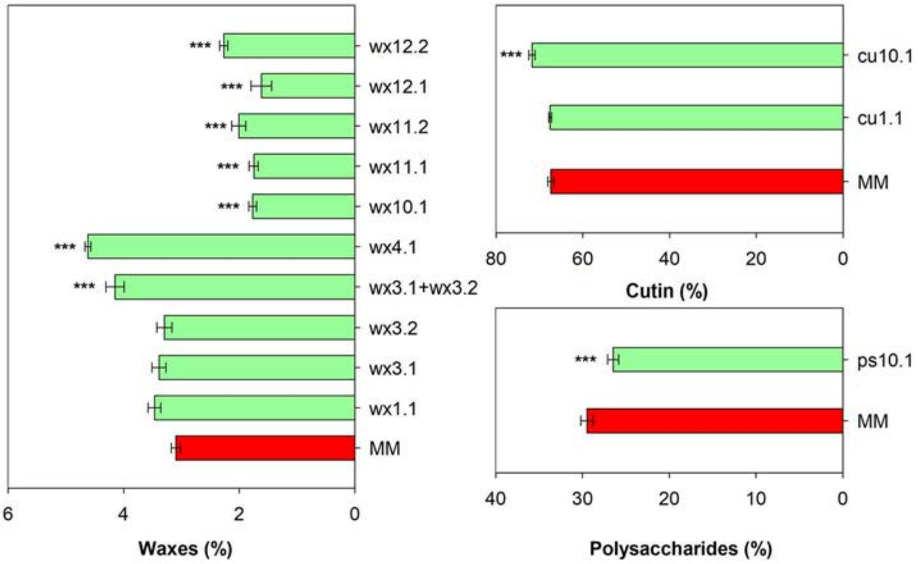


Fig. I-9: Comparison between the parental MM and the SP lines with QTLs related to the percentage of cutin, polysaccharides and waxes. Red ripe fruits were analyzed. Data are presented as means ± SE. Asterisks indicate significant differences with MM (****p* ≤ 0.001). *cu*, QTL percentage of cutin; *ps*, QTL percentage of polysaccharides; *wx*, QTL percentage of waxes.

3.5.4 Cuticle Color

A total of twenty-four QTLs were identified for the cuticle color traits between both populations (Fig. I-10). It should be noted that cuticle color QTLs colocalized with most of the QTLs identified for cuticle phenolics, except for *ph12.1*, and with the two QTLs for the amount of cuticle with highest additive effect, *cm3.2* and *cm11.2* (Fig. I-11). In this sense, the highest increase in L*, around 8%, was observed for *L11.1* and *L12.1*, which coincides with the lines harboring *cm11.2* and *ph12.2* with lowest amount of cuticle and percentage of phenolics, respectively (Fig. I-7, I-8 and I-10). The rest of the QTLs with lyc effect showed similar increases. *L4.1* showed a decrease in L* that coincided with the already mentioned increase in phenolics. The lowest decrease in C* (~11%) was observed for *C12.1* while the rest of the lines showed similar reductions. No differences with MM were detected for *C3.1* or *C4.1*, despite the later showing significant differences in the previous analysis. Regarding H°, the highest increase was observed for *H12.1* and the combined effect of *H7.1+H7.2* followed by *H11.1* while the rest of the lines with lyc effect showed similar values. In this case the significant decrease previously detected for *H4.1* was also observed. Study of the individual and combined QTLs for color present in chromosome 7 only showed additive effect for H° but not the other color parameters.

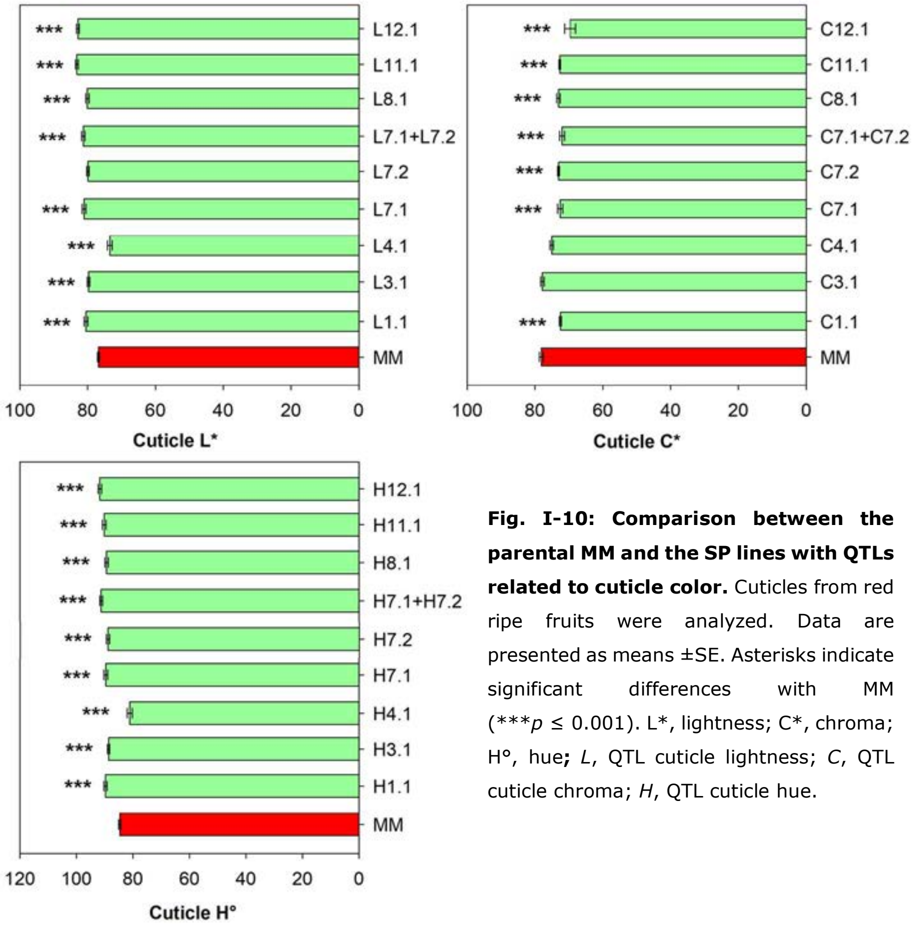
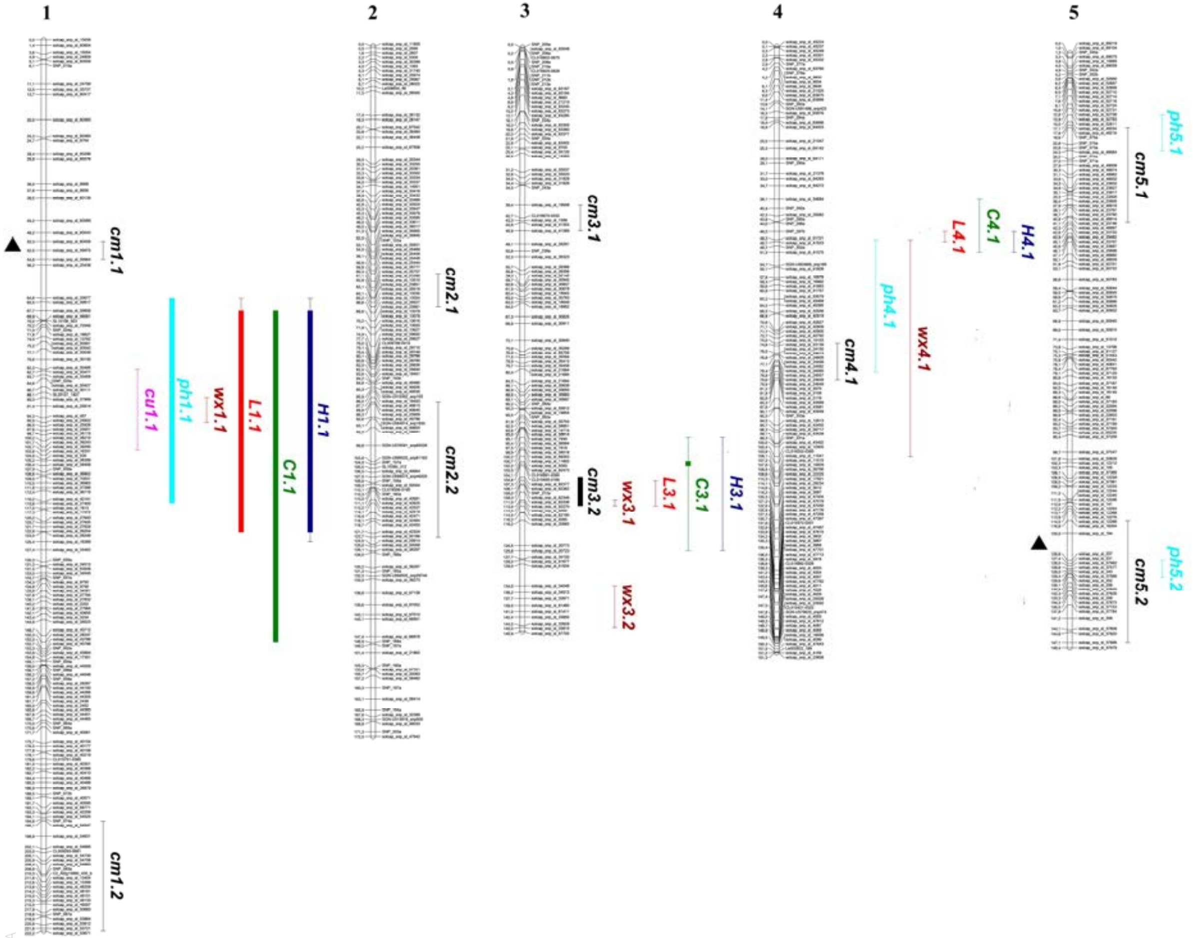


Fig. I-10: Comparison between the parental MM and the SP lines with QTLs related to cuticle color. Cuticles from red ripe fruits were analyzed. Data are presented as means ±SE. Asterisks indicate significant differences with MM (***$p \leq 0.001$). L*, lightness; C*, chroma; H°, hue**;** *L*, QTL cuticle lightness; *C*, QTL cuticle chroma; *H*, QTL cuticle hue.

As a summary, Fig.I-11 shows a compilation of all the genomic regions identified that are involved in the different cuticle traits studied.


1
2
3
4
5
cm1.1
cu1.1
ph1.1
wx1.1
L1.1
C1.1
H1.1
cm1.2
cm2.1
cm2.2
cm3.1
cm3.2
wx3.1
L3.1
C3.1
H3.1
wx3.2
cm4.1
ph4.1
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cm5.1
ph5.2
cm5.2

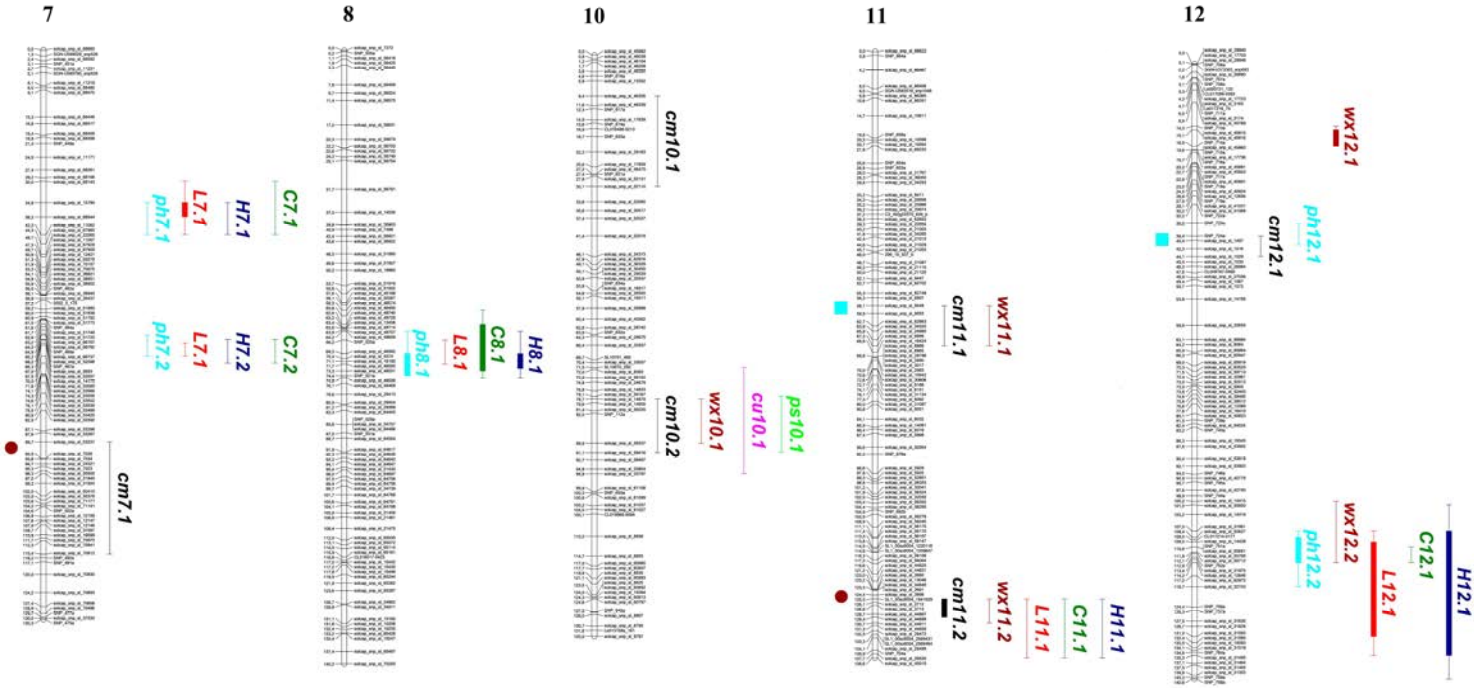

Fig. I-11: Map of the QTL regions identified in the RIL and IL populations for the different cuticle related traits. Bold lines represent QTL regions with $p < 0.001$. Symbols with the same shape and color located to the left of chromosomes denote regions with epistatic interactions. Symbol color indicates the trait. Numbers to the left of chromosomes indicate genetic distances expressed in cM (Kosambi mapping function). RIL, recombinant inbred line; IL, inbred line; *cm*, QTL amount of cuticle; *cu*, QTL percentage of cutin; *ps*, QTL percentage of polysaccharides; *ph*, QTL percentage of phenolics; *wx*, QTL percentage of waxes; *L*, QTL cuticle lightness; *C*, QTL cuticle chroma; *H*, QTL cuticle hue.

3.6 Analysis of the Genomic Regions

Genes located within each QTL region were investigated to identify candidate genes that were reported in the literature to affect cuticle synthesis (Tab. I-4). Surprisingly, only a few cuticle genes were identified in the QTLs. Despite the overlap between *ph1.1* and *wx1.1*, each showed a different peak marker. *MYB12*, a transcription factor known to affect the phenolic metabolic pathway was closest to the *ph1.1* and *cu1.1* peak marker whereas *LONG CHAIN ACYL SYNTHETASE 1* (*LACS1*), a gene involved in wax and cutin biosynthesis, was closer to the *wx1.1* peak. Several *β-KETOACYL-COENZYME A SYNTHASE* (*KCS*) genes, involved in the elongation of very long chain fatty acids, were identified in the QTLs. *ECERIFERUM6* (*CER6*) was located in the peak of *cm2.2*, *FIDDLEHEAD* (*FDH*) and *KCS11-like1*, a gene very similar to *A. thaliana KCS11*, were located in the *ph8.1* peak marker or close to it, respectively; also, *KCS11-like2* was located within *wx12.1*. Additionally, putative tomato orthologs of *A. thaliana* wax genes were also identified in QTLs related to the amount of cuticle, although far from the peak markers.

Comparative expression analyses of some putative candidate genes related to waxes and phenolics of MM and the corresponding SP lines are shown in Fig. I-12. The SP line harboring *ph1.1* showed a drastic decrease of *MYB12* expression during ripening compared with MM, 2.8-fold at breaker and 32-fold at red ripe. *LACS1* only showed a small decrease at breaker followed by an 8.7-fold reduction at red ripe. *FDH* expression in MM increased during growth with a notable maximum at breaker whereas in the SP line with *ph8.1* the expression level did not change with growth but displayed a 5-fold decrease in expression at breaker compared to MM. On the other hand, the *ph8.1* line showed a small increase of *KCS11-like1* expression compared to MM during ripening. The SP line containing *wx12.1* showed an increased expression of *KCS11-like2* compared to MM, especially during the whole fruit growth period until breaker.

Tab. I-4: Cuticle related genes reported in the literature identified in the QTL regions. Genes located on the peak marker, or <5 genes apart from the peak marker, are shown in bold. Underlined genes are located 10-20 genes far from the peak marker. Genes in regular case are located >20 genes apart from the peak marker. When more than on QTL is shown, the gene/s indicated are located within the overlapping regions. *cm*, QTL amount of cuticle; *ph*, QTL percentage of phenolics; *cu*, QTL percentage of cutin; *wx*, QTL percentage of waxes; Arabidopsis, *Arabidopsis thaliana*; tomato, *Solanum lycopersicum*.

QTL	Gene symbol	Gene locus	Species	Literature
ph1.1/wx1.1/cu1.1	***LACS1***	Solyc01g079240	Arabidopsis	Lü *et al.* (2009)
	MYB12	Solyc01g079620	tomato	Adato *et al.* (2009)
ph1.1	*DET1*	Solyc01g056340	tomato	Davuluri *et al.* (2005)
cm1.2	*CER9*	Solyc01g107880	Arabidopsis	Lü *et al.* (2012)
	LACS2	Solyc01g109180	Arabidopsis	Schnurr *et al.* (2004)
cm2.2	***CER6***	Solyc02g085870	tomato	Leide *et al.* (2007)
	CER13	Solyc02g086500	Arabidopsis	Rashotte *et al.* (2001)
ph4.1/wx4.1	*PAS2*	Solyc04g014370	Arabidopsis	Bach *et al.* (2008)
cm5.1/ph5.1	*GL1*	Solyc05g008250	Arabidopsis	Xia *et al.* (2010)
cm5.2	*CER10*	Solyc05g054490	Arabidopsis	Rashotte *et al.* (2001)
	CYP77A6	Solyc05g055400	Arabidopsis	Li-Beisson *et al.* (2009)
cm7.1	*TAGL1*	Solyc07g055920	tomato	Giménez *et al.* (2015)
ph8.1	***FDH***	Solyc08g067260	Arabidopsis	Voisin *et al.* (2009)
	KCS11-like 1	Solyc08g067410	tomato	-
wx12.1	*KCS11-like 2*	Solyc12g006820	tomato	-

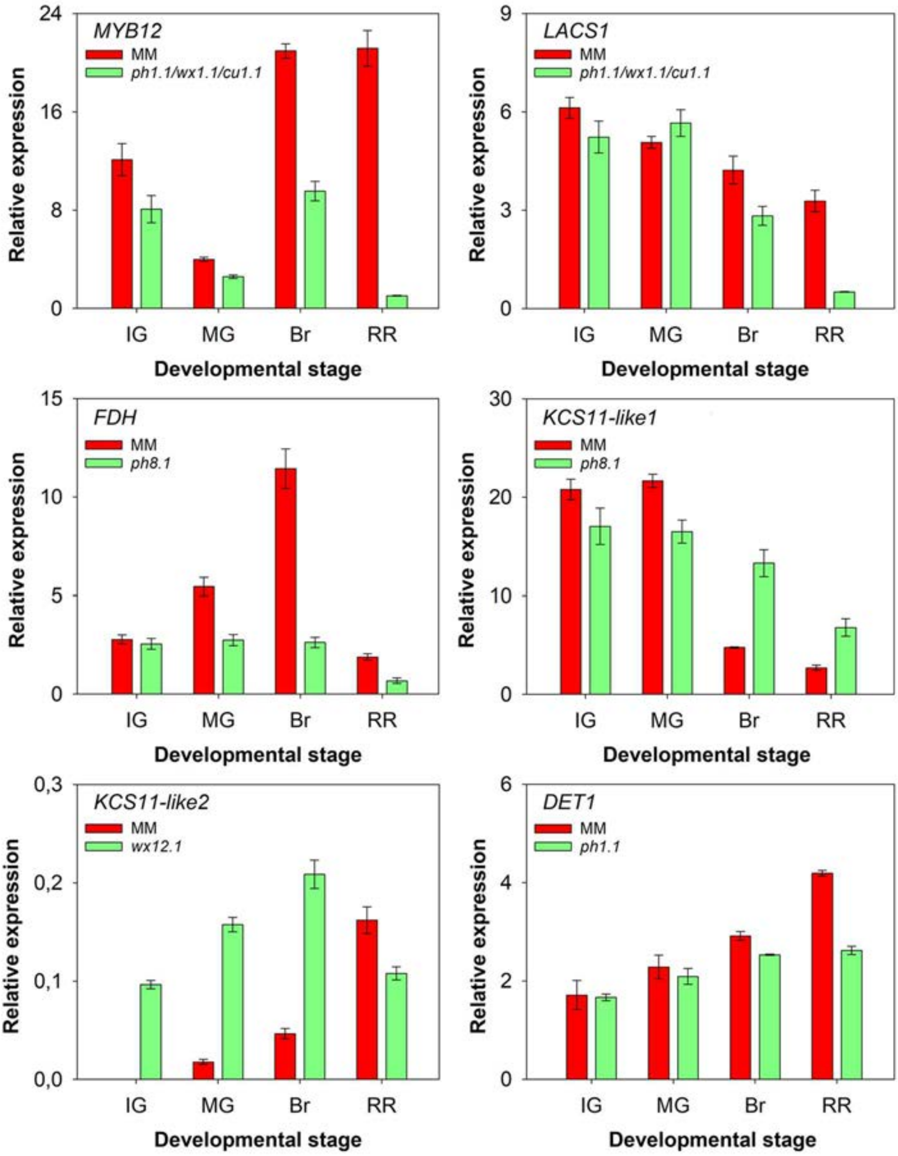

Fig. I-12: Relative expression throughout epicarp development. MM and the SP lines containing the QTLs *ph1.1*/*wx1.1*/*cu1.1*, *ph8.1* and *wx12.1* were analyzed for the putative candidate genes *MYB12*, *LACS1*, *FDH*, *KCS11-like1*, *KCS11-like2* and *DET1*. Data are presented as means ± SE. IG, immature green; MG, mature green; Br, breaker; RR, red ripe; *cu*, QTL percentage of cutin; *ph*, QTL percentage of phenolics; *wx*, QTL percentage of waxes.

Inspection of changes in the mRNA sequences for these genes between the parental lines showed that the differences present produced conserved protein changes, with the only exception of *LACS1* with a L183 (MM) to S183 (TO-937) change.

The SP line harboring the pim allele of *MYB12*, a gene known to significantly reduce cuticle phenolics (Adato *et al.*, 2009), did not show the highest reduction in phenolics, as it would be expected from the results of the QTL analysis. Therefore, it is possible that an additional gene/s within the QTL is modulating its effect. Expression analysis of *DE-ETIOLATED1* (*DET1*), a gene that has been reported to increase phenolic accumulation in tomato fruits and is also located within *ph1.1*, was also studied. However, only a 1.6-fold reduction was detected at red ripe between MM and the SP line harboring *ph1.1* (Fig. I-12). Further studies were carried out related to the potential role of *MYB12* in changes on the percentage of waxes and cutin, two QTLs that could not be validated with the SP population. Hence, red ripe cuticles of Ailsa Craig and the isogenic line Ailsa Craig *y/y* were analyzed (Tab. I-5). As it has been established, *MYB12* is the gene responsible for the *colorless fruit epidermis* "*y*" mutation in Ailsa Craig (Adato *et al.*, 2009). The mutant showed a significantly lower percentage of cutin and an increase in polysaccharides and waxes. As it was already reported (Adato *et al.*, 2009), the percentage of cuticle phenolics displayed a 10-fold reduction in the mutant in comparison with the wildtype.

Tab. I-5: Percentages of cuticle components present in Ailsa Craig wt and Ailsa Craig *y/y*. Red fruits were analyzed. Data are presented as means ± SE. Asterisks indicate significant differences ($p \leq 0.001$). wt, wild type; *y*, *colorless fruit epidermis* mutation.

Component	Ailsa Craig wt	Ailsa Craig *y/y*
% cutin	67.71 ± 0.12	56.95 ± 0.77 ***
% polysaccharides	29.21 ± 0.09	38.85 ± 0.77 ***
% waxes	3.07 ± 0.02	4.37 ± 0.04 ***
% phenolics	6.91 ± 0.30	0.62 ± 0.07 ***

Some genes related to cuticle waxes have been reported to be upregulated in tomato fruit peel at breaker in different *MYB12* mutant alleles (Adato *et al.*, 2009; Fernández-Moreno *et al.*, 2016). To investigate if the observed downregulation of the pim allele of *MYB12* was enough to differentially express these genes, their expression throughout was studied in MM and the

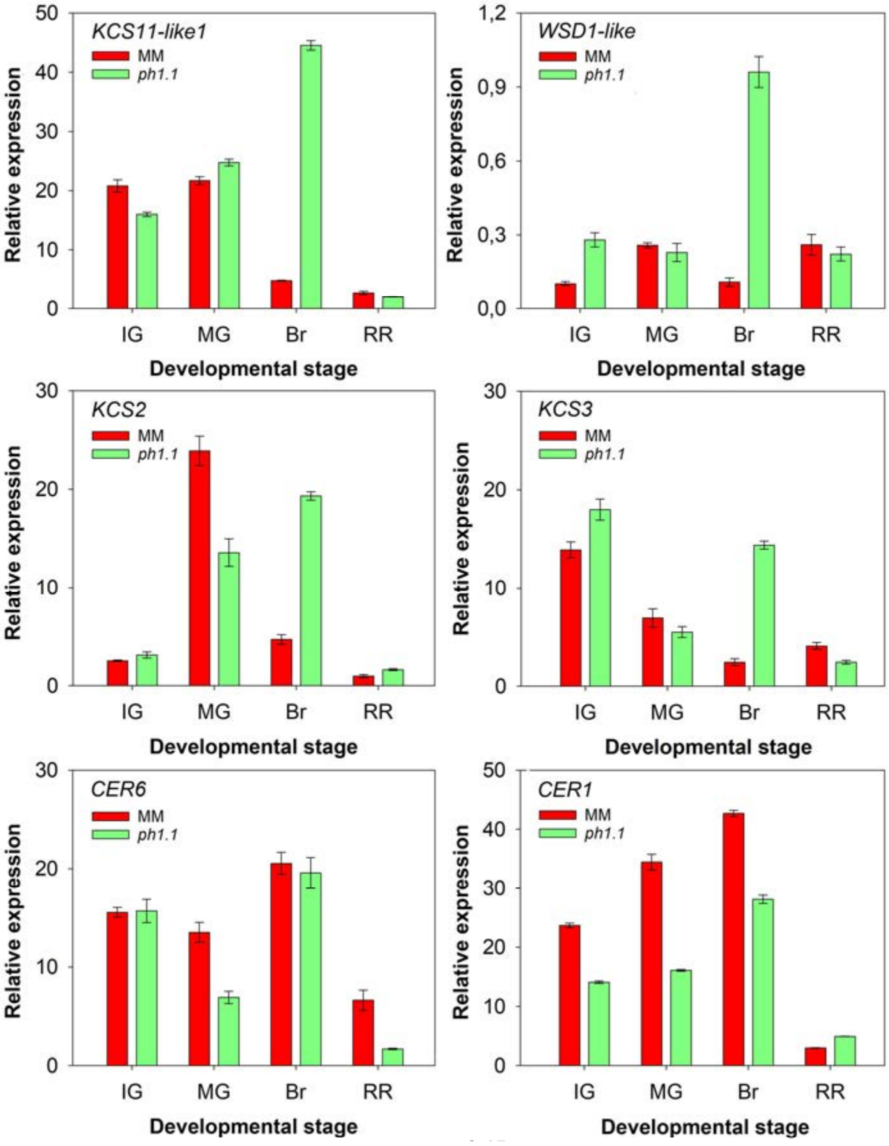

Fig. I-13: **Relative expression throughout epicarp development.** MM and the SP line containing *ph1.1* were analyzed for the genes putatively related to cuticle wax biosynthesis that are over or under-expressed in several pink tomato mutants. *KCS11-like1*, *CER6*, *WSD1*, *WSD1-like1*, *WSD1-like2*, *CER1*, *KCS2*, and *KCS3*. Data are presented as means ± SE. IG, immature green; MG, mature green; Br, breaker; RR, red ripe; *ph*, QTL percentage of phenolics.

line harboring *ph1.1*/*wx1.1*/*cu1.1* (Fig. I-13). *KCS11-like1*, *KCS2*, *KCS3* and *WDS1-like*, a gene very similar to the *A. thaliana WSD1* (*WAX ESTER*

SYNTHASE/ACYL-CoA:DIACYLGLYCEROL ACYLTRANSFERASE), showed a transient increase in their expression at breaker. On the other hand, no increase in *CER6* was observed and *CER1* showed lower expression than MM throughout development.

4. DISCUSSION

4.1 Natural Variation of Tomato Fruit Cuticle Shows a Combination of Additive and Epistatic Traits

A comprehensive cuticle analysis has been carried out in two populations RIL and SP inbred line, thus favoring the detection of additional QTLs that were not significant or present in the RIL population. Understanding the interaction of these epistatic QTLs with the genetic background is important in plant breeding for the introgression of specific traits. A combination of additive and epistatic interactions was detected for all the traits analyzed. Moreover, the transgressive inheritance found in the RIL population for most of the characters analyzed indicated the presence of QTLs with a positive contribution to the trait in both parents. Of the twenty-two genomic regions identified spanning ten of the twelve chromosomes, some were specific and only affected one or two traits and others were more complex and produced global changes comprising many of the parameters studied. Uncovering the complex network of genetic interactions responsible for cuticle synthesis allows for a better understanding of its natural variability, provides an insight into the evolution of the traits and their potential for breeding purposes.

QTL analysis of a tomato IL population derived from the interspecific cross between cultivated tomato and the green-fruited species *S. pennellii* found three QTLs for the amount of specific wax and cutin components and another one for cuticle thickness (Fernández-Moreno *et al.*, 2017). Except for the thickness QTL, none of the other regions coincide with any of the QTLs herein identified. The thickness QTL coincided with the *cm7.1* region found here. *TOMATO AGAMOUS-LIKE1* (*TAGL1*) was postulated as one of the possible candidate genes for this trait (Fernández-Moreno *et al.*, 2017). Indeed, modification of *TAGL1* expression in tomato has been shown to lead to significant changes in cuticle mass, thickness and invagination (Giménez *et al.*, 2015).

Of the numerous known genes involved in phenolic synthesis and regulation only *MYB12* and *DET1* were located within one of the QTLs found for cuticle phenolics. Similarly, several genes have been found to participate in cuticle deposition (Hen Avivi *et al.*, 2014) however, none of these genes locate within the QTLs identified for any of the cuticle traits herein studied. Several genes involved in wax accumulation in *A. thaliana* were identified in QTLs related to cuticle deposition (Tab. I-4). Only two genes, *LACS2* and the cytochrome P450 monooxygenase *CYP77A6*, reported to alter cutin deposition in *A. thaliana* were located within cuticle QTLs. However, since none of these genes are located close to the QTL peak markers, their role as candidate genes needs to be further analyzed. Three *KCS* genes were identified in the peak markers of QTLs unrelated to *waxes*, *cm2.2* and *ph8.1*. In tomato, *cer6* mutant has been shown to affect the amount of cuticle and reduce the synthesis of *n*-alkanes while increasing triterpenoids without altering the total amount of waxes (Leide *et al.,* 2007). This would agree with its role as candidate gene for *cm2.2*. Of the two *KCS* located around *ph8.1* peak marker, the notable reduction of *FDH* expression during ripening in the TO-937 allele indicates that this is the most likely candidate gene. Indeed, the maximum expression at breaker coincided with the massive incorporation of phenolics to the cuticle, although waxes also show a comparatively modest increase during ripening in MM (España *et al.,* 2014a). Mutation of *FDH* in *A. thaliana* displayed an increase in cutin and waxes (Voisin *et al.,* 2009) that was not observed in the *ph8.1* line. This apparent unlikely role of a *KCS* in cuticle phenolics could be associated with transport. It is still unknown how phenolics are transported to the cuticle, although there are several reports of phenolics esterified to cutin and waxes (Karabourniotis and Liakopoulos, 2005; Leide *et al.,* 2018). It is plausible that they are transported already esterified to a fatty acid instead of the bonding occurring within the cuticle matrix. In this context, *FDH* could be involved in the synthesis of the fatty acid domain. Putative orthologs of *KCS11* have been postulated as candidate genes for wax accumulation in eucalypt and banana leaves (Gosney *et al.,* 2016; Sampangi-Ramaiah *et al.,* 2016). The notably increased expression throughout development of the TO-937 allele of *KCS11-like2* and the reduction in waxes observed in the *wx12.1* line suggests that this gene could be indirectly involved in wax synthesis through the amount of substrate available for waxes. In this regard, the ubiquitous and relatively low expression of *KCS11* in *A. thaliana* has been suggested as an indication of a general role in the synthesis of very-long-fatty acids required for growth and development (Kim *et al.,* 2013).

4.2 Genetic Interaction Between Cuticle Phenolics and Waxes

Cuticle phenolics and waxes play important biophysical roles that have great agronomic importance for fruit growth on the vine and also during postharvest (Lara *et al.,* 2019). However, a negative correlation between cuticle waxes and phenolics, based on the modification of the phenolic metabolic pathway, either silencing *CHS*, the first committed enzyme of the flavonoid pathway, or favoring the accumulation of anthocyanins in the fruit by expression of the transcription factors *ROSEA* and *DELILA*, has been reported (Heredia *et al.,* 2015). The nature of this relation could have a critical impact in plant breeding since it would imply the necessity to choose between increasing mechanical resistance provided by phenolics and reduced water permeability driven by waxes. A small but significant correlation was observed within the RIL population ($r= -0.275$, $P < 0.01$) between the percentage of phenolics and waxes. Analysis of the colocation of waxes and phenolics indicate that this relation is based on the QTLs present in chromosome 1 and 12 with overlapping regions for both compounds. However, identification of QTLs that modify either phenolics or waxes, such as *ph7.1*, *ph7.2*, *ph8.1* and *wx12.1*, indicates that there are alternative genetic strategies to avoid a compromise between increased phenolics and waxes. Moreover, colocation of QTLs with similar effect for waxes and phenolics on chromosome 4 suggests a complex interaction between these pathways. In this sense, it has recently been reported a Kelch repeat F-box protein (SMALL AND GLOSSY LEAVES1) that negatively regulates both wax and phenylpropanoid biosynthesis in *A. thaliana* (Kim *et al.,* 2019; Yu *et al.,* 2019).

MYB12 was identified as the candidate gene behind *ph1.1* since a notable downregulation of the pim allele was detected during ripening, coinciding with the phenotypic differences observed in the cuticle. Cuticle analysis of Ailsa Craig *y/y* showed a significant decrease in phenolics and cutin and increase in waxes and polysaccharides compared to the wildtype (Tab. I-5), in agreement with the overlap of the QTLs for phenolics, waxes and cutin and the almost significant QTL for polysaccharides present in chromosome 1. The reported increase in various *y* mutant alleles of several enzymes related to wax biosynthesis during ripening (Adato *et al.*, 2009; Fernández-Moreno *et al.,* 2016) supports a role of *MYB12* in regulating the phenolic and fatty acid biosynthetic pathways and would explain the percentual differences observed in Ailsa Craig *y/y* for all cuticle components. However, it does not explain the absence of differences in waxes and cutin observed in the line harboring *MYB12*, especially since most of these wax-related genes were also upregulated. Also, the reduced effect on phenolics (Fig. I-8) observed for the line containing the pim allele of *MYB12*, despite *ph1.1* being the major QTL with the highest additive effect, could be the result of an interaction between *MYB12* and other region/s within this wide QTL that could be masking its individual effect. In this regard *DET1*, a regulator of light signal transduction whose downregulation increases flavonoid accumulation in tomato fruits, is the only gene with a known effect in the phenolic pathway located within *ph1.1* (Davuluri *et al.,* 2005). However, *DET1* expression only showed a small decrease at red ripe and it is unclear if it could be enhancing phenolic accumulation. This lesser than expected effect on phenolics could be behind the absence of differences for waxes and cutin observed in the line harboring the overlapping QTLs *ph1.1*, *wx1.1* and *cu1.1* and would imply that a certain degree of phenolic reduction is needed for the effect on lipids to be significant. Additionally, an interaction between *MYB12* and *LACS1*, the gene closest to *wx1.1* peak marker, could also explain the results on waxes and cutin. Although *LACS1* expression has not been reported to be modified in the transcriptomic analysis of "*y*" mutants (Fernández-Moreno *et al.,* 2016), the observed downregulation of the *LACS1* pim allele during ripening indicates a potential role as candidate gene for waxes and cutin. Analysis of the *A. thaliana LACS* family has uncovered a complex network of redundant activities, with overlapping functions of *LACS1* and *LACS2* on cutin and wax biosynthesis and *LACS1* and *LACS4* on wax biosynthesis (Lü *et al.,* 2009; Jessen *et al.,* 2011); hence the absence of effect on waxes and cutin could also be due to the involvement of other *LACS* genes. Individualized analysis of both genes and potential compensating roles of other *LACS* genes needs to be addressed to clarify their effects.

4.3 The Role of Phenolics in Cuticle Color

From all the cuticles from different species studied thus far, tomato fruit cuticle is the only one reported that exhibits a special characteristic that is, a significant change from a cream color during growth to orange-yellow at ripe (España *et al.*, 2014a,b). This change coincides with the ripening process. Naringenin chalcone has been shown to be responsible for the orange-yellow color of the ripe tomato fruit cuticle and, in turn, for the red color of ripe tomatoes (Adato *et al.,* 2009). Pink tomatoes do not accumulate this flavonoid at ripe and their cuticles display the same cream color as prior to ripening (Adato *et al.,* 2009). Colocalization of cuticle color and phenolic QTLs was observed in chromosomes 1, 4, 7, 8 and 12 and their effects were similar that is, a decrease in phenolics was accompanied by an increase in L* and H° and a decrease in C*, implying more lightness, less saturation and a shift from orange towards a yellow-cream hue. This supports the significant correlation observed between percentage of phenolics and L* (r = -0.7.01 **), C* (r = 0.668 **) and H° (r = -0.738 **) observed in the RIL population. However not all the QTLs for phenolics colocalized with color QTLs. This was the case of *ph12.1*, *ph5.1* and *ph5.2*, all with pim effect. Whereas no effect was observed in the SP population for *ph5.1* and *ph5.2*, *ph12.1* showed a significantly higher phenolic content. It has been reported an increase in colorless phenolic acids, mainly *p*-coumaric and *p*-hydroxy benzoic acids, as well as naringenin chalcone during ripening in tomato fruit cuticle (España *et al.*, 2014a). Thus, it is possible that *ph12.1* increases the accumulation of phenolic acids without significantly modifying that of naringenin chalcone. This would be in contrast with *ph4.1* where the increase in phenolics was associated with a lower lightness and more orange hue, most probably due to a significant increase in naringenin chalcone. Regarding the absence of effect of *ph5.1* and *ph5.2* in the SP population despite being significant in the RIL population QTL analysis, it could be that these QTLs interact epistatically with other genomic regions, what will be analyzed in Chapter II.

Besides phenolics, cuticle color QTLs colocalized with the two major cuticle QTLs, *cm11.2* and *cm3.2* thus indicating that the amount of cuticle contributes to color as well. Nevertheless, this relationship between the amount of cuticle and color is not straightforward since not all the QTLs that reduced or increased the amount of cuticle influenced color. It is probable that there is a minimum of cuticle that needs to be decreased (or even increased) before displaying changes in color. In this sense it is interesting to point out the effect of the line carrying *ph4.1*/*cm4.1*/*wx4.1*/*L4.1*/*C4.1*/*H4.1*. In this line the reduction of cuticle is slightly higher than that observed in *cm3.2* (Fig. I-7) and yet the increase in phenolics counteracted the putative effect of the decrease of cuticle and still displayed changes to darker and more orange cuticle color. Comparison of the effect of the amount of cuticle and percentage of phenolics on cuticle color indicates that, broadly, QTLs with the highest effect in phenolics, *ph12.2* and *ph7.1*+*ph7.2*, showed the highest effect in color, whereas the rest had a similar effect to the amount of cuticle QTLs. Yet, as in the case of phenolics, none of the QTLs for color exhibited values close to those of TO-937.

Tomato fruit color is an important quality trait since it is associated with the accumulation of lycopene in the flesh and flavonoids in the peel, including the cuticle. Pink tomatoes are the result of the lack of naringenin chalcone accumulation during ripening (Adato *et al.*, 2009; Ballester *et al.*, 2009; España *et al.*, 2014b). However, the impact on fruit color of a reduction or increase in naringenin chalcone has not been addressed. A few QTLs related to external fruit color have been reported in the SP population here employed (Barrantes *et al.,* 2016). Only one of these QTLs was located in a line (SP_7-3) that also carries two of the phenolic QTLs here identified, *ph7.1+ph7.2*. Yet, this external fruit color QTL, *ec_a*7.1*, did not translate into differences in the CIE L*C*H° color space. This could be related to the fact that the CIE L*a*b* is a poor uniform color space for small color differences (Luo *et al.*, 2001). From a breeder's perspective, it is important to know whether there are genetic factors (genes) other than carotenoids that could visually modify fruit color appearance. Identifying such genetic factors or QTLs for fruit color properties will provide tomato breeders with new information on an important fruit quality trait.

CHAPTER II:

QUANTITATIVE TRAIT LOCI PYRAMIDING FOR CUTICLE FLAVONOIDS RELATED TRAITS

1. INTRODUCTION

Tomato fruit flavonoids accumulate predominantly in the tomato fruit peel (Hunt and Baker, 1980). The most abundant one is naringenin chalcone. It accumulates to approximately 1% of the peel dry weight during ripening (Muir *et al.,* 2001) and it is responsible for the orange-yellow color that displays the cuticle at red ripe (Adato *et al.,* 2009). Naringenin chalcone is the only flavonoid that is transported and incorporated to the outer cuticle matrix (Hunt and Baker, 1980; Slimestad and Verheul, 2009). The synthesis of cuticle flavonoids related to the nutritional and organoleptic quality of the tomato fruit is the result of coordinated activities that involve many of the primary and secondary metabolism pathways regulated by developmental, physiological and environmental signals. It is therefore necessary to identify genes or QTLs that regulate cuticle flavonoids pathways and thus regulate processes important for tomato agronomic traits. Many studies have demonstrated the contribution of cuticle flavonoids in several important and complex agronomic traits including fruit stiffness (Matas *et al.,* 2004; España *et al.,* 2014a), the final color of the tomato fruit (Adato *et al.,* 2009), fruit dullness (Verhoeyen *et al.,* 2002), and many nutritional and organoleptic quality traits that have big impact on the consumer purchase decision. Consequently, fruit quality in terms of both nutritional and organoleptic has become a major focus of most traditional and innovative tomato breeding approaches. Such traits usually exhibit quantitative variation influenced by environmental conditions and controlled by several genes that are difficult to identify (Schauer *et al.,* 2008).

Previously (see chapter I) we identified, localized and validated nine QTLs for the percentage of phenolics, chromosomes 1, 4, 5, 7, 8 and 12, and estimated their additive effects. That study showed that no SP line appeared to decrease the cuticle phenolic content to the level of the lower-flavonoid content parent (TO-937) and a few SP lines were found to be superior to the higher flavonoid content parent (MM), indicating the complex inheritance of the traits. In this sense, genome-wide analysis of epistatic QTLs in the RIL population using ICImapping software rendered just a few significant epistatic QTL regions (see Fig. I-3). However, additional epistatic effects would be needed to explain the wide segregation observed in the RIL population. Therefore, the study of combined QTLs will allow the detection of new interactions between genomic regions.

To further understand the genetics of cuticle phenolics, we pyramided several of the phenolic QTL alleles of the accession of *S. pimpinellifolium* into the genetic background of MM. The development of modern plant molecular techniques and quantitative genetics in the last two decades has dramatically widened the applicability of gene pyramiding. Our pyramiding strategy consisted of combining more than one QTL for cuticle phenolics in the genetic background of the cultivated tomato MM to produce new genetic materials with higher or lesser cuticle flavonoid content in comparison with MM. It is a useful method in applied breeding to assemble multiple desirable QTLs involved in many agronomic traits into a single genotype. The method is commonly used for self-pollinated crops to improve an existing elite cultivar through the introgression of a few genes of large effects from wild species. The validity of this approach for quantitative traits was first demonstrated by pyramiding two QTLs involved in plant height and grain number in rice (Ashikari *et al.,* 2005). The creation of new lines combining different QTL regions, SP_(QTL+QTL), and their phenotyping will improve our understanding of combined effects of the QTLs in the genetic background of the cultivated tomato. Moreover, the study of SP_(QTL+QTL) lines will help identify the effects, either additive or epistatic, of the combined QTLs on the amount and percentage of cuticle phenolics as well as on cuticle color.

2. MATERIALS AND METHODS

2.1 Plant Material and Greenhouse Experiments

Eight SP lines from the genomic library of ILs developed by Barrantes *et al.* (2014) carrying QTLs for cuticle phenolics were employed for the generation of homozygous lines combining two introgressions harboring QTLs for the percentage of phenolics. These SP lines (SP_1-2; SP_4-2; SP_5-3; SP_5-5; SP_7-3; SP_8-1; SP_12-2 and SP_12-4) were grown in spring-summer 2013 and manually cross-pollinated to obtain F_1 hybrids. A list of the crosses carried out is presented in Tab. II-1. In winter 2013, the resulting SP_(QTL+QTL)F_1 hybrids were grown and self-pollinated to obtain the corresponding F_2 individuals, SP_(QTL+QTL)F_2. Twenty-two SP_(QTL+QTL)F_1 and twenty-three SP_(QTL+QTL)F_2 were generated.

Tab. II-1: List of the SP_(QTL+QTL) lines accumulating QTLs for cuticle phenolics. Lines combining QTLs were obtained after crossing individual SP lines and marker selection of double or triple homozygous in the F_2 populations derived from each cross. QTL, quantitative trait loci; *ph*, QTL percentage of phenolics.

Season	Crosses	SP_(QTL+QTL)
Winter 2015	SP_1-2 x SP_8-1	SP_(*ph1.1*+*ph8.1*)
	SP_1-2 x SP_5-5	SP_(*ph1.1*+*ph5.2*)
	SP_4-2 x SP_5-3	SP_(*ph4.1*+*ph5.1*)
	SP_4-2 x SP_8-1	SP_(*ph4.1*+*ph8.1*)
	SP_4-2 x SP_7-3	SP_(*ph4.1*+*ph7.1*+*ph7.2*)
	SP_4-2 x SP_12-4	SP_(*ph4.1*+*ph12.2*)
	SP_5-3 x SP_1-2	SP_(*ph5.1*+*ph1.1*)
	SP_5-5 x SP_7-3	SP_(*ph5.2*+*ph7.1*+*ph7.2*)
	SP_7-3 x SP_8-1	SP_(*ph7.1*+*ph8.1*)
		SP_(*ph7.1*+*ph7.2*+*ph8.1*)
	SP_7-3 x SP_12-4	SP_(*ph7.1*+*ph7.2*+*ph12.2*)
	SP_8-1 x SP_5-3	SP_(*ph8.1*+*ph5.1*)
	SP_8-1 x SP_5-5	SP_(*ph8.1*+*ph5.2*)
	SP_8-1 x SP_12-4	SP_(*ph8.1*+*ph12.2*)
	SP_12-2 x SP_1-2	SP_(*ph12.1*+*ph1.1*)
	SP_12-2 x SP_5-3	SP_(*ph12.1*+*ph5.1*)
	SP_12-4 x SP_1-2	SP_(*ph12.2*+*ph1.1*)
	SP_12-4 x SP_5-3	SP_(*ph12.2*+*ph5.1*)
	SP_12-4 x SP_5-5	SP_(*ph12.2*+*ph5.2*)
Summer 2016	SP_4-2 x SP_1-2	SP_(*ph4.1*+*ph1.1*)
	SP_4-2 x SP_5-5	SP_(*ph4.1*+*ph5.2*)
	SP_5-3 x SP_7-3	SP_(*ph5.1*+*ph7.1*+*ph7.2*)
	SP_12-4 x SP_12-2	SP_(*ph12.2*+*ph12.1*)

In spring 2014, the SP_(QTL+QTL)F_2 populations were sown in seed plug trays of 51 cells, each of 60 mL depth. Two trays were sown per SP_(QTL+QTL)F_2. Trays were placed in a glasshouse and leaf samples from each plantlet were taken for DNA extraction and subsequent MAS. In summer 2014, the marker-selected plants from each SP_(QTL+QTL)F_2 population were transplanted to soil in a multi-tunnel polyethylene plastic house. Plants were vibrated daily to ensure fruit set. Some crosses could not be obtained in summer 2013 and the corresponding SP lines were grown again in summer 2014 to obtain the SP_(QTL+QTL)F_1 hybrids and subsequent SP_(QTL+QTL)F_2 in winter 2014. In spring 2015, seeds from these remaining SP_(QTL+QTL)F_2 were sown and the individuals carrying the desired combination of markers selected following the same procedure mentioned above. These marker selected plants were transplanted to soil and vibrated daily to ensure fruit set. These later obtained SP_(QTL+QTL) lines correspond to those shown in Tab.II-1 under the heading summer 2016.

The first set of SP_(QTL+QTL) lines was grown and analyzed in winter 2015 and the second one in summer 2016. In both instances the SP lines carrying phenolic QTLs, the nSP lines carrying *ph7.1* (a228) and *ph7.2* (a239) individualized, the corresponding SP_(QTL+QTL) lines, TO-937, and MM were sown. Fifteen plants per genotype and 30 for MM were grown in a multi-tunnel polyethylene plastic house. The lines were grown following a randomized complete design of three replications. Each replication consisted of rows and every row contained four blocks, each containing five plants of the same line. Three additional blocks of MM plants were randomly distributed. Plants were vibrated daily to ensure fruit set and flowers labelled at anthesis for the identification of the developmental stage. Fruits were harvested at all stages of development (IG, MG, Br and RR), except fruits from nSPs that were harvested at the RR stage only. A minimum of 30 fruits per line and developmental stage were collected except for TO-937 for which the number of collected fruits was around 100-150 fruits per stage. Fruits from the first truss were not harvested.

All the experiments described above were conducted in a multi-tunnel polyethylene plastic house 60 m long x 24 m wide. The within-row and between-row spacing in this greenhouse was 0.5 m and 1.5 m respectively. The glasshouse used as nursery was equipped with a fan and a pad evaporative cooling system to prevent excessive heat in summer and a water-heating system for winter. Plants were transplanted to soil at the four-true leaf growth stage.

2.2 Selection of the SP_(QTL+QTL) Lines

The twenty-three SP_(QTL+QTL) lines were selected by MAS using SNP markers resolved by HRM as following: SP line carrying QTL for the percentage of phenolics was crossed with another SP line harboring another QTL for the percentage of phenolics (Fig. II-1). Individuals from SP_(QTL+QTL)F_2 generations carrying more than one QTL-peak markers as homozygous were selected using the list of SNP markers shown in Anx. II-1 and then phenotypically characterized.

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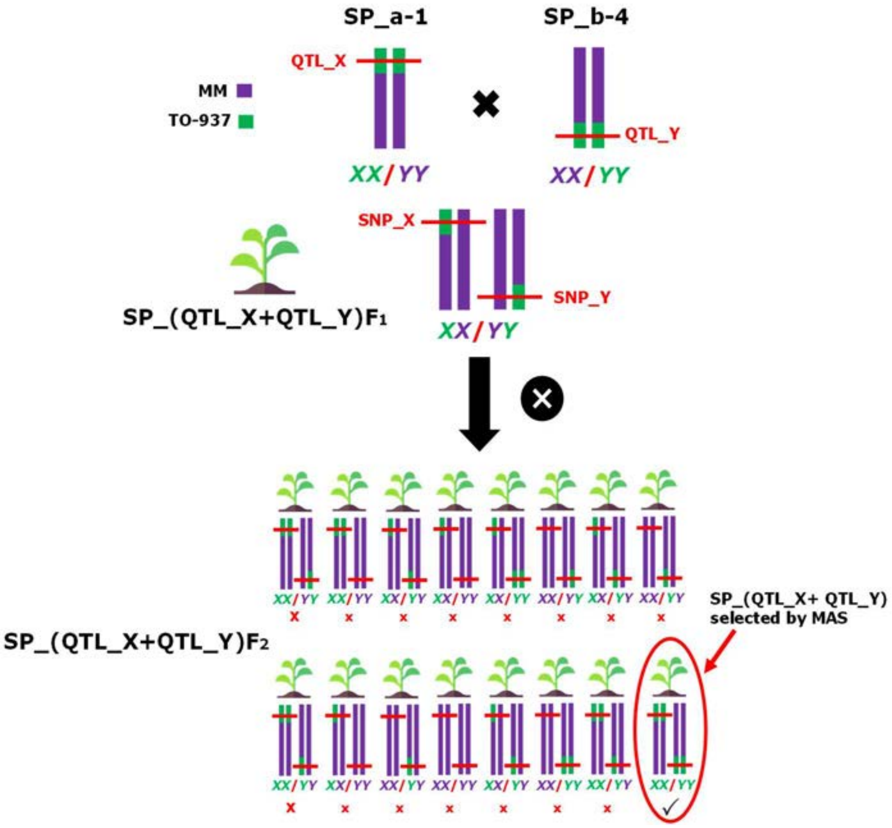

Fig. II-1: A scheme describing the pyramiding process to create and select twenty-three SP_(QTL+QTL) lines using MAS and two generations: SP_(QTL+QTL)F$_1$ and SP_(QTL+QTL)F$_2$. Two SP lines: SP_a-1 and SP_b-4 holding respectively at homozygous condition QTL-*X* and QTL-*Y* were crossed. QTL-*X* and QTL-*Y* are both identified as QTLs for the percentage of phenolics trait. Among the sixteen possible allele combinations generated in the SP_(QTL+QTL)F$_2$, only SP_(QTL_*X*+QTL_*Y*) with four TO-937 alleles: double homozygotes *XX*/*YY* were selected using peak markers SNP_X and SNP_Y. SP: *Solanum pimpinellifolium*; a: chromosome a; b: chromosome b; MAS, marker assisted selection; QTL, quantitative trait loci; SNP, single nucleotide polymorphism.

3 RESULTS

3.1 Phenotypic Performance of the SP_(QTL+QTL) Lines

3.1.1 Percentage of Phenolics

The SP_(QTL+QTL) lines obtained by crossing two SP lines holding introgressions that contain QTL with lyc and/or pim effect for the percentage of phenolics trait with the aim to obtain different combinations of homozygous *ph*-QTLs in the same MM genetic background. The MAS resulted in a total of twenty-three SP_(QTL+QTL) lines (nineteen lines in winter 2015 and four lines in summer 2016) (Tab. II-1). Each SP_(QTL+QTL) holds more than one QTL for the percentage of phenolics at homozygous state. The phenotypic performance of the SP_(QTL+QTL) lines for the percentage of cuticle phenolics, the amount of cuticle, the amount of phenolics and three cuticle color parameters L*, C* and H° were evaluated. The nSPs a228_*ph7.1* and a239_*ph7.2* were used in our phenotypic study to compare the single effect to the combined effects of their QTLs in the corresponding SP lines and SP_(QTL+QTL) lines.

Among the nineteen SP_(QTL+QTL) lines tested in winter 2015, only eleven SP_(QTL+QTL) lines show significant differences when compared to MM (Fig. II-2). Among the latter eleven SP_(QTL+QTL) lines, seven SP_(QTL+QTL) lines show significant decrease in their percentage of cuticle phenolics. The SP_(QTL+QTL) lines that have the lowest percentage of cuticle phenolics among all SP_(QTL+QTL) lines are SP_(*ph7.1*+*ph7.2*+*ph8.1*), SP_(*ph7.1*+*ph7.2*+*ph12.2*); SP_(*ph12.2*+*ph1.1*); SP_(*ph8.1*+*ph12.2*); SP_(*ph7.1*+*ph8.1*); SP_(*ph5.2*+*ph7.1*+*ph7.2*) and SP_(*ph12.2*+*ph5.2*). Most of the latter SP_(QTL+QTL) lines combine more than one QTLs with positive lyc effect. SP_(*ph7.1*+*ph7.2*+*ph8.1*) and SP_(*ph7.1*+*ph7.2*+*ph12.2*), displaying the lowest percentage of phenolics, combine respectively *ph7.1*, *ph7.2*, *ph8.1* and *ph7.1, ph7.2*, *ph12.2*, QTLs with the highest percentage of phenotypic variance (Tab. I-2). Moreover, SP_(QTL+QTL) lines harboring 3 QTLs with positive lyc effect have less phenolics than SP_(QTL+QTL) lines with 2 QTLs with positive lyc effect. On the other hand, three SP_(QTL+QTL) lines

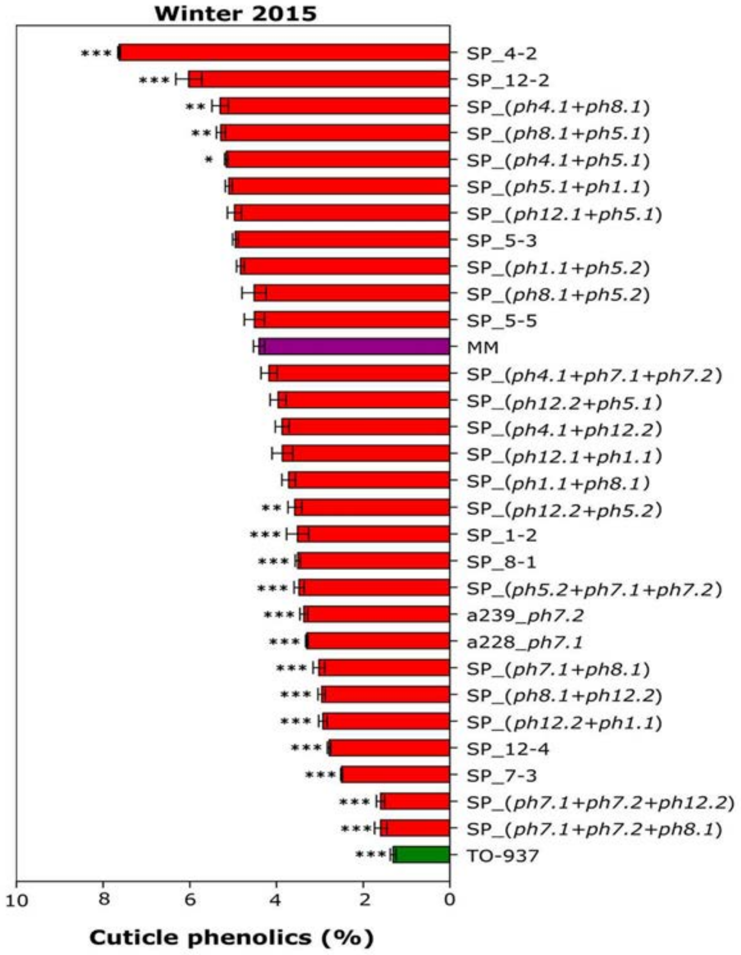

Fig. II-2: Comparison between the SP_(QTL+QTL) lines, the SP lines derived from, nSPs: a228_*ph7.1* and a239_*ph7.2*, TO-937 and MM during winter 2015 for the percentage of cuticle phenolics trait. Cuticles from red ripe fruits were analyzed. Data are presented as means ±SE. Asterisks indicate significant differences between all lines and MM (**p*<0.05, ***p*<0.01, ****p*<0.001). SP_1-2; SP_4-2; SP_5-3; SP_5-5; SP_7-3; SP_8-1; SP_12-2 and SP_12-4 harbor respectively *ph1.1*, *ph4.1*, *ph5.1*, *ph5.2*, *ph7.1+ph7.2*, *ph8.1*, *ph12.1* and *ph12.2*. nSPs: a228 and a239 harbor respectively *ph7.1* and *ph7.2.* SP, *Solanum pimpinellifolium*; QTL, quantitative trait loci; *ph*, QTL percentage of phenolics.

SP_(*ph4.1+ph5.1*), SP_(*ph8.1+ph5.1*) and SP_(*ph4.1+ph8.1*), show significant increase in their percentage of cuticle phenolics (Fig. II-2). Among the four SP_(QTL+QTL) lines tested in summer 2016, SP_(QTL+QTL) lines holding *ph4.1*, QTL with positive pim effect, show significant increase in its percentage of cuticle phenolics when compared to MM e.g. [SP_ (*ph4.1+ph1.1*) and SP_(*ph4.1+ph5.1*)] (Fig. II-3). SP_(*ph12.2+ph12.1*) and SP_(*ph5.1+ph7.1+ph7.2*) show significant decrease in their percentage of cuticle phenolics when compared to MM (Fig. II-3).

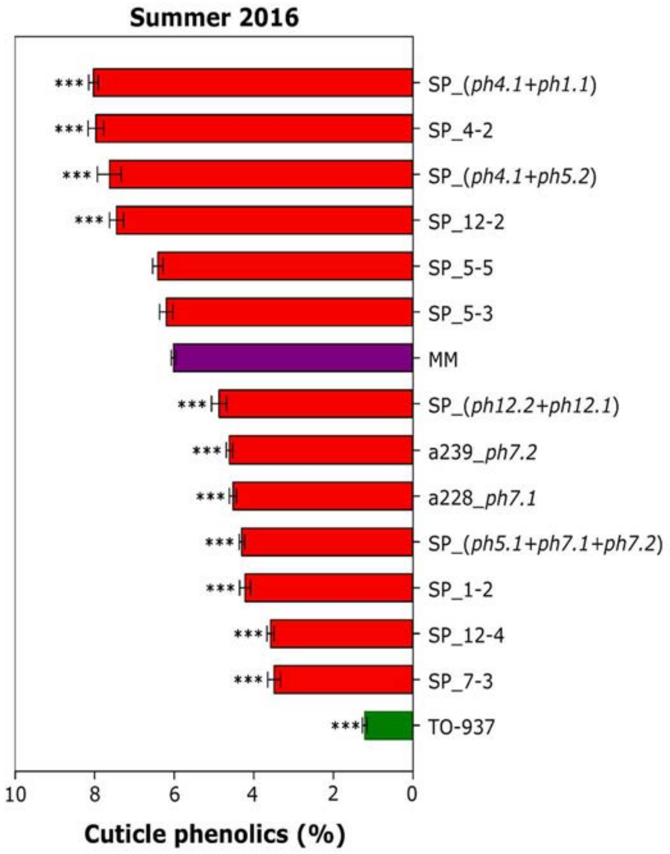

Fig. II-3: Comparison between the SP_(QTL+QTL) lines, the SP lines derived from, TO-937 and MM during summer 2016 for the percentage of cuticle phenolics trait. Cuticles from red ripe fruits were analyzed. Data are presented as means ±SE. Asterisks indicate significant differences between all lines and MM (**p*<0.05, ***p*<0.01, ****p*<0.001). SP_1-2; SP_4-2; SP_5-3; SP_5-5; SP_7-3; SP_12-2 and SP_12-4 harbor respectively *ph1.1*, *ph4.1*, *ph5.1*, *ph5.2*, *ph7.1+ph7.2*, *ph12.1* and *ph12.2*. SP, *Solanum pimpinellifolium*; QTL, quantitative trait loci; *ph*, QTL percentage of phenolics.

Detailed interpretation will be given hereafter to try understand the phenotypic performance for the percentage of cuticle phenolics of the SP_(QTL+QTL) lines tested in winter 2015 and summer 2016 to identify the type of genetic effects (either additive or epistatic additive x additive) between the combined QTLs in the MM genetic background. The results of SP_(QTL+QTL) lines in winter 2015 and summer 2016 are described in the following pages but, as an advance, the QTL effects found are summarized in Table II-2.

Tab. II-2: A summary table of the SP_(QTL+QTL) lines of winter 2015 and summer 2016, describing and resuming observations of the different interactions between combined QTLs in the MM genetic background for the percentage of cuticle phenolics trait. Epistasis tested by univariate general linear, complete 2 x 2 factorial model analysis. Significant epistasis is considered when * for $p \leq 0.05$; ** for $p \leq 0.01$; *** for $p \leq 0.001$ and ns for non-significant epistatic interaction. lyc, lycopersicum; pim, pimpinellifolium; QTL, quantitative trait loci; *ph*, QTL percentage of phenolics.

SP_(QTL+QTL)	**Individual additive effects**	**Combined effect**	**Combined additive effect**	**Epistatic**	**Observations**
SP_(*ph7.1*+*ph7.2*+*ph8.1*)	lyc+lyc+lyc	lyc	lyc	ns	Combined QTL effect different to individual lines. Positive additive effect of *ph7.1*+*ph7.2* lyc in *ph8.1* lyc and in *ph8.1* pim. SP_(QTL+QTL) reached TO-937 percentage phenolics.
SP_(*ph7.1*+*ph7.2*+*ph12.2*)	lyc+lyc+lyc	lyc	lyc	ns	Combined QTL effect different to individual lines. Positive additive effect of *ph7.1*+*ph7.2* lyc in *ph12.2* lyc and in *ph12.2* pim. SP_(QTL+QTL) reached TO-937 percentage phenolics.
SP_(*ph12.2*+*ph1.1*)	lyc+lyc	lyc	-	**	Combined QTL effect similar to SP_12-4. Positive additive x additive effect of *ph12.2* lyc in *ph1.1* lyc.
SP_(*ph8.1*+*ph12.2*)	lyc+lyc	lyc	-	***	Combined QTL effect similar to SP_12-4. Positive additive x additive effect of *ph8.1* lyc in *ph12.2* lyc.
SP_(*ph7.1*+*ph8.1*)	lyc+lyc	lyc	-	***	Combined QTL effect similar to individual lines SP_8-1, a228_*ph7.1* and a239_*ph7.2*. Positive additive x additive effect of *ph7.1* lyc in *ph8.1* lyc.

Tab. II-2 cont. I

SP_(QTL+QTL)	Individual additive effects	Combined effect	Combined additive effect	Epistatic	Observations
SP_(*ph5.2*+*ph7.1*+*ph7.2*)	pim+lyc	lyc	-	***	Combined QTL effect different to individual lines, but similar to a228_ph7.1 and a239_ph7.2. Positive additive x additive effect of *ph7.1*+*ph7.2* lyc in *ph5.2* lyc.
SP_(*ph12.2*+*ph5.2*)	lyc+pim	lyc	-	*	Combined QTL effect different to individual lines. Positive additive x additive effect of *ph12.2* lyc in *ph5.2* lyc.
SP_(*ph5.1*+*ph7.1*+*ph7.2*)	pim+lyc	lyc	lyc	ns	Combined QTL effect different to individual lines. Positive additive x additive effect of *ph5.1* pim in *ph7.1*+*ph7.2* pim.
SP_(*ph12.2*+*ph12.1*)	lyc+pim	lyc	lyc	ns	Combined QTL effect different to individual lines. Positive additive x additive effect of *ph12.2* lyc in *ph12.1* lyc and *ph12.1* pim.
SP_(*ph1.1*+*ph8.1*)	lyc+lyc	nul	-	**	Combined QTL effect similar to individual lines. Positive additive x additive effect of *ph8.1* lyc in *ph1.1* lyc.
SP_(*ph12.1*+*ph1.1*)	pim+lyc	nul	-	ns	Combined QTL effect similar to SP_1-2. Positive additive x additive effect of *ph12.1* pim in *ph1.1* lyc.

Tab. II-2 cont. II

SP_(QTL+QTL)	Individual additive effects	Combined effect	Combined additive effect	Epistatic	Observations
SP_(*ph4.1+ph12.2*)	pim+lyc	nul	nul	ns	Combined QTL effect different to individual lines. Positive additive x additive effect of *ph4.1* pim in *ph12.2* lyc and in *ph12.2* pim.
SP_(*ph12.2+ph5.1*)	lyc+pim	nul	-	ns	Combined QTL effect different to individual lines. Positive additive x additive effect of *ph12.2* lyc in *ph5.1* lyc and *ph5.1* pim.
SP_(*ph4.1+ph7.1+ph7.2*)	pim+lyc	nul	nul	ns	Combined QTL effect different to individual lines. Positive additive x additive effect of *ph4.1* pim in *ph7.1+ph7.2* lyc and in *ph7.1+ph7.2* pim.
SP_(*ph8.1+ph5.2*)	lyc+pim	nul	-	***	Combined QTL effect similar to SP_5-5. Positive additive x additive effect of *ph8.1* lyc in *ph5.2* lyc.
SP_(*ph1.1+ph5.2*)	lyc+pim	nul	-	**	Combined QTL effect similar to SP_5-5. Positive additive ex additive effect of *ph1.1* lyc in *ph5.2* lyc.
SP_(*ph12.1+ph5.1*)	pim+pim	nul	-	***	Combined QTL effect similar to SP_5-3. Positive additive x additive effect of *ph12.1* pim in *ph5.1* lyc.

Tab. II-2 cont. III

SP_(QTL+QTL)	Individual additive effects	Combined effect	Combined additive effect	Epistatic	Observations
SP_(*ph12.1*+*ph5.1*)	pim+pim	nul	-	***	Combined QTL effect similar to SP_5-3. Positive additive x additive effect of *ph12.1* pim in *ph5.1* lyc.
SP_(*ph4.1*+*ph5.1*)	pim+pim	nul	-	***	Combined QTL effect similar to SP_5-3. Positive additive x additive effect of *ph4.1* pim in *ph5.1* lyc.
SP_(*ph8.1*+*ph5.1*)	lyc+pim	pim	-	***	Combined QTL effect similar to SP_5-5. Positive additive x additive effect of *ph8.1* lyc in *ph5.1* lyc.
SP_(*ph4.1*+*ph8.1*)	pim+lyc	pim	pim	ns	Combined QTL effect different to individual lines. Positive additive x additive effect of *ph4.1* pim in *ph8.1* lyc and *ph8.1* pim.
SP_(*ph4.1*+*ph5.2*)	pim+pim	pim	-	*	Combined QTL effect different to individual lines. Positive additive x additive effect of *ph4.1* pim in *ph5.2* lyc
SP_(*ph4.1*+*ph1.1*)	pim+lyc	pim	-	***	Combined QTL effect similar to SP_4-2. Positive additive x additive effect of *ph4.1* pim in *ph1.1* lyc

SP_(QTL+QTL) lines generated from crossing SP_7-3 and SP_8-1 were selected by MAS: SP_(*ph7.1*+*ph7.2*+*ph8.1*) and SP_(*ph7.1*+*ph8.1*) (Tab. II-1). SP_(*ph7.1*+*ph7.1*+*ph8.1*) has the lowest significant percentage of phenolics among the SP_(QTL+QTL) lines of winter 2015 when compared to MM (Fig. II-2). Moreover, SP_(*ph7.1*+*ph7.2*+*ph8.1*) displays significantly less percentage of phenolics than SP_7-3, SP_8-1, SP_(*ph7.1*+*ph8.1*), a228_*ph7.1* and a239_*ph7.2* (Fig. II-4). Interestingly, SP_(*ph7.1*+*ph7.2*+*ph8.1*) shows no significant difference from TO-937. Moreover, the percentage of phenolics in a228_*ph7.1* and a239_*ph7.2* is not significantly different from SP_8-1 (Fig. II-4), indicating that separately/individually in the MM genetic background, QTLs: *ph7.1*, *ph7.2* and *ph8.1* have equal negative effects. Moreover, results also show that the combined effect of *ph7.1*, *ph7.2* and *ph8.1* exceed the combined effect of (*ph7.1*+*ph7.2*) and (*ph7.1*+*ph8.1*). Epistasis plot for SP_(*ph7.1*+*ph7.2*+*ph8.1*) shows no crossing or no parallel interaction between QTLs: (*ph7.1*+*ph7.2*) and *ph8.1* (Fig. II-5). Statistically, no significant epistatic interaction between (*ph7.1*+*ph7.2*) and *ph8.1* was found. Thus, QTLs *ph7.1*, *ph7.2* and *ph8.1* act additively when combined in

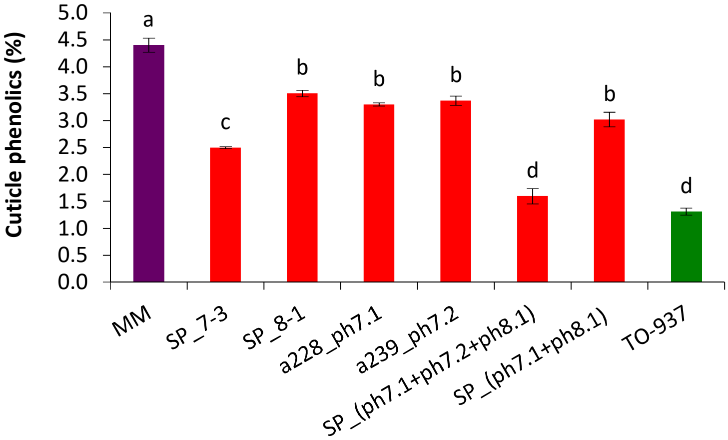

Fig. II-4: Comparison between SP_(*ph7.1*+*ph7.2*+*ph8.1*), SP_(*ph7.1*+*ph8.1*), a228_*ph7.1*, a239_*ph7.2*, SP_7-3, SP_8-1, TO-937 and MM for the percentage of cuticle phenolics trait. Cuticles from red ripe fruits were analyzed. Data are presented as means ±SE. Letters indicate significant differences between lines at $p<0.05$. SP_7-3 and SP_8-1 harbor respectively *ph7.1*+*ph7.2* and *ph8.1*. SP, *Solanum pimpinellifolium*; *ph*, QTL percentage of phenolics.

the MM genetic background to significantly reduce the percentage of cuticle phenolics.

As for SP_(*ph7.1+ph8.1*), results show that its percentage of phenolics is significantly lower than MM and SP_7-3 (Fig. II-4), but not significantly different from SP_8-1, a228_*ph7.1* and a239_*ph7.2* (Fig. II-4). These results indicate that one of the QTLs involved might be suppressed. Epistasis plot for SP_(*ph7.1+ph8.1*) shows a non-parallel crossing interaction (Fig. II-5), indicating an epistatic interaction between *ph7.1* and *ph8.1* that is confirmed statistically. These results show that one of the QTLs involved (*ph7.1* or *ph8.1*) masks the other QTL while combined in the same MM genetic background.

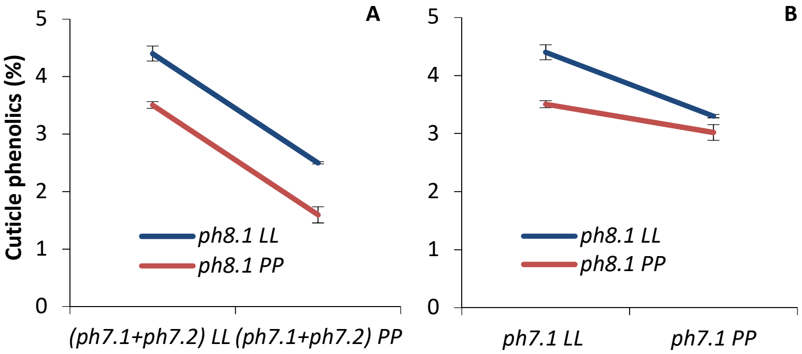

Fig. II-5: **Epistasis interaction plots for the percentage of cuticle phenolics trait between**: **A.** (*ph7.1+ph7.2*) and *ph8.1*. **B.** *ph7.1* and *ph8.1*. Data are presented as means ±SE, expressed in au for the possible allele combination: {LL/LL; LL/PP; PP/LL; PP/PP} found at interacting QTLs on two different chromosomes. The maternal QTL is indicated on the X axis and the paternal QTL is indicated by the color of the plot lines. L, *Solanum lycopersicum* allele; P, *Solanum pimpinellifolium* allele; *ph*, QTL percentage of phenolics.

Similar results were seen in SP_(*ph7.1+ph7.2+ph12.2*), where significant decrease in the percentage of phenolics was observed when compared to MM (Fig. II-2). Moreover, SP_(*ph7.1+ph7.2+ph12.2*) phenolics content is also significantly lower than SP_7-3, SP_12-4, a228_*ph7.1* and a239_*ph7.2* phenolics contents (Anx. II-2.A). No significant changes in the percentage of phenolics were seen between TO-937 and SP_(*ph7.1+ph7.2+ph12.2*) (Anx. II-2.A). These results show that the combined effect of *ph7.1, ph7.2* and *ph12.2* significantly exceed the individual effects of *ph7.1*, *ph7.2*, *ph12.2* and the combined effect of *ph7.1* and *ph7.2.* In addition to that, these results show that the low percentage of phenolics of TO-937 can also be reached by the combination of three QTLs: *ph7.1*, *ph7.2* and *ph12.2*. As for the nSPs: a228_*ph7.1* and a239_*ph7.2*, a significant difference exists between them and SP_12-4 (Anx. II-2.A), indicating that the individual effect of *ph12.2* is significantly bigger than the individual effects of *ph7.1* and *ph7.2*. The epistasis plot shows no clear crossing interaction (Anx. II-2.B) and statistically, no significant epistatic interaction was found between (*ph7.1*+*ph7.2*) and *ph12.2.* Thus, the latter QTLs act as three additive QTLs when combined in the MM genetic background to significantly decrease the percentage of phenolics.

The lowest significant percentage of phenolics among the SP_(QTL+QTL) lines holding two QTLs with positive lyc effect for the percentage of phenolics in winter 2015 were seen in SP_(*ph12.2*+*ph1.1*) and SP_(*ph8.1*+*ph12.2*). The two SP_(QTL+QTL) lines display significant decreases in their percentage of phenolics when compared to MM (Fig. II-2). SP_(*ph12.2*+*ph1.1*) and SP_(*ph8.1*+*ph12.2*) show no significant differences when compared to SP_12-4 and their percentage of phenolics are significantly lower than SP_1-2 and SP_8-1 respectively (Fig. II-6.A; Anx. II-3.A). The epistasis plots show a clear non-parallel interactions for SP_(*ph12.2*+*ph1.1*) and SP_(*ph8.1*+*ph12.2*) (Fig. II-6.B; Anx. II-3.B). Statistically, significant epistatic interactions were found between *ph12.2* and *ph1.1* and between *ph8.1* and *ph12.2*. These results show that *ph12.2* masks *ph1.1* and *ph.8.1* in the MM genetic background.

SP_(*ph12.2*+*ph12.1*) shows significant decrease in its percentage of phenolics when compared to MM (Fig. II-3). Moreover, its percentage of phenolics is significantly lower than SP_12-2 and significantly higher than SP_12-4 (Anx. II-4.A). As for the epistasis plot, no crossing interaction was seen (Anx. II-4.B). Statistically, no epistatic interaction was found between *ph12.2* and *ph12.1*. These results indicate that *ph12.2* and *ph12.1* act additively in the MM genetic background. And since SP_(*ph12.2*+*ph12.1*)'s final additive effect is negative, this indicate that *ph12.2* positive lyc effect is significantly bigger than *ph12.1* positive pim effect.

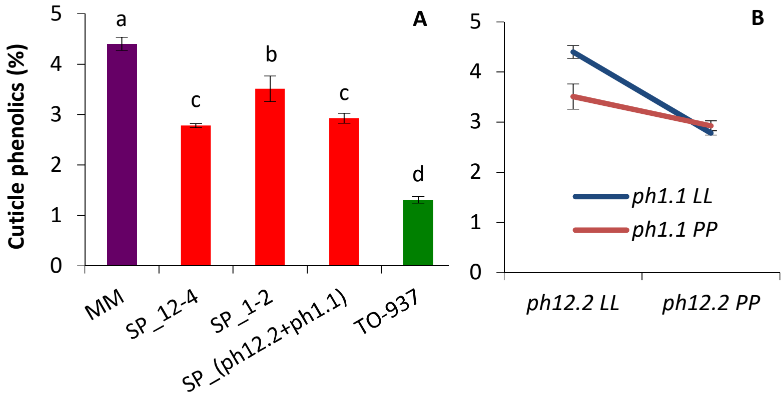

Fig. II-6: A. Comparison between SP_(*ph12.2+ph1.1*), SP_12-4, SP_1-2, TO-937 and MM for the percentage of cuticle phenolics trait. Cuticles from red ripe fruits were analyzed. Data are presented as means ±SE. Letters indicate significant differences between lines at $p<0.05$. SP_12-4 and SP_1-2 harbor respectively *ph12.2* and *ph1.1.* **B. Epistasis interaction plot for the percentage of cuticle phenolics trait between *ph12.2* and *ph1.1.*** Data are presented as means ±SE, expressed in au for the possible allele combination: {LL/LL; LL/PP; PP/LL; PP/PP} found at interacting QTLs on two different chromosomes. The maternal QTL is indicated on the X axis and the paternal QTL is indicated by the color of the plot lines. L, *Solanum lycopersicum* allele; P, *Solanum pimpinellifolium* allele; *ph*, QTL percentage of phenolics.

The first non-significant decrease in the percentage of cuticle phenolics that occurs among the SP_(QTL+QTL) lines of winter 2015 was observed in SP_(*ph1.1*+*ph8.1*) (Fig. II-2). No significant differences were found between SP_(*ph1.1*+*ph8.1*) and its corresponding SP lines: SP_1-2 and SP_8-1 (Fig. II-7.A). The epistasis plot shows a crossing interaction (Fig. II-7.B), and statistically, an epistatic interaction was confirmed between *ph1.1* and *ph8.1*. These results indicate that one of the QTLs (*ph1.1* or *ph8.1*) suppresses the other QTL when combined in the MM genetic background despite that both QTLs have positive lyc effects for the percentage of cuticle phenolics trait. It is noteworthy to mention that there are no significant differences in the percentage of phenolics between SP_1-2 and SP_8-1 suggesting *ph1.1* and *ph8.1* have equal positive lyc effects in the MM genetic background (Fig. II-7.A).

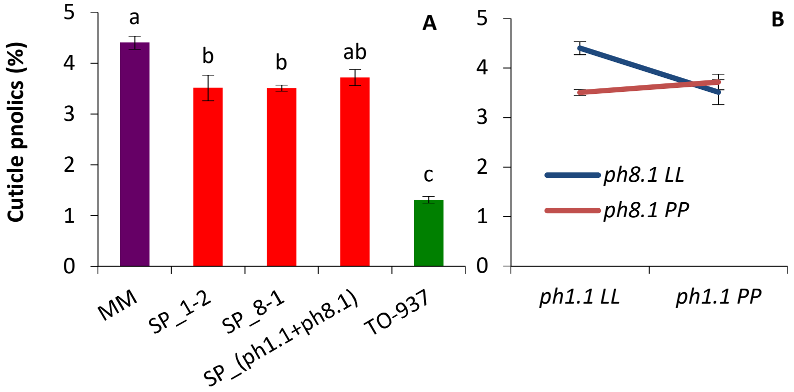

Fig. II-7: A. Comparison between SP_(*ph1.1+ph8.1*), SP_1-2, SP_8-1, TO-937 and MM for the percentage of cuticle phenolics trait. Cuticles from red ripe fruits were analyzed. Data are presented as means ±SE. Letters indicate significant differences between lines at $p<0.05$. SP_1-2 and SP_8-1 harbor respectively *ph1.1* and *ph8.1.* **B. Epistasis interaction plot for the percentage of cuticle phenolics trait between *ph1.1* and *ph8.1.*** Data are presented as means ±SE, expressed in au for the possible allele combination: {LL/LL; LL/PP; PP/LL; PP/PP} found at interacting QTLs on two different chromosomes. The maternal QTL is indicated on the X axis and the paternal QTL is indicated by the color of the plot lines. L, *Solanum lycopersicum* allele; P, *Solanum pimpinellifolium* allele; *ph*, QTL percentage of phenolics.

In SP_(*ph12.1+ph1.1*), no significant changes in the percentage of cuticle phenolics were seen when compared to MM (Fig. II-2) and when compared to SP_1-2 (Anx. II-5.A). However, SP_(*ph12.1+ph1.1*) has significantly less percentage of cuticle phenolics than SP_12-2 (Anx. II-5.A). The epistasis plot shows no clear interaction (Anx. II-5.B). Indeed, statistically, no significant epistatic interaction was seen between *ph1.1* and *ph12.1*, indicating that *ph1.1* and *ph12.1* act additively in the MM genetic background.

Two SP_(QTL+QTL) lines: SP_(*ph4.1+ph12.2*) and SP_(*ph4.1+ph7.1+ph7.2*) show no significant differences when compared to MM (Fig. II-2). However, they both show significant differences from their corresponding SP lines. SP_(*ph4.1+ph12.2*) and SP_(*ph4.1+ph7.1+ph7.2*) have significantly less phenolics than SP_4-2 and significantly more phenolics than SP_12-4 and SP_7-3 respectively (Fig. II-8.A; Anx. II-6.A). In both epistasis plots, no crossing interactions were seen (Fig. II-8.B; Anx. II-6.B). The non-parallel appearance at these plots was not statistically confirmed, no significant epistatic interactions were found between *ph4.1* & (*ph7.1*+*ph7.2*) and between *ph4.1* & *ph12.2*. These results show that *ph4.1* & (*ph7.1*+*ph7.2*) and *ph4.1* and *ph12.2* act additively in the MM genetic background. *ph4.1* positive pim effect partly neutralizes both (*ph7.1*+*ph7.2*) and *ph12.2* positive lyc effects.

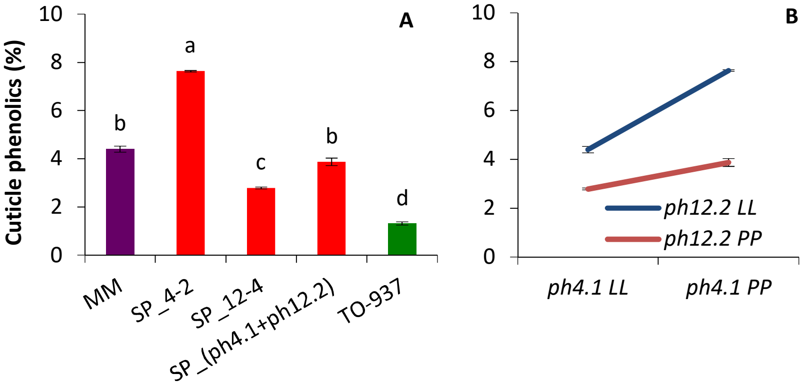

Fig. II-8: A. Comparison between SP_(*ph4.1*+*ph12.2*), SP_4-2, SP_12-4, TO-937 and MM for the percentage of cuticle phenolics trait. Cuticles from red ripe fruits were analyzed. Data are presented as means ±SE. Letters indicate significant differences between lines at $p<0.05$. SP_4-2 and SP_12-4 harbor respectively *ph4.1* and *ph12.2*. **B. Epistasis interaction plot for the percentage of cuticle phenolics trait between *ph4.1* and *ph12.2.*** Data are presented as means ±SE, expressed in au for the possible allele combination: {LL/LL; LL/PP; PP/LL; PP/PP} found at interacting QTLs on two different chromosomes. The maternal QTL is indicated on the X axis and the paternal QTL is indicated by the color of the plot lines. L, *Solanum lycopersicum* allele; P, *Solanum pimpinellifolium* allele; *ph*, QTL percentage of phenolics.

SP_(*ph4.1*+*ph8.1*) and SP_(*ph4.1*+*ph1.1*) have the highest percentage of phenolics among SP_(QTL+QTL) of 2015 and 2016 respectively (Fig. II-2; Fig. II-3). Each of them holds two significant QTLs with contrary additive effects for the percentage of phenolics traits: *ph4.1* with positive pim effect and *ph8.1* and *ph1.1* with positive lyc effect. However, SP_(*ph4.1*+*ph8.1*) and SP_(*ph4.1*+*ph1.1*) show significant increases when compared to MM (Fig. II-2; Fig. II-3). SP_(*ph4.1*+*ph8.1*) and SP_(*ph4.1*+*ph1.1*) have significantly more percentage of phenolics than SP_8-1 and SP_1-2 respectively (Fig. II-9.A; Anx. II-7.A). Only SP_(*ph4.1*+*ph8.1*) has significantly less phenolics than SP_4-2. As for the epistasis plot no clear interaction was seen for SP_(*ph4.1*+*ph8.1*) (Fig. II-9.B) and statistically, no significant epistatic interaction was found between *ph4.1* and *ph8.1* indicating that *ph4.1* and *ph8.1* act additively in the MM genetic background. On the other hand, the epistasis plot between *ph4.1* and *ph1.1* shows a non-parallel interaction (Anx. II-7.B) and statistically a significant epistatic interaction was confirmed. These results show that *ph4.1* masks *ph1.1* in the MM genetic background.

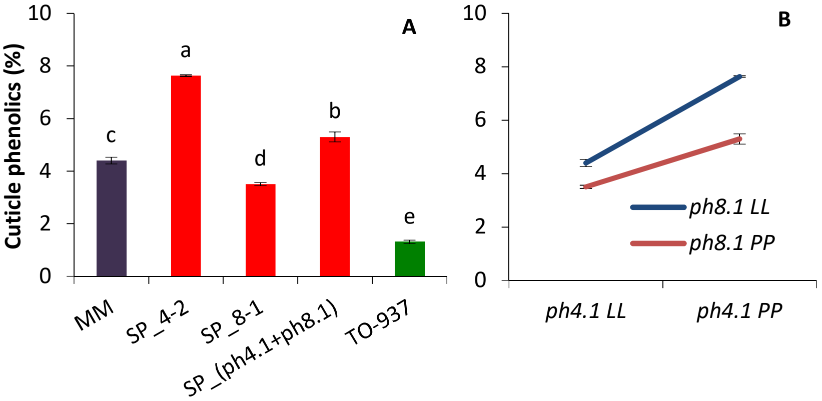

Fig. II-9: A. Comparison between SP_(*ph4.1*+*ph8.1*), SP_4-2, SP_8-1, TO-937 and MM for the percentage of cuticle phenolics trait. Cuticles from red ripe fruits were analyzed. Data are presented as means ±SE. Letters indicate significant differences between lines at $p<0.05$. SP_4-2 and SP_8-1 harbor respectively *ph4.1* and *ph8.1.* **B. Epistasis interaction plot for the percentage of cuticle phenolics trait between *ph4.1* and *ph8.1.*** Data are presented as means ±SE, expressed in au for the possible allele combination: {LL/LL; LL/PP; PP/LL; PP/PP} found at interacting QTLs on two different chromosomes. The maternal QTL is indicated on the X axis and the paternal QTL is indicated by the color of the plot lines. L, *Solanum lycopersicum* allele; P, *Solanum pimpinellifolium* allele; *ph*, QTL percentage of phenolics.

All the following SP_(QTL+QTL) harbor at least *ph5.1* or *ph5.2*. Unlike the latter SP_(QTL+QTL), their percentage of cuticle phenolics were evaluated during the four developmental stages (IG, MG, Br, RR).

SP_(*ph5.2*+*ph7.1*+*ph7.2*) starts to show significant difference from MM at MG, when both SP_5-5 and SP_7-3 show significant difference from MM (Anx. II-8.A). At Br, when SP_5-5 is no longer different from MM, SP_(*ph5.2*+*ph7.1*+*ph7.2*) is also no longer different from MM (Anx. II-8.A). However, At RR, SP_7-3 and SP_(*ph5.2*+*ph7.1*+*ph7.2*) shows significant increase when compared from MM (Anx. II-8.A). As for SP_(*ph12.2*+*ph5.2*), it starts to show significant changes from MM at IG together with SP_5-5

(Fig. II-10.A). These significant changes disappear during MG and Br when nor SP_12-4 or SP_5-5 harboring respectively *ph12.2* and *ph5.2* show significant changes. However, at RR, SP_(*ph12.2*+*ph5.2*) shows significant decrease together with SP_12-4 (Fig. II-10.A). These results show that when combined, *ph5.2* contributes to the positive pim effect of SP_(*ph5.2*+*ph7.1*+*ph7.2*) and SP_(*ph12.2*+*ph5.2*) at early stages. However, *ph7.1*+*ph7.2* and *ph12.2* are responsible for the positive lyc effect at late stages. Their individual effect is similar to their combined effect despite that *ph5.2* moderates but does not neutralize *ph7.1*+*ph7.2* or

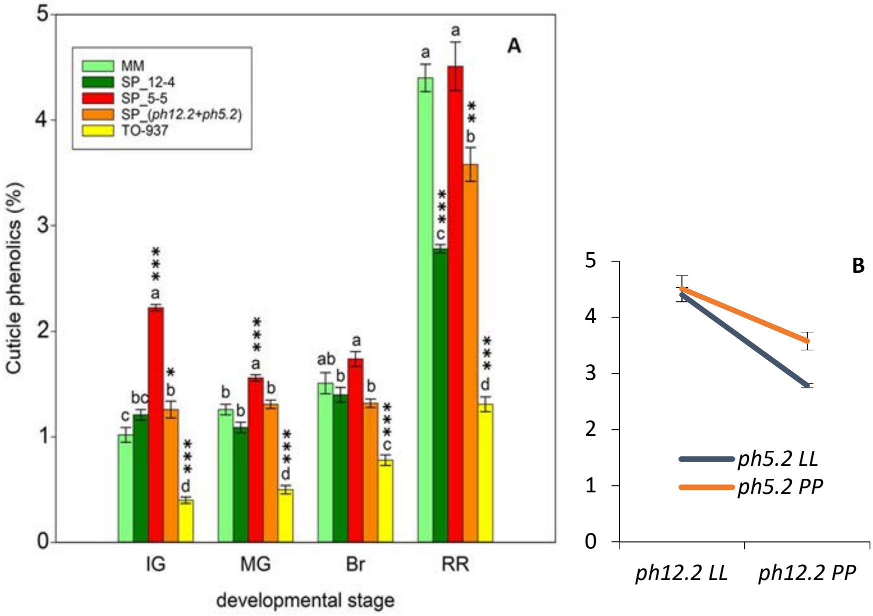

Fig. II-10: A. Comparison between SP_(*ph12.2*+*ph5.2*), SP_12-4, SP_5-5, TO-937 and MM for the percentage of cuticle phenolics trait at IG, MG, Br and RR stages. Fruit cuticles from all developmental stages were analyzed. Data are presented as means ±SE. Asterisks indicate significant differences with MM (*p<0.05; **p<0.01; ***p<0.001). Letters indicate significant differences between lines at p<0.05. SP_12-4 and SP_5-5 harbor respectively *ph12.2* and *ph5.2*. **B. Epistasis interaction plot for the percentage of cuticle phenolics trait between *ph12.2* and *ph5.2* at RR stage.** Data are presented as means ±SE, expressed in au for the possible allele combination: {LL/LL; LL/PP; PP/LL; PP/PP} found at interacting QTLs on two different chromosomes. The maternal QTL is indicated on the X axis and the paternal QTL is indicated by the color of the plot lines. L, *Solanum lycopersicum* allele; P, *Solanum pimpinellifolium* allele; *ph*, QTL percentage of phenolics; IG, immature green; MG, mature green; Br, breaker; RR, red ripe.

ph12.2 at RR when combined in the MM genetic background. The epistasis plots shows non-parallel interactions (Fig. II-10.B; Anx. II-8.B) that were statistically confirmed.

SP_(*ph12.2*+*ph5.1*) shows significant decrease only at RR (Anx. II-9.A). Moreover, its percentage of phenolics is significantly different from SP_12-4 harboring *ph12.2* and SP_5-3 harboring *ph5.1* (Anx. II-9.A). These results show that *ph12.2* positive lyc effect is mainly responsible for the final positive lyc effect of SP_(*ph12.2*+*ph5.1*) at all stages. The epistasis plot shows no clear interaction (Anx. II-9.B) and no significant epistatic interaction was confirmed indicating that *ph12.2* and *ph5.1* act additively in the MM genetic background.

SP_(*ph1.1*+*ph5.2*) and SP_(*ph8.1*+*ph5.2*) show no significant differences from MM at almost all stages (Fig. II-11.A; Anx. II-10.A), except at IG, where only SP_(*ph8.1*+*ph5.2*) shows slightly significant increase (Anx. II-10.A). As for the epistasis plots, non-parallel interactions were seen for both SP_(QTL+QTL)s (Fig. II-11.B; Anx. II-10.B). Statistically, significant epistatic interactions between *ph1.1* & *ph5.2* and between *ph8.1* & *ph5.2* were confirmed. These results show that *ph5.2* suppresses *ph1.1* and *ph8.1* in the MM genetic background for the percentage of phenolics trait not only at RR but also at IG, MG and Br.

SP_(*ph12.1*+*ph5.1*) and SP_(*ph4.1*+*ph5.1*) show no significant differences when compared to MM at all stages (Anx. II-11.A; Anx-12.A). Moreover, only at RR, SP_(*ph4.1*+*ph5.1*) and SP_(*ph12.1*+*ph5.1*) have significantly less percentage of phenolics than SP_4-2 and SP_12-2 respectively (Anx. II-11.A; Anx. II-12.A). As for the epistasis plots, crossing interactions were seen for both SP_(QTL+QTL)s (Anx. II-11.B; Anx. II-12.B) and statistically significant epistatic interactions were found between *ph4.1* and *ph5.1* and between *ph12.1* and *ph5.1*. These results indicate that *ph5.1* suppresses greatly the positive pim effects of *ph4.1* and *ph12.1* only at RR stage.

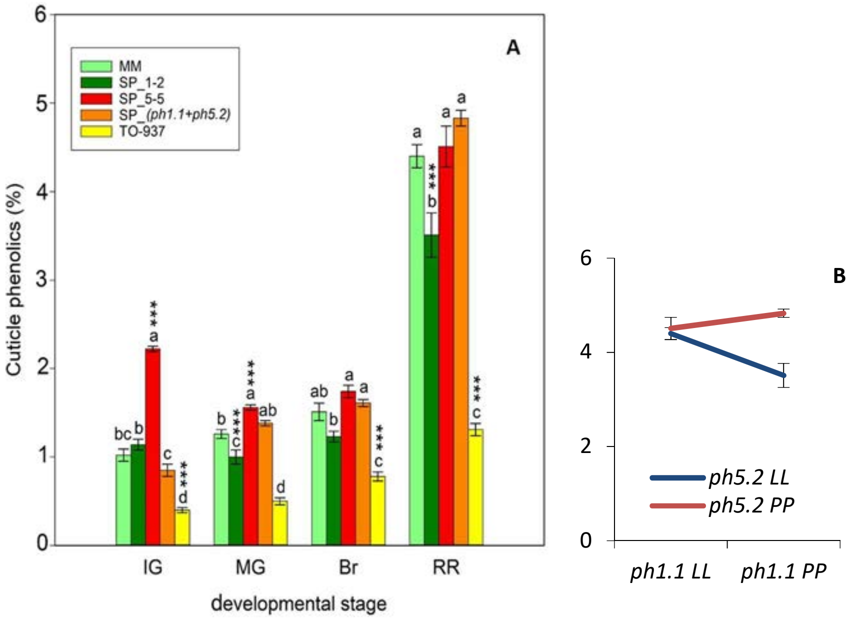

Fig. II-11: A. Comparison between SP_(*ph1.1+ph5.2*), SP_1-2, SP_5-5, TO-937 and MM for the percentage of cuticle phenolics trait at IG, MG, Br and RR stages. Fruit cuticles from all developmental stages were analyzed. Data are presented as means ±SE. Asterisks indicate significant differences with MM (*$p<0.05$; **$p<0.01$; ***$p<0.001$). Letters indicate significant differences between lines at $p<0.05$. SP_1-2 and SP_5-5 harbor respectively *ph1.1* and *ph5.2.* **B. Epistasis interaction plot for the percentage of cuticle phenolics trait between *ph1.1* and *ph5.2* at RR stage*.*** Data are presented as means ±SE, expressed in au for the possible allele combination: {LL/LL; LL/PP; PP/LL; PP/PP} found at interacting QTLs on two different chromosomes. The maternal QTL is indicated on the X axis and the paternal QTL is indicated by the color of the plot lines. L, *Solanum lycopersicum* allele; P, *Solanum pimpinellifolium* allele; *ph*, QTL percentage of phenolics; IG, immature green; MG, mature green; Br, breaker; RR, red ripe.

At IG, SP_(*ph8.1+ph5.1*) shows increase in its percentage of phenolics when compared to MM, but similar to SP_5-3. At MG, it shows significant decrease, similar to SP_8-1. At Br, it shows no significant changes from MM and it has an intermediate percentage of phenolics between SP_8-1 and SP_5-3 (Fig. II-12.A). However, at RR, SP_(*ph8.1+ph5.1*) shows significant increase when compared to MM and to SP_5-3. These results indicate that at IG, *ph5.1* is responsible for the pim positive effect in SP_(*ph8.1+ph5.1*) whereas, at MG, *ph8.1* positive lyc effect is activated and masked totally *ph5.1* when combined. The epistasis plot at RR shows non-parallel interaction (Fig. II-12.B). Statistically, significant epistatic interaction was confirmed between *ph8.1* and *ph5.1*, indicating that *ph5.1* might be activated by *ph8.1* in the MM genetic background to increase the percentage of phenolics in the cuticle at RR stage.

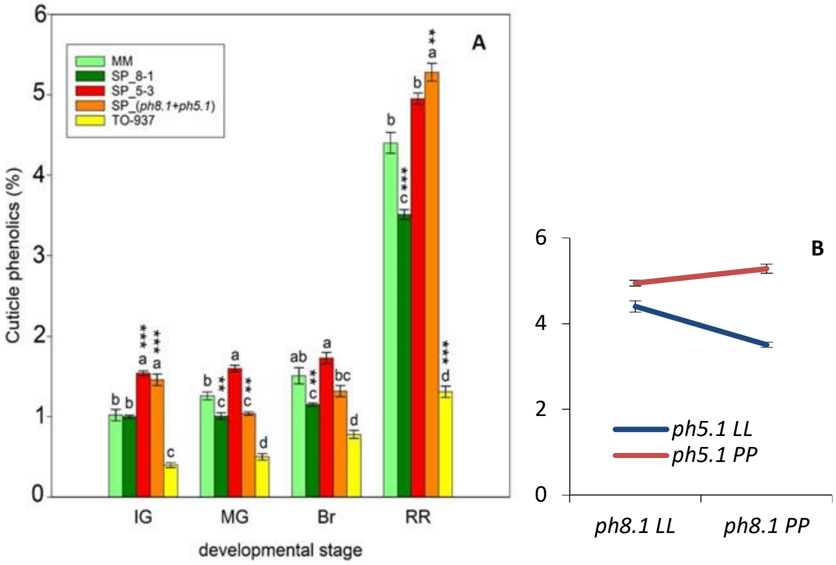

Fig. II-12: A. Comparison between SP_(*ph8.1+ph5.1*), SP_8-1, SP_5-3, TO-937 and MM for the percentage of cuticle phenolics trait at IG, MG, Br and RR stages. Fruit cuticles from all developmental stages were analyzed. Data are presented as means ±SE. Asterisks indicate significant differences with MM (**p*<0.05; ***p*<0.01; ****p*<0.001). Letters indicate significant differences between lines at *p*<0.05. SP_8-1 and SP_5-3 harbor respectively *ph8.1* and *ph5.1.* **B. Epistasis interaction plot for the percentage of cuticle phenolics trait between *ph8.1* and *ph5.1* at RR stage.** Data are presented as means ±SE, expressed in au for the possible allele combination: {LL/LL; LL/PP; PP/LL; PP/PP} found at interacting QTLs on two different chromosomes. The maternal QTL is indicated on the X axis and the paternal QTL is indicated by the color of the plot lines. L, *Solanum lycopersicum* allele; P, *Solanum pimpinellifolium* allele; *ph*, QTL percentage of phenolics; IG, immature green; MG, mature green; Br, breaker; RR, red ripe.

SP_(*ph5.1+ph1.1*) shows significant increase in its percentage of phenolics when compared to MM only at RR (Anx. II-13.A). Its epistasis plot shows a non-parallel interaction (Anx. II-13.B) and statistically a significant epistatic interaction was confirmed. These results show that *ph1.1* might activate *ph5.1* when combined in the MM genetic background at RR, even if the final increase of the SP_(QTL+QTL) was not significant.

SP_(*ph5.1+ph7.1+ph7.2*) starts to show significant difference from MM at IG, together with SP_5-3 (Fig. II-13.A). At MG, both SP_(*ph5.1+ph7.1+ph7.2*) and SP_5-3 show slightly significant increase from MM. However, At Br and RR, SP_(*ph5.1+ph7.1+ph7.2*) shows significant decrease when compared from MM, similarly to SP_7-3 (Fig. II-13.A). These results show that when combined, *ph5.1* contributes to the positive pim effect of SP_(*ph5.2+ph7.1+ph7.2*) at early stages. However, *ph7.1+ph7.2* is responsible for the positive lyc effect at late stages. The epistasis plot shows no crossing interaction (Fig. II-13.B) and statistically no significant epistatic interaction was confirmed indicating that *ph5.1* and *ph7.1+ph7.2* act additively when combined in the MM genetic background.

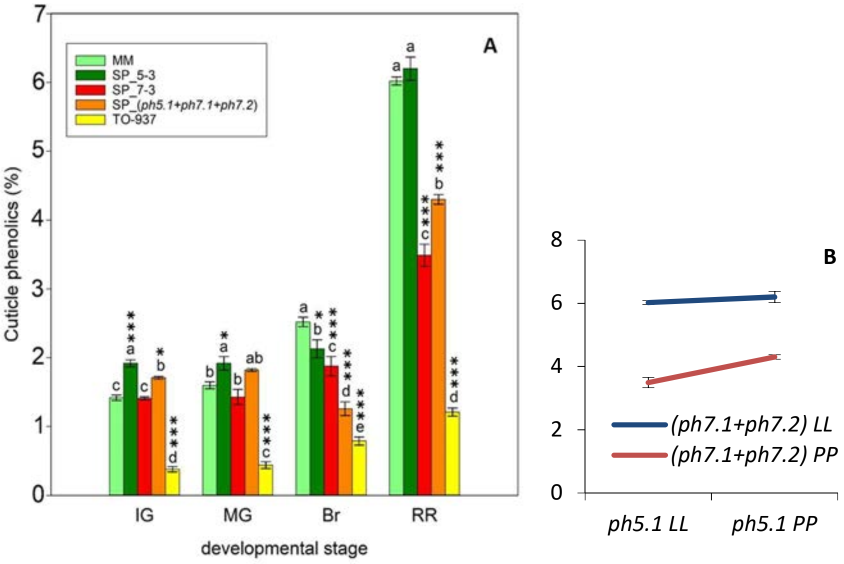

Fig. II-13: A. Comparison between SP_(*ph5.1+ph7.1+ph7.2*), SP_5-3, SP_7-3, TO-937 and MM for the percentage of cuticle phenolics trait at IG, MG, Br and RR stages. Fruit cuticles from all developmental stages were analyzed. Data are presented as means ±SE. Asterisks indicate significant differences with MM (**p*<0.05; ***p*<0.01; ****p*<0.001). Letters indicate significant differences between lines at *p*<0.05. SP_5-3 and SP_7-3 harbor respectively *ph5.1* and *ph7.1+ph7.2.* **B. Epistasis interaction plot for the percentage of cuticle phenolics trait between *ph5.1* and *ph7.1+ph7.2* at RR stage.** Data are presented as means ±SE, expressed in au for the possible allele combination: {LL/LL; LL/PP; PP/LL; PP/PP} found at interacting QTLs on two different chromosomes. The maternal QTL is indicated on the X axis and the paternal QTL is indicated by the color of the plot lines. L, *Solanum lycopersicum* allele; P, *Solanum pimpinellifolium* allele; *ph*, QTL percentage of phenolics; IG, immature green; MG, mature green; Br, breaker; RR, red ripe.

SP_(*ph4.1+ph5.2*) displays significant increase in its percentage of phenolics when compared to MM at all the stages (Fig. II-14.A). Moreover, at early stages (IG and MG), SP_(*ph4.1+ph5.2*) shows no significant difference from SP_5-5, whereas at late stages (Br and RR), SP_(*ph4.1+ph5.2*) is not significantly different from SP_4-2 (Fig. II-14.A). As for the epistasis plot, a crossing interaction was seen (Fig. II-14.B). Statistically, a significant epistatic interaction was found between *ph4.1* and *ph5.2*. All these results indicate that *ph4.1* suppresses *ph5.2* in the MM

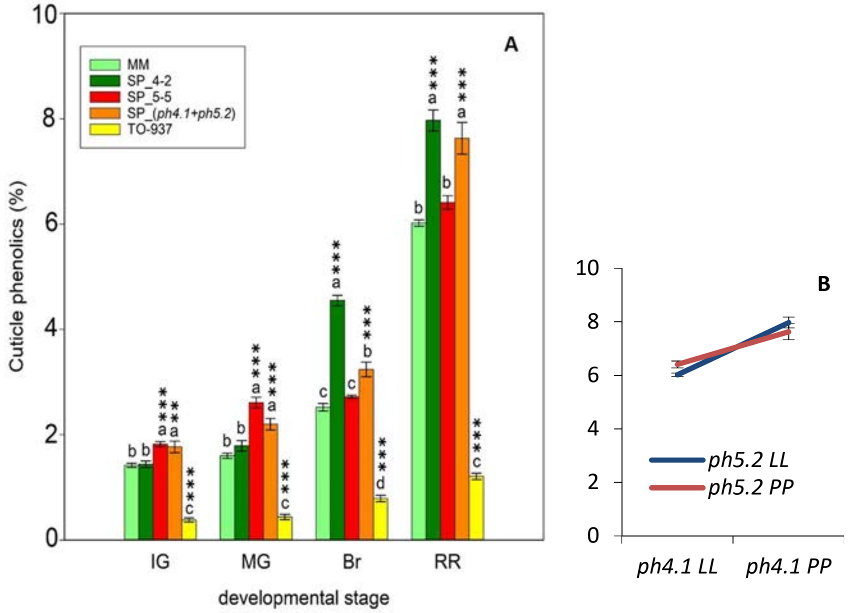

Fig. II-14: A. Comparison between SP_(*ph4.1+ph5.2*), SP_4-2, SP_5-5, TO-937 and MM for the percentage of cuticle phenolics trait at IG, MG, Br and RR stages. Fruit cuticles from all developmental stages were analyzed. Data are presented as means ±SE. Asterisks indicate significant differences with MM (**p*<0.05; ***p*<0.01; ****p*<0.001). Letters indicate significant differences between lines at *p*<0.05. SP_4-2 and SP_5-5 harbor respectively *ph4.1* and *ph5.2*. **B. Epistasis interaction plot for the percentage of cuticle phenolics trait between *ph4.1* and *ph5.2* at RR stage.** Data are presented as means ±SE, expressed in au for the possible allele combination: {LL/LL; LL/PP; PP/LL; PP/PP} found at interacting QTLs on two different chromosomes. The maternal QTL is indicated on the X axis and the paternal QTL is indicated by the color of the plot lines. L, *Solanum lycopersicum* allele; P, *Solanum pimpinellifolium* allele; *ph*, QTL percentage of phenolics; IG, immature green; MG, mature green; Br, breaker; RR, red ripe.

genetic background for the percentage of phenolics trait not only at RR, but also at Br where *ph4.1* is effective.

3.1.2 Amount of Cuticle

To study the amount of phenolics in the fruit cuticles of RR tomato fruits, the total amount of cuticle formed by the phenolics-related SP_(QTL+QTL) lines, the SP lines derived from and the nSPs: a228_*ph7.1* and a239_*ph7.2* during winter 2015 and summer 2016 were measured for calculations. But this trait has interest itself since reveals the putative genetic relationships (either by pleiotropy or by linkage) between the two traits, especially when several of the phenolic QTLs colocalized with cuticle amount QTLs (those QTLs contained in SP_5-3, SP_5-5, and SP_12-2, see chapter I).

Among the nineteen SP_(QTL+QTL) lines tested in winter 2015, only eight SP_(QTL+QTL) lines show significant changes in their amount of cuticle when compared to MM (Fig. II-15). Surprisingly, the SP_(QTL+QTL) lines with the lowest amount of cuticle and the highest amounts of cuticle are derived from SP_1-2, SP_7-3, SP_8-1 and SP_12-4, SP lines that show no significant changes in their amount of cuticle (Fig. II-15). As for the SP_(QTL+QTL) lines derived from SP_5-5 (the SP line with the highest amount of cuticle among all SP lines), none of them show significant increase in their amount of cuticle when compared to MM (Fig. II-15).

As for the SP_(QTL+QTL) lines tested in summer 2016, three out four SP_(QTL+QTL)s show significant decrease when compared to MM. The SP_(QTL+QTL) lines are: {SP_(*ph4.1+ph1.1*); SP_(*ph5.1+ph7.1+ph7.2*); SP_(*ph12.2+ph12.1*)} (Fig. II-16). It is noteworthy to mention that a239_*ph7.2* has significantly less amount of cuticle than MM only in summer (Fig. II-16).

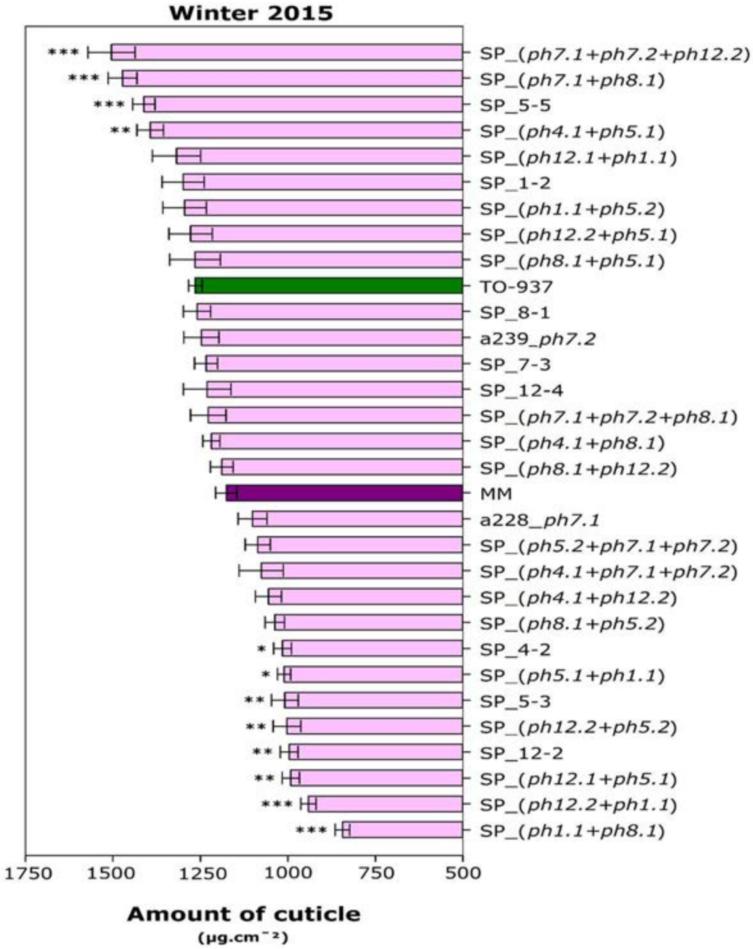

Fig. II-15: Comparison between the SP_(QTL+QTL) lines, the SP lines derived from, nSPs: a228_*ph7.1* and a239_*ph7.2*, TO-937 and MM during winter 2015 for the amount of cuticle trait. Cuticles from red ripe fruits were analyzed. Data are presented as means ±SE. Asterisks indicate significant differences between all lines and MM (*p<0.05, **p<0.01, ***p<0.001). SP_1-2; SP_4-2; SP_5-3; SP_5-5; SP_7-3; SP_8-1; SP_12-2 and SP_12-4 harbor respectively *ph1.1*, *ph4.1*, *ph5.1*, *ph5.2*, *ph7.1*+*ph7.2*, *ph8.1*, *ph12.1* and *ph12.2*. nSPs: a228 and a239 harbor respectively *ph7.1* and *ph7.2.* SP, *Solanum pimpinellifolium*; QTL, quantitative trait loci; *ph*, QTL percentage of phenolics.

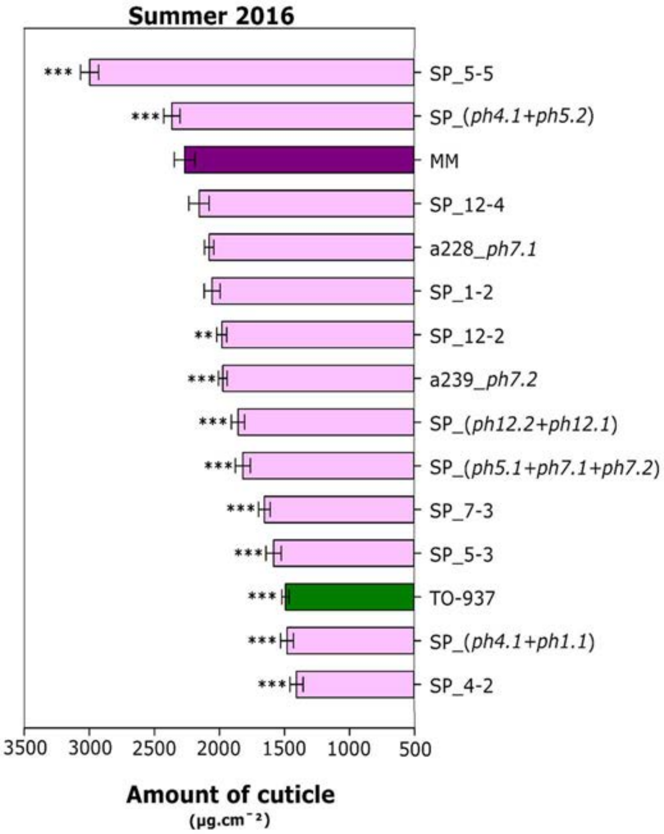

Fig. II-16: Comparison between the SP_(QTL+QTL) lines, the SP lines derived from, TO-937 and MM during summer 2016 for the amount of cuticle trait. Cuticles from red ripe fruits were analyzed. Data are presented as means ±SE. Asterisks indicate significant differences between all lines and MM (**p*<0.05, ***p*<0.01, ****p*<0.001). SP_1-2; SP_4-2; SP_5-3; SP_5-5; SP_7-3; SP_12-2 and SP_12-4 harbor respectively *ph1.1*, *ph4.1*, *ph5.1*, *ph5.2*, *ph7.1+ph7.2*, *ph12.1* and *ph12.2*. SP, *Solanum pimpinellifolium*; QTL, quantitative trait loci; *ph*, QTL percentage of phenolics.

3.1.3 Amount of Phenolics

The amount of phenolics trait is defined as the quantity of phenolics in one µg.cm^{-2} of cuticle. It is a complex trait that depends on two traits: the percentage of phenolics and the amount of cuticle. The results of the SP lines tested in winter 2015 for the amount of phenolics trait show significant decrease when compared to MM in lines: SP_7-3, SP_12-4 and SP_8-1 (Fig. II-17). These SP lines hold QTLs with negative effects for the percentage of cuticle phenolics. Lines such as SP_1-2 and SP_5-3 show no significant changes in their amount of phenolics when compared to MM (Fig. II-17), despite holding *ph1.1* and *cm5.1* respectively. Moreover, lines such as SP_12-2, SP_5-5 and SP_4-2 show significant increases in their amount of phenolics when compared to MM (Fig. II-17). The latter lines hold QTLs with positive effects for the percentage of phenolics and/or the amount of cuticle. The overall results indicate that both traits affect equally the final amount of phenolics. It is noteworthy to mention that none of the SP lines has reached down the low amount of phenolics of TO-937, including SP_3-3 (Chapter I) and SP_7-3, holding respectively (*cm3.1+cm3.2*) and (*ph7.1+ph7.2*) (Fig. II-17). However, by combining *ph7.1* and *ph7.2* with *ph8.1* or *ph12.2* as in SP_(*ph7.1+ph7.2+ph8.1*) and SP_(*ph7.1+ph7.2+ph12.2*), the amounts of cuticle phenolics can reach down the TO-937 amount of phenolics (Fig. II-17).

In summer 2016, all lines including SP_(QTL+QTL) lines, SP lines and a228_*ph7.1* and a239_*ph7.2* show significant changes in their amount of cuticle phenolics when compared to MM (Fig. II-18). This is because all latter lines show significant changes whether in their percentage of cuticle phenolics or in their amount of cuticle.

Almost all SP_(QTL+QTL) lines of winter 2015 have similar phenotypic performance for the percentage and amount of phenolics in the cuticle of RR tomato fruits. Some exceptions exist and their results will be interpreted here after.

SP_(*ph1.1+ph8.1*) shows significant decrease in the amount of phenolics when compared to MM (Fig. II-17). Moreover, it shows no significant changes in its percentage of cuticle phenolics (Fig. II-2), however, it has the lowest significant amount of cuticle among the SP_(QTL+QTL)s of winter 2015. Thus, the significant decrease in the amount of phenolics is due to the significant decrease in the amount of cuticle and not the percentage of phenolics. Another interesting result was seen in SP_(*ph7.1+ph8.1*) where no significant changes in the amount of phenolics were seen (Figf. II.17) despite having a significant low percentage of phenolics (Fig. II-2). This can be explained by the fact that the significant high amount of cuticle of SP_(*ph7.1*+*ph8.1*) (Fig. II-15) prevents the decrease in the amount of phenolics.

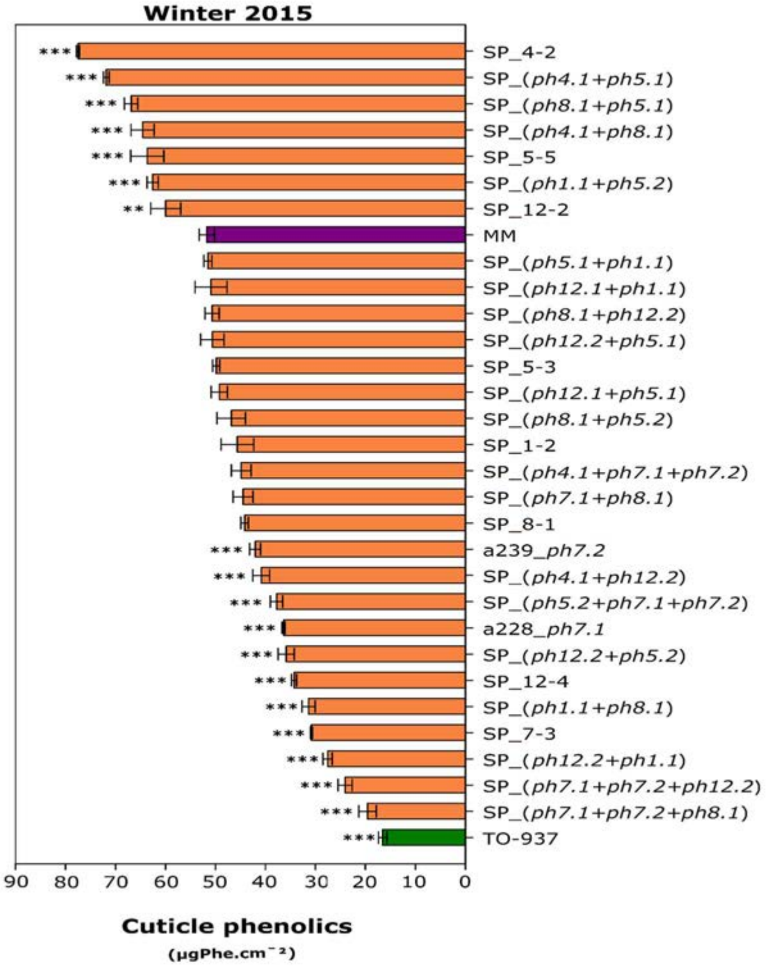

Fig. II-17: Comparison between the SP_(QTL+QTL) lines, the SP lines derived from, nSPs: a228_*ph7.1* and a239_*ph7.2*, TO-937 and MM during winter 2015 for the amount of cuticle phenolics trait. Cuticles from red ripe fruits were analyzed. Data are presented as means ±SE. Asterisks indicate significant differences between all lines and MM (**p*<0.05, ***p*<0.01, ****p*<0.001). SP_1-2; SP_4-2; SP_5-3; SP_5-5; SP_7-3; SP_8-1; SP_12-2 and SP_12-4 harbor respectively *ph1.1*, *ph4.1*, *ph5.1*, *ph5.2*, *ph7.1*+*ph7.2*, *ph8.1*, *ph12.1* and *ph12.2*. nSPs: a228 and a239 harbor respectively *ph7.1* and *ph7.2.* SP, *Solanum pimpinellifolium*; QTL, quantitative trait loci; *ph*, QTL percentage of phenolics.

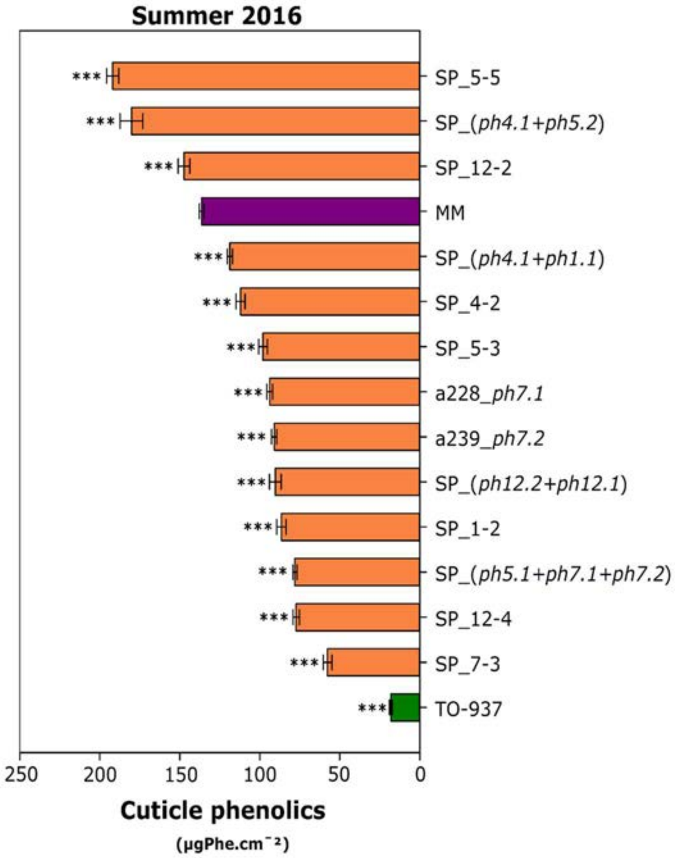

Fig. II-18: Comparison between the SP_(QTL+QTL) lines, the SP lines derived from, TO-937 and MM during summer 2016 for the amount of cuticle trait. Cuticles from red ripe fruits were analyzed. Data are presented as means ±SE. Asterisks indicate significant differences between all lines and MM (**p*<0.05, ***p*<0.01, ****p*<0.001). SP_1-2; SP_4-2; SP_5-3; SP_5-5; SP_7-3; SP_12-2 and SP_12-4 harbor respectively *ph1.1*, *ph4.1*, *ph5.1*, *ph5.2*, *ph7.1+ph7.2*, *ph12.1* and *ph12.2*. SP, *Solanum pimpinellifolium*; QTL, quantitative trait loci; *ph*, QTL percentage of phenolics.

3.1.4 Cuticle Color Parameters

Almost all SP_(QTL+QTL) lines of winter 2015 that show significant differences in their percentage of phenolics, display significant changes in their cuticle color parameters when compared to MM (Fig. II-19; 20; 21). SP_(QTL+QTL) lines with significant low percentage of phenolics and thus combining *ph* QTLs with lyc positive effects are mostly the ones that show significant high cuticle Lightness L* (Fig. II-19), significant low cuticle

Chroma C* (Fig. II-20) and significant high cuticle Hue H° (Fig. II-21). SP_(QTL+QTL) lines with significant high percentage of phenolics and thus combining QTLs with pim positive effects are the ones that show significantly lower L* (Fig. II-19), significantly higher C* (Fig. II-20) and significantly lower H° (Fig. II-21). However, this does not always apply to SP_(QTL+QTL) lines showing no significant changes in their cuticle phenolics content. For example, SP_(QTL+QTL) lines holding *ph* QTLs with lyc and pim positive effects such as SP_ (*ph4.1*+*ph12.2*) and SP_(*ph4.1*+*ph7.1*+*ph7.2*) do show significant increase in their L* (Fig. II-19). It is noteworthy to mention that SP_(QTL+QTL) lines holding three *ph* QTLs with positive lyc effects for the percentage of phenolics are the ones who have the highest L* and H° (Fig. II-19; 21) and the lowest C* (Fig. II-20). Moreover, L*s of SP_(QTL+QTL) lines such as SP_(*ph12.2*+*ph1.1*) and SP_(*ph8.1*+*ph12.2*), holding more than one *ph* QTL with lyc positive effect, surpass TO-937 L*.

In SP_(QTL+QTL) lines of summer 2016, all SP_(QTL+QTL) lines showing differences in their percentage of phenolics, display also significant changes in their L* C* and H°, except SP_(*ph4.1*+*ph1.1*). SP_(*ph4.1*+*ph1.1*) shows significant increase in its percentage of phenolics and significant decrease in its amount of cuticle. This may explain the non-significant changes in its cuticle color parameters (Fig. II-22; 23; 24).

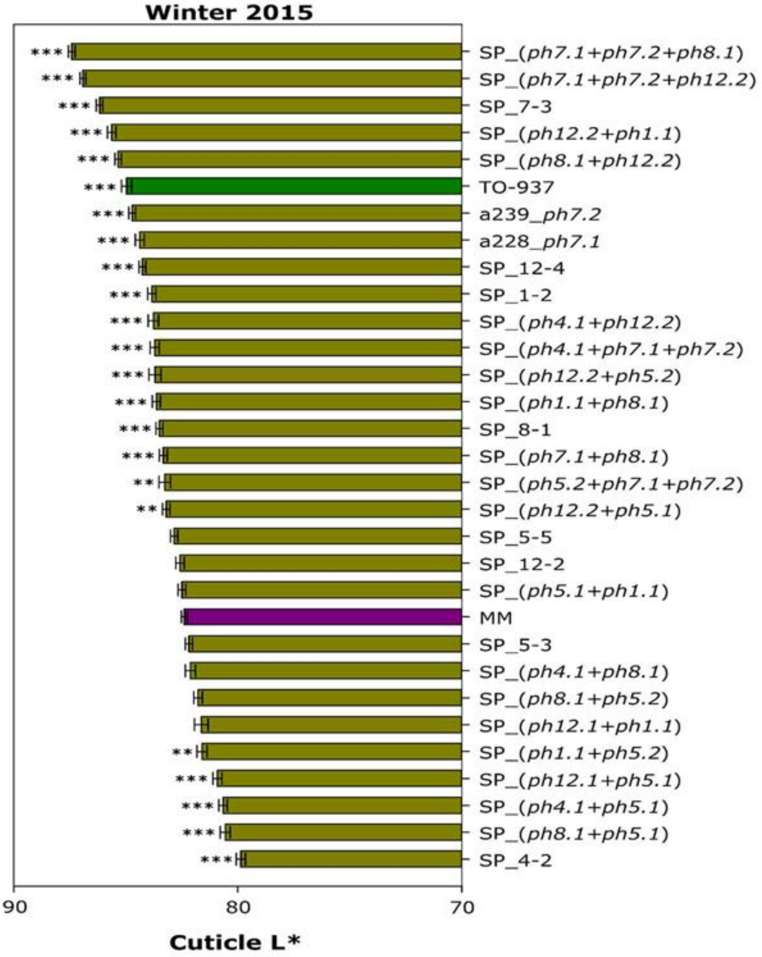

Fig. II-19: Comparison between the SP_(QTL+QTL) lines, the SP lines derived from, nSPs: a228_*ph7.1* and a239_*ph7.2*, TO-937 and MM during winter 2015 for the cuticle L* trait. Cuticles from red ripe fruits were analyzed. Data are presented as means ±SE. Asterisks indicate significant differences between all lines and MM (*p<0.05, **p<0.01, ***p<0.001). SP_1-2; SP_4-2; SP_5-3; SP_5-5; SP_7-3; SP_8-1; SP_12-2 and SP_12-4 harbor respectively *ph1.1*, *ph4.1*, *ph5.1*, *ph5.2*, *ph7.1+ph7.2*, *ph8.1*, *ph12.1* and *ph12.2*. nSPs: a228 and a239 harbor respectively *ph7.1* and *ph7.2.* L*, lightness; SP, *Solanum pimpinellifolium*; QTL, quantitative trait loci; *ph*, QTL percentage of phenolics.

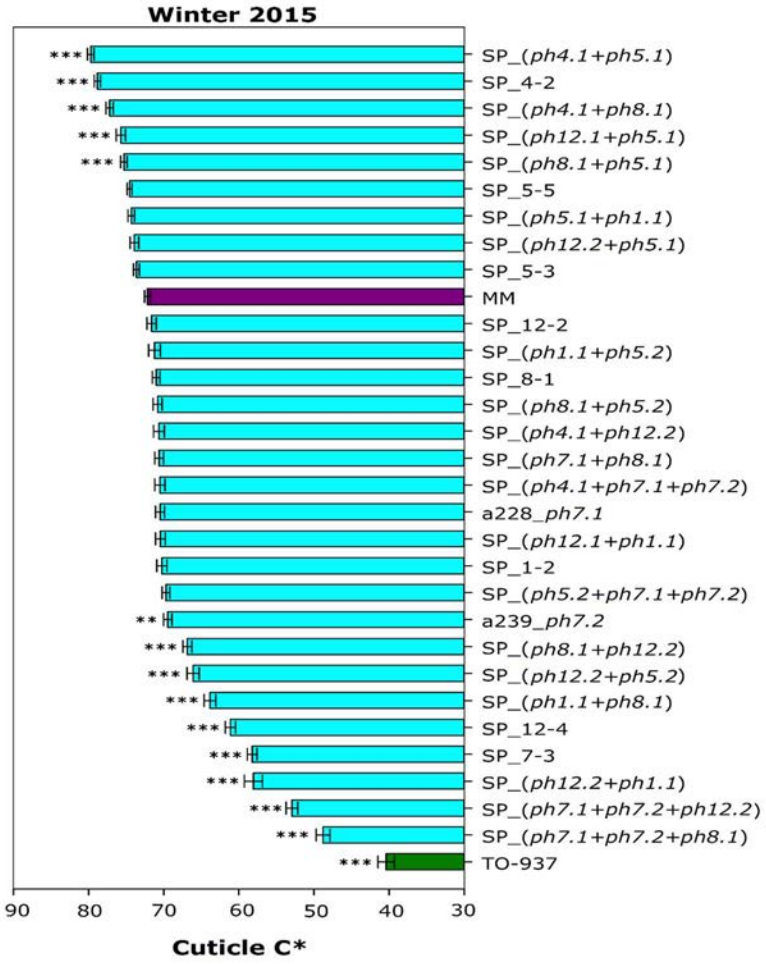

Fig. II-20: Comparison between the SP_(QTL+QTL) lines, the SP lines derived from, nSPs: a228_*ph7.1* and a239_*ph7.2*, TO-937 and MM during winter 2015 for the cuticle C* trait. Cuticles from red ripe fruits were analyzed. Data are presented as means ±SE. Asterisks indicate significant differences between all lines and MM (*$p<0.05$, **$p<0.01$, ***$p<0.001$). SP_1-2; SP_4-2; SP_5-3; SP_5-5; SP_7-3; SP_8-1; SP_12-2 and SP_12-4 harbor respectively *ph1.1*, *ph4.1*, *ph5.1*, *ph5.2*, *ph7.1+ph7.2*, *ph8.1*, *ph12.1* and *ph12.2*. nSPs: a228 and a239 harbor respectively *ph7.1* and *ph7.2.* C*, chroma; SP, *Solanum pimpinellifolium*; QTL, quantitative trait loci; *ph*, QTL percentage of phenolics.

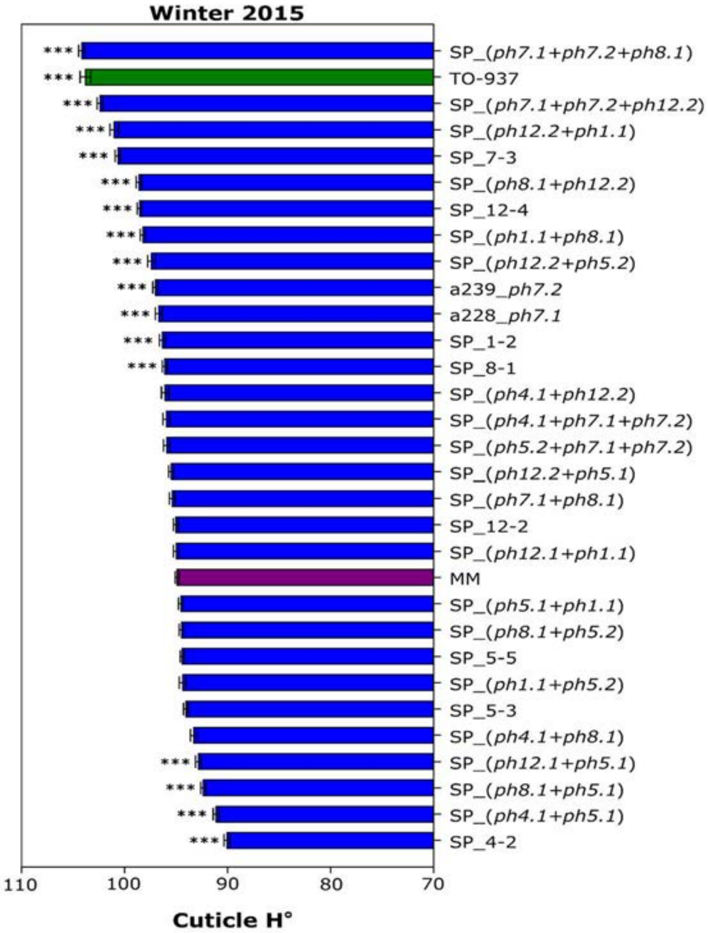

Fig. II-21: Comparison between the SP_(QTL+QTL) lines, the SP lines derived from, nSPs: a228_*ph7.1* and a239_*ph7.2*, TO-937 and MM during winter 2015 for the cuticle H° trait. Cuticles from red ripe fruits were analyzed. Data are presented as means ±SE. Asterisks indicate significant differences between all lines and MM (*$p<0.05$, **$p<0.01$, ***$p<0.001$). SP_1-2; SP_4-2; SP_5-3; SP_5-5; SP_7-3; SP_8-1; SP_12-2 and SP_12-4 harbor respectively *ph1.1*, *ph4.1*, *ph5.1*, *ph5.2*, *ph7.1+ph7.2*, *ph8.1*, *ph12.1* and *ph12.2*. nSPs: a228 and a239 harbor respectively *ph7.1* and *ph7.2.* H°, hue; SP, *Solanum pimpinellifolium*; QTL, quantitative trait loci; *ph*, QTL percentage of phenolics.

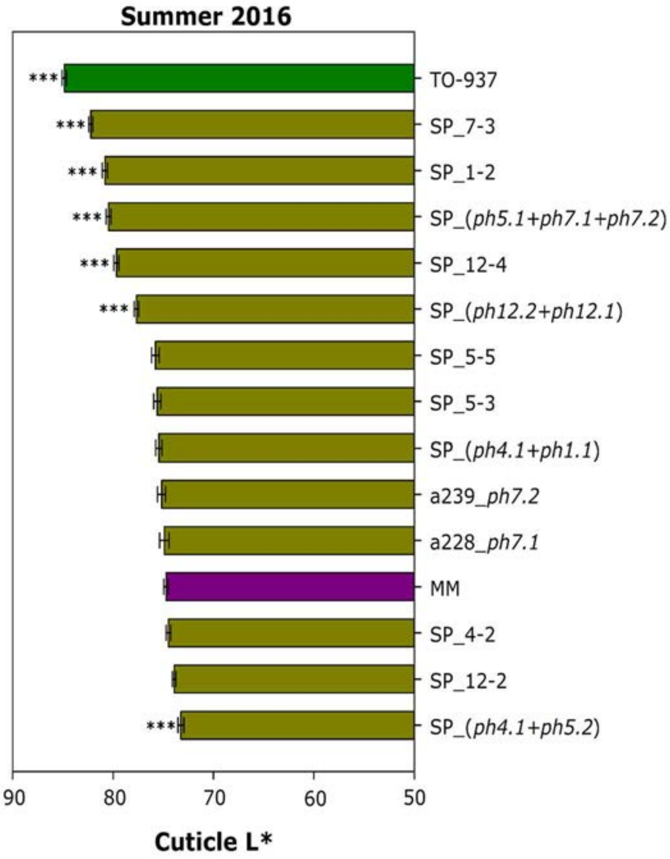

Fig. II-22: Comparison between the SP_(QTL+QTL) lines, the SP lines derived from, TO-937 and MM during summer 2016 for the cuticle L* trait. Cuticles from red ripe fruits were analyzed. Data are presented as means ±SE. Asterisks indicate significant differences between all lines and MM (*$p<0.05$, **$p<0.01$, ***$p<0.001$). SP_1-2; SP_4-2; SP_5-3; SP_5-5; SP_7-3; SP_12-2 and SP_12-4 harbor respectively *ph1.1*, *ph4.1*, *ph5.1*, *ph5.2*, *ph7.1+ph7.2*, *ph12.1* and *ph12.2*. L*, lightness; SP, *Solanum pimpinellifolium*; QTL, quantitative trait loci; *ph*, QTL percentage of phenolics.

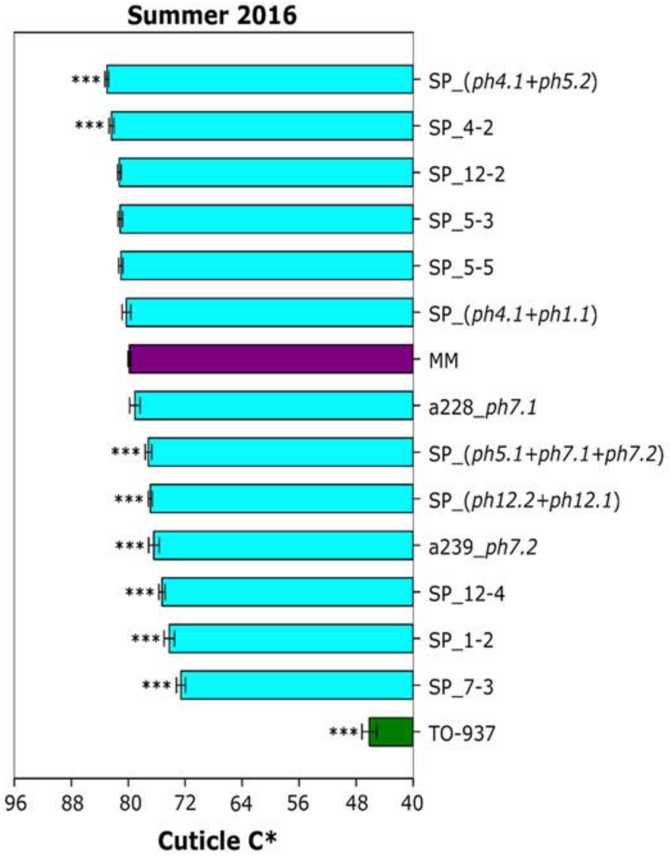

Fig. II-23: Comparison between the SP_(QTL+QTL) lines, the SP lines derived from, TO-937 and MM during summer 2016 for the cuticle C* trait. Cuticles from red ripe fruits were analyzed. Data are presented as means ±SE. Asterisks indicate significant differences between all lines and MM (**p*<0.05, ***p*<0.01, ****p*<0.001). SP_1-2; SP_4-2; SP_5-3; SP_5-5; SP_7-3; SP_12-2 and SP_12-4 harbor respectively *ph1.1*, *ph4.1*, *ph5.1*, *ph5.2*, *ph7.1+ph7.2*, *ph12.1* and *ph12.2*. C*, chroma; SP, *Solanum pimpinellifolium*; QTL, quantitative trait loci; *ph*, QTL percentage of phenolics.

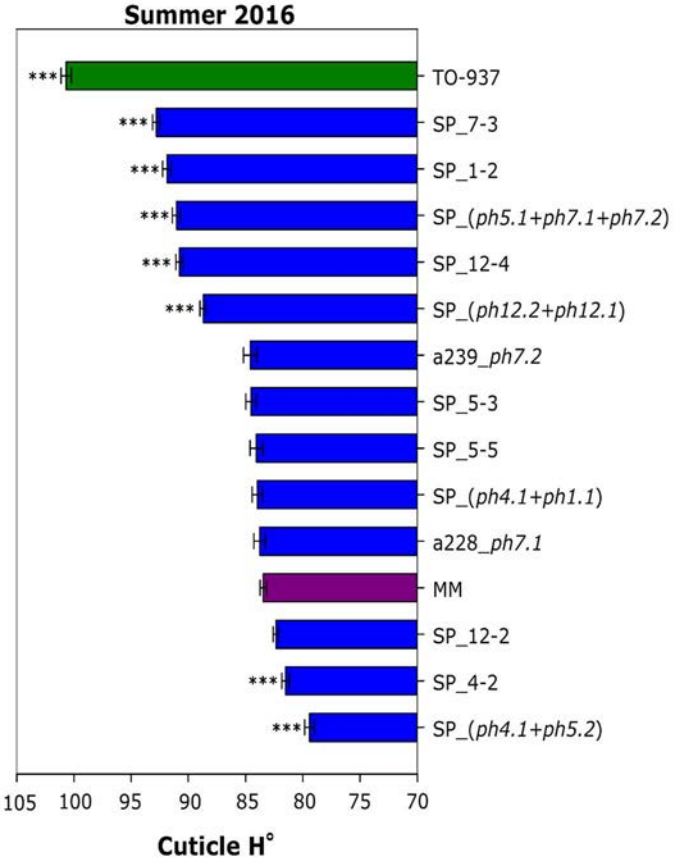

Fig. II-24: Comparison between the SP_(QTL+QTL) lines, the SP lines derived from, TO-937 and MM during summer 2016 for the cuticle H° trait. Cuticles from red ripe fruits were analyzed. Data are presented as means ±SE. Asterisks indicate significant differences between all lines and MM (*$p<0.05$, **$p<0.01$, ***$p<0.001$). SP_1-2; SP_4-2; SP_5-3; SP_5-5; SP_7-3; SP_12-2 and SP_12-4 harbor respectively *ph1.1*, *ph4.1*, *ph5.1*, *ph5.2*, *ph7.1+ph7.2*, *ph12.1* and *ph12.2*. H°, hue; SP, *Solanum pimpinellifolium*; QTL, quantitative trait loci; *ph*, QTL percentage of phenolics.

3.2 Seasonable Phenotypic Comparison for the Percentage of Phenolics Trait

The effects of the nine QTLs regions identified previously (see chapter I), were also investigated during two seasons, winter and summer, to study the effect of environmental factors on the expression of this trait.

Similar expression patterns of the QTLs were seen among all SP lines holding the nine regions for the percentage of phenolics trait in winter and in summer (Fig. II-25). However, all lines, except TO-937, have more phenolics in their cuticles in summer than in winter. These results show that in summer, the cuticle accumulates more phenolics than in winter.

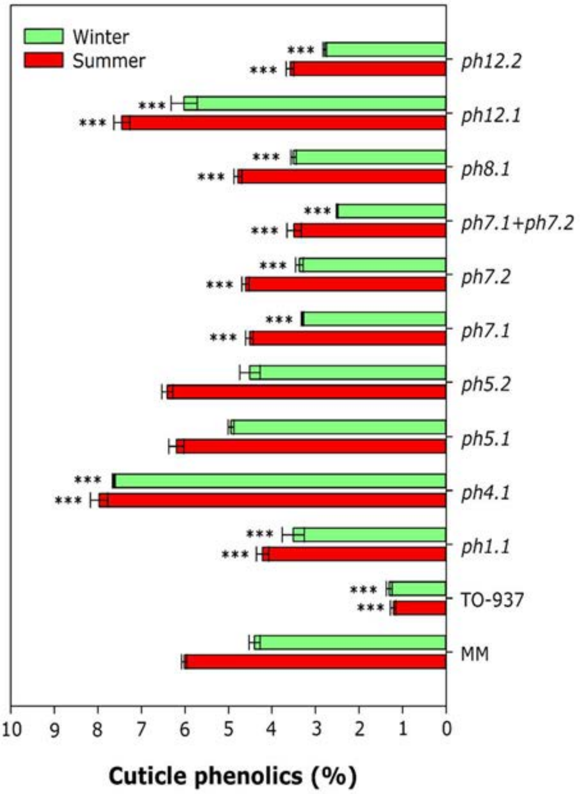

Fig. II-25: Percentages of cuticle phenolics from fruits harvested in winter and in summer. The parental line MM and the SP lines harboring QTL regions related to cuticle phenolics are shown. Data are presented as means ± SE. Asterisks indicate significant differences with MM (*$p<0.05$; **$p<0.01$; ***$p<0.001$). QTL, quantitative trait loci; *ph*, QTL percentage of phenolics.

4. DISCUSSION

Transgressive inheritance in both directions has been observed for most of the studied cuticle traits including cuticle phenolics in the RIL population indicating the presence of positive-effect genes related to cuticle flavonoid accumulation in both parents. Regarding the IL population, no SP line appeared to have inferior percentage of cuticle phenolics to the lower-flavonoid content parent TO-937 and few lines were found to have superior percentage to the higher flavonoid content parent MM. Thus, pyramiding different genomic regions containing QTLs was used as a strategy to create lines with higher or lesser flavonoid content than the parental lines and so corroborate the effects of the studied QTLs. Moreover, the identification of candidate genes involved in the variation of cuticle phenolics, through QTLs mapping experiments has led to the identification of individuals carrying favorable alleles at these *loci* that will provide genetic material for the development of new improved varieties. Localization of the effects to flavonoids, accompanied by testing specific interactions between phenolics QTLs, can improve the ability to select for specific QTL for crop improvement and can be used as a good basis for implementation of a QTL pyramiding strategy to bring flavonoid-specific QTL that perform additively into the same line.

QTL pyramiding approach has also enormous potential to improve our understanding of interactions among QTLs especially epistatic interactions. By definition, epistasis is the interaction between nonallelic genes whereby one gene interferes with the phenotypic expression of another gene (Bateson, 1907). If there are interactions among the objective genes/QTLs, breeding strategy must be addressed by the incorporation of the epistasis (Jahufer *et al.,* 2002; Dwivedi *et al.,* 2007). Carlborg and Haley (2004) showed that epistasis is a common response to selection in breeding programs. Therefore, genetic epistatic interactions should be considered in crop breeding strategies. For multiple genetic traits, the potential number of epistatic interactions is very large. Many classical quantitative genetic studies have established the importance of epistasis (Spickett and Thoday 1966; Falconer, 1981; Mather and Jinks, 1982; Pooni *et al.,* 1987). In maize and rice, evidence of epistasis and its importance to performance has been well documented (Lamkey *et al.,* 1995; Yu *et al.,* 1997; Li *et al.,* 1997; Lukens and Doebley, 1999; Luo *et al.,* 2001; Xing *et al.,* 2002; Hinze and Lamkey, 2003; Mei *et al.,* 2005).

Gene pyramiding is defined as a method aimed at assembling multiple desirable genes/QTLs from multiple parents into a single genotype (Ye and Smith, 2008). The end product of a gene/QTL pyramiding program is a genotype with all the target genes/QTLs fixed in homozygous condition (Ye and Smith, 2008). The advent of molecular marker technology and the availability of an IL population made the latter process easier. SNPs markers are not only very helpful for precise identification and mapping of QTLs underlying traits of agronomic interest as seen before but also for pyramiding QTLs/genes (Mekonnen *et al.,* 2017). Marker assisted QTL/gene pyramiding has not been used only for pyramiding major or qualitative disease resistance genes in plants but also for quality traits. So far, numerous crops including rice, wheat, cotton and tomato, with high yield and durable/broad spectrum resistance to pathogens, insect pests and herbicides have been developed by pyramiding multiple genes/QTLs of interest using MAS to contribute to increased crop production and productivity to maintain food security globally (Tanksley *et al.,* 1989; Cox *et al.,* 1993; Liu *et al.,* 2000; Gur *et al.,* 2011; Rigano *et al.,* 2014; Mekonnen *et al.,* 2017). The efficacy of pyramiding to improve tomato traits has been previously reported. For example, Gur *et al.* (2011) developed genotypes carrying three independent yield-promoting genomic regions introduced from *S. pennellii* that resulted in a hybrid with 50 % higher yields than leading commercial varieties in multiple environments and irrigation regimens. Moreover, introgressions originating from *S. pennellii* were introduced into lines of processing tomato. Sacco *et al.* (2013) created novel tomato lines by pyramiding QTLs increasing fruit ascorbic acid and phenolics content in the genetic background of the cultivated line M82. Pyramidation-constructed lines in the latter study revealed significant increases in total phenolics, phenolic acids, ascorbic acid, and total antioxidant activity compared to their parental lines IL_7-3 and IL_12-4 and to the cultivated line M82. Rigano *et al.* (2014) using the same latter material, demonstrated that it is possible to incorporate favorable wild species QTLs in the cultivated genetic background to obtain genotypes with higher nutritional value.

Some cuticle-related QTLs were previously identified and mapped in tomato and cucumber using different populations (Fernández-Moreno *et al.,* 2017; Rett-Cadman *et al.,* 2019), but no attention was paid to the accumulation of those QTL into one genetic background/line so far. In the present study, we used MAS to directly join several phenolics QTLs. The SP_(QTL+QTL) lines were obtained in double-homozygote condition so facilitating determination of the inheritance mode of each QTL for the cuticle phenolics content. The generated SP_(QTL+QTL) lines show increased, decreased or similar phenolics content than MM. The pyramiding of the percentage of cuticle phenolics QTLs showed that no SP_(QTL+QTL) line holding a combination of two QTLs with positive lyc effects lowered the percentage of cuticle phenolics to the level of TO-937. Only SP_(QTL+QTL) lines with a combination of a minimum of 3 QTLs with additive positive lyc effects can reduce to the TO-937 percentage of phenolics level, e.g., SP_(*ph7.1+ph7.2+ph8.1*) and SP_(*ph7.1+ph7.2+ph12.2*). Moreover, no SP_(QTL+QTL) line (during summer or winter) holding two phenolics QTLs with additive positive lyc effect can surpass the additive combined lyc positive effects of *ph7.1* and *ph7.2*. In other words, no combination among the SP_(QTL+QTL) lines shows significantly more cuticle phenolics than SP_7-3 that itself is considered a line with two QTLs with positive lyc effect for the percentage of cuticle phenolics. SP_(*ph7.1+ph7.2+ph8.1*) and SP_(*ph7.1+ph7.2+ph12.2*) have pale cuticle and their cuticle color parameters are not significantly different from TO-937. The hypothetical combination for example of *ph7.1*, *ph7.2, ph8.1* and *ph12.2* in one genetic background may generate a colorless cuticle that could lead to pink tomato fruits (Adato *et al.,* 2009) despite not presenting the *y* mutation. Pink tomatoes are highly appreciated by consumers especially in the Japanese market. Moreover, the significant increase in the amount of cuticle seen only in SP_(*ph7.1+ph7.2+ph12.2*) and SP_(*ph7.1+ph8.1*) but not in SP_(*ph7.1+ph7.2+ph8.1*), probably indicates that ph7.2 region blocks/interacts negatively with ph8.1 region which may have a positive pim effect on the amount of cuticle trait. It is striking that despite the differences in the amount of cuticle between SP_(*ph7.1+ph7.2+ph8.1*) and SP_(*ph7.1+ph7.2+ph12.2*), their final cuticle color parameters are not significantly different indicating that phenolic content is the main factor in the determination of cuticle color. As for the percentage of phenolics in SP_(*ph7.1+ph8.1*), it is not clear which phenolics QTL is causing the final effect. Thus, it is interesting to do further investigation to figure out which QTL is suppressed/masked: *ph7.1* or *ph8.1* or simply, *ph7.1* could be inactive without *ph7.2* in the MM genetic background. The dissection of the interactions between *ph7.1*, *ph7.2* and *ph8.1* would be highly important and possible since the required material is available.

As mentioned previously in chapter I, *ph1.1* is the QTL with the highest additive effect according to the QTL analysis. However, the percentage of phenolics of SP_(QTL+QTL) harboring at least *ph1.1* has modest decrease in the percentage of phenolics. When combined in the MM genetic background, *ph1.1* effect is totally suppressed by *ph12.2* and *ph8.1*, QTLs with positive lyc additive effect. These results confirmed what is been suggested previously in this work. *MYB12*, the identified candidate gene behind *ph1.1* could be masking its own effect by interacting with *ph8.1* and *ph12.2* regions. Thus, further analysis should be done to study the composition of the cuticle including percentage of waxes, cutin and polysaccharides and the expression level of genes such as *MYB12* (Cr. 1*)*, *FDH* and *KCS11 like1* (Cr. 8) and *KCS11 like2* (Cr. 12) in the lines SP_(*ph12.2+ph1.1*) and SP_(*ph1.1+ph8.1*) to understand the interactions between involved regions.

The amounts of cuticle and the amounts of phenolics of the latter SP_(QTL+QTL) lines are the lowest among all SP_(QTL+QTL) lines. It seems that there is an interaction between pim regions containing *ph1.1*, *ph8.1* and *ph12.2* to reduce drastically the amount of cuticle. Contrarily to what is seen in combinations such as *ph7.1+ph7.2* & *ph8.1* and *ph7.1+ph7.2* & *ph12.2* concerning the cuticle color parameters traits, the significant differences in the amount of cuticle seen in SP_(*ph12.2+ph1.1*) and SP_(*ph1.1+ph8.1*) contribute to the significant changes in the cuticle color L*, C* and H° traits, together with the percentage of phenolics trait. It is not clear whether the changes in the amount of cuticle in the different phenolics QTL+QTL combinations have consequences also on the cuticle architecture including cuticle invagination, thickness and density and thus on the cuticle color parameters. Comparison of RIL 82 and RIL 84, derived from the same parental lines MM and TO-937, shows that significantly different cuticle masses can display similar thickness and invagination (Barraj Barraj *et al.,* 2021). At the same time, significant differences in cuticle thickness can be observed in lines with similar cuticle mass and invagination (RIL 12 vs. RIL 83), as well as significant changes in the degree of invagination without affecting cuticle mass or thickness (RIL 40 vs. RIL 142) (Barraj Barraj *et al.,* 2021).

QTLs exhibiting opposite phenotypic additive effects on cuticle phenolics trait were also combined. The performances of SP_(QTL+QTL) lines carrying contrary additive effect on cuticle phenolics do not always exhibit phenolic content equal to those of the parental line MM. Thus, the sum of their effects was not always nullified. For example, *ph4.1* & *ph7.1+ph7.2* and *ph4.1* & *ph12.2* neutralize each other as in SP_(*ph4.1+ph7.1+ph7.2*) and SP_(*ph4.1+ph12.2*) respectively, indicating that *ph4.1*, (*ph7.1+ph7.2*) and *ph12.2* individual effects are equal in the MM genetic background. Moreover, *ph12.2* positive lyc effect is stronger than the individual effects of *ph7.1* and *ph7.2*. Similarly, in SP_(*ph12.1+ph1.1*), *ph12.1* and *ph1.1* has opposite but equal effects. However, it is not the case in SP_(*ph4.1+ph1.1*) SP_(*ph4.1+ph8.1*) and SP_(*ph12.2+ph12.1*), where two types of genetic effects exist: epistatic and additive. In SP_(*ph4.1+ph8.1*) and SP_(*ph12.2+ph12.1*), *ph4.1* positive pim effect is stronger than *ph8.1* positive lyc effect and *ph12.2* positive lyc effect is stronger than *ph12.1* positive pim effect respectively, leaving the remaining final effect in both SP_(QTL+QTL) lines like that of to the strongest QTLs *ph4.1* and *ph12.2*. As in SP_(*ph4.1+ph1.1*), *ph1.1* positive lyc effect is masked by *ph4.1* positive pim effect. This may indicate that *ph1.1* additive lyc effect might be strongly affected by epistatic interactions with the genetic background *loci* since the selection of SP_(QTL+QTL) lines may lead to obtaining different QTLs surrounding regions than the original introgression in SP_1-2. Thus, it is not clear whether the effect of *ph1.1* retains its magnitude and stability under different background conditions because it is possible that *ph1.1* showed QTL × QTL and/or QTLs x background interactions, even if its single effect is additive. The expressions of QTLs for complex traits are strongly affected by the genetic backgrounds (Mei *et al.,* 2006; Zheng *et al.,* 2011). Therefore, further investigation through the dissection of the introgressed regions of SP_(*ph4.1*+*ph1.1*) into reduced sub-SP_(QTL+QTL) will facilitate the understanding of the QTL effects and their different epistatic interactions. The newly created SP_(QTL+QTL) lines will be useful source to study the epistatic interactions between the ´new´ introgressed donor segment and the rest of the recurrent parent genome which gives an important clue about differences in performance of the same set of QTLs under variable backgrounds conditions. Moreover, it is also useful to make a comparison study on genomic level using different SNPs markers via MAS between the SP_(QTL+QTL) and the corresponding SP lines to identify background differences.

Among all SP_(QTL+QTL) lines, no combination surpasses the individual effect of *ph4.1* for the percentage of cuticle phenolics trait. Even a combination harboring *ph4.1* and other phenolics QTL with additive/epistatic positive pim effect such as *ph5.1*, *ph5.2* and *ph12.1*, show no increase in the percentage of phenolics significantly higher than that of SP_4-2. Interestingly, for *ph5.1* that is considered an epistatic QTL, its positive effect is only seen when combined to *ph8.1*, QTL with additive positive lyc effect, indicating that *ph8.1* pim region activates *ph5.1* or in other words, *ph5.1* pim effect is masked by *ph8.1* lyc region. However, *ph5.1*+*ph8.1* combined positive pim effect was detected only at two developmental stages IG and RR. This could indicate that *ph5.1* pim region is the one activating and thus, converting *ph8.1* positive lyc effect into pim effect. Probably, this hypothesis is only possible when the increase caused by *ph5.1* is significant. A SP_(QTL+QTL) line such as SP_(*ph8.1*+*ph5.1*) showing superior performances for their cuticle phenolics (percentage and amount) is useful material for breeding programs. Line displaying significant increase in phenolics contents without changes in the amount of cuticle (and probably in its cuticle thickness) could provide tools for breeding programs working on the improvement of the nutritional value and the mechanical resistance, traits that are partially controlled by the phenolic component of the cuticle. Surprisingly, no combination including *ph5.2*, epistatic QTL with positive pim effect, caused significant increase in the percentage of phenolics, even a combination with *ph4.1* did not display a percentage of phenolics higher than that of SP_4-2. It is not clear whether *ph5.2* pim region interacts only with pim regions causing decreases in the percentage of phenolics by masking them. Hence, the generation of SP_(*ph12.1*+*ph5.2*) and SP_(*ph5.1*+*ph5.2*) is needed to study the interactions between combined QTLs. However, it is clear that *ph5.2* is inhibiting/masking additive positive lyc ph QTLs such as *ph1.1* and *ph8.1* and preventing any effects. In combinations with *ph7.1*+*ph7.2* and *ph12.2*, *ph5.2* can be probably masked by these two phenolics QTLs with high additive positive lyc effects and thus, the remaining positive lyc effects seen in SP_(*ph5.2*+*ph7.1*+*ph7.2*) and SP_(*ph5.2*+*ph12.2*) are caused by *ph7.1*+*ph7.2* and *ph12.2* reduced effects under different background conditions. In general, QTLs with relatively big effects are more stable than those with small effects (Tanksley, 1993; Khan *et al.,* 2012). However, El Soda *et al.* (2014) demonstrated that main QTLs effects can be reduced or nullified under different conditions.

The introgression of QTLs/genes and its combinations do not always result in desirable improvement for the targeted trait. In some cases, the effect of identified genetic *loci* in a QTL/gene pyramiding program is not sufficient to fully explain the desired phenotypic variation due to various interactions occurring for a complex quantitative trait. The possible interactions between the genetic make-up, epistasis, pleiotropy and linkage among the introgressed *loci*, with the genetic background could have negative/positive effects on the expression of the traits that should never be underestimated (Xu and Crouch, 2008; Bovill *et al.,* 2010; Sandhu *et al.,* 2018). Consequently, the novel genotypes could be used as genetic material for breeding schemes aimed at generating new hybrids or improved varieties with higher nutritional levels, resistant to cracking, with long shelf life etc. However, to use these lines as breeding material to improve for example the nutritional value trait, it will be important to understand if introgressed regions from wild species determine taste alterations and/or allergenic effects. It is also crucial to test the performance of the set of QTLs pyramided to check its stability and magnitude under different environmental conditions, thus facilitating the use of introgressed regions as a source of favorable alleles for higher phenolics for example.

To conclude, it is noteworthy to mention that when the targets are QTLs with moderate or small effects, pyramiding may be less successful. However, in selection for phenolics, it is important to be able to detect QTLs of small magnitude, because these may play an important role in achieving the ultimate selection response in a breeding program. The results obtained herein reinforce the importance of epistasis investigations in marker trait association studies, as the individual effect of a marker as locus depends on the marker genotype at other interacting loci. In fact, a favorable allele at one locus may be an unfavorable one in a different genetic background, and vice versa (Holland, 2001). Thus, this must be taken in consideration, especially for phenolics, due to the several possible interactions between the multiple alleles from different loci. Furthermore, the results indicate that epistatic interaction plays an important role in controlling the expression of complex traits. Thus, the utilization of marker assisted selection in different plant breeding programs must take epistatic effects into consideration. In addition to that, inaccurate QTL localizations result in the need to select for more markers loci covering large genomic segments to be certain that target QTL alleles are retained in selected progeny. Moreover, QTL effects may be specific to a particular combination of introgression and genetic background. Finally, our results show that many different QTLs are needed to be pyramided to achieve a significant improvement emphasizing the polygenic and complex nature of the studied traits.

CONCLUSIONS

CONCLUSIONS

1. Results from the performed QTL analysis show that the genetic control of the studied cuticle traits is polygenic and complex. For most traits the two parental lines contributed with positive and negative allelic effect QTL regions. Moreover, additive and epistatic interactions were detected.

2. Despite the negative correlation between cuticle phenolic and wax contents reported in the literature and also observed in the RIL population here studied, a number of QTLs that independently control these two traits were identified. This will allow improving the fruit cuticle mechanical resistance driven by phenolics without compromising its water barrier properties determined by waxes.

3. The genetic control of the very low phenolic content of the cuticle of the *S. pimpinellifolium* accession here studied implies the combination of multiple QTL regions with small effects. However, the effects of several of these minor *loci* were masked by epistatic interactions which make difficult its precise control in breeding. The identification of QTLs with epistatic as well as additive interactions would help understanding the complex network of genetic interactions responsible for cuticle synthesis and would provide insights of the traits and their potential for breeding purposes.

4. Most of QTL regions identified in this study had not been previously reported. Only a small set of them colocalized with cuticle genes already described in the literature that were postulated as candidate genes.

RESUMEN EXTENDIDO EN ESPAÑOL

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La cutícula vegetal, como interfaz entre la planta y el medio ambiente, juega un papel en el desarrollo de la planta, la calidad del fruto y la postcosecha. Entre las funciones con relevancia agronómica que ejerce, se encuentran la protección frente a la pérdida de agua y la radiación UV, su función como regulador térmico, y la protección mecánica frente a patógenos y estreses abióticos, ya sean de origen ambiental o causados por los propios tejidos internos de la planta. En suma, la cutícula vegetal participa en la protección contra la penetración de hongos, el agrietado de los frutos y otras estructuras, la deshidratación y la fusión de los órganos. Además, tiene un papel relevante en el control del desarrollo de los órganos. La cutícula está formada por una matriz lipídica de polihidroxi ácidos grasos esterificados denominada cutina, ensamblada con los polisacáridos procedentes de la pared de la célula epidérmica, que además contiene compuestos fenólicos y ceras. Las ceras son una mezcla de lípidos solubles y triterpenoides los cuales pueden encontrarse dentro de la matriz de la cutícula o depositados en la superficie exterior. Las ceras tienen un papel fundamental en el control de la pérdida de agua de los tejidos internos y también pueden conferir cierta resistencia mecánica. En la cutícula del fruto del tomate, las ceras juegan un pequeño papel en la resistencia mecánica, siendo la fracción fenólica el principal modulador de la rigidez, deformación y resistencia de la cutícula. Esta fracción fenólica está compuesta por derivados de ácidos cinámicos presentes en la cutícula ya desde los primeros estadios del desarrollo del fruto y que aumenta significativamente durante la maduración con la incorporación del flavonoide chalconaringenina, responsable del color amarillo anaranjado de la cutícula en tomates rojos maduros. La chalconaringenina constituye aproximadamente el 1% del peso seco de la piel del fruto de tomate maduro y es el único flavonoide que se transporta e incorpora a la cutícula. La síntesis de flavonoides, de importancia en la calidad nutricional y organoléptica del fruto del tomate, está regulada por señales del desarrollo, fisiológicas y ambientales. Por lo tanto, la identificación de genes o QTLs (*loci* de caracteres cuantitativos) que regulen la síntesis de flavonoides y otros compuestos fenólicos que se acumulan en la cutícula es un aspecto importante para la calidad del fruto. Numerosos estudios han demostrado la contribución de los flavonoides de la cutícula en varios caracteres agronómicos importantes y complejos, incluida la rigidez del fruto, el color final del fruto del tomate, el brillo del fruto, todos ellos caracteres relacionados con la calidad externa del fruto que tienen un gran impacto en la decisión de compra del consumidor. Puesto que los flavonoides también participan en la calidad nutricional del fruto en términos tanto nutricionales como organolépticos, la modificación de la acumulación de flavonoides en la cutícula afectará también a dichos caracteres organolépticos. Es importante mencionar que la calidad organoléptica se ha convertido en un foco importante en los programas de mejora genética de tomate. Estos caracteres suelen presentar variaciones cuantitativas influenciadas por las condiciones ambientales y controladas por varios genes que son difíciles de identificar.

Es fundamental entender la base genética de la composición y el color de la cutícula para la mejora genética de los cultivos. Aunque se han identificado numerosos genes relacionados con la biosíntesis de la cutícula, la mayoría de ellos se han descubierto tras el análisis de poblaciones mutagenizadas o el silenciamiento de genes. Sin embargo, su participación en la variación genética presente en poblaciones naturales, cultivares y especies silvestres estrechamente relacionadas con los cultivos es incierta. Pocos estudios se han centrado en el análisis de la variabilidad de la cutícula en poblaciones naturales, siendo las ceras el principal carácter estudiado en varias especies. Los análisis de genes implicados en caracteres anatómicos y biofísicos de la cutícula, muchos de gran importancia para el rendimiento de la planta y la calidad del fruto, son escasos. En este sentido cabe mencionar la relación encontrada entre la pérdida de agua en postcosecha en pimiento con las diferencias en la composición de ceras y cantidad de cutina, o la identificación de QTLs asociados al grosor y grado de invaginación de la cutícula en tomate y pepino.

La mejora genética del tomate se basa en gran medida en la explotación de la variación natural de las especies silvestres emparentadas con el tomate cultivado. El estudio de algunas especies silvestres de tomate ha demostrado la existencia de variabilidad para algunos caracteres anatómicos y químicos de la cutícula. Sin embargo, su genética solo se ha estudiado en líneas derivadas de *Solanum pennellii* Dun., una especie que presenta frutos maduros de color verde. En este trabajo, se ha explorado por primera vez la variabilidad de la cutícula de una especie silvestres de fruto rojo en una población de líneas recombinantes consanguíneas (RIL) y una población de líneas de introgresión (IL) ambas derivadas del cruce interespecífico entre una variedad de tomate cultivado, *S. lycopersicum* L. 'Moneymaker' (MM) y una entrada de su pariente silvestre más cercano *S. pimpinellifolium* L., 'TO-937'.

El trabajo que se describe en esta tesis se inicia a partir de un estudio preliminar de la cutícula de las 169 líneas de la población RIL así como de sus parentales MM y TO-937. En dicho estudio previo se aislaron las cutículas de frutos rojo maduro y se estudió la cantidad de cutícula y de cada uno de sus componentes mayoritarios (ceras, polisacáridos, cutina y fenoles), además de su color empleando el espacio de color CIEL*C*H° donde L* indica la luminosidad, C* la croma o saturación y H° el tono. Ese estudio permitió identificar variabilidad entre los parentales para casi todos los caracteres de cutícula analizados, con la excepción de la cantidad de cutina y de polisacáridos. Sin embargo, la población RIL sí mostró variabilidad para todos los caracteres de cutícula, incluyendo cantidad de cutina y de polisacáridos, al igual que reveló la existencia de herencia transgresiva para varios caracteres como es el caso de la cantidad de cutícula, fenoles y ceras. Partiendo de esta información, se establece como objetivo general de esta tesis analizar la arquitectura genética que controla los diferentes caracteres de composición de cutícula, prestando especial atención a los fenoles y al color de la cutícula con vistas a aprovechar la diversidad genética natural disponible en la especie silvestre para transferir a variedades comerciales los alelos beneficiosos que afectan los caracteres asociados de la cutícula. Para alcanzar este objetivo principal, se establecieron cuatro objetivos específicos:

1. Localizar las regiones genómicas que controlan los caracteres involucrados en la composición y el color de la cutícula mediante

la realización de un análisis QTL utilizando poblaciones de cartografiado genético de líneas recombinantes consanguíneas (RIL) y líneas de introgresión (IL) derivadas del cruce de *S. lycopersicum* x *S. pimpinellifolium*.

2. Validar los QTLs identificados mediante una caracterización fenotípica de las líneas de introgresión que los contienen y un nuevo conjunto de subIL con introgresiones acortadas.
3. Identificar genes candidatos dentro de las regiones genómicas de los QTLs a partir de la información disponible en la bibliografía tanto en tomate como en otras especies y el análisis bioinformático de dichas regiones genómicas. Posteriormente, validar la hipotética relación funcional entre los genes candidatos y los QTLs mediante análisis de los niveles de expresión.
4. Crear nuevas líneas de introgresión que porten dos o tres QTLs implicados en el porcentaje de fenoles de cutícula para estudiar los efectos (epistáticos o aditivos) de las distintas combinaciones de QTLs.

Para el análisis de QTL de la población RIL se empleó un mapa de ligamiento con 4,700 marcadores SNP (single nucleotide polymorphism o polimorfismo de nucleótido único) polimórficos entre los dos parentales que se redujo posteriormente a 1,302 marcadores SNP informativos con el fin de realizar, además de análisis de QTLs únicos, análisis de QTLs epistáticos. Se detectaron un total de 60 QTLs para los diferentes caracteres estudiados en ambas poblaciones. El análisis de QTLs para los diferentes componentes de la cutícula y caracteres de color reveló una naturaleza poligénica compleja, combinando interacciones aditivas y epistáticas entre los diferentes QTLs. El análisis de la población IL permitió por un lado validar los QTLs identificados en la población RIL y por otro identificar QTLs nuevos que bien no eran significativos en la población RIL o no aparecieron en el análisis de QTLs la población RIL. La herencia transgresiva encontrada en la población RIL para la mayoría de los caracteres analizados indicó la presencia de QTLs con una contribución positiva al carácter en ambos padres. De las regiones genómicas identificadas, las cuales abarcan diez de los doce cromosomas, algunas eran específicas y solo afectaban a uno o dos caracteres, mientras que otras eran más complejas y producían cambios globales que comprendían a la mayoría de los parámetros estudiados. Descubrir e interpretar la compleja red de interacciones genéticas responsables de la síntesis de la cutícula permitirá una mejor comprensión de su variabilidad natural, y ayudará en la comprensión de cómo han evolucionado estos caracteres y cuál sería su potencial para fines de mejora.

Adicionalmente, se procedió a acortar tres líneas SP (*Solanum pimpinellifolium*) con el objetivo de separar distintos QTLs que se encontraban dentro de la misma introgresión. Así, la SP_3-2 se acortó para separar los QTLs de cantidad de cutícula *cm3.1* y *cm3.2*. Por otro lado, el acortamiento de la SP_7-2 permitió estudiar el efecto individualizado de *ph7.1* y *ph7.2*. Para ello se partió de líneas F_2 procedentes del cruce entre las correspondientes líneas SP con MM. En cada una de estas poblaciones F_2 se seleccionó con la ayuda de marcadores moleculares aquellas plantas que llevaban cada uno de los QTLs individualizados y posteriormente se identificaron con marcadores adicionales los bordes de las introgresiones en estas nuevas líneas SP. Se generaron cinco nSP a partir de SP_3-2: b10, b22, b40, b73 y b168. Las nSP b10 y b73 albergaron *cm3.1*, b22 y b40 *cm3.2* mientras que b168 lleva ambos QTLs. El análisis de la cantidad de cutícula para estas líneas mostró que las líneas con QTLs individuales tenían una reducción significativa en comparación con el MM parental, mientras que la combinación de *cm3.1+cm3.2* mostró una cantidad aún menor de cutícula que cada QTL individualizado. Además, el análisis del porcentaje de ceras mostró que b22, b40 o b168, todos ellos con *wx3.1*, tenían una cantidad de ceras similar a MM. En cuanto al color de la cutícula, se observó un aumento significativo de L* y H° en las líneas portadoras de QTLs para el color: b20, b40 y b168. Como se observó previamente en la población de IL, no se observaron diferencias para la cutícula C*. Se generaron tres nSP a partir de SP_7-2: a228, a239 y a203 que albergan *ph7.1*, *ph7.2* y *ph7.1+ph7.2,* respectivamente. La comparación del porcentaje de fenoles con MM mostró que cada QTL individual tuvo una reducción significativa y la línea que combina *ph7.1+ph7.2* mostró una reducción más fuerte en fenoles. También todas las líneas mostraron un aumento significativo en L* y H° mientras que una disminución en C* en comparación con MM con la excepción de C* en a228.

Para investigar los QTLs observados en SP_11-1 para la cantidad de cutícula y el porcentaje de ceras, se procedió a acortar esta línea siguiendo la estrategia previamente descrita, obteniéndose tres nuevas líneas SP solapantes c33, c49 y c199. Desafortunadamente, se observó una región entre los marcadores Solcap_62807 y Solcap_724 que no estaba presente en forma pim en ninguna de las tres líneas nSP generadas. Por lo tanto, la introgresión presente en c199 no fue la misma que la de SP_11-1. La comparación de MM con estas líneas nSP mostró una cantidad similar de cutícula y de porcentaje de ceras. Esto sugiere una de varias posibilidades: que el QTL detectado previamente en la SP_11-1 no fuera real o que estuviera localizado en la región no presente como pim en ninguna de las líneas nSP. Una explicación alternativa es que la población de líneas SP se analizó en verano, mientras que las líneas nSP se probaron en otoño-invierno. Dado que la deposición de la cutícula se ve afectada por las condiciones ambientales, siendo la alta temperatura e irradiación y la baja humedad relativa parámetros ambientales que favorecen la mayor síntesis y acumulación de cutícula y de todos sus componentes, es posible que este QTL se vea afectado significativamente por las condiciones ambientales y su efecto solo sea detectable en condiciones de primavera o verano. Para comprobar si la falta de efecto detectado en las líneas nSP pudiera ser efecto del ambiente, se volvieron a estudiar estas líneas junto con MM y la SP_11-1 en verano de 2018. Los resultados confirmaron la disminución en la cantidad de cutícula y porcentaje de ceras para SP_11-1 pero no para ninguna de las líneas nSP, lo que descartaría un efecto del ambiente y sugiere la localización de los QTLs *cm11.1* y *wx11.1* en la región comprendida entre los marcadores Solcap_62807 y Solcap_724. Para la validación de estos QTLs sería necesario generar nuevas líneas nSP derivadas de SP_11-1 que contuvieran esta región y que permitieran estudiar adecuadamente su efecto sobre la cantidad de cutícula y porcentaje de ceras.

Para la cantidad de cutícula se detectaron 15 QTLs. El mayor efecto sobre la cantidad de cutícula se observó en la línea SP que alberga *cm11.2* con una reducción de la cantidad de cutícula de dos veces en comparación con MM. El siguiente mayor efecto se detectó en *cm4.1* y en la combinación *cm3.1+cm3.2*. Cada uno de los QTLs del cromosoma 3 mostró una reducción similar en la cutícula, ~ 38%, mientras que la combinación mostró un pequeño efecto aditivo, reduciendo en un 45% la cantidad de cutícula. Por otro lado, el efecto pim de *cm5.2* produjo un aumento del 12-14% en la cantidad de cutícula. Aunque ninguna SP línea llevaba *cm10.2* individualizado, la comparación de *cm10.1+cm10.2* con *cm10.1* mostró un aumento en la cantidad de cutícula a niveles casi similares a los de MM, lo que indica que el efecto pim de *cm10.2* fue capaz de contrarrestar el efecto lyc de *cm10.1.*

Con respecto al porcentaje de fenoles se identificaron nueve regiones QTL en los cromosomas 1, 4, 5, 7, 8 y 12. A pesar del muy bajo porcentaje de fenoles presentes en la cutícula de TO-937, se identificaron cuatro QTLs con efecto pim: *ph4.1*, *ph5.1*, *ph5.2* y *ph12.1*. En concreto, los QTLs con efecto pim, *ph4.1* y *ph12.1*, mostraron un aumento de 30 y 20% en fenoles, respectivamente, en comparación con MM. Sin embargo, los otros dos, *ph5.1* y *ph5.2* no mostraron ninguna diferencia con MM. En cuanto a los QTLs con efecto lyc, la mayor reducción de fenoles, alrededor del 40%, se observó en *ph12.2* y en el efecto combinado de *ph7.1+ph7.2*, mientras que los restantes QTLs mostraron una reducción similar en el porcentaje de fenoles en rojo maduro. Sorprendentemente *ph1.1*, a pesar de ser el QTL con mayor efecto aditivo, mostró una reducción intermedia en compuestos fenólicos, comparable a *ph7.1*, *ph7.2* y *ph8.1.* Se observó un efecto aditivo notable para los QTL ubicados en el cromosoma 7, con la combinación *ph7.1+ph7.2* mostrando un porcentaje mucho menor de compuestos fenólicos que *ph7.1* o *ph7.2* por separado.

En el caso del porcentaje de ceras se identificaron nueve QTLs, seis con efecto pim y tres con efecto lyc. De los QTLs con efecto pim, solo *wx4.1* y la combinación *wx3.1+wx3.2* mostraron un porcentaje significativamente mayor de ceras que MM, mientras que las líneas que llevan *wx3.1* o *wx3.2* no mostraron ningún efecto significativo sobre las ceras. La mayor reducción de ceras se detectó en las líneas que llevaban *wx10.1*, *wx11.1* y *wx12.1*, con *wx11.2* y *wx12.2* mostrando una reducción bastante similar en las ceras. Sin embargo, la línea que alberga *wx1.1* no mostró ninguna diferencia con MM para ceras, y los otros dos QTLs con efecto pim, *wx11.2* y *wx12.2* mostraron un efecto opuesto. Dos de estos QTLs, *wx1.1* y *wx12.2*, se colocalizaron con *ph1.1* y *ph12.2* y mostraron efectos opuestos. Mientras que *wx1.1* y *wx12.2* exhibieron un efecto pim, *ph1.1* y *ph12.2* tuvieron efecto lyc. Sin embargo, los otros QTL para fenólicos no se asociaron con regiones genómicas relacionadas con la acumulación de ceras. El análisis de la colocación de ceras y fenólicos indica que la relación negativa entre porcentaje de fenoles y ceras de cutícula descrita en la bibliografía se basa en los QTL presentes en los cromosomas 1 y 12, ya que en ambos casos las regiones de los QTLs de fenoles y ceras solapan y, como se ha indicado, tienen efectos opuestos. Por otro lado, la identificación de QTL que modifican el porcentaje de fenoles o bien de ceras, como es el caso de *ph7.1*, ph7.2, *ph8.1* y *wx12.1*, indica que existen estrategias genéticas alternativas para evitar un compromiso entre el aumento de fenoles y ceras de la cutícula. Además, la colocación de un QTL con un efecto similar para ceras y fenoles en el cromosoma 4 sugiere una interacción compleja entre la acumulación de ambos compuestos. Como se observó anteriormente, a pesar de la identificación de un QTL para porcentaje de cutina en el cromosoma 1, la línea SP correspondiente tuvo valores similares a MM y solo los QTLs *cu10.1* y *ps10.1* mostraron diferencias significativas con MM.

Para los caracteres de color de la cutícula se identificaron un total de 24 QTLs entre ambas poblaciones. Cabe señalar que los QTLs de color de la cutícula colocalizaron con la mayoría de los QTLs identificados para fenoles de cutícula, a excepción de *ph12.1*, y con los dos QTLs para la cantidad de cutícula con mayor efecto aditivo, *cm3.2* y *cm11.2*. En este sentido, el mayor aumento de L* (mayor luminosidad, es decir, un color más claro), en torno al 8%, se observó para los QTLs *L11.1* y *L12.1*, coincidiendo con las líneas con menor cantidad de cutícula y menor porcentaje de fenoles, respectivamente. El resto de los QTLs con efecto lyc mostraron incrementos similares. *L4.1* mostró una disminución de L* que coincidió con el aumento de fenoles ya mencionado. La menor disminución en C* (~ 11%) se observó para *C12.1* mientras que el resto de las líneas mostraron reducciones similares. No se detectaron diferencias con MM en las líneas SP que llevaban *C3.1* o *C4.1.* En cuanto a H°, el mayor incremento se observó para *H12.1* y el efecto combinado de *H7.1+H7.2* seguido de *H11.1* mientras que el resto de las líneas con efecto lyc mostraron valores similares. El estudio de los QTLs individuales y combinados para el color presente en el cromosoma 7 mostró un efecto aditivo solo para H°.

Una vez identificados y validados los QTLs, se procedió al análisis bioinformático de dichas regiones genómicas aprovechando que el genoma del tomate está secuenciado y anotado (https://solgenomics.net/) y que además disponemos del genoma resecuenciado de los parentales estudiados en esta tesis, MM y TO-937. En un primer paso se recogieron todos los genes relacionados con la síntesis de cutícula y de sus componentes cutina, polisacáridos, fenoles y ceras previamente identificados en la bibliografía y se identificaron los posibles ortólogos en tomate de aquellos genes estudiados en otras especies. En un segundo paso se localizó la posición en el genoma de todos estos genes para estudiar si alguno de ellos colocalizaba en las regiones genómicas de los QTLs detectados y validados. Sorprendentemente, de los numerosos genes de cutícula, cutina, fenoles y ceras citados en la bibliografía, solo unos pocos estaban presentes en las regiones de algún QTL de este estudio. Este sí fue el caso de *MYB12*, un factor de transcripción que se sabe que modifica la ruta de los fenilpropanoides y que es el gen responsable de la mutación *colorless fruit epidermis* (*y*) en tomate. *LONG CHAIN ACYL CoA SYNTHETASE 1* (*LACS1*) es un gen involucrado en la biosíntesis de ceras y cutina en *Arabidopsis thaliana*. Ambos genes, *MYB12* y *LACS1*, se sitúan justo en el marcador del pico de los QTLs *ph1.1*, *cu1.1* y *wx1.1* o muy cerca. Por otro lado, *ECERIFERUM6* (*CER6*), un gen cuyo efecto en la biosíntesis de ceras ha sido estudiado en *A. thaliana* y en tomate, donde además se ha visto un efecto en la cantidad de cutícula, colocaliza con el marcador del pico de *cm2.2*. Otros dos genes de ceras, *FIDDLEHEAD* (*FDH*) y *β-KETOACYL-CoA SINTHASE*

11 (*KCS11*), colocalizan con el pico del QTL de fenoles *ph8.1*. Adicionalmente, también se identificó algún ortólogo de algún otro gen de ceras de *A. thaliana* en la región de QTLs relacionados con la cantidad de cutícula, aunque lejos del marcador del pico. A continuación, se procedió al estudio de todos los genes presentes en cada una de las regiones de los QTLs identificándose en algún caso genes pertenecientes a alguna familia génica relacionada con la síntesis de ceras o cutina, como el caso de *KCS11-like2* en *wx12.1*.

La expresión a lo largo del desarrollo de los genes candidatos identificados y presentes en la región cercana a los picos de algunos QTLs fue analizada por RT qPCR en el parental MM y la línea SP correspondiente. La línea SP que alberga la introgresión pim del QTL *ph1.1* mostró una disminución drástica de la expresión de *MYB12* durante la maduración en comparación con MM. Esta disminución fue de 2,8 veces en el estadio pintón y hasta 32 veces en el de rojo maduro. En cambio, la expresión de *LACS1* fue similar entre MM y la línea SP durante el desarrollo, y solo en el estadio final de rojo maduro se observó una disminución de 8.7 veces. En el caso de *ph8.1*, la expresión de *FDH* aumentó en MM durante el desarrollo alcanzando su máximo en pintón y luego decayendo en rojo maduro. Sin embargo, la línea SP correspondiente mostró un nivel bajo de expresión a lo largo de todo el desarrollo y disminuyó aún más durante la maduración. Por otro lado, la expresión de *KCS11-like1* mostró un comportamiento distinto, con un pequeño aumento durante la maduración con respecto al parental MM. Finalmente, la línea SP que contiene el QTL para ceras *wx12.1* mostró una mayor expresión de *KCS11-like2* en comparación con MM, especialmente durante todo el período de crecimiento hasta el inicio de la maduración en pintón.

En el segundo apartado de esta tesis se ha analizado el efecto en la cutícula de la acumulación de dos o más QTLs de fenoles. Para ello se han cruzado las ocho SP líneas que contienen los nueve QTLs de porcentaje de fenoles entre sí, de forma que las introgresiones quedaran en heterocigosis en los híbridos F_1. Mediante marcadores moleculares de tipo SNP desarrollados en este trabajo se comprobó si efectivamente las plantas F_1

contenían las introgresiones en heterocigosis, se autofecundaron las que resultaron positivas y se obtuvieron las progenies F_2 correspondientes y, en ellas, mediante los mismos marcadores, se seleccionaron las plantas homocigotas para las distintas combinaciones de QTLs deseadas. En total se generaron 23 líneas de las cuales 18 tenían combinaciones fijadas de dos QTLs y cinco tenían combinaciones de tres QTLs. Estas líneas se nombraron SP_(QTL+QTL) y se fenotiparon en este estudio durante dos temporadas (en el invierno de 2015 y en el verano de 2016) con el fin de mejorar la comprensión de los efectos combinados de los QTLs en el fondo genético del cultivar de tomate. Además, el estudio de las líneas SP_(QTL+QTL) nos ha ayudado a identificar los efectos, ya fuesen aditivos o epistáticos, de los QTL combinados en muchos caracteres relacionados con la cutícula, incluido el % de fenoles, la cantidad de cutícula, etc.

Las líneas SP_(QTL+QTL) generadas mostraron tres tipos de efectos en el contenido de fenoles: aumento, reducción o similar respecto al de MM. Entre las 19 líneas SP_(QTL+QTL) del invierno de 2015, solo 11 líneas SP_(QTL+QTL) mostraron diferencias significativas con MM. Entre ellas, siete líneas SP_(QTL+QTL) mostraron una disminución significativa del % de fenoles de cutícula. Las líneas SP_(QTL+QTL) con el porcentaje más bajo de fenoles de cutícula fueron SP_(*ph7.1+ph7.2+ph8.1*); SP_(*ph7.1+ph7.2+ph12.2*); SP_(*ph12.2+ph1.1*); SP_(*ph8.1+ph12.2*); SP_(*ph7.1+ph8.1*); SP_(*ph5.2+ph7.1+ph7.2*) y SP_(*ph12.2+ph5.2*). La mayoría de las últimas líneas SP_(QTL+QTL) combinan más de un QTL con efecto lyc positivo. SP_(*ph7.1+ph7.2+ph8.1*) y SP_(*ph7.1+ph7.2+ph12.2*), que muestran el porcentaje más bajo de fenoles, combinan respectivamente *ph7.1*, *ph7.2*, *ph8.1* y *ph7.1*, *ph7.2*, *ph12.2*, QTLs con el % más alto de variación fenotípica. Además, las líneas SP_(QTL+QTL) que contienen tres QTLs con efecto lyc positivo tienen menos fenoles que las líneas SP_(QTL+QTL) con dos QTLs con efecto lyc positivo. Por otro lado, tres líneas SP_(QTL+QTL): SP_(*ph4.1+ph5.1*), SP_(*ph8.1+ph5.1*) y SP_(*ph4.1+ph8.1*), muestran un aumento significativo en su % de fenoles de cutícula. Entre las cuatro líneas SP_(QTL+QTL) del verano de 2016, las SP_(QTL+QTL) que contienen *ph4.1*, QTL con efecto pim positivo, muestran un aumento significativo en el % de fenoles de cutícula en comparación con MM, por ejemplo, SP_(*ph4.1+ph1.1*) y SP_(*ph4.1+ph5.1*). SP_(*ph12.2+ph12.1*) y SP_(*ph5.1+ph7.1+ph7.2*) muestran una disminución significativa en el % de fenoles de cutícula en comparación con MM.

En conjunto, los resultados son interesantes porque resaltan que en la acumulación de fenoles en la cutícula del fruto se requerirían el concurso de varios genes localizados en distintas regiones genómicas y que lo esperable según el análisis de QTLs realizado en las poblaciones RIL e IL no siempre se cumple en el sentido de que combinaciones de QTLs importantes muestran poco efecto una vez se han colocado en el fondo genético del parental de la especie cultivada, mientras que aparecen efectos relativamente altos e inesperados al combinar regiones en principio no tan importantes según los resultados en la población RIL o IL. Por ello, es necesario realizar un análisis exhaustivo de posibles interacciones epistáticas tanto en las RILs como en las combinaciones entre QTLs en las ILs.

La introgresión de QTLs / genes y sus combinaciones no siempre dan como resultado una mejora en el carácter de interés. En algunos casos, el efecto de los *loci* identificados en un programa de acumulación (piramidación) de genes/QTLs no es suficiente para explicar completamente la variación fenotípica deseada, debido a la ocurrencia de interacciones génicas para estos caracteres cuantitativos complejos. Las posibles interacciones, ya sean la epistasis entre los *loci* introgresados o de cada uno de ellos con el fondo genético de la especie cultivada, además de los posibles efectos pleiotrópicos, podrían tener efectos negativos / positivos en la expresión de los caracteres que nunca deben subestimarse. En consecuencia, los nuevos genotipos generados en este trabajo podrían usarse como material genético para programas de mejora genética destinados a generar nuevos híbridos o variedades mejoradas con niveles nutricionales más altos, resistentes al agrietado, con una larga vida útil, etc. También es crucial probar el rendimiento del conjunto de QTLs combinados para comprobar su estabilidad y magnitud en diferentes condiciones ambientales, facilitando así el uso de regiones introgresadas como fuente de alelos favorables para cantidades de fenoles superiores, por ejemplo.

Para concluir, cabe mencionar que cuando los QTLs tienen efectos moderados o pequeños, la búsqueda de una combinación genética que mejore el carácter puede tener menos éxito. Sin embargo, en la selección de fenoles, es importante poder detectar QTLs de pequeña magnitud, ya que pueden desempeñar un papel importante en el logro de la respuesta de selección final en un programa de mejora genética. Los resultados obtenidos en este trabajo refuerzan la importancia de las investigaciones de la epistasis en los estudios de asociación de caracteres de marcadores, ya que el efecto individual de un marcador en el *locus* de interés depende del genotipo del marcador en otros *loci* que interactúan. De hecho, un alelo favorable en un *locus* puede ser uno desfavorable en un fondo genético diferente y viceversa. Por lo tanto, esto debe tenerse en cuenta, especialmente para los fenoles, debido a las diversas interacciones posibles entre los múltiples alelos de diferentes *loci*. Además, los resultados indican que la interacción epistática juega un papel importante en el control de la expresión de caracteres complejos. Por tanto, para la utilización de la selección asistida por marcadores en diferentes programas de mejora genética debe tenerse en cuenta los efectos epistáticos. Nuestros resultados muestran que se necesita un buen número de diferentes QTLs combinados en una misma línea de introgresión para conseguir lograr fenotipos equivalentes a los de las líneas parentales.

Este trabajo ha identificado varios QTLs de efectos mayores y menores implicados en la composición y el color de la cutícula, que colocalizan con genes candidatos previamente estudiados, lo que indica que las regiones genómicas donde residen dichos QTLs pueden ser muy relevantes para la mejora de muchas características agronómicas interesantes relacionadas con la cutícula y que influyen en gran medida en la aceptación de los frutos de tomate en los mercados para fresco y para procesado. El trabajo demuestra que la genética de la síntesis y regulación de los componentes bioquímicos de la cutícula es de naturaleza muy compleja. La identificación de QTLs con efectos epistáticos, así como los efectos aditivos revelados, ayudarán a entender la compleja red de interacciones genéticas responsables de los caracteres estudiados y permitirán una mejor comprensión de las posibilidades de la variabilidad natural en la mejora de estos caracteres.

La mayoría de los QTLs identificados en este estudio son totalmente novedosos y no previamente reportados en la literatura. En este trabajo se sugiere que existe una amplia variabilidad genética y alélica en las especies silvestres del tomate, que está disponible para la mejora de la calidad del fruto relacionada con la cutícula, mediante, por ejemplo, la modulación de los niveles de compuestos fenólicos o ceras haciendo uso de los alelos de *S. pimpinellifolium* en los QTLs de los caracteres estudiados. Así, con una estrategia de piramidación bien diseñada, los QTLs involucrados en compuestos fenólicos podrían combinarse en un mismo fondo genético para así obtener líneas interesantes punto de partida para desarrollar cultivares que presenten cutículas de determinadas características biomecánicas que les confieran mayor resistencia al agrietado, o en cuanto a su color o su firmeza.

Finalmente, este trabajo demuestra que el resultado combinado de QTLs en las líneas SP_(QTL+QTL) no siempre ha sido el esperado, pues se han observado casos en los que combinaciones de QTLs (por ejemplo, algunos de los de compuestos fenólicos) con un mismo efecto aditivo en el mismo sentido no han presentado la esperada suma de efectos aditivos en la línea que los combinaba, y que por tanto algunos de estos QTLs pueden perder su efecto individual cuando interactúan epistáticamente con otros QTLs, remarcando de nuevo la naturaleza extremadamente compleja de las bases genéticas de los caracteres estudiados.

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ANNEX I

Anx. I-1: SNP markers and primers designed to select nSPs derived from SP_3-2. Asterisks indicate the solcap marker corresponding to the QTL peaks that were used in the first step of selection. MAS, marker assisted selection; nSPs, newly created SP lines; SNP, single nucleotide polymorphism.

Marker	SNP	Forward and Reverse Primers (5'-3')
Solcap_55055	[T/C]	ACGTGCTGTTGTTGATTCAGA CGCTCACAAGGGTCTGTAAT
CL016675-0242	[T/C]	TTGCTCTTGAGTGGTGGAAC TCAACATCCTGATCAGAAACGA
Solcap_41353	[T/C]	TGCCCTCAACTAGAAACAACTC GCTTTGCGAGAGTCCCATTT
Solcap_26399	[A/G]	GGGTTCACTCTCTGGATCTCC CAAATCCTTTCGCAACGCCA
Solcap_18583	[T/C]	CCTGAAACATTGGCTTTTACTCGT GTTGATGGCATTGAAGGAGCA
Solcap_36888	[A/G]	GTGAAGCCAAGCAAAAGTGT ACTTTCTTCCCTGATCAAGCA
Solcap_30833	[A/G]	CCCCATCAAATAAGGGTGCC GTTTCACGAATTTCTGGCATTTT
Solcap_35697	[A/G]	TGACATACAATTCCCTGAGCTAGA TTCCTGCACAATTTGAGTATGCG
Solcap_62377	[T/G]	AAGCTACCCGGATTGCACC CCGTTGGGTTGCCTAAGATA
Solcap_9292*	[A/C]	GAAATGGCAAGGGAATTCAA CTTTTCTTGCATTATTCACTTCATC
Solcap_62180	[T/C]	TCCACAGATTCCAGGAAGTATT TTTTGGATTCCGGAACTTTG

Anx. I-2: SNP markers and primers designed to select nSPs derived from SP_7-2. Asterisks indicate the solcap marker corresponding to the QTL peaks that were used in the first step of selection. MAS, marker assisted selection; nSPs, newly created SP lines; SNP, single nucleotide polymorphism.

Marker	SNP	Forward and Reverse Primers (5'-3')
Solcap_68725	[T/C]	TGAAGCTCAGATGGATGATGAGA TTCCTGAAAATCTGCCACCA
Solcap_68446	[A/G]	AGAAAACCACCGTTCATGTTGT GATGGAGTGGAAGTGCAAAGA
Solcap_100437	[T/C]	ATCAGCTTCAACAAATCCGTTT CCCTGACTCAATCGATCATGG
SNP_446a*	[A/G]	AGAGGTGCCTTGAGTTGGAA ACGAGATCATCACTGTGGGA
Solcap_52220	[A/G]	GGATGCATCAGAATACGACGTT TGGAATTTCTCAGTTCATCACA
Solcap_38851	[T/C]	AGGAAACTACATCACTTATCTAAAAGG ATCTCCCAAGGCACAGATCA
Solcap_52557*	[T/C]	TCAAACACCTTTGGATCTTTCA TGAGTTCACATGATTCAGTTTACG
Solcap_53582	[T/G]	GCAGGCACCTAACAGTTCAC TGGTAGTGAACTAGTGGGCG
Solcap_53393	[T/C]	AAGAGCAAGGACGCCTTCA GGCTGGTAGACTTTCTGAACC
Solcap_31228	[A/C]	GAAATGGCAAGGGAATTCAA CTTTTCTTGCATTATTCACTTCATC
Solcap_66622	[T/C]	TTGTTCCAGCCCCTTCATCA AAGGGGTGAAGACTGCAAGT
Solcap_20977	[A/G]	GTGTTAACATCGCCGGTGAG CAAGAGGCTGCGATTGACTT
Solcap_20996	[A/G]	TCTCGGCGTCTTCAAATTCG CAGGTTGTTCATGGGATGCA

Anx. I-3: SNP markers and primers designed to select nSPs derived from SP_11-1. MAS, marker assisted selection; nSPs, newly created SP lines; SNP, single nucleotide polymorphism.

Marker	SNP	Forward and Reverse Primers (5'-3')
Solcap_21011	[T/C]	GGAAACGGTTGTCTAGAAATGGT
		TGCCTGGTCCTTCTATCGTT
Solcap_21079	[A/G]	TCTGGGTGCTCTGAACTTCG
		TGCGACTTCTATCAAACACCT
Solcap_9467	[A/C]	TGAATAATGATGCACTAGAGCCA
		GACGATCTGAACTTGTTTGGGA
Solcap_62807	[A/C]	AAACTGAAGCCAAGCACCTT
		AGTGGTATCATCTTTGTTCTCCA
Solcap_34345	[A/C]	TCTCAGTCACAGCCATCTTCT
		TTTGGTAAAGTAAGGTGCAGAGT
Solcap_6899	[T/C]	CCTCGTCAGCTCCTTTTGA
		ATGTCTGTGGTGGTCAAGGA
Solcap_724	[T/C]	GTAATGTCCTGCTTCTTTCCTGT
		GTGAAGTTCGACATTGACACCA
Solcap_12406	[T/C]	TACAATCCACTGTCGCCACT
		TTTTGAAACTCGGATGGGCA
Solcap_6114	[A/G]	GTCACCTCAGTACATGGGATAC
		GAAGGCCGAGGTGTCAGTAT
Solcap_100133	[T/G]	GCATGATCTGTTGAGTGTCATC
		TTTCGATCTGCAGTCAAACATT

Anx. I-4: Primers used for RT-qPCR. [1] this work, [2] Rueda Herrera, (2018), [3] Expósito-Rodríguez *et al.* (2008).

Gene symbol	Gene locus	Forward and Reverse Primers (5'-3')
MYB12	Solyc01g079620[1]	AAAGTGGGCATCAAGAGAGG GTAACGACCTCCAAGAGCCT
LACS1	Solyc01g079240[1]	AAGGATGGAAAGTGGGGTCC CCAGGTTCAATTCCGTGAGC
DET1	Solyc01g056340[1]	TGGCTGACATTTTCCTGCAA AGCAAGAGTAACACTGTACAACT
CER6	Solyc02g085870[2]	GGTTCTTCCAGCTTCAGAGC ATAACAGCTCTTCCACCAGC
CER1	Solyc03g117800[1]	ACGGATCAATAAGCGTTATGTACA GCTGGTAGAGACTATGAGGAATG
WSD1-like	Solyc07g053890[1]	ATATCCAACGAGCGATGCAG TTTGGCGGGAAGGAAATGTG
FDH	Solyc08g067260[1]	GAGTTACGTCCCGAGATCCA TTCATCCAGAGCTCCGAACA
KCS11-like 1	Solyc08g067410[1]	TGAGGCTGTGACTAATGTACCG GACAAAGAAGGGGTCGGGTT
KCS2	Solyc09g083050[1]	TCGTGTTACAAGCCCAAACCT TCCTCAGTGAAAATCCCGGC
KCS3	Solyc11g072990[1]	AGCTTGTATGCCATCGTTAGC ATGAGGCTAGCACTACACCC
KCS11-like 2	Solyc12g006820[1]	GGTGCAGACGACAAGTGTTT GCCACTGCCATGAGTTCTTT
CAC	Solyc08g006960[3]	CCTCCGTTGTGATGTAACTGG ATTGGTGGAAAGTAACATCATCG
EXP	Solyc07g025390[3]	GCTAAGAACGCTGGACCTAATG TGGGTGTGCCTTTCTGAATG
SAND	Solyc03g115810[3]	TTGCTTGGAGGAACAGACG GCAAACAGAACCCCTGAATC

Anx. I-5: Amount of cuticle of the SP inbred line population and both parental lines MM and TO-937. Cuticle from red ripe fruits were analyzed. Data are presented as means ±SE. Asterisks indicate significant differences with MM (***$p \leq 0.001$).

Line	μg·cm^{-2} cuticle	Line	μg·cm^{-2} cuticle
MM	2154.11 ± 44.77	SP_6-5	1947.04 ± 63.66
TO-937	1629.07 ± 34.99 ***	SP_7-1	1978.74 ± 56.74
SP_1-1	1606.91 ± 33.40 ***	SP_7-2	1961.00 ± 45.74
SP_1-2	2061.67 ± 71.14	SP_7-3	1563.57 ± 52.75 ***
SP_1-3	1948.20 ± 41.42	SP_7-4	1974.40 ± 79.41
SP_1-4	1798.07 ± 68.47 ***	SP_8-1	1428.00 ± 48.42 ***
SP_2-1	1691.91 ± 52.37 ***	SP_8-2	2125.19 ± 62.69
SP_2-2	1689.00 ± 45.51 ***	SP_8-3	1963.28 ± 63.28
SP_2-3	1690.39 ± 38.29 ***	SP_8-4	1951.62 ± 56.54
SP_2-4	1621.36 ± 55.38 ***	SP_9-1	1964.23 ± 56.01
SP_2-5	1610.40 ± 53.86 ***	SP_9-2	2017.74 ± 43.95
SP_3-2	1499.32 ± 57.71 ***	SP_9-3	1979.26 ± 35.57
SP_3-3	1487.77 ± 53.66 ***	SP_9-4	1944.07 ± 67.89
SP_3-4	1973.01 ± 40.92 ***	SP_10-1	2063.07 ± 34.29
SP_4-1	1949.44 ± 67.12	SP_10-2	1722.86 ± 59.32 ***
SP_4-2	1428.85 ± 55.47 ***	SP_10-3	1905.07 ± 61.01
SP_4-3	1322.82 ± 63.22 ***	SP_10-4	1954.62 ± 51.47
SP_4-4	1928.89 ± 65.50	SP_10-5	1962.49 ± 5 0.22
SP_5-1	1957.27 ± 60.24	SP_10-6	1997.37 ± 63.27
SP_5-2	1651.92 ± 44.86 ***	SP_11-1	1380.96 ± 46.10 ***
SP_5-3	1651.33 ± 62.98 ***	SP_11-2	1387.37 ± 29.03 ***
SP_5-4	1761.21 ± 55.14 ***	SP_11-3	1384.25 ± 64.50 ***
SP_5-5	2463.25 ± 61.26 ***	SP_11-4	1302.49 ± 72.90 ***
SP_6-1	2076.42 ± 69.49	SP_12-1	2086.36 ± 58.52 ***
SP_6-2	2026.39 ± 75.50	SP_12-2	1627.61 ± 68.24 ***
SP_6-3	1995.98 ± 60.26	SP_12-3	1549.80 ± 70.41 ***
SP_6-4	1970.09 ± 54.80	SP_12-4	1991.41 ± 71.05

Anx. I-6: Percentage of cuticle phenolics of the SP inbred line population and both parental lines MM and TO-937. Cuticle from red ripe fruits were analyzed. Data are presented as means ±SE. Asterisks indicate significant differences with MM (***p* ≤ 0.01; ****p* ≤ 0.001).

Line	% phenolics	Line	% phenolics
MM	4.75 ± 0.14	SP_6-5	4.51 ± 0.11
TO-937	1.36 ± 0.08 ***	SP_7-1	3.90 ± 0.07 ***
SP_1-1	4.46 ± 0.11	SP_7-2	3.54 ± 0.15 ***
SP_1-2	3.76 ± 0.09 ***	SP_7-3	3.13 ± 0.08 ***
SP_1-3	4.79 ± 0.15	SP_7-4	4.99 ± 0.17
SP_1-4	4.39 ± 0.05	SP_8-1	3.88 ± 0.10 ***
SP_2-1	4.49 ± 0.03	SP_8-2	4.44 ± 0.16
SP_2-2	4.55 ± 0.14	SP_8-3	4.76 ± 0.17
SP_2-3	4.70 ± 0.09	SP_8-4	4.86 ± 0.16
SP_2-4	4.67 ± 0.12	SP_9-1	4.77 ± 0.10
SP_2-5	4.57 ± 0.07	SP_9-2	4.76 ± 0.07
SP_3-2	4.86 ± 0.07	SP_9-3	5.11 ± 0.02
SP_3-3	4.58 ± 0.06	SP_9-4	5.21 ± 0.18
SP_3-4	4.34 ± 0.07	SP_10-1	5.24 ± 0.06
SP_4-1	4.86 ± 0.05	SP_10-2	4.98 ± 0.12
SP_4-2	6.23 ± 0.22 ***	SP_10-3	4.70 ± 0.17
SP_4-3	6.33 ± 0.15 ***	SP_10-4	5.10 ± 0.05
SP_4-4	5.07 ± 0.15	SP_10-5	4.47 ± 0.13
SP_5-1	4.64 ± 0.29	SP_10-6	4.48 ± 0.07
SP_5-2	4.81 ± 0.16	SP_11-1	4.78 ± 0.06
SP_5-3	4.58 ± 0.04	SP_11-2	5.09±0.09
SP_5-4	4.85 ± 0.02	SP_11-3	4.93 ± 0.17
SP_5-5	4.87 ± 0.03	SP_11-4	4.86 ± 0.09
SP_6-1	4.78 ± 0.13	SP_12-1	4.36 ± 0.12
SP_6-2	4.55 ± 0.10	SP_12-2	5.36 ± 0.03 **
SP_6-3	4.55 ± 0.05	SP_12-3	5.43 ± 0.07 **
SP_6-4	4.56 ± 0.12	SP_12-4	3.92 ± 0.04 ***

Anx. I-7: Percentage of cuticle waxes of the SP inbred line population and both parental lines MM and TO-937. Cuticles from Red ripe fruits were analyzed. Data are presented as means ±SE. Asterisks indicate significant differences with MM (****p* ≤ 0.001).

Line	% waxes	Line	% waxes
MM	2.57 ± 0.27	SP_6-5	2.39 ± 0.20
TO-937	4.09 ± 0.05 ***	SP_7-1	2.49 ± 0.20
SP_1-1	2.27 ± 0.18	SP_7-2	2.47 ± 0.24
SP_1-2	2.37 ± 0.25	SP_7-3	2.68 ± 0.14
SP_1-3	2.33 ± 0.18	SP_7-4	2.44 ± 0.25
SP_1-4	2.32 ± 0.10	SP_8-1	2.40 ± 0.35
SP_2-1	2.45 ± 0.11	SP_8-2	2.35 ± 0.18
SP_2-2	2.47 ± 0.32	SP_8-3	2.55 ± 0.20
SP_2-3	2.53 ± 0.11	SP_8-4	2.41 ± 0.33
SP_2-4	2.60 ± 0.20	SP_9-1	2.54 ± 0.33
SP_2-5	2.42 ± 0.18	SP_9-2	2.46 ± 0.35
SP_3-2	2.45 ± 0.08	SP_9-3	2.45 ± 0.26
SP_3-3	3.53 ± 0.22 ***	SP_9-4	2.41 ± 0.11
SP_3-4	2.51 ± 0.18	SP_10-1	2.44 ± 0.26
SP_4-1	2.76 ± 0.12	SP_10-2	2.44 ± 0.19
SP_4-2	3.55 ± 0.14 ***	SP_10-3	1.60 ± 0.13 ***
SP_4-3	3.59 ± 0.28 ***	SP_10-4	1.45 ±0 .06 ***
SP_4-4	2.54 ± 0.36	SP_10-5	2.52 ± 0.26
SP_5-1	2.47 ± 0.19	SP_10-6	2.55 ± 0.19
SP_5-2	2.73 ± 0.14	SP_11-1	1.72 ± 0.09 ***
SP_5-3	2.43 ± 0.29	SP_11-2	1.77 ± 0.05 ***
SP_5-4	2.62 ± 0.28	SP_11-3	1.62 ± 0.11 ***
SP_5-5	2.46 ± 0.25	SP_11-4	1.73 ± 0.14 ***
SP_6-1	2.62 ± 0.25	SP_12-1	1.85 ± 0.14 ***
SP_6-2	2.40 ± 0.27	SP_12-2	1.64 ± 0.13 ***
SP_6-3	2.59 ± 0.27	SP_12-3	1.66 ± 0.11 ***
SP_6-4	2.46 ± 0.05	SP_12-4	1.61 ± 0.09 ***

Anx. I-8: Percentage of cutin of the SP inbred line population and both parental lines MM and TO-937. Cuticles from red ripe fruits were analyzed. Data are presented as means ±SE. Asterisks indicate significant differences with MM (****p* ≤ 0.001).

Line	% cutin	Line	% cutin
MM	68.34 ± 0.62	SP_6-5	68.24 ± 0.37
TO-937	67.18 ± 0.10	SP_7-1	68.42 ± 0.78
SP_1-1	68.56 ± 0.88	SP_7-2	68.49 ± 0.45
SP_1-2	67.85 ± 0.83	SP_7-3	68.34 ± 0.58
SP_1-3	68.15 ± 0.42	SP_7-4	67.92 ± 0.70
SP_1-4	68.48 ± 0.78	SP_8-1	68.55 ± 0.20
SP_2-1	68.85 ± 0.80	SP_8-2	68.55 ± 0.79
SP_2-2	68.05 ± 1.00	SP_8-3	68.57 ± 0.21
SP_2-3	68.25 ± 0.33	SP_8-4	68.46 ± 0.45
SP_2-4	68.57 ± 0.40	SP_9-1	68.37 ± 0.55
SP_2-5	68.39 ± 0.58	SP_9-2	68.45 ± 0.37
SP_3-2	68.07 ± 0.35	SP_9-3	68.73 ± 0.11
SP_3-3	67.36 ± 0.19	SP_9-4	68.28 ± 0.23
SP_3-4	67.98 ± 0.40	SP_10-1	68.65 ± 0.85
SP_4-1	68.16 ± 0.49	SP_10-2	68.32 ± 0.51
SP_4-2	67.51 ± 0.06	SP_10-3	71.98 ± 0.48 ***
SP_4-3	67.40 ± 0.40	SP_10-4	72.44 ± 0.39 ***
SP_4-4	68.45 ± 0.47	SP_10-5	68.42 ± 0.23
SP_5-1	68.50 ± 0.22	SP_10-6	68.47 ± 0.66
SP_5-2	67.76 ± 0.63	SP_11-1	69.10 ± 0.31
SP_5-3	67.97 ± 0.39	SP_11-2	68.92 ± 0.43
SP_5-4	68.43 ± 0.58	SP_11-3	69.24 ± 0.35
SP_5-5	68.20 ± 0.46	SP_11-4	69.13 ± 0.40
SP_6-1	67.71 ± 0.56	SP_12-1	68.89 ± 0.61
SP_6-2	68.34 ± 0.83	SP_12-2	69.38 ± 0.47
SP_6-3	68.06 ± 0.37	SP_12-3	69.26 ± 0.13
SP_6-4	68.79 ± 0.34	SP_12-4	69.42 ± 0.48

Anx. I-9: Percentage of cuticle polysaccharides of the SP inbred line population and both parental lines MM and TO-937. Cuticles from red ripe fruits were analyzed. Data are presented as means ±SE. Asterisks indicate significant differences with MM (****p* ≤ 0.001).

Line	% polysaccharides	Line	% polysaccharides
MM	29.09 ± 0.64	SP_6-5	29.37 ± 0.56
TO-937	28.73 ± 0.10	SP_7-1	29.09 ± 0.63
SP_1-1	29.18 ± 0.78	SP_7-2	29.04 ± 0.25
SP_1-2	29.78 ± 0.58	SP_7-3	28.97 ± 0.62
SP_1-3	29.52 ± 0.56	SP_7-4	29.64 ± 0.69
SP_1-4	29.20 ± 0.75	SP_8-1	29.05 ± 0.21
SP_2-1	28.70 ± 0.72	SP_8-2	29.10 ± 0.66
SP_2-2	29.48 ± 0.70	SP_8-3	28.88 ± 0.33
SP_2-3	29.22 ± 0.39	SP_8-4	29.13 ± 0.15
SP_2-4	28.83 ± 0.23	SP_9-1	29.10 ± 0.58
SP_2-5	29.18 ± 0.41	SP_9-2	29.09 ± 0.39
SP_3-2	29.48 ± 0.34	SP_9-3	28.82 ± 0.26
SP_3-3	29.11 ± 0.31	SP_9-4	29.31 ± 0.33
SP_3-4	29.51 ± 0.49	SP_10-1	28.91 ± 0.64
SP_4-1	29.08 ± 0.59	SP_10-2	29.24 ± 0.44
SP_4-2	28.94 ± 0.15	SP_10-3	26.42 ± 0.39 ***
SP_4-3	29.01 ± 0.37	SP_10-4	26.11 ± 0.35 ***
SP_4-4	29.01 ± 0.28	SP_10-5	29.06 ± 0.22
SP_5-1	29.03 ± 0.40	SP_10-6	28.98 ± 0.56
SP_5-2	29.52 ± 0.71	SP_11-1	29.18 ± 0.31
SP_5-3	29.60 ± 0.38	SP_11-2	29.31 ± 0.47
SP_5-4	28.95 ± 0.38	SP_11-3	29.14 ± 0.46
SP_5-5	29.34 ± 0.35	SP_11-4	29.14 ± 0.50
SP_6-1	29.68 ± 0.31	SP_12-1	29.26 ± 0.74
SP_6-2	29.26 ± 0.61	SP_12-2	28.98 ± 0.35
SP_6-3	29.35 ± 0.10	SP_12-3	29.08 ± 0.21
SP_6-4	28.75 ± 0.35	SP_12-4	28.97 ± 0.56

Anx. I-10: Percentage of cuticle lightness (L*) of the SP inbred line population and both parental lines MM and TO-937. Cuticles from red ripe fruits were analyzed. Data are presented as means ±SE. Asterisks indicate significant differences with MM (***$p \leq 0.001$).

Line	Cuticle L*	Line	Cuticle L*
MM	78.03 ± 0.31	SP_6-5	78.08 ± 0.27
TO-937	83.33 ± 0.18 ***	SP_7-1	82.74 ± 0.21 ***
SP_1-1	79.21 ± 0.34	SP_7-2	82.58 ± 0.15 ***
SP_1-2	85.87 ± 0.38 ***	SP_7-3	82.66 ± 0.18 ***
SP_1-3	78.38 ± 0.22	SP_7-4	77.51 ± 0.19
SP_1-4	77.93 ± 0.18	SP_8-1	82.53 ± 0.19 ***
SP_2-1	77.91 ± 0.13	SP_8-2	79.03 ± 0.39
SP_2-2	77.63 ± 0.15	SP_8-3	78.79 ± 0.26
SP_2-3	78.89 ± 0.38	SP_8-4	77.79 ± 0.16
SP_2-4	77.64 ± 0.23	SP_9-1	78.83 ± 0.27
SP_2-5	77.96 ± 0.14	SP_9-2	78.48 ± 0.17
SP_3-2	80.63 ± 0.08 ***	SP_9-3	77.45 ± 0.36
SP_3-3	80.54 ± 0.07 ***	SP_9-4	78.92 ± 0.26
SP_3-4	77.46 ± 0.17	SP_10-1	77.44 ± 0.18
SP_4-1	77.47 ± 0.24	SP_10-2	77.69 ± 0.17
SP_4-2	73.00 ± 0.29 ***	SP_10-3	78.71 ± 0.14
SP_4-3	73.01 ± 0.18 ***	SP_10-4	78.02 ± 0.17
SP_4-4	78.31 ± 0.38	SP_10-5	78.84 ± 0.35
SP_5-1	77.44 ± 0.17	SP_10-6	78.56 ± 0.11
SP_5-2	77.41 ± 0.32	SP_11-1	77.94 ± 0.13
SP_5-3	77.36 ± 0.20	SP_11-2	80.50 ± 0.07 ***
SP_5-4	77.34 ± 0.23	SP_11-3	80.22 ± 0.14 ***
SP_5-5	77.88 ± 0.15	SP_11-4	79.89 ± 0.20 ***
SP_6-1	77.33 ± 0.23	SP_12-1	78.79 ± 0.40
SP_6-2	77.46 ± 0.14	SP_12-2	77.00 ± 0.31
SP_6-3	79.59 ± 0.47	SP_12-3	78.96 ± 0.49
SP_6-4	77.73 ± 0.25	SP_12-4	85.23 ± 0.29 ***

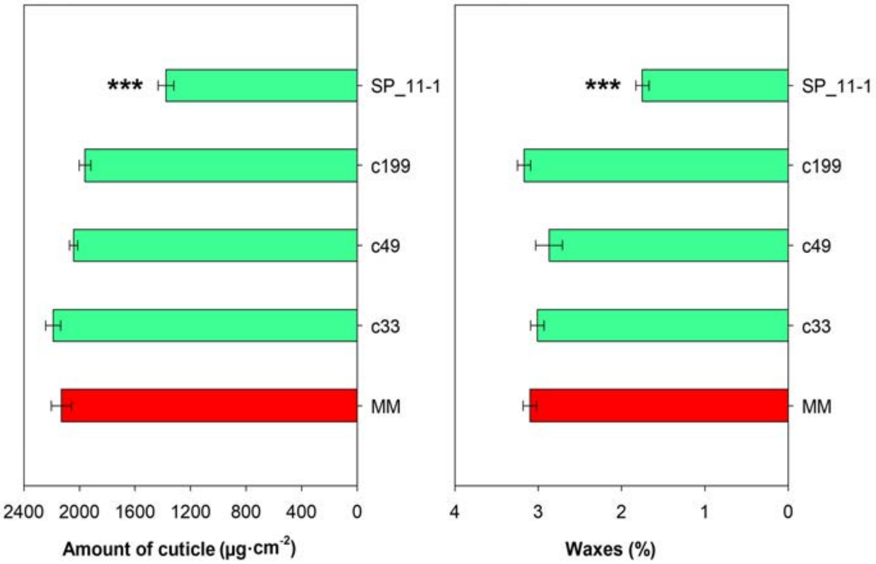
Anx. I-11: Percentage of cuticle chroma (C*) of the SP inbred line population and both parental lines MM and TO-937. Cuticles from red ripe fruits were analyzed. Data are presented as means ±SE. Asterisks indicate significant differences with MM (****p* ≤ 0.001).

Line	Cuticle C*	Line	Cuticle C*
MM	73.70 ± 1.13	SP_6-5	74.97 ± 0.55
TO-937	50.29 ± 0.57 ***	SP_7-1	69.00 ± 0.64 ***
SP_1-1	72.49 ± 0.65	SP_7-2	68.54 ± 0.40 ***
SP_1-2	65.24 ± 1.85 ***	SP_7-3	68.52 ± 0.80 ***
SP_1-3	74.24 ± 1.21	SP_7-4	75.09 ± 0.39
SP_1-4	74.76 ± 0.63	SP_8-1	67.24 ± 0.86 ***
SP_2-1	74.98 ± 0.76	SP_8-2	74.35 ± 0.68
SP_2-2	74.60 ± 0.56	SP_8-3	74.57 ± 0.52
SP_2-3	73.11 ± 0.35	SP_8-4	74.71 ± 0.46
SP_2-4	74.90 ± 0.79	SP_9-1	74.58 ± 0.26
SP_2-5	74.33 ± 0.82	SP_9-2	75.44 ± 0.84
SP_3-2	74.40 ± 0.36	SP_9-3	75.48 ± 0.49
SP_3-3	74.92 ± 0.68	SP_9-4	75.50 ± 0.55
SP_3-4	73.41 ± 0.70	SP_10-1	74.64 ± 0.61
SP_4-1	73.62 ±0.50	SP_10-2	74.44 ± 0.39
SP_4-2	81.74 ± 0.52 ***	SP_10-3	75.49 ± 0.52
SP_4-3	82.50 ± 0.68 ***	SP_10-4	75.50 ± 0.21
SP_4-4	74.96 ± 0.66	SP_10-5	73.51 ± 0.50
SP_5-1	74.08 ± 0.86	SP_10-6	74.64 ± 0.34
SP_5-2	74.89 ± 0.36	SP_11-1	74.26 ± 0.22
SP_5-3	74.89 ± 0.36	SP_11-2	68.48 ± 0.61 ***
SP_5-4	74.70 ± 0.40	SP_11-3	67.38 ± 0.21 ***
SP_5-5	74.98 ± 0.26	SP_11-4	68.03 ± 0.79 ***
SP_6-1	74.99 ± 0.45	SP_12-1	75.47 ± 0.54
SP_6-2	75.02 ± 0.92	SP_12-2	74.44 ± 0.31
SP_6-3	75.64 ± 0.60	SP_12-3	73.90 ± 0.96
SP_6-4	74.02 ± 0.40	SP_12-4	67.23 ± 0.50 ***

Anx. I-12: Percentage of cuticle hue (H°) of the SP inbred line population and both parental lines MM and TO-937. Cuticles from red ripe fruits were analyzed. Data are presented as means ±SE. Asterisks indicate significant differences with MM (**p* ≤ 0.05; ***p* ≤ 0.01; ****p* ≤ 0.001).

Line	Cuticle H°	Line	Cuticle H°
MM	86.01 ± 0.51	SP_6-5	85.67 ± 0.48
TO-937	99.94 ± 0.10 ***	SP_7-1	92.49 ± 0.90 ***
SP_1-1	88.05 ± 0.54	SP_7-2	92.30 ± 0.69 ***
SP_1-2	96.53 ± 1.22 ***	SP_7-3	91.72 ± 0.55 ***
SP_1-3	86.03 ± 0.52	SP_7-4	84.87 ± 0.37
SP_1-4	86.12 ± 0.94	SP_8-1	92.37 ± 0.71 ***
SP_2-1	86.60 ± 0.40	SP_8-2	86.82 ± 0.61
SP_2-2	86.32 ± 0.71	SP_8-3	86.34 ± 0.67
SP_2-3	88.23 ± 0.55	SP_8-4	86.50 ± 0.22
SP_2-4	85.13 ± 0.70	SP_9-1	87.65 ± 0.37
SP_2-5	86.12 ± 0.60	SP_9-2	86.23 ± 0.37
SP_3-2	90.20 ± 0.27 ***	SP_9-3	86.14 ± 0.44
SP_3-3	89.75 ± 0.19 ***	SP_9-4	85.77 ± 0.49
SP_3-4	85.33 ± 0.32	SP_10-1	85.04 ± 0.60
SP_4-1	84.32 ± 0.39	SP_10-2	84.97 ± 0.40
SP_4-2	77.15 ± 0.69 ***	SP_10-3	86.39 ± 0.26
SP_4-3	75.03 ± 0.62 ***	SP_10-4	84.69 ± 0.39
SP_4-4	84.52 ± 0.42	SP_10-5	87.74 ± 0.47
SP_5-1	84.78 ± 0.80	SP_10-6	85.34 ± 0.43
SP_5-2	85.42 ± 0.43	SP_11-1	86.05 ± 0.27
SP_5-3	85.42 ± 0.43	SP_11-2	89.59 ± 0.27 ***
SP_5-4	85.74 ± 0.50	SP_11-3	88.92 ± 0.34 *
SP_5-5	84.68 ± 0.41	SP_11-4	89.95 ± 0.52 ***
SP_6-1	86.37 ± 0.26	SP_12-1	87.06 ± 0.52
SP_6-2	85.46 ± 0.57	SP_12-2	85.29 ± 0.50
SP_6-3	88.18 ± 0.74	SP_12-3	85.95 ± 0.94
SP_6-4	84.63 ± 0.45	SP_12-4	96.73 ± 1.51 ***

Anx. I-13: Amount of cuticle and percentage of waxes of the nSP lines derived from SP_11-1. Cuticles from red ripe fruits were analyzed. Data are presented as means ±SE. Asterisks indicate significant differences with MM (***$p \leq 0.001$).

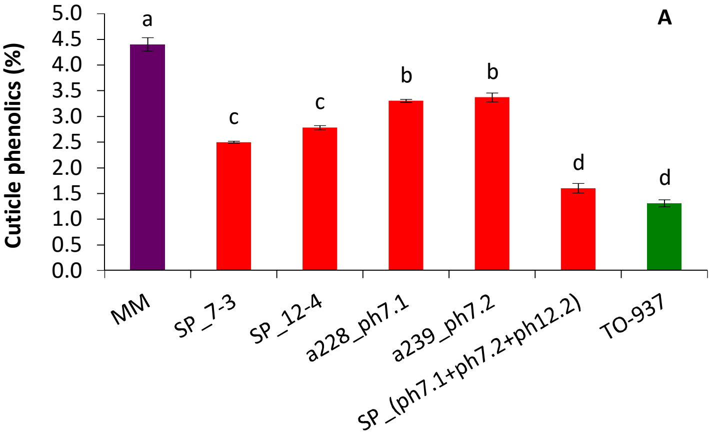


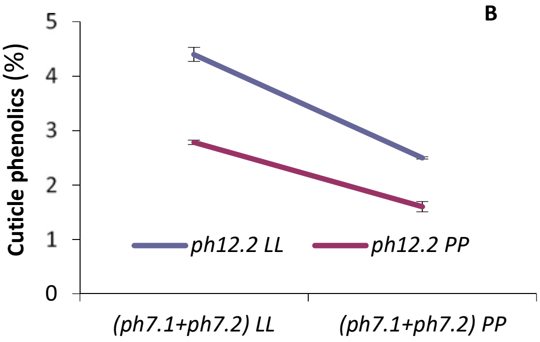
ANNEX II

Anx. II-1: List of primers designed for the selection of the double and triple homozygous SP_(QTL+QTL) lines. SP, *Solanum pimpinellifolium*; QTL, quantitative trait locus; *ph*, QTL percentage of phenolics; SNP, single nucleotide polymorphism.

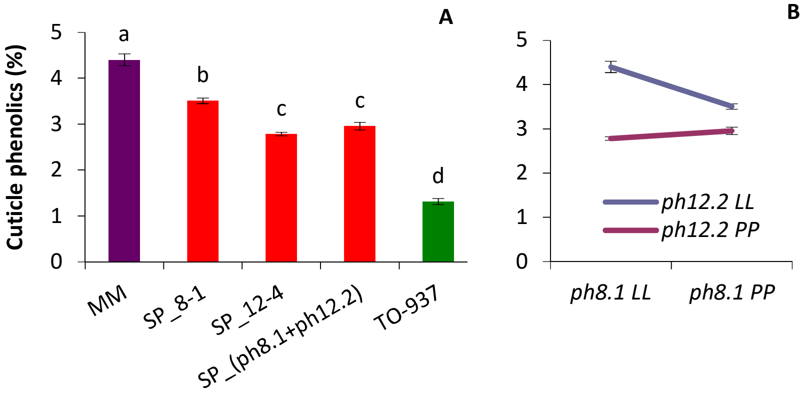
SP lines	QTL	Solcap marker	SNP	Forward and Reverse Primers (5'-3')
SP_1-2	*ph1.1*	Solcap_457	[A/G]	CTGGGTCGTCGGATAACTCA GTGACCCACTACCAGACGAA
SP_4-2	*ph4.1*	SNP_302a	[T/C]	AAAGGGTTAATCGCAGTGACC TAACAGGAAGAGACAAAACGGT
SP_5-3	*ph5.1*	Solcap_49234	[A/G]	TCTTATTTGAAGGCTTGGGAAA TATGCCATCCATCTGATCCA
SP_5-5	*ph5.2*	Solcap_37626	[A/G]	TCATCAAATGGTCAGTCAGGTC GGATATGCCCTCCCACATGC
SP_7-3	*ph7.1*	SNP_446a	[A/G]	AGAGGTGCCTTGAGTTGGAA ACGAGATCATCACTGTGGGA
SP_7-3	*ph7.2*	Solcap_52557	[T/C]	TCAAACACCTTTGGATCTTTCA TGAGTTCACATGATTCAGTTTACG
SP_8-1	*ph8.1*	Solcap_48592	[A/G]	CCACAGTACGTGCTTCTTGG CATGCTTTTTGGAAGTTCAGAG
SP_12-2	*ph12.1*	Solcap_45860	[A/C]	TAGCTTGTTGCATGGGGAAC AGCAGTAGCAGCAACAGCAG
SP_12-4	*ph12.2*	Solcap_55706	[A/G]	CGCGTATAGTGATGAAAGGAAGA AACTGGTAAGGTAGGCGCAA

Anx. II-2: A. Comparison between SP_(*ph7.1+ph7.2+ph12.2*), a228_*ph7.1*, a239_*ph7.2*, SP_7-3, SP_12-4, TO-937 and MM for the percentage of cuticle phenolics trait. B. Epistasis interaction plot for the percentage of cuticle phenolics trait between (*ph7.1+ph7.2*) and *ph12.2* at RR stage. A. Cuticles from red ripe fruits were analyzed. Data are presented as means ±SE. Letters indicate significant differences between lines at $p<0.05$. SP_7-3 and SP_12-4 harbor respectively *ph7.1+ph7.2* and *ph12.2*. **B.** Data are presented as means ±SE, expressed in au for the possible allele combination: {LL/LL; LL/PP; PP/LL; PP/PP} found at interacting QTLs on two different chromosomes. The maternal QTL is indicated on the X axis and the paternal QTL is indicated by the color of the plot lines. L, *Solanum lycopersicum* allele; P, *Solanum pimpinellifolium* allele; *ph*, QTL percentage of phenolics; RR, red ripe.

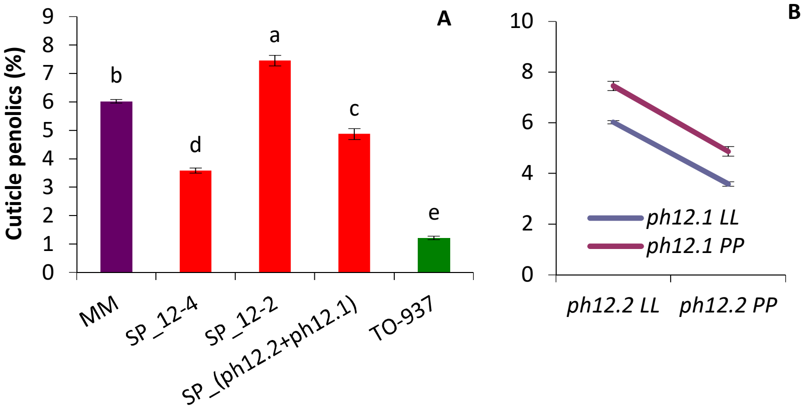




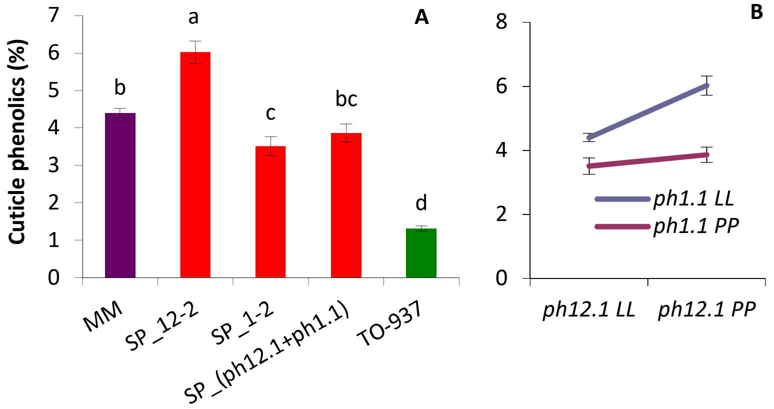
Anx. II-3: A. Comparison between SP_(*ph8.1*+*ph12.2*), SP_8-1, SP_12-4, TO-937 and MM for the percentage of cuticle phenolics trait. B. Epistasis interaction plot for the percentage of cuticle phenolics trait between *ph8.1* and *ph12.2* at RR stage. A. Cuticles from red ripe fruits were analyzed. Data are presented as means ±SE. Letters indicate significant differences between lines at *p*<0.05. SP_8-1 and SP_12-4 harbor respectively *ph8.1* and *ph12.2*. **B.** Data are presented as means ±SE, expressed in au for the possible allele combination: {LL/LL; LL/PP; PP/LL; PP/PP} found at interacting QTLs on two different chromosomes. The maternal QTL is indicated on the X axis and the paternal QTL is indicated by the color of the plot lines. L, *Solanum lycopersicum* allele; P, *Solanum pimpinellifolium* allele; *ph*, QTL percentage of phenolics; RR, red ripe.



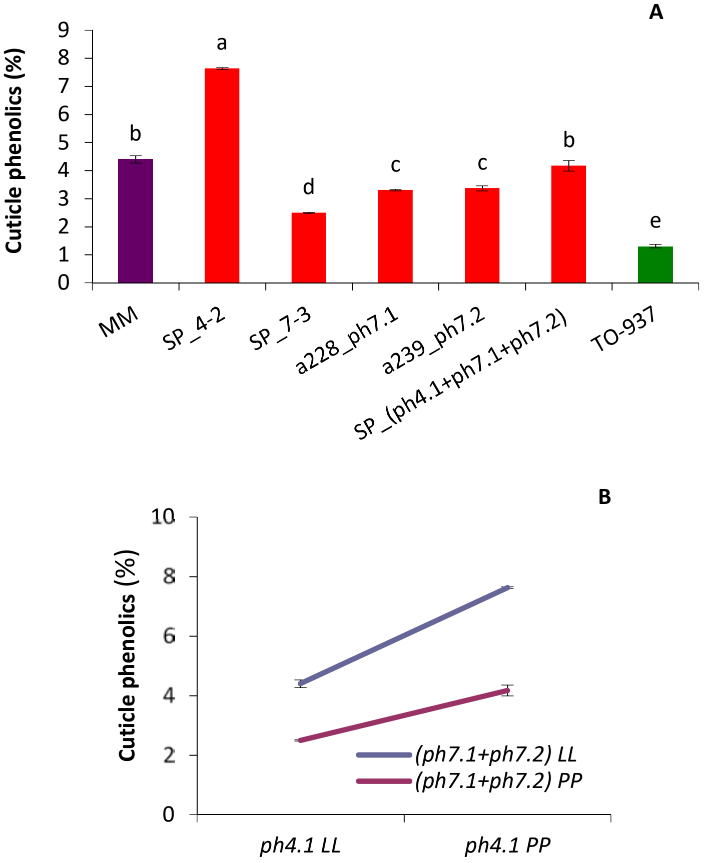
Anx. II-4: A. Comparison between SP_(*ph12.2+ph12.1*), SP_12-4, SP_12-2, TO-937 and MM for the percentage of cuticle phenolics trait. B. Epistasis interaction plot for the percentage of cuticle phenolics trait between *ph12.2* and *ph12.1* at RR stage. A. Cuticle red ripe fruits were analyzed. Data are presented as means ±SE. Letters indicate significant differences between lines at p<0.05. SP_12-4 and SP_12-2 harbor respectively *ph12.2* and *ph12.1*. **B.** Data are presented as means ±SE, expressed in au for the possible allele combination: {LL/LL; LL/PP; PP/LL; PP/PP} found at interacting QTLs on two different chromosomes. The maternal QTL is indicated on the X axis and the paternal QTL is indicated by the color of the plot lines. L, *Solanum lycopersicum* allele; P, *Solanum pimpinellifolium* allele; *ph*, QTL percentage of phenolics; RR, red ripe.



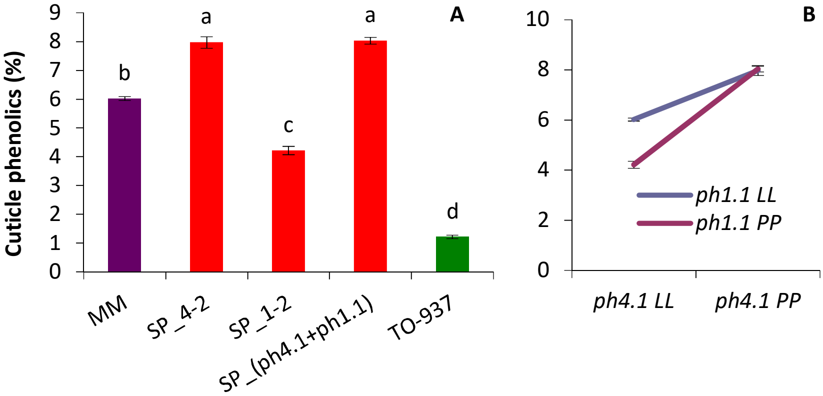
Anx. II-5: A. Comparison between SP_(*ph12.1*+*ph1.1*), SP_12-2, SP_1-2, TO-937 and MM for the percentage of cuticle phenolics trait. B. Epistasis interaction plot for the % of cuticle phenolics trait between *ph12.1* and *ph1.1* at RR stage. A. Red ripe fruits were analyzed. Data are presented as means ±SE. Letters indicate significant differences between lines at $p<0.05$. SP_12-2 and SP_1-2 harbor respectively *ph12.1* and *ph1.1* **B.** Data are presented as means ±SE, expressed in au for the possible allele combination: {LL/LL; LL/PP; PP/LL; PP/PP} found at interacting QTLs on two different chromosomes. The maternal QTL is indicated on the X axis and the paternal QTL is indicated by the color of the plot lines. au, arbitrary units; L, Solanum lycopersicum allele; P, Solanum pimpinellifolium allele; *ph*, QTL percentage of phenolics; RR, red ripe.



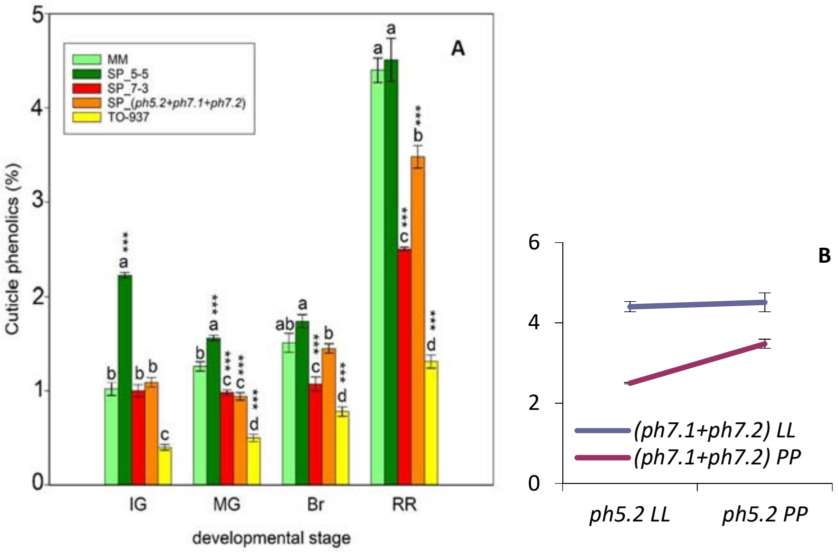
Anx. II-6: A. Comparison between SP_(*ph4.1+ph7.1+ph7.2*), SP_4-2, SP_7-3, a228_*ph7.1*, a239_*ph7.2*, TO-937 and MM for the percentage of cuticle phenolics trait. B. Epistasis interaction plot for the percentage of cuticle phenolics trait between *ph4.1* and (*ph7.1+ph7.2*) at RR stage. A. Cuticles from red ripe fruits were analyzed. Data are presented as means ±SE. Letters indicate significant differences between lines at $p<0.05$. SP_4-2 and SP_7-3 harbor respectively *ph4.1* and *ph7.1+ph7.2* **B.** Data are presented as means ±SE, expressed in au for the possible allele combination: {LL/LL; LL/PP; PP/LL; PP/PP} found at interacting QTLs on two different chromosomes. The maternal QTL is indicated on the X axis and the paternal QTL is indicated by the color of the plot lines. L, *Solanum lycopersicum* allele; P, *Solanum pimpinellifolium* allele; *ph*, QTL percentage of phenolics; RR, red ripe.



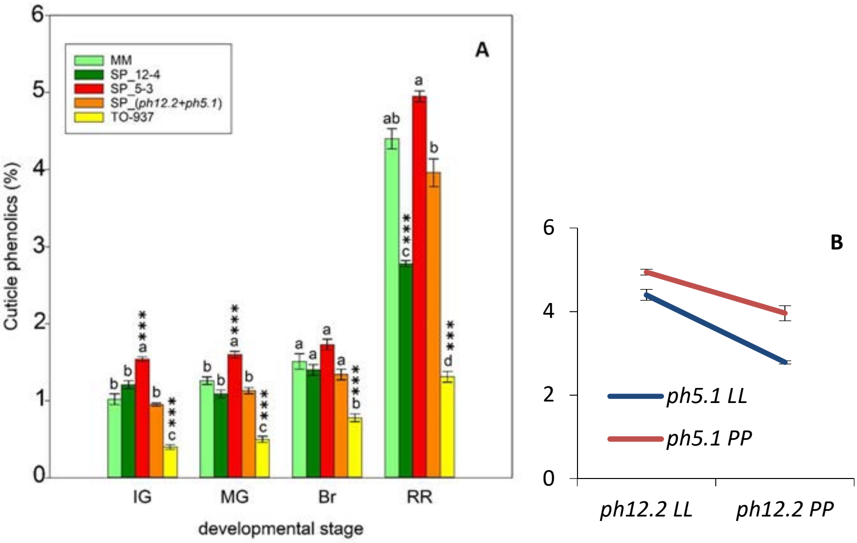
Anx. II-7: A. Comparison between SP_(*ph4.1*+*ph1.1*), SP_4-2, SP_1-2, TO-937 and MM for the percentage of cuticle phenolics trait. B. Epistasis interaction plot for the percentage of cuticle phenolics trait between *ph4.1* and *ph1.1* at RR stage. A. Cuticles from red ripe fruits were analyzed. Data are presented as means ±SE. Letters indicate significant differences between lines at $p<0.05$. SP_4-2 and SP_1-2 harbor respectively *ph4.1* and *ph1.1* **B.** Data are presented as means ±SE, expressed in au for the possible allele combination: {LL/LL; LL/PP; PP/LL; PP/PP} found at interacting QTLs on two different chromosomes. The maternal QTL is indicated on the X axis and the paternal QTL is indicated by the color of the plot lines. L, *Solanum lycopersicum* allele; P, *Solanum pimpinellifolium* allele; *ph*, QTL percentage of phenolics; RR, red ripe.



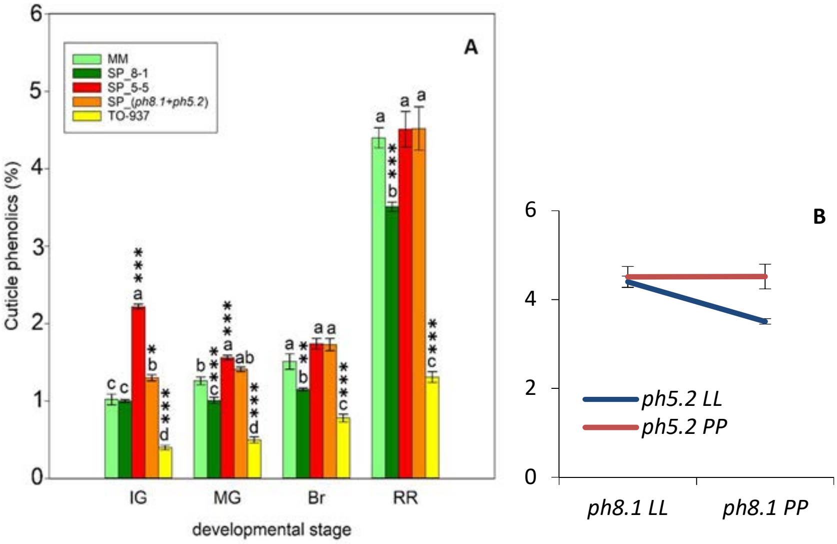
Anx. II-8: A. Comparison between SP_(*ph5.2*+*ph7.1*+*ph7.2*), SP_5-5, SP_7-3, TO-937 and MM for the percentage of cuticle phenolics trait at IG, MG, Br and RR stages. B. Epistasis interaction plot for the percentage of cuticle phenolics trait between *ph5.2* and *ph7.1*+*ph7.2* at RR stage. A. Fruit cuticles from all developmental stages were analyzed. Data are presented as means ±SE. Asterisks indicate significant differences with MM (**p*<0.05; ***p*<0.01; ****p*<0.001). Letters indicate significant differences between lines at *p*<0.05. SP_5-5 and SP_7-3 harbor respectively *ph5.2* and *ph7.1*+*ph7.2* **B.** Data are presented as means ±SE, expressed in au for the possible allele combination: {LL/LL; LL/PP; PP/LL; PP/PP} found at interacting QTLs on two different chromosomes. The maternal QTL is indicated on the X axis and the paternal QTL is indicated by the color of the plot lines. L, *Solanum lycopersicum* allele; P, *Solanum pimpinellifolium* allele; *ph*, QTL percentage of phenolics; IG, immature green; MG, mature green; Br, breaker; RR, red ripe.



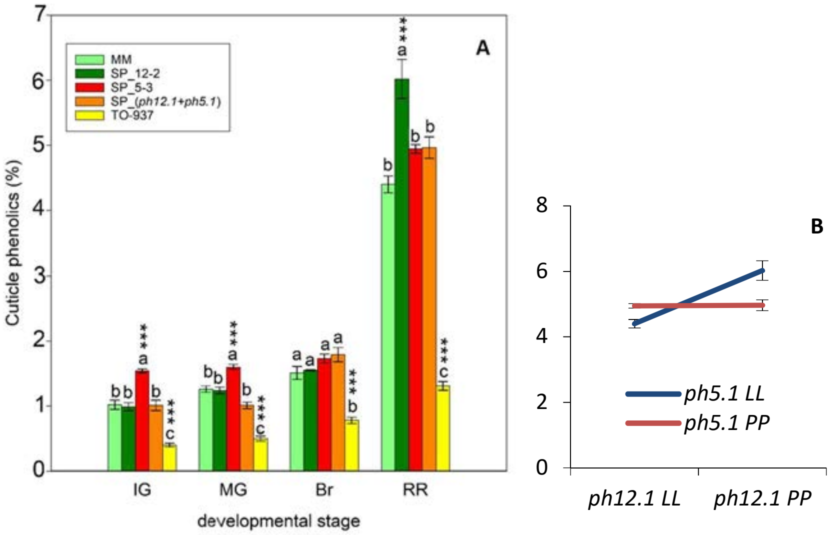
Anx. II-9: A. Comparison between SP_(*ph12.2+ph5.1*), SP_12-4, SP_5-3, TO-937 and MM for the percentage of cuticle phenolics trait at IG, MG, Br and RR stages. B. Epistasis plot interaction for the percentage of cuticle phenolics trait between *ph12.2* and *ph5.1* at RR stage. A. Fruit cuticles from all developmental stages were analyzed. Data are presented as means ±SE. Asterisks indicate significant differences with MM (*$p<0.05$; **$p<0.01$; ***$p<0.001$). Letters indicate significant differences between lines at $p<0.05$. SP_12-4 and SP_5-3 harbor respectively *ph12.2* and *ph5.1* **B.** Data are presented as means ±SE, expressed in au for the possible allele combination: {LL/LL; LL/PP; PP/LL; PP/PP} found at interacting QTLs on two different chromosomes. The maternal QTL is indicated on the X axis and the paternal QTL is indicated by the color of the plot lines. L, *Solanum lycopersicum* allele; P, *Solanum pimpinellifolium* allele; *ph*, QTL percentage of phenolics; IG, immature green; MG, mature green; Br, breaker; RR, red ripe.



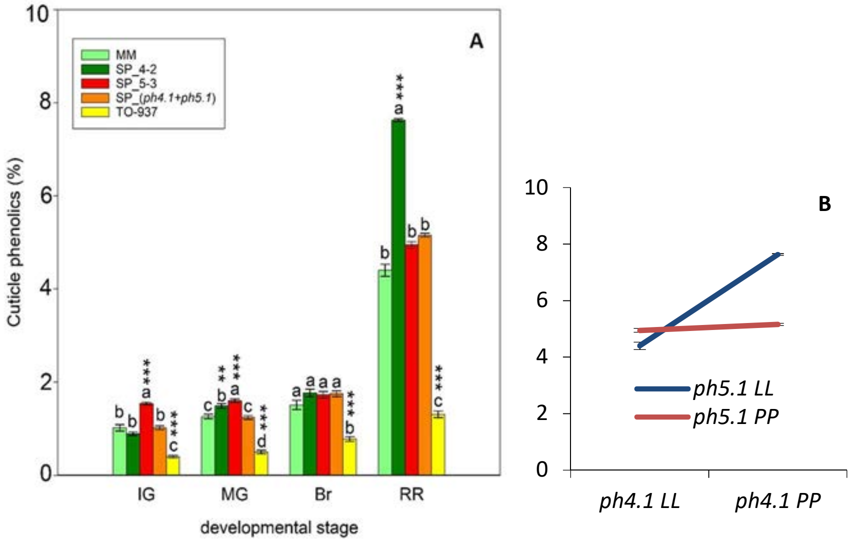
Anx. II-10: A. Comparison between SP_(*ph8.1*+*ph5.2*), SP_8-1, SP_5-5, TO-937 and MM for the percentage of cuticle phenolics trait at IG, MG, Br and RR stages. B. Epistasis plot interaction for the percentage of cuticle phenolics trait between *ph8.1* and *ph5.2* at RR stage. A. Fruit cuticles from all developmental stages were analyzed. Data are presented as means ±SE. Asterisks indicate significant differences with MM (**p*<0.05; ***p*<0.01; ****p*<0.001). Letters indicate significant differences between lines at *p*<0.05. SP_8-1 and SP_5-5 harbor respectively *ph8.1* and *ph5.2* **B.** Data are presented as means ±SE, expressed in au for the possible allele combination: {LL/LL; LL/PP; PP/LL; PP/PP} found at interacting QTLs on two different chromosomes. The maternal QTL is indicated on the X axis and the paternal QTL is indicated by the color of the plot lines. L, *Solanum lycopersicum* allele; P, *Solanum pimpinellifolium* allele; *ph*, QTL percentage of phenolics; IG, immature green; MG, mature green; Br, breaker; RR, red ripe.



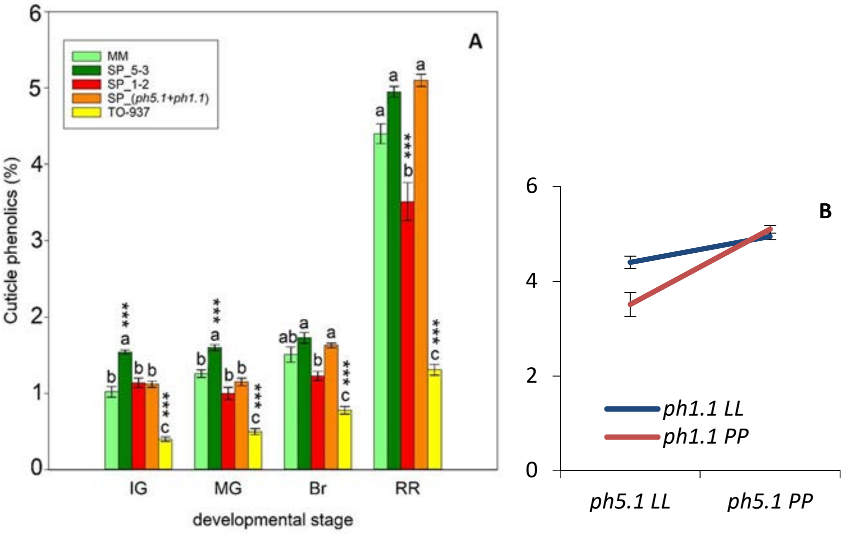
Anx. II-11: A. Comparison between SP_(*ph12.1*+*ph5.1*), SP_12-2, SP_5-3, TO-937 and MM for the percentage of cuticle phenolics trait at IG, MG, Br and RR stages. B. Epistasis plot interaction for the percentage of cuticle phenolics trait between *ph12.1* and *ph5.1* at RR stage. A. Fruit cuticles from all developmental stages were analyzed. Data are presented as means ±SE. Asterisks indicate significant differences with MM (*$p<0.05$; **$p<0.01$; ***$p<0.001$). Letters indicate significant differences between lines at $p<0.05$. SP_12-2 and SP_5-3 harbor respectively *ph12.1* and *ph5.1* **B.** Data are presented as means ±SE, expressed in au for the possible allele combination: {LL/LL; LL/PP; PP/LL; PP/PP} found at interacting QTLs on two different chromosomes. The maternal QTL is indicated on the X axis and the paternal QTL is indicated by the color of the plot lines. L, *Solanum lycopersicum* allele; P, *Solanum pimpinellifolium* allele; *ph*, QTL percentage of phenolics; IG, immature green; MG, mature green; Br, breaker; RR, red ripe.



Anx. II-12: A. Comparison between SP_(*ph4.1*+*ph5.1*), SP_4-2, SP_5-3, TO-937 and MM for the percentage of cuticle phenolics trait at IG, MG, Br and RR stages. B. Epistasis plot interaction for the percentage of cuticle phenolics trait between *ph4.1* and *ph5.1* at RR stage. A. Fruits from all developmental stages were analyzed. Data are presented as means ±SE. Asterisks indicate significant differences with MM (**p*<0.05; ***p*<0.01; ****p*<0.001). Letters indicate significant differences between lines at *p*<0.05. SP_4-2 and SP_5-3 harbor respectively *ph4.1* and *ph5.1* **B.** Data are presented as means ±SE, expressed in au for the possible allele combination: {LL/LL; LL/PP; PP/LL; PP/PP} found at interacting QTLs on two different chromosomes. The maternal QTL is indicated on the X axis and the paternal QTL is indicated by the color of the plot lines. L, *Solanum lycopersicum* allele; P, *Solanum pimpinellifolium* allele; *ph*, QTL percentage of phenolics; IG, immature green; MG, mature green; Br, breaker; RR, red ripe.



Anx. II-13: A. Comparison between SP_(*ph1.1*+*ph5.1*), SP_1-2, SP_5-3, TO-937 and MM for the percentage of cuticle phenolics trait at IG, MG, Br and RR stages. B. Epistasis plot interaction for the percentage of cuticle phenolics trait between *ph1.1* and *ph5.1* at RR stage. Fruit cuticles from all developmental stages were analyzed. Data are presented as means ±SE. Asterisks indicate significant differences with MM (*$p<0.05$; **$p<0.01$; ***$p<0.001$). Letters indicate significant differences between lines at $p<0.05$. SP_1-2 and SP_5-3 harbor respectively *ph1.1* and *ph5.1* **B.** Data are presented as means ±SE, expressed in au for the possible allele combination: {LL/LL; LL/PP; PP/LL; PP/PP} found at interacting QTLs on two different chromosomes. The maternal QTL is indicated on the X axis and the paternal QTL is indicated by the color of the plot lines. L, *Solanum lycopersicum* allele; P, *Solanum pimpinellifolium* allele; *ph*, QTL percentage of phenolics; IG, immature green; MG, mature green; Br, breaker; RR, red ripe.



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