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Specific characteristics of the apple fruit cuticle: Investigation of early and late season cultivars ‘Prima’ and ‘Florina’ (*Malus domestica* Borkh.)

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ABSTRACT

The cuticle is a heterogeneous extracellular layer, whose composition varies between plant species, cultivars and organs. Specific structural and compositional characteristics indicate the domesticated apple (*Malus domestica* Borkh.) as a suitable non-model plant system for comprehensive analyses of the cuticularization of epidermal cell walls. Our findings focused on the cuticular deposition of apple fruits, offering a continuous, thick and robust but also lenticel-containing experimental system for functional studies. In detail, the cuticular wax and cutin composition and the water permeance of apple fruits were comparatively analyzed in two cultivars, which also demonstrates the cultivar-specific plasticity of cuticle formation.

Despite significant differences in cuticle thickness and weight, fruits of the early and late season apple cultivars ‘Prima’ and ‘Florina’ accumulated on average 95 µg cm^{-2} cuticular waxes mainly consisting of pentacyclic triterpenoids like ursolic acid (≥70%). The average chain length of the aliphatic wax fraction of ‘Florina’ and ‘Prima’ was about 29.1. The cutin matrix averaged 995 µg cm^{-2} dominated by hydroxylated hexadecanoic and octadecanoic acid monomers (mixed C_{16}/C_{18} type). Although the hydrophobic cuticle is the main barrier in limiting transpirational water loss across the outermost plant surface, chemical differences between both cultivars were not related to the water permeance. ‘Prima’ and ‘Florina’ fruit surfaces had an average permeance for water of 5×10^{-5} m s^{-1}.

It is hypothesized that compositional differences such as a higher amount of alkanoic acids, primary alkanols and alkanals in the cuticular wax mixture and modifications in the cutin matrix in the late season cultivar ‘Florina’ compared to early season cultivar ‘Prima’ represent cultivar-specific patterns in cuticle formation of apple fruits.

1. Introduction

The cuticle, a hydrophobic layer, covers almost all primary aerial plant organs and, thus, mediates plant interaction with its surrounding environment. It is extracellularly deposited on the outer epidermal cell wall and plays critical roles in plant physiology and development, as maintaining mechanical strength, shielding ultraviolet radiation and most notably preventing desiccation by limiting non-stomatal water loss, enabling the maintenance of an adequate water status and cellular activities (Tsubaki et al., 2013; Martin and Rose 2014; Schuster et al., 2016). The efficacy of the protective cuticular barrier is influenced by the physical and chemical properties of its components (Jeffree 1996; Bernard and Joubès 2013).

The plant cuticle associated with the underlying polysaccharide cell wall is chemically and structurally heterogeneous in nature. It is mainly composed of a polymeric cutin matrix impregnated with cuticular waxes. At the interface, hydrophilic cell wall polysaccharides penetrate the cuticle (Kolattukudy 2001; López-Casado et al., 2007; Fich et al., 2016). This region is termed as ‘cuticular layer’ compared to the overlying, outermost ‘cuticle proper’, which is supposed to be free from intermingled, fibrous polysaccharides (Jeffree, 2006; Fernández et al., 2016, 2017).

Cuticular waxes comprise a complex mixture of homolog series of very-long-chain alkanoic acids (≥20 carbon units) and their derivatives, besides secondary metabolites with ring structures, such as pentacyclic triterpenoids, and are responsible for the establishment of the main transport-limiting barrier of cuticular membranes (Kunst and Samuels 2003; Burghardt and Riederer 2006; Yeats and Rose 2013). Characteristic cutin monomers are for the most part C_{16} and C_{18} alkanoic acids carrying a hydroxyl group in the ω-position and hydroxyl or

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epoxy groups located in the mid-chain position (Kolattukudy 1980; Heredia 2003; Stark and Tian 2006). The composition and absolute amount of esterified cutin monomers and components in the cuticular wax mixture can vary between plant species, even between organs of the same plant and throughout developmental stages (Jeffree 1996; Samuels et al., 2008; Buschhaus and Jetter 2011). The ultrastructure of the cuticle is highly ordered. However, its precise three-dimensional polymeric assembly and the specific composition of the different layers within the cuticle are only partially understood (Jeffree 2006).

Most studies of cuticular biosynthesis, composition, structure and function have focused in *Arabidopsis thaliana* (L.) Heynh. vegetative organs, especially leaves, which are amphistomatous and have an extremely thin cuticle (20 nm to 30 nm), making it a limited model plant system of cuticle research (Jenks et al., 2002; Schreiber and Schönherr 2009). On the other hand, the adoption of *Solanum lycopersicum* L. fruits as model system have been shown to be important for cuticular studies, since these astomatous fleshy berries provide a high amount of uniform cuticular material (Domínguez et al., 2008; Isaacson et al., 2009; Martin and Rose 2014). Nevertheless, there is only little information for the most fruit crops. Ongoing research about cuticular characteristics of different fruit types and the significance of their specific cuticle profiles is still needed (Veraverbeke et al., 2001; Belge et al., 2014; Fernández et al., 2016).

Some reports on the cuticle of the crisp, hard and juicy pome fruits of *Malus domestica* Borkh. are available. Studies of cuticular composition and architecture have revealed that there are not only substantial differences between *Solanum lycopersicum* and *Malus domestica* fruits; there are also high variabilities between *Malus domestica* cultivars (Markley and Sando 1931). It is generally accepted that *Malus domestica* fruits possess a cuticle with a mixed C_{16}/C_{18} type cutin matrix mainly composing of saturated and unsaturated dihydroxy hexadecanoic, trihydroxy and epoxy hydroxy octadecanoic acids and a triterpenoid-rich cuticular wax mixture (Eglinton and Hunneman 1968; Baker and Holloway 1970; Holloway 1973; Morice and Shortland 1973; Veraverbeke et al., 2001; Verardo et al., 2003; Dong et al., 2012). Cuticular waxes of *Malus domestica* fruits are dominated by pentacyclic triterpenoids such as ursolic acid and oleanolic acid, which are common triterpenoids in plants (Belding et al., 1998). Pentacyclic triterpenoids differ in biosynthesis from very-long-chain aliphatic cuticular constituents such as *n*-alkanes, alkanals, primary alkanols, secondary alkanols and alkanones that have a common biosynthetic pathway.

The first pathways underlying the cuticle biosynthesis are identical for the aliphatic components of cuticular waxes and cutin and involve the *de novo* biosynthesis of C_{16} and C_{18} alkanoic acids in the plastids of epidermal cells. To generate cutin monomers, C_{16} and C_{18} alkanoic acids are modified in the endoplasmic reticulum. These processes involve activating the alkanoic acids by conjugation to coenzyme A, oxidation of the terminal and/or mid-chain carbon units, rarely elongation processes and the acyl transfer from coenzyme A to glycerol (Fich et al., 2016). Likewise in the endoplasmic reticulum, plastid-derived long-chain precursors are elongated to very-long-chain alkanoic acids (≥20 carbon units) and, subsequently, converted by reduction, decarbonylation, hydroxylation, and oxidation reactions in characteristic cuticular wax compounds (Kunst and Samuels 2003; Yeats and Rose 2013).

The first committed biosynthetic pathway of pentacyclic triterpenoids is the cyclization of 2,3-oxidosqualene arising by the isoprenoid pathway. Subsequently, various rearrangements, which diversify the core triterpenoid structure and impart functional properties, include the addition of hydroxyl, ketone, epoxy, formyl and carboxyl groups. In plants, triterpenoids with an ursane-, an oleanane- and a lupane-type carbon skeleton derived from α-amyrin, β-amyrin and lupeol are the most widely distributed pentacyclic triterpenoids. Modification of β-amyrin leads to the formation of oleanolic acid. Ursolic acid is generated by uvaol, the precursor of which is α-amyrin (Phillips et al., 2006; Jäger et al., 2009; Pensec et al., 2014).

The presence of pentacyclic triterpenoids as biologically active compounds is of interest due to their wide range of antioxidant and antimicrobial properties (He and Liu 2007; Szakiel et al., 2012; Woźniak et al., 2015). Fruits of the domesticated species *Malus domestica* are one of the essential sources of triterpenoids (Boyer and Liu 2004). Nevertheless, the current knowledge of variable components and absolute amounts and their functional significance to the cuticular barrier properties remains limited, notwithstanding that the chemical plasticity of the cuticle has been studied extensively (Pollard et al., 2008). However, by restricting the transpirational water loss, the cuticular barrier contributes to the fruit freshness, appearance and firmness (Róth et al., 2007; Albert et al., 2013; Costa 2016).

This study is focused on the hydrophobic cuticle of *Malus domestica* fruits and, in particular, its microscopic, functional and chemical characteristics. Fruit cuticular wax and cutin accumulation of two scab resistant *Malus domestica* cultivars that possess different ripening traits and different modes of post-harvest storability were analyzed. ‘Florina’ (origin: France) and ‘Prima’ (origin: USA) represent a winter and a summer cultivar. Medium-sized to sized fruits of ‘Florina’ are harvested at the end of October and, subsequently, can be stored for three months or more, whereas medium-sized fruits of ‘Prima’ are not suitable for prolonged storage after harvest in September to October (Dayton et al., 1954). It may be hypothesized that these characteristics reflect variations in cuticular deposition by affecting the barrier properties against water loss, gas exchange and pathogenic invasion (Horrocks 1964; Kerstiens 1996; Riederer and Schreiber 2001; Martin and Rose 2014).

2. Materials and methods

2.1. Plant material

Trees of apple (*Malus domestica* Borkh.) cultivars ‘Florina’ and ‘Prima’ were grown in an orchard at the Experimental Research Farm of Szent István University, Soroksár. The apple fruits were harvested at the full ripening stage, at 120 days after pollination of the early season cultivar ‘Prima’ and at 145 days after pollination of the late season cultivar ‘Florina’. Fruits were taken from four sides of several trees, to minimize microclimate effects.

2.2. Determination of surface area, epidermal cell density, lenticel density, fresh weight and contact angle

The surface areas of apple fruits were determined according to the pixel values of a planar surface produced by slicing the fruit in comparison to a reference area (Adobe Photoshop 7.0.1). The epidermal cell density was estimated by counting the cell number on nail polish imprints from fruit surfaces using a Leica microscope system. The lenticel density was determined by counting the total number of lenticels on isolated fruit cuticular membranes using a binocular microscope (Leica Microsystems). Fresh weights of apple fruits were measured using a balance with a precision of 0.1 g (Sartorius AC210S). Contact angle measurements with a droplet of 1 µl deionized water were recorded at room temperature on an OCA 15 contact angle system with SCA20 software (DataPhysics Instruments).

2.3. Isolation of cuticular membranes

Cuticular membranes were enzymatically isolated from apple fruits by incubating epidermal discs at room temperature with 1% pectinase (Trenolin Super DF, Erbslöh) and 1% cellulase (Celluclast, Novo Nordisk AIS) in 20 mM citric acid, pH 3.0, containing 1 mM sodium azide to prevent microbial growth. The enzyme solution was exchanged weekly. Isolated cuticular membranes were extensively washed in deionized water and air-dried.