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A Focus on the Biosynthesis and Composition of Cuticle in Fruits

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ABSTRACT: Cuticles are plant structures, composed mostly by lipidic layers, synthesized by nonwoody aerial plant organs and deposited on the surface of outer epidermal cell walls. Although its significance has been often disregarded, cuticle deposition modifies organ chemistry, influences mechanical properties, and plays a central role in sensing and interacting with the surrounding environment. Even though some research has been undertaken addressing cuticle biosynthesis and composition in vegetative plant tissues, comparatively less information is available regarding cuticle composition in the epidermis of fruits. However, recent work points to a role for cuticles in the modulation of fruit quality and postharvest performance, indicating that current models for the investigation of fruit development, metabolism, and quality need to integrate a comprehensive knowledge of the cuticle layer. This paper provides an overview of recent findings and observations regarding cuticle biosynthesis and composition in fruits from species of agronomic and economic relevance. Important, but often neglected differences in cuticle composition and biosynthesis patterns among diverse fruit species are described herein to generate an atlas of what is currently known about fruit cuticles and to highlight what remains to be explored. Emphasis is placed on the need to investigate each genetic background considering its own specificities, to permit correlations with the particular physiology of each species considered. Both specific composition and changes during maturation and ripening are reviewed.

KEYWORDS: *cuticle, biosynthesis, composition, cutin, fruit, wax*

INTRODUCTION: GENERAL FEATURES OF CUTICLE COMPOSITION

The cuticle is synthesized by the epidermis of fruits and other nonwoody aerial plant organs. This mostly lipidic layer has been traditionally considered to cover the surface of outer epidermal cell walls, even though recent findings suggest that the cuticle may be interpreted rather as a lipidized cell wall region.[1] In any case, the cuticle modifies the chemical and mechanical properties of the plant organ, hence playing a central role in the interaction with the environment[2] and decisively affecting plant development. A major component shaping the cuticle is cutin, a lipidic polyester composed primarily of hydroxylated and epoxy-hydroxylated C_{16} and C_{18} esterified fatty acids,[3] which originate commonly from $C_{16:0}$ and $C_{18:1}$ precursors synthesized in the plastids.[4−7] The most common C_{16} cutin monomers are 9,16- and 10,16-dihydroxyhexadecanoic acids, whereas 9,10,18-trihydroxyoctadecanoic and 9,10-epoxy,18-hydroxyoctadecanoic acids are the most characteristic monomers of the C_{18} class.[7] In some plant species, C_{16} or C_{18} fatty acids may predominate, whereas in other cases a mixed composition of both monomer classes has been observed. In addition to fatty acid derivatives, cutin also contains variable amounts of phenolic compounds, dicarboxylic acids, and glycerol.[8]

Cutin constitutes the insoluble polymeric scaffold in which amorphous intracuticular waxes are embedded, with both crystalline and amorphous epicuticular waxes coating the plant surface. These cuticular waxes are hydrophobic compounds, some of which can be extracted in organic solvents such as chloroform or hexane.[9] They exist as complex mixtures showing compositional variability among and within plant species,[4] as well as organ-to-organ differences.[9] These mixtures include long- and very long-chain ($>C_{18}$) fatty acids, hydrocarbons, alcohols, aldehydes, ketones, esters, triterpenes, sterols, and flavonoids. In some instances, the different lipid classes found in cuticular waxes may be present as homologous series, whereas in other cases a single chain length may dominate. This aspect is very relevant, as the structures and properties of the wax crystals formed in each case will be dependent on, among other factors, their composition. Potential hydrophobic interactions between cutin and cuticular waxes are favored by the fact that both are fatty acid derivatives and share the same biosynthetic origin in the plastids and the same pool of precursors.

Some cuticles may also contain cutan, a nonester and nonhydrolyzable core matrix comprising a network of aliphatic compounds linked by ether bonds which remain after cutin hydrolysis.[10] Additional cuticular compounds include significant amounts of polysaccharides[1] and flavonoids, which confer the structure particular mechanical characteristics. It has been reported, for instance, that polysaccharides incorporated into the cutin matrix are responsible for properties such as the elastic modulus or stiffness, whereas phenolics have been suggested to account for cutin rigidity.[11,12] In turn, the cutin matrix imparts extensibility; hence, the heterogeneous composition of cuticles conveys a range of viscoelastic and strain-hardening properties that modulate the mechanical behavior of plant organs, allowing them to expand and helping to stiffen the epidermal cell walls, which are, in comparison,

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Table 1. Main *n*-Alkane and Triterpenoid Components of Cuticular Waxes and Predominant Cutin Monomers Reported in Fruit Species According to Botanical Family

botanical family	fruit type	ripening type	predominant wax components		predominant cutin monomers	refs
Solanaceae						
tomato (*Solanum lycopersicum*)	berry	climacteric	*n*-hentriacontane (C_{31})	triterpenoid alcohols (*α*-, *β*-, and *δ*-amyrin)	C_{16} monomers (10,16-dihydroxy $C_{16:0}$)	69, 71−80, 134
pepper (*Capsicum* sp.)	berry	nonclimacteric	*n*-hentriacontane (C_{31})	triterpenoid alcohols (*α*- and *β*-amyrin)	C_{16} monomers (9(10),16-dihydroxy $C_{16:0}$)	81, 82, 84
eggplant (*Solanum melongena*)	berry	nonclimacteric	*n*-hentriacontane (C_{31})	triterpenoid alcohols (*α*- and *β*-amyrin)	not reported	82
wild tomato (*Solanum* Sect. *Lycopersicon*)	berry	climacteric	*n*-hentriacontane (C_{31}) (in most species studied)	triterpenoid alcohols (*α*-, *β*-, and *δ*-amyrin)	C_{16} monomers (10,16-dihydroxy $C_{16:0}$)	79
Rosaceae						
apple (*Malus* × *domestica*)	pome	climacteric	*n*-nonacosane (C_{29})	triterpenoid acids (ursolic acid)	C_{18} monomers (tetrahydroxy $C_{18:0}$)	60, 85−89
Asian pear (*Pyrus bretchneideri*)	pome	climacteric	*n*-nonacosane (C_{29})	triterpenoid alcohols (*α*-amyrin)	not reported	90
peach (*Prunus persica*)						
melting	drupe	climacteric	*n*-tricosane (C_{23}) *n*-pentacosane (C_{25}) *n*-heptacosane (C_{27})	triterpenoid acids (ursolic acid, oleanolic acid)	C_{18} monomers (*ω*-hydroxy $C_{18:1}$)	92
nonmelting	drupe	climacteric	*n*-pentacosane (C_{25})	triterpenoid acids (ursolic acid)	C_{18} monomers (*ω*-hydroxy $C_{18:1}$)	91, 92
plum (*Prunus domestica*)	drupe	climacteric	*n*-nonacosane (C_{29})	triterpenoid acids (oleanolic acid)	not reported	135
strawberry (*Fragaria* × *ananassa*)	etaerio	nonclimacteric	not reported	not reported	C_{16} monomers (9(10),16-dihydroxy $C_{16:0}$)	62
sweet cherry (*Prunus avium*)	drupe	nonclimacteric	*n*-nonacosane (C_{29})	triterpenoid acids (ursolic acid)	cultivar-dependent (dihydroxy $C_{16:0}$ or dihydroxy $C_{18:x}$ reported mainly)	3, 93, 94
black chokeberry (*Aronia melanocarpa*)	berry	nonclimacteric	not reported	not reported	C_{18} monomers (*ω*-hydroxy-9,10-epoxy $C_{18:1}$ and *ω*-hydroxy-9,10-epoxy $C_{18:0}$)	62
cloudberry (*Rubus chamaemorus*)	syncarp	nonclimacteric	not reported	not reported	C_{16} monomers (74% of 9(10),16-dihydroxy $C_{16:0}$)	62
raspberry (*Rubus idaeus*)	syncarp	climacteric	not reported	not reported	C_{16} monomers (9(10),16-dihydroxy $C_{16:0}$)	62
rosehip (*Rosa rugosa*)	etaerio	nonclimacteric	not reported	not reported	C_{16} monomers (79% of 9(10),16-dihydroxy $C_{16:0}$)	62
rowanberry (*Sorbus aucuparia*)	po*μ*e	nonclimacteric	not reported	not reported	C_{16} monomers (9(10),16-dihydroxy $C_{16:0}$)	62
Rutaceae						
grapefruit (*Citrus* × *paradisi*)	hesperidium	nonclimacteric	detailed composition not reported		C_{16} monomers (*ω*-hydroxy-10-oxo $C_{16:0}$)	61, 64, 96, 98
lemon (*Citrus limon*)	hesperidium	nonclimacteric	*n*-hentriacontane (C_{31}) *n*-nonacosane (C_{29})	not reported	C_{16} monomers (dihydroxy $C_{16:0}$ and *ω*-hydroxy-oxo $C_{16:0}$)	64, 98
mandarin (*Citrus reticulata*)	hesperidium	nonclimacteric	detailed composition not reported		C_{16} monomers (dihydroxy $C_{16:0}$ and *ω*-hydroxy-oxo $C_{16:0}$)	64, 97, 98
orange (*Citrus sinensis*)	hesperidium	nonclimacteric	*n*-hentriacontane (C_{31}) *n*-nonacosane (C_{29})	triterpenoid alcohols (*α*- and *β*-amyrin)	C_{16} monomers (dihydroxy $C_{16:0}$ and *ω*-hydroxy-oxo $C_{16:0}$)	64, 95, 98
Vitaceae						
grape (*Vitis vinifera*)	berry	nonclimacteric	*n*-pentacosane (C_{25}) *n*-heptacosane (C_{27}) *n*-nonacosane (C_{29})	triterpenoid acids (oleanolic acid)	not reported	65, 66, 99−102
Oleaceae						
olive (*Olea europaea*)	drupe	nonclimacteric	*n*-heptacosane (C_{27})	triterpenoid acids (oleanolic acid)	not reported	105
Ericaceae						
bilberry (*Vaccinium myrtillus*)	berry	nonclimacteric	not reported	not reported	C_{18} monomers (9,10,18-trihydroxy $C_{18:0}$ and *ω*-hydroxy-9,10-epoxy $C_{18:0}$)	106
cranberry (*Vaccinium oxycoccos*)	berry	nonclimacteric	not reported	not reported	C_{18} monomers (*ω*-hydroxy-9,10-epoxy $C_{18:0}$)	106
crowberry (*Empetrum nigrum*)	drupe	nonclimacteric	not reported	not reported	C_{16} monomers (9(10),16-dihydroxy $C_{16:0}$)	62

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Table 1. continued

botanical family	fruit type	ripening type	predominant wax components		predominant cutin monomers	refs
Ericaceae						
lingonberry (*Vaccinium vitis-idaea*)	berry	nonclimacteric	not reported	not reported	C_{18} monomers (ω-hydroxy-9,10-epoxy $C_{18:0}$ and 9,10,18-trihydroxy $C_{18:0}$)	106
Grossulariaceae						
black currant (*Ribes nigrum*)	berry	nonclimacteric	not reported	not reported	C_{16} monomers (10(9,8),16-dihydroxy $C_{16:0}$)	106
Elaeagnaceae						
sea buckthorn (*Hippophaë rhamnoides*)	berry	nonclimacteric	not reported	not reported	C_{18} monomers (ω-hydroxy-9,10-epoxy $C_{18:1}$ and ω-hydroxy-9,10-epoxy $C_{18:0}$)	106

more elastic.[2] Some of these properties are also dependent, at least to some extent, upon the external conditions to which the plant organ is exposed, particularly temperature and humidity.[13−15]

The composition and biosynthesis of cutin[7,16] and cuticular waxes,[9,17−19] cuticle permeability properties,[20−22] and cuticle and cutin biomechanics[2,23,24] have been covered in published reviews and will not be addressed here in detail. However, because the majority of work investigating cuticle biosynthesis and composition has been conducted on vegetative plant tissues and, in contrast, little information is available in relation to the epidermis of fruits, this review adds a further perspective to the current knowledge of the roles of cuticle in plant development. The minimization of water loss has been generally considered the main function of the fruit cuticle, but there is ample experimental evidence that, in addition to water-proofing, it may also play a major role in modulating protection against biotic and abiotic factors, fruit appearance, and textural properties, thus significantly affecting major traits related to postharvest quality.[25] The preservation of all these functions requires structural integrity of the cuticle throughout fruit expansion and development, meaning that a finely tuned mechanism to control cuticular composition and structure must also be a part of the developmental program. Despite their physiological relevance in determining fruit quality attributes and the economic implications of their maintenance throughout the commercial production and distribution chain of fruit, significant questions remain to be answered, including the mechanisms of cuticle biosynthesis and the specific wax and cutin (Table 1) composition of fruit cuticles. This paper reviews currently available information on the specific composition and biochemical changes in cuticles during maturation and ripening of fruits. Such knowledge may help frame putative cuticle impacts when the developmental process of a given fruit is to be investigated.

PATHWAYS AND REGULATION OF CUTICLE BIOSYNTHESIS: A BRIEF OVERVIEW

Fatty acids are precursors for both cutin and wax biosynthesis. These fatty acid precursors are thought to be synthesized de novo in plastids as the result of fatty acid synthase (FAS) activity, which involves, sequentially, the condensation of acetyl CoA with malonyl-acyl carrier protein (ACP), the reduction of 3-ketoacyl-ACP, the dehydration of 3-hydroxyacyl-ACP, and finally the reduction of *trans*-Δ^2-enoyl-ACP.[19,26,27] This fatty acyl primer is further extended by repeated condensations, thus yielding long-chain fatty acids (C_{16} and C_{18}). The precursors for wax production are mainly saturated fatty acids (C_{16} and C_{18}), whereas cutin is a polymer of predominantly $C_{16:0}$ and $C_{18:x}$ hydroxy fatty acids,[6] and hence the partitioning of these long-chain fatty acid precursors into wax or cutin biosynthesis pathways is likely to be a key regulatory point controlling the amount and specific composition of these two types of cuticle lipids.

Whereas de novo fatty acid biosynthesis is ubiquitous, cutin is synthesized exclusively by epidermal cells. Current understanding of the specific pathway that leads to the production of cutin monomers was established essentially 40 years ago by Kolattukudy and co-workers through studies on apple and *Vicia faba* leaves,[6,28] but many remaining knowledge gaps[29,30] still preclude a comprehensive understanding of the biosynthesis and polymerization of cutin monomers including the polymerization site itself, the monomer transport mechanisms, and the enzymes involved in their assembly. Even so, two recent papers have shed some light on these aspects by identifying an acyltransferase responsible for cutin polymerization in fruit cuticles of a range of *Solanum* species.[31,32] The necessary hydroxylation (at both internal and terminal positions) and epoxydation reactions are catalyzed by enzymes showing typical characteristics of the cytochrome P450 (CYP) enzyme family, among which the CYP77A, CYP86A, and CYP94B subfamilies comprise members that catalyze the hydroxylation either at midchain positions or at the terminal methyl group (ω-hydroxylases) on aliphatic fatty acid chains.[33−37] An effective function in cutin biosynthesis has been confirmed for some fatty acid ω-hydroxylases through the physiological and biochemical characterization of the corresponding mutants.[38] Distinctive substrate specificities[39,40] and expression patterns in response to environmental conditions[41] have been revealed, too. Long-chain acyl-CoA synthetases (LACS) are also required for cutin production,[42] indicating that these precursors need to be activated (CoA-bound) prior to the establishment of the cutin network. The ω-hydroxyl groups in the monomers form ester bonds, whereas a variable portion of the midchain hydroxyl groups is involved in ester-type cross-links, thus giving rise to and holding together the final polymeric complex.[7,16] The establishment of these ester linkages is catalyzed by acyltransferases, which transfer the activated cutin monomers to the free hydroxyl groups present in the cutin polymer[28] in a reaction purported to require ATP and a cutin primer, and indeed two glycerol-3-phosphate acyltransferases, GPAT4 and GPAT8, have been proved necessary for cutin assembly.[43] Phenolic esters such as those of ferulic and *p*-coumaric acids also contribute, although to a minor extent, to the establishment of the cutin polymer.[44]

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Because genetic and external factors are known to cause changes in the amount and composition of wax, a strict regulation of the underlying biosynthetic pathways is to be expected. The characterization of mutants with deficient or altered wax coatings has contributed to shedding light on wax biosynthesis,[9,45−47] which is likewise restricted to epidermal tissues.[19,48,49] Waxes are often rich in very long-chain fatty acids (VLCFA) esterified to very long-chain alcohols, and hence the elongation of C_{16} and C_{18} fatty acid precursors is required for the synthesis of these wax components. Further extension of C_{16} and C_{18} fatty acids requires ACP elimination by an acyl-ACP thioesterase and activation to fatty acyl-CoA esters, which is catalyzed by long-chain acyl-CoA synthetases.[19,26] Chain elongation is biochemically analogous to de novo synthesis, the four consecutive reactions being catalyzed by ATP-dependent fatty acid elongases (FAE),[18,50−52] which require malonyl-CoA rather than malonyl-ACP as the two-carbon donor. Because elongases catalyze the primary reaction characterizing wax biosynthesis, they have been assigned an essential role in the process and have thus been the object of considerable research effort. The biochemistry and genetics of these elongation systems have been reviewed elsewhere[9,17,19,50,53−55] and will not be considered herein.

In addition to lipids derived from fatty acids, cuticles are also composed of lipids derived from isoprene, which primarily include the triterpenoids present in cuticular waxes. Triterpenoid biosynthesis involves the joining of six isoprene units to yield the hydrocarbon precursor squalene, which is then activated into 2,3-epoxysqualene and then cyclized. The cyclization of 2,3-epoxysqualene represents the first committed step in triterpenoid biosynthesis, which is carried out by oxidosqualene cyclases (OSCs). The diversity of existing OSCs and the large range of possible rearrangements they can catalyze eventually result in a great diversity of triterpenoid structures even though they start from the same substrate.[56]

Wax and cutin secretion to organ surfaces is also a key aspect of cuticle formation, because these lipophilic compounds must be exported from the cytoplasm after biosynthesis, cross the hydrophilic apoplast, and finally be assembled. The delivery to the outer tissues apparently involves lipid transfer proteins (LTP), which are expressed in plant epidermal tissues and secreted to the outside of the cell wall and cuticle.[17,18,57,58] Some members of the ATP-binding cassette (ABC) transporter family have been actually demonstrated to be required for cuticular lipid secretion.[19,59]

THE FRUIT CUTICLE: ARE ALL SPECIES EQUAL?

Many studies on chemical composition of cuticles have been published taking as a model leaves and other plant vegetative tissues. The significant differences observed for over 40 years in cuticle composition of different organs of the same plant, regarding both cutin[60−63] and waxes,[64−66] provide clear evidence that the assumption that fruit cuticle composition can be inferred from that of leaves cannot be sustained.

Taking tomato as a model species, for example, published information on the composition of cuticular waxes and cutin monomers supports the existence of such differences. Noticeable differences in total wax and cutin loads have been observed between fruits and leaves of the 'Micro-Tom' cultivar.[67−69] Total cutin load was reported to amount to 2.5 μg cm^{-2} in leaves[68] and to as high as 586.1 μg cm^{-2} in fruits.[69] Such quantitative differences were found for cuticular waxes as well: total loads of 3.5 and 15 μg cm^{-2} were reported for leaves and fruits, respectively,[67] although total wax amount in fruits of the same cultivar has been recently shown to reach even higher values.[69] More importantly, substantial compositional differences have been also observed between leaves and fruits, in addition to differences in total wax and cutin loads. For waxes, the main compositional differences were found for the percentage of branched alkanes (approximately 22 vs. 8% of total cuticular waxes in leaves and fruit, respectively), whereas the percentages for *n*-alkanes and triterpenoids were similar in both cases,[67,69] and significant amounts of alcohols and fatty acids were found for fruit cuticles.[69] As to cutin monomers, the chief differences in composition between leaves and fruits referred to the amount and type of hydroxy-acids and to the percentage of dicarboxylic acids.[68,69]

Tomato fruit cuticles provide a valuable model for fruit cuticle research, as they are astomatous and comparatively thick, which means that it is possible to isolate considerable amounts of "unperforated" material for biochemical, physical, or structural characterization.[70] Albeit less intensively, cuticles of some other fruit species, mostly corresponding to crops of economic relevance, have also been investigated and revealed significant differences across genotypes in their properties. Because fruit cuticles modulate several traits of economic importance,[25,70] the understanding of the functions and impact of this outer layer on quality, storage potential, and shelf life of produce will require its characterization as a preliminary step.

To highlight interspecific differences in the chemical composition of fruit cuticles, the following subsections review published literature to reveal the variation in the main components of cutin and waxes that exists across different fruit types. With the purpose of revealing possible compositional similarities within fruits of species related taxonomically, this information has been organized according to botanical families. The many unknowns still existing in relation to fruit cuticle composition are highlighted in the summary of available information for each listed fruit species presented in Table 1.

Solanaceae. *Tomato (Solanum lycopersicon).* The cuticle composition of tomato, widely used as a model species for research related to different aspects of fruit development, has been investigated with considerably more intensity in comparison with other fruit species. Cuticular waxes of the 'Micro-Tom' cultivar consist predominantly of very long-chain alkanes and triterpenoids.[67,69,71] The C_{31} alkane *n*-hentriacontane was prevalent in the epicuticular wax film, whereas in the intracuticular wax compartment it was mixed with the pentacyclic triterpenoid alcohols α-, β-, and δ-amyrin. For the 'Ailsa Craig' cultivar, a similar composition of cuticular waxes was reported,[72] 55−60% of the total amount consisting of *n*-alkanes, among which *n*-hentriacontane was also shown as the most abundant, followed by *n*-nocacosane (C_{29}) and *n*-tritriacontane (C_{33}). Amyrins (α, β, and δ) together accounted for about 18% of wax components in this cultivar. When the cutin composition was analyzed, the C_{16} cutin monomer 10,16-dihydroxy $C_{16:0}$ acid was found to account for about 74% of total monomers. The prevalence of *n*-hentriacontane, amyrins, and 10,16-dihydroxy $C_{16:0}$ acid in the composition of tomato cuticles was confirmed in subsequent studies on 'Ailsa Craig',[73−77] 'M82',[76−79] and 'John Baer' and 'Pearson'.[80] However, when cuticles of seven different species of wild tomato were analyzed, substantial diversity in the microscopic morphology of the cuticle and the underlying epidermal cell layer, as well as in the content of cutin and waxes, was found.[79] The cuticular wax content of the wild species surpassed that of

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cultivated tomato by up to 7-fold, and variability in the amount of wax esters and triterpenoid isomers was also found. It can be speculated that selection for this species has favored a particular cuticle composition that may be associated with more favorable agronomic characteristics.

Pepper (Capsicum sp.). Kissinger and co-workers[81] examined the cuticles of 10 *Capsicum* sp. cultivars differing in quality attributes such as pungency and fruit size. Globally, the cuticle was found to be particularly rich in C_{16} cutin monomers, the main component in quantitative terms being 9(10),16-dihydroxyhexadecanoic acid, whereas 9,10-epoxy-18-hydroxyoctadecanoic acid was the main representative of the C_{18} monomer type. The major aliphatic constituents of cuticular waxes were *n*-alkanes, in which *n*-hentriacontane was the predominant single compound. Significant amounts of methyl-branched alkanes and of the triterpenoids α- and β-amyrin were also detected. A concurrently published study on 12 bell pepper cultivars also identified *n*-hentriacontane and triterpenoid alcohols as the dominating wax components in these fruit.[82] A later work also disclosed that pepper fruit cuticles contain a cutan-like fraction.[83] More recently, the cuticle lipid composition in a near-isogenic backcross population (BC_2F_2) obtained between an inbred line of *C. annuum* and a wild line of *C. chinense*, which markedly differ in postharvest water loss rates, has been analyzed.[84] Interestingly, the major components of cuticular waxes and cutin were the same as mentioned above,[81] but significant differences in absolute and relative amounts within wax classes and cutin monomers were found between the parents and among the segregating BC_2F_2 population. Such approaches are needed for other fruit species, particularly when quality at harvest and during postharvest is affected by phenomena related with cuticle dynamics, such as water loss or microcracking. This would help identify metabolic signatures associated with particular physiological traits and quality attributes.

Eggplant (Solanum melongena). The surface waxes of eggplant fruit have been also examined in three cultivars.[82] In this study, waxes were extracted in *tert*-butylmethyl ether and chromatographed through a silica gel column, from which two fractions (termed fractions 1 and 2) were eluted using hexane/toluene and methanol, respectively. For all three cultivars, *n*-alkanes (C_{23}−C_{36}) predominated in fraction 1, which represented 77% of all waxes recovered. The dominating compound in this fraction (up to 18%) was *n*-hentriacontane, followed by *n*-tritriacontane. The triterpenoid components were eluted with fraction 2, amyrins and other triterpenols being the main compound detected, similar to the observations for other species within the Solanaceae family. No information was reported on cutin monomers.

Rosaceae. *Apple (Malus × domestica).* The first reports on cuticle composition of apple fruit date back to the 1970s. Three cultivars ('Dougherty', 'Granny Smith', and 'Sturmer') were studied in relation to the composition of their surface waxes,[85] and it was found that *n*-nonacosane and nonacosan-10-ol were prominent in all three cultivars among the constituent hydrocarbons and secondary alcohols, respectively. In contrast, the main primary alcohols differed among cultivars, with *n*-hexacosanol (C_{26}) predominating in 'Sturmer', whereas *n*-tetracosanol (C_{24}) was the most abundant in 'Dougherty' and 'Granny Smith' fruits. Waxes from 'Sturmer' also differed from the other two cultivars studied in regard to the main fatty acids present, showing a comparatively higher content of hexadecanoic and lower content of octadecadienoic acid. Subsequent studies on other cultivars[86,87] confirmed *n*-nonacosane as the main alkane in apple fruit cuticles, but also provided a broader overview of cultivar-specific variability in wax composition of apple cuticles. The analysis of cuticular waxes of 17 cultivars[86] showed triterpenoids as the dominating wax fraction, ursolic acid accounting for 30−72% of total waxes, and furthermore revealed that secondary alcohols were the most cultivar-specific compounds—more so than primary alcohols—contrasting with previous observations.[85] In addition to *n*-nonacosane, surface waxes of 'Red Fuji' apples included important amounts of nonacosan-10-ol and the ketone nonacosan-10-one.[87] Similar results indicative of cultivar-related variability were reported by other researchers as well.[88,89] In the latter case, the secondary alcohol nonacosan-10-ol was found to be the compound explaining the main differences in surface characteristics of 'Jonagold', 'Jonagored', and 'Elstar' fruit.

With respect to cutin monomers, the C_{18}-hydroxylated class of monomers was found to account for 73% of total cutin monomers in 'Golden Delicious',[60] which was much higher than the amounts in leaves and flower parts from this variety, and it was thus hypothesized that the content of C_{18} monomers was related to the rate of expansion of different organs. This finding emphasizes the need to assess compositional differences between particular tissues, as stated above. When the aliphatic components of apple fruit cuticles were characterized using elemental analysis, ^{13}C nuclear magnetic resonance (NMR), and Fourier transform infrared spectroscopy, they were shown to contain lower percentages of the cutan-like fraction than those of pepper fruit and to display slightly different chemical structure, too.[83]

Pear (Pyrus sp.). Despite the economic importance of the world trade of both European and Asian pears, only the latter have been investigated in relation to cuticle composition and uniquely in regard to their waxes. A total of 54 compounds were identified in the cuticular waxes of 'Pingguoli' Asian pear,[90] the main constituents observed being pentacyclic triterpene alcohols (32%), fatty acids (27.8%), and alkanes (25.9%). The composition of each of these three major compound families was dominated by α-amyrin, hexadecanoic acid, and C_{19}−C_{31} *n*-alkanes, respectively. To the best of our knowledge, there is no published information on the cutin composition of pear cuticles, thus also offering ample field for further research.

Peach (Prunus persica). Surprisingly, very limited research has been reported in regard to the cuticle composition of climacteric stone fruits such as peach, apricot, or plum, despite its potential relevance for water loss and decay susceptibility of these fruit species. Peach skin was taken as a model for studying properties of pubescent surfaces concerning water−plant surface interactions,[91] and cuticle composition was analyzed for the content of the main component types. Peach cuticles were found to be composed of 53% cutan, 27% waxes, 23% cutin, and 1% hydroxycinnamic acids, whereas trichomes contained 15% waxes and 19% cutin, filled with 63% polysaccharide material associated with hydroxycinnamic acid derivatives and flavonoids. Nevertheless, the detailed composition of each of these fractions was not described. We have recently analyzed the composition of waxes and cutin monomers in cuticles isolated enzymatically from melting- and non-melting-type peach fruit[92] and identified the $C_{18:1}$ derivative 18-hydroxyoctadecenoic acid as the most abundant cutin monomer (17−20% at harvest, depending on the cultivar), whereas the triterpenes ursolic acid and oleanolic

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acid were prominent among the components of cuticular waxes, accounting together for 44−52% at harvest. In contrast to most published studies on fruit cuticles, in which *n*-nonacosane and *n*-hentriacontane were reported to be the main alkane components of cuticular waxes (Table 1), we identified *n*-tricosane (C_{23}), *n*-pentacosane (C_{25}), and *n*-heptacosane (C_{27}) as the predominant alkanes in peach fruit, with substantial cultivar-related differences in the total content of these compounds (10−24% at harvest).

Sweet Cherry (Prunus avium). Considerably more reports than for peach exist for sweet cherry cuticles, although most of them have been mainly concerned with its physical and mechanical properties as related to water permeability and disorders to which these fruit are particularly prone, such as cracking. An early study on ripe 'Bing' cherries[93] revealed ursolic acid and *n*-nonacosane as prominent components of cuticular waxes of fruit. This was confirmed when the composition of both cutin and waxes of four sweet cherry cultivars was studied during on-tree fruit development and maturation.[3] The most abundant constituents of waxes at fruit maturity were the triterpenes ursolic and oleanolic acids, which together accounted for 76% of total wax, followed by the alkanes *n*-nonacosane and *n*-heptacosane and the secondary alcohol nonacosan-10-ol. These data essentially agree with our own observations on 'Celeste' and 'Somerset' cherries, showing ursolic acid (roughly 50% of total waxes) to be the major triterpene, *n*-nonacosane and *n*-heptacosane the most abundant alkanes (7−11%), and *n*-tricosanol (C_{23}) and *n*-triacontanol (C_{30}) the main primary alcohols detected in cuticular wax.[94] Although these studies agree as to the major components of cuticular waxes in all of the cultivars assessed, they show discrepancies for other types of wax constituents. Specifically, cuticular waxes of 'Bing' cherries are rich in hexadecanoic, octadecanoic, octadecenoic, and octadecadienoic acids and contain significant amounts of sitosterol.[93] Because dehydrated fruit skins were used as the source of wax, when these compounds were not found in a later study,[3] it was concluded that their detection might have been an artifact derived from contamination with membrane lipids. However, the analysis of the cuticular waxes of 'Celeste' and 'Somerset' fruit also revealed significant contents of octadecadienoic acid and phytosterols,[94] despite the fact that enzymatically isolated cuticles were used as the source material for wax extraction. Therefore, these discrepancies are likely to reflect actual cultivar-related differences in cuticular wax composition.

Although published reports have shown only minor quantitative differences in wax composition among cherry cultivars, discrepancies have been found as to cutin monomer composition. The cutin fraction of mature 'Kordia' fruit consisted mainly of C_{16} (69.5%) and, to a much lesser extent, C_{18} (19.4%) monomers, the most abundant components being 9(10),16-dihydroxyhexadecanoic and 9,10,18-trihydroxyoctadecanoic acids.[3] However, we have found that the cutin fraction of 'Celeste' and 'Somerset' cherry fruit is composed mainly of C_{18} monomers (65.3 and 57.9%, respectively),[94] which contrasts with previous observations in this species and shows cultivar-related differences in cuticle composition.

Soft Fruit Species. The composition of the cutin fraction of some berries within the Rosaceae family has been investigated.[62] The cutin composition of strawberry (*Fragaria* × *ananassa*), raspberry (*Rubus idaeus*), and rowanberry (*Sorbus aucuparia*) fruit was shown to be dominated by 9(10),16-dihydroxyhexadecanoic acid, although all three species displayed wider variety of cutin monomers in comparison with non-Rosaceae berry fruits that were analyzed in the same work. A similar cutin composition was observed in rosehip (*Rosa rugosa*), black chokeberry (*Aronia melanocarpa*), and cloudberry (*Rubus chamaemorus*) fruits,[62] although additional compounds such as C_{15} monomers and α,ω-dicarboxylic acids with midchain hydroxyl groups were also found. Additionally, strawberry and raspberry cutin proved to be highly resistant to depolymerization, and this observation was suggested to arise from the presence of a particularly high amount of cutan-type compounds. The cutin/cutan ratio is likely to affect significantly the pre- and postharvest evolution of fruit quality attributes, including their value as a dietary fiber, and this topic is thus worthy of more detailed examination. Similarly, no published studies are available for cuticular wax composition of these species, thus offering ample opportunity for further research.

Rutaceae. *Citrus Fruit (Citrus sp.).* In orange, triterpenoids constitute the most abundant wax fraction found in mature fruits, mainly consisting of α-amyrin, β-amyrin, lupeol acetate, and friedelin.[95] Additionally, aldehydes are also major constituents of the cuticular waxes in orange and lemon fruit, together with alkanes, primary alcohols, and fatty acids. The predominant compounds are reportedly *n*-nonacosane and *n*-hentriacontane among the alkanes and *n*-tetracosanal (C_{24}), *n*-hexacosanal (C_{26}), and *n*-octacosanal (C_{28}) among the aldehydes.[64,95] Terpenoids and aldehydes were likewise reported to be the main compound families present in epicuticular waxes of grapefruit, with some differences observed according to the canopy position,[96] which supports the assumption that the environmental conditions cause cuticle modifications and affect fruit development. In contrast, mandarin fruit waxes are dominated by alkanes, followed by esters > ketones > aldehydes > fatty acids > primary alcohols > triterpenes.[97] The detailed compositions of each of these classes of component were not reported, but some differences were also found according to the canopy position of fruit.

We are aware of only two published studies on cutin components of citrus fruit. The fruit cuticles of four different species (orange, lemon, grapefruit, and mandarin) were reported to contain less cutin (59−67%) than those of their leaves (79−82%).[98] The major monomeric constituents detected were dihydroxy C_{16} (30−62%) and 16-hydroxy-oxo C_{16} acids. Similarly, when the cutin monomer composition was analyzed in different tissues of grapefruit, including fruit peel, leaf, juice sac, and inner seed coat, it was found that 16-hydroxy-10-oxo C_{16} acid was a major component in the fruit peel.[61] Substantial tissue-associated variation was observed, as 10,16-dihydroxy C_{16} acid and its positional isomers were the main cutin monomers in leaves, the composition of the juice sac cutin was dominated by dihydroxy C_{16}, hydroxy-oxo C_{16}, hydroxy-epoxy C_{18}, and trihydroxy C_{18} acids, and ω-hydroxy and dicarboxylic C_{16} and $C_{18:1}$, ω-hydroxy-epoxy C_{18} and trihydroxy C_{18} acids were the major components of the inner seed coat cutin.

Vitaceae. *Grape (Vitis vinifera).* Similarly to citrus fruit, aldehydes have also been found to be important constituents in waxes of grapes, where the straight-chain, even-chain length type predominates. Early in 1965, two published works[65,99] reported that the surface waxes of ripe sultana grapes consist largely of the triterpenoid oleanolic acid, with lesser amounts of *n*-alcohols (mainly C_{24}, C_{26}, and C_{28}), *n*-aldehydes (largely C_{28} and C_{30}), esters, free acids (predominantly C_{24}, C_{26}, and C_{28}), and *n*-hydrocarbons (mainly C_{25}, C_{27}, C_{29}, and C_{31}). A

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considerable amount (around 30%) of oleanolic acid was also found in cuticle waxes of 'Palomino fino' grapes,[100] although surprisingly *n*-alkanes represented <5% of total mass and, remarkably, no aldehydes were identified. Oleanolic acid was also the dominant component in cuticular waxes of 'Pinot noir' grapes at all stages of fruit development,[101] as well as in eight different cultivars investigated in a recent study,[102] in which triterpenoid contents were found to range from 42% to as much as 80% of total cuticular waxes. Due to their relevance for fermentative processes and their health-promoting properties,[103] major triterpenes (oleanolic acid and β-sitosterol) in grape waxes have been targeted for study and suggested to be a suitable tool to characterize grape varieties.[104] When the cuticular wax of fruit of the European wild vine (*Vitis vinifera* ssp. *sylvestris*) was analyzed, only minor quantitative differences were found in some of the wax fractions in comparison with the cultivated vine (*V. vinifera* ssp. *vinifera*),[66] suggesting their conservation during evolution.

We are not aware of any published studies on the cutin composition of grape berry cuticles, although the evolution of total cutin amounts during fruit maturation has been reported.[101] Taking into account its commercial importance with regard to both fresh consumption of table grapes and winemaking, further studies addressing this topic are desirable.

Oleaceae. *Olive (Olea europaea).* Because the procedure for olive oil extraction may dissolve an important part of the surface waxes, and given its economic importance, a number of published studies have analyzed wax compounds present in the oil. In contrast, little information is available on the intact fruit. When chloroform-soluble waxes of 'Coratina' olives at the green and black maturity stages were extracted, substantial compositional differences were found in each case.[105] Triacylglycerols (17 and 25% in green and black fruit, respectively) and triterpene acids (38 and 26%, respectively) were the predominant families of wax compounds in the fruit. Triterpenols accounted for 14% of total waxes in green fruit, whereas only traces were detected in ripe samples. The major triterpene in both cases was oleanolic acid (70 and 83%, correspondingly). Similar to the observations in grape (see previous subsection), *n*-alkanes were only a small part of the total wax mass (3 and 7% in green and black fruit, respectively), dominated by C_{27} or C_{29}, depending on the maturity stage.

Ebenaceae. *Persimmon (Diospyros kaki).* The total amount of waxes and cutin in fruit cuticles isolated from 27 astringent and nonastringent cultivars of persimmon has been published recently,[12] although the chemical composition of each fraction was not analyzed in detail. The cuticles of these fruit were composed of 22−38% (337−770 μg cm^{-2}) of wax and 33−56% (578−1378 μg cm^{-2}) of cutin. Whereas significant cultivar-related differences in cuticle composition were found, in agreement with other fruit species, these differences were not related to fruit astringency. Additional studies should enable the determination of which are the quality traits, in each fruit species, that are modulated by cuticle subtleties as well as the mechanisms by which this regulation occurs.

Others: Grossulariaceae, Ericaceae, Elaeagnaceae. No published information is available regarding the cuticular wax composition of soft fruit species within these families, and only the composition of cutin monomers has been analyzed and reported. Studies on six non-Rosaceae berry types, namely, sea buckthorn (*Hippophaë rhamnoides*), black currant (*Ribes nigrum*), cranberry (*Vaccinium oxycoccos*), lingonberry (*Vaccinium vitis-idaea*), bilberry (*Vaccinium myrtillus*), and crowberry (*Empetrum nigrum*), have revealed notable differences in total cutin loads (6−47%) across species.[62,106] The predominant cutin monomers were C_{16} and C_{18} ω-hydroxy acids with midchain functionalities, among which epoxy groups were particularly frequent in sea buckthorn and cranberry (71 and 60% over total cutin monomers, respectively). Cuticles of these fruits also contained significant amounts of C_{15} monomers and α,ω-dicarboxylic acids.

Conclusion. This survey shows that fruit cuticles display considerable variability among species and illustrates the inappropriateness of generalizations on fruit cuticle properties. Yet a complete overview of cuticle constituents is available in only a few instances (Table 1). Although *n*-alkanes and triterpenoids are prominent components of cuticular waxes in all of the fruit species for which such information exists, fruit cuticle types seem to emerge according to their main alkane (C_{29} vs C_{31}) and triterpenoid (triterpenoid acids vs triterpenoid alcohols) components. Some grouping is also apparent according to the predominant cutin monomers (C_{16} vs C_{18}). On the basis of the scarce information currently available, these groupings do not seem to show any correspondence with fruit type (berries vs drupes) or with ripening pattern (climacteric vs nonclimacteric). However, this review reveals clear similarities in fruit cuticle composition among phylogenetically close species (Table 1). For example, the investigated species within the Solanaceae family display C_{31} and triterpenoid alcohols as the main *n*-alkane and triterpenoid components of cuticular waxes, respectively, with dihydroxy $C_{16:0}$ as the predominant cutin monomer. In contrast, C_{29} and triterpenoid acids have been reported as the main wax components in most of the species examined within the Rosaceae, with the predominant cutin monomers displaying wider variation. Cutin composition in the cuticles of citrus fruit is reportedly dominated by C_{16} monomers, whereas insufficient information is available to allow generalizations on predominant wax compounds within this botanical family (Table 1).

Recent studies conducted on *Arabidopsis* have identified proteins specifically involved in the elongation of compounds longer than C_{28} and C_{30}, which are moreover expressed differentially in an organ- and tissue-specific manner.[107−111] Although no similar information has been reported for edible fruits, the question arises whether potential differences in the expression of homologous fruit genes or proteins may be related to the dissimilarities in the chain length of the predominant cuticular alkanes across botanical families (Table 1). The potential relationship of these differences with cuticle structure and properties, and hence, with the horticultural attributes of each fruit type, requires intensive research. The implications of cuticle composition and structure for postharvest potential and for the design of handling strategies are huge.[25] In the absence of these studies, any careless generalization is likely to lead to oversimplification and to failure of any postharvest procedure designed on such a weak basis.

CUTICLE BIOSYNTHESIS IN FRUITS

Cuticle Biosynthesis during Fruit Maturation and Ripening. The process of cuticle, cutin, and cuticular wax formation during on-tree fruit development has been studied essentially from the ultrastructural,[101,112] biomechanical/biophysical,[113−116] and biochemical points of view.[3,73,86,87,95,102,117] It has frequently been observed that

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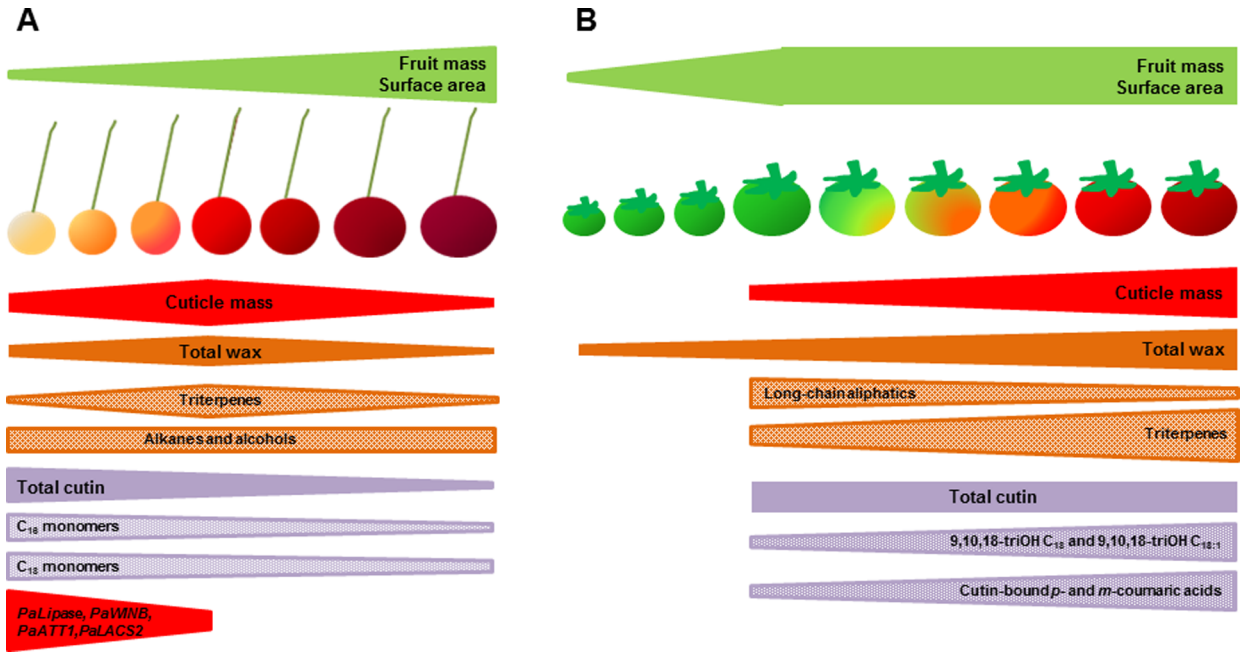

Figure 1. Changes in cuticle deposition and composition during the development of (A) sweet cherry and (B) tomato as an example of a nonclimacteric and a climacteric fruit species, respectively. The figure is intended uniquely as an illustration of the profound dissimilarities reported among different fruit species. The literature survey undertaken in this review indicates that the fate of cuticular compound families during fruit development differs noticeably among fruit species, and therefore these patterns should not be taken as representative of each ripening type. The figure was drawn from data reported in refs 3, 71−73, and 118−120. Changes are expressed per unit of surface area.

cuticle deposition ceases at a given developmental stage, generally corresponding to the transition between growth and ripening, giving rise to decreased amounts of cuticle per unit surface area. This favors increased surface strain as fruit expands, and thus the formation of micro- or macroscopic cracks, the latter having major economic consequences for some commercially important fruit crops. We are not aware of any cause/effect studies relating cuticle composition to the development of this disorder, but some genes are down-regulated concomitantly with arrested cuticle deposition in fruit species particularly prone to this disorder, such as sweet cherry,[118] which may provide the tools to investigate this aspect.

Generalizations on a particular biological event among different species should be treated with caution, as different genetic backgrounds often possess specific mechanisms to cope with environmental stimuli. For this reason, to tackle the compositional evolution of specific cuticular components during fruit development we inspected the available literature on those fruit species for which such information has been published. This survey indicates that any temptation to simplify or generalize the fate of different cuticular compound families during fruit development should be ruled out.

For instance, even the commonly observed trend of early cessation of cuticle deposition is not found in all cases, a notorious exception being tomato (Figure 1). Although long-chain aliphatics were actually found to decrease in developing tomato fruit, the extent of triterpene increases overcompensated for that reduction.[119] A continuous increase in the wax coverage during the developmental course from mature green to red ripe fruits in the 'Micro-Tom' cultivar has also been reported.[71] The total amount of wax and the proportion of alkadienes increased during ripening of 'Ailsa Craig' tomato from the mature green to the red ripe stages, whereas the total amount of cutin monomers per unit of surface area and the relative proportions of the corresponding constituents remained approximately constant during the same period, except for increases in 9,10,18-trihydroxyoctadecanoic and 9,10,18-trihydroxyoctadecenoic acids.[72] This continuous increase in total cuticular waxes during tomato maturation and ripening from as early as the small green stage was subsequently confirmed, with a progressive rise in the absolute content of all wax classes, with the exception of isoalkanes.[63] The amounts of most cutin monomers as well as that of *p*-coumaric and *m*-coumaric acids also increased throughout development. A substantial increase in the phenolic content of the cuticles of 'Ailsa Craig', 'Alicante', and 'Grower's pride' tomatoes was also observed in an earlier work,[120] the major part of the flavonoids detected in ripe fruit being bound to the cutin matrix.

A steady decrease in the proportion of primary alcohols and a progressive increase in esters in cuticular waxes were found during the maturation of grape berries, whereas the content in cutin per surface unit decreased sharply between berry set and véraison.[101] Total triterpenoid contents have also been observed to decline in grapes during fruit ripening.[102] In contrast, a survey on 17 different apple cultivars[86] disclosed increases in the percentage of primary alcohols in the epicuticular waxes as fruit development progressed. More recently, the alkanes *n*-nonacosane and *n*-heptacosane, the alkene *n*-nonacosene, and the secondary alcohol nonacosan-10-ol were observed to increase as fruit reached the mature stage, whereas nonacosan-10-one decreased during the same period.[87] For sweet cherry fruit, it was found that the amount of triterpenes per unit area decreased, with alkanes and alcohols remaining approximately constant throughout fruit development (Figure 1). On the other hand, the amounts of C_{16} and C_{18} cutin monomers per unit area declined, even though divergent trends were shown for different compounds within

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Table 2. Tomato Fruit Genotypes Impaired or Altered in Cuticle-Related Genes

genotype	cuticle-related phenotype	refs
alcobaça	altered wax profiles (throughout development) and cuticle lipid compositions (early stages of development); higher relative amounts of C_{18} monomers	73
cutin deficient (*cd1*, *cd2*, *cd3*)	dramatic reduction in cutin content, altered cuticle architecture, increased stiffness of cuticle surface, enhanced water permeability	31, 77, 136
delayed fruit deterioration (*dfd*)	altered cuticle composition and architecture	72
GDSL1-silenced lines[a]	reduced thickness of fruit cuticle, decreased content of cutin monomers, decreased wax load	32
nonripening (*nor*)	altered wax profiles (throughout development) and cuticle lipid compositions (early stages of development); higher relative amounts of C_{18} monomers	73, 137
positional sterile (*ps*)	increased water loss, severe depletion of *n*-alkanes and aldehydes, increased triterpenoids and sterol derivatives, occurrence of alkyl esters not present in the wild-type fruit; similar wax load, unaffected cutin monomer composition	134
ripening inhibitor (*rin*)	altered wax profiles (throughout development) and cuticle lipid compositions (early stages of development); higher relative amounts of C_{18} monomers	73
slcer6 loss-of-function	deficiency in *n*-alkanes and aldehydes longer than C_{30}, increase in intracuticular triterpenoids	67, 71
SlSHN3-silenced lines	reduction in cuticular lipids, lower cutin content, and altered architecture of fruit surface	78
sticky peel (*pe*)[b]	severe reduction in cutin biosynthesis and altered wax deposition	75
sticky peel/light green double mutant (*pe lg*)	glossy soft fruit, severely reduced thickness of fruit cuticle, enhanced water permeability	134, 136
y	reduced thickness of fruit cuticle, lower cutin content, and decreased elasticity	138
16 glossy and 8 dull mutants	altered amount and/or composition of wax and cutin, cuticle thickness, and surface aspect of the fruit	69

[a]GDSL1 is allelic to CD1. [b]PE is allelic to CD2.

each monomer family.[3] The compositional changes in cuticular waxes during fruit development have also been investigated in 'Newhall' orange fruit, and it was found that waxes are secreted continuously during ripening and that decreases in the amount of all major wax fractions were related to the glossy fruit flavedo phenotype of the so-termed 'glossy Newhall' mutant.[95] The mutant fruits have dramatically reduced aldehyde and alkane contents associated with a severe loss of wax crystals. No information was reported on changes in the amount of total cutin or cutin monomers.

This handful of available studies exemplifies how modifications in cuticle composition during fruit maturation and ripening are variable among different fruits. Similar investigations, undertaken on a wide range of additional fruit species, may help identify putative common trends within particular fruit types.

Insights into the Molecular Aspects of Cuticle Biosynthesis in Fruits. Little information exists on the regulation of cuticle formation in fruits at the molecular level. In fact, only a few recent papers have reported the identification of genes related to cuticular wax or cutin biosynthesis in these organs, most of them conducted in tomato as the model species. Cuticle deposition involves a large number of steps and interconnected routes that determine its amount, composition, and correct structural assembly.[7,9,16−19] The identification of the key control points is rendered a difficult task by the multilevel regulation of these pathways, which may involve gene expression, protein modification, and cell environmental changes. A feasible way to tackle this subject is to use mutants altered in fruit cuticle attributes in comparison with the wild-type phenotype. Some of such cuticle mutants exist for tomato (Table 2), and their comprehensive analysis may help detect candidate key genes, proteins, metabolites, and pathways associated with cuticle biosynthesis and functions. The current availability and affordable cost of -omics technologies paves the way for such "systems biology" approaches.

Using this strategy, the gene *LeCER6* (currently referred to as *SlCER6*, owing to the renaming of tomato to *Solanum lycopersicum*), a homologue of *AtCER6*, which codes for a very long-chain fatty acid β-ketoacyl-CoA synthase, has been identified,[67] and a LeCER6 loss-of-function mutant was obtained by reverse genetic techniques. Associated functional characterization showed that waxes of the mutant fruit were deficient in *n*-alkanes and aldehydes longer than C_{30}, whereas hydrocarbons with shorter or branched chains were unaffected. Mutant waxes were also significantly richer in intracuticular terpenoids. Interestingly, these modifications were reflected in cuticle permeability, unquestionably demonstrating a major role of these compounds in the transpiration barrier properties of the cuticle.[67] When the developmental course of maturation and ripening was followed, both in the wild type and in the *LeCER6* mutant, the deficiency was discernible as early as the mature green stage.[71] Comparative transcriptome and metabolome analyses in tomato showed that 17% of 574 peel-associated transcripts were putatively related to cuticle component-generating metabolic pathways.[74] Among these, the expression of *LeCER6* in both fruit exocarp and endocarp was shown to increase progressively during maturation and ripening, supporting the putative association of this gene with cuticular wax formation.

More recently, a homology-based approach has been employed to isolate two oxidosqualene cyclases (OSC) involved in the biosynthesis of triterpene alcohols and expressed exclusively in fruit epidermis, one of which was demonstrated to be a product-specific β-amyrin synthase.[121] When the transcription ratios of these OSCs, designated SlTTS1 and SlTTS2, were compared among three tomato cultivars ('Micro-Tom', 'M82',"and 'Ailsa Craig') differing in their triterpenoid profile, it was found that transcriptional control of these two OSCs accounted only partially for these differences, suggesting the involvement of other factors such as differences in catalytic activity or the presence of additional OSCs.

A few fruit-expressed genes related to cutin deposition have been also identified taking advantage of altered genetic backgrounds (Table 2). The characterization of a tomato mutant designated *cutin deficient 1* (*cd1*), showing only 5−10% cutin content in comparison with the wild-type fruit,[77] revealed

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that CD1 is an extracellular acyltransferase with polyester synthesis activity required for cutin accumulation in vivo, demonstrating it to be a cutin synthase directly involved in cutin polymerization.[31] The protein is localized in the cuticle, specifically at the interface with the cell wall, and the expression of its coding gene parallels spatial and temporal patterns of cuticle deposition in the fruit. Similarly, the characterization of the *sticky peel* (*pe*) tomato mutant, which suffers from severe reduction in cutin biosynthesis and alterations in wax deposition, allowed the identification and partial characterization of CD2, a putative transcription factor suggested to act upstream of several metabolic pathways involved in the biosynthesis of a range of surface tissue-associated molecules.[75] A tomato transcription factor (SlSHN3) that regulates fruit cuticle formation and epidermal patterning has also been characterized recently.[78] Silencing the *SlSHN3* gene resulted in remarkable morphological alterations of fruit epidermis and a significant reduction in cuticular lipids. Similarly, a mutation in *SlCYP86A69*, one of the SlSHN3 target genes, led to severe cutin deficiency and altered architecture of the fruit surface. SlCYP86A69 was characterized to possess NADPH-dependent ω-hydroxylation activity, with a preference for oleic acid ($C_{18:1}$) to yield the cutin monomer 18-hydroxyoctadecenoic acid.[78]

In this context, a recent work[69] has shown the potential of a simple phenotypic attribute such as fruit brightness for the identification of cuticle mutants, thus providing an additional tool for the discovery of new genes putatively involved in cuticle biosynthesis. A mutant collection of the 'Micro-Tom' cultivar was screened for altered fruit brightness, which led to the isolation of 16 glossy and 8 dull mutants displaying concomitant differences in cuticle composition, thickness, or structure. Whereas glossy mutants were altered in cutin load and/or composition, dull mutants differed from the wild-type fruit in the characteristics of the epidermis. Surprisingly, no relationship was found between fruit brightness and wax load variations.

Considerably less research effort at the molecular level has been focused on fruits other than tomato. The identification of several candidate genes putatively involved in cuticle formation during development and maturation of 'Regina' sweet cherry fruit revealed that 13 of 18 identified sequences showed expression patterns restricted to fruit exocarp and correlated with cuticle deposition rates.[118] This study recognized some genes that deserve further research as to a major role in cuticle formation, including two transcription factors, one lipase, one LTP, one ABC transporter, two CYP86A hydroxylases, two LACS, and one GPAT. More recently, two of these candidate genes, *PaLACS2* (a putative long-chain acyl-CoA synthetase) and *PaATT1* (a putative cytochrome P450 monooxygenase), were suggested to be major genes involved in cutin biosynthesis on the basis of the effects of their ectopic expression in *Arabidopsis thaliana*.[122]

A similar approach was adopted in apple (cv. 'Prima' and 'Florina') fruit, based on sequence homology studies and transcription profiling.[123] Expression patterns of the selected genes showed that they are active in, and in some cases specific to, fruit skin. The genes identified included some members involved in the FAE complex, wax and cutin modifications, one LTP, one ABC transporter, and one transcription factor. However, these apple and sweet cherry genes were identified on the basis of transcriptomic and homology data uniquely, and an actual involvement in cuticle formation remains to be confirmed. Even so, the proteins encoded by three full-length expressed sequence tag sequences identified in apple cDNA libraries as likely to encode triterpene synthases (*MdOSC1*, *MdOSC2*, and *MdOSC3*) have been actually demonstrated to possess triterpene synthase activity by transient expression in *Nicotiana benthamiana* leaves and by expression in *Pichia methanolica*.[124] Interestingly, MdOCS1 was shown to produce α- and β-amyrin in a 5:1 ratio and, thus, to be the only triterpene synthase identified to date for which α-amyrin accounts for >80% of the total product. This finding implies that MdOCS1 is primarily an α-amyrin synthase, and this is interesting in the light of earlier indications that the main component of the triterpene fraction in apple fruit cuticle is ursolic acid (Table 1), the precursor of which is α-amyrin. The expression levels for these *MdOSC* genes in 'Royal Gala' apple showed preferential expression in the fruit peel and also significantly dissimilar expression levels among tissues, which were reflected in different contents of ursolic and oleanolic acids. The increasing availability of genomic sequences for nonmodel fruit species will provide the basis for shedding light on the current knowledge of cuticle formation in these commodities.

METHODOLOGICAL FOOTPRINT IN CUTICLE COMPOSITION STUDIES

Cuticle components are usually extracted, identified, and quantified following similar methodologies, and therefore major compositional discrepancies found between fruit species are not likely to arise from technical variations across different research groups. This is particularly clear when a given team has found such dissimilarities after using the same methodology for the analysis of cuticles obtained from different fruit species. Even so, it is worth pointing out some methodological aspects that may have some influence on reported results. Indeed, many of the studies reviewed herein were conducted >30 years ago and used different technological solutions.

Waxes are usually extracted by dipping the starting material in an organic solvent such as chloroform, methanol, dichloromethane, hexane, petroleum ether, or toluene, at room temperature or at the boiling point of the solvent, in a single or in a two-step extraction to separate epi- and intracuticular waxes. However, although the sequential extraction with different organic solvents has often been used as an approach to differentiate between epi- and intracuticular waxes, such differential extraction procedures are not selective enough as the resulting extracts are cross-contaminated. For an accurate quantification of the components of both compartments, methods allowing the mechanical removal of epicuticular waxes have been demonstrated to be preferable for this separation, such as those involving the use of adhesives (glycerol, water, nitrocellulose, gum arabic), followed by the stripping off or brushing of the outer film so obtained.[125,126] The starting material, in turn, can involve intact fruit samples, dehydrated fruit skins, or enzymatically isolated cuticles, all of which methodologies have associated weaknesses. For instance, plant cuticles were shown to sorb large amounts of hexadecanoic and octadecanoic acids when incubated in cell slurries obtained through enzymatic digestion of fruits and leaves,[127] thus placing under suspicion the common method of estimating intracuticular wax composition from cuticles isolated enzymatically. Also, the remarkable differences in skin structure found across fruit types (such as those between apple and citrus fruit) may underlie a part of these discrepancies, together with chemical dissimilarities such as pH, which might influence the

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extraction of individual compounds. Additional features of fruit skin structure that may prove problematic or account for detected interspecific differences include thickness, flexibility, or the presence of stomata, lenticels, or trichomes.

Gas chromatography (GC) separation of the extracted compounds has been employed since the early 1960s, but the subsequent introduction of capillary columns increased analysis robustness. Compounds in the extracts may be injected directly, with previous chemical derivatization of active functional groups, or with previous hydrolysis of high-molecular-weight esters to release moieties that are then detected. In addition, compound detection by flame ionization (FID) or by mass spectrometry (MS) can result in quantitative and qualitative differences and hence justify some discrepancies.

Effective compound identification can be achieved by the comparison of Kovats retention indices and mass spectral data between the analyses and appropriate standards processed under matching experimental conditions. As standards for cuticle components are not always available commercially, the identification may be impaired or biased with respect to results obtained by other researchers if the group has the possibility to produce them in house. On the other hand, GC-MS identification is not free from drawbacks. For instance, identification is done by comparing the recorded EI-MS spectra with those available in public databases or with those obtained from reference compounds and then interpreting the fragmentation patterns in terms of chemical structure. Yet the spectra of many wax and cutin constituents are not available in MS libraries. In addition, rearrangements and isomerization may occur in unsaturated hydrocarbons, which might lead to the obtained spectra not showing the expected characteristic fragmentation configurations.[128] Even so, chemical techniques such as stereospecific oxidation and trimethylsilylation followed by gas−liquid chromatography coupled to MS have been employed successfully for the determination of double-bond positions of unsaturated fatty acids.[129,130] The use of more sensitive and up-to-date methodologies, such as two-dimensional GC, tandem chromatography arrangements, high-temperature GC (HTGC), HTGC-MS, or infrared and Raman microspectroscopic analysis,[131,132] would provide a valuable complementary tool to add to the current chemical standard analytical methods employed in bulk composition of plant cuticle analyses and hence allow a more accurate comparison between different plant materials, at both the intra- and interspecific levels.

CONCLUDING REMARKS AND FUTURE PROSPECTS

Biological developmental processes in plants are strictly controlled at various levels by cell composition and architecture. Organ- and cell-surrounding structures, such as the cell wall and the cuticle, are now recognized as highly dynamic developmental controllers, at both the signaling and the metabolic levels, as they provide the first communication level in response to conditions imposed on the plant by the surrounding environment. Despite the enormous efforts and advances in understanding cell organization, some cell components have received less attention from the scientific community, even though they are of paramount importance for plant development. Cell walls have been intensively studied and shown to vary in their composition, metabolism, and dynamics among different fruit species and even genotypes of the same species.[133] In contrast, a large number of gaps in knowledge still exist for almost every step of the putative pathways for the biosynthesis of plant cuticle.[7,9,16−19] Differences in cuticle composition between vegetative organs and fruits have been reported, but it is unclear whether exactly the same biosynthetic pathways and regulating mechanisms operate in fruit tissues, and there are many unknowns in regard to cuticle composition of fruits themselves (Table 1).

In this review, we highlight the variability proven to exist across individual fruit species and even across different genotypes of the same species. Such observations raise questions on the precise role of cuticles and on how they act as factors determining organ development and in coping with environmental cues. Answering this challenge will entail further efforts of fundamental research aiming at devising a generalized model of cuticle structure, together with a comprehensive atlas of differences between organisms, organ and cell types, and a track of metabolic regulation, to translate this knowledge into a practical understanding of the resulting biophysical properties. Future perspectives include understanding their impact and a putative premise of manipulating biological features useful to human activity, such as growing rates, respiration and water dynamics, protection against pathogens, or quality preservation of agronomic products.

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Notes

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ABBREVIATIONS USED

ABC, ATP-binding cassette; ACP, acyl carrier protein; CD, cutin deficient; CYP, cytochrome P450; DFD, delayed fruit deterioration; EI, electron ionization; FAE, fatty acid elongase; FAS, fatty acid synthase; FID, flame ionization detector; GC, gas chromatography; GDSL-lipase, Gly-Asp-Ser-Leu esterase/acylhydrolase; GPAT, glycerol-3-phosphate:acyl-CoA acyltransferase; HTGC, high-temperature gas chromatography; LACS, long-chain acyl-CoA synthetase; LTP, lipid transfer protein; MS, mass spectrometry; OSC, oxidosqualene cyclase; VLCFA, very long-chain fatty acids

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