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Ontogenetic variation in chemical and physical characteristics of adaxial apple leaf surfaces

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Abstract

The reaction of plants to environmental factors often varies with developmental stage. It was hypothesized, that also the cuticle, the outer surface layer of plants is modified during ontogenesis. Apple plantlets, *cv*. Golden Delicious, were grown under controlled conditions avoiding biotic and abiotic stress factors. The cuticular wax surface of adaxial apple leaves was analyzed for its chemical composition as well as for its micromorphology and hydrophobicity just after unfolding of leaves ending in the seventh leaf insertion. The outer surface of apple leaves was formed by a thin amorphous layer of epicuticular waxes. Epidermal cells of young leaves exhibited a distinctive curvature of the periclinal cell walls resulting in an undulated surface of the cuticle including pronounced lamellae, with the highest density at the centre of cells. As epidermal cells expanded during ontogenesis, the upper surface showed only minor surface sculpturing and a decrease in lamellae. With increasing leaf age the hydrophobicity of adaxial leaf side decreased significantly indicated by a decrease in contact angle.

Extracted from plants, the amount of apolar cuticular wax per area unit ranged from only 0.9 μg cm^{-2} for the oldest studied leaf to 1.5 μg cm^{-2} for the youngest studied leaf. Differences in the total amount of cuticular waxes per leaf were not significant for older leaves. For young leaves, triterpenes (ursolic acid and oleanolic acid), esters and alcohols were the main wax components. During ontogenesis, the proportion of triterpenes in total mass of apolar waxes decreased from 32% (leaf 1) to 13% (leaf 7); absolute amounts decreased by more than 50%. The proportion of wax alcohols and esters, and alkanes to a lesser degree, increased with leaf age, whereas the proportion of acids decreased. The epicuticular wax layer also contained α-tocopherol described for the first time to be present also in the epicuticular wax. The modifications in the chemical composition of cuticular waxes are discussed in relation to the varying physical characteristics of the cuticle during ontogenesis of apple leaves.

Keywords: *Malus domestica*; Rosaceae; Leaf; Surface wax; Micromorphology; Chemical composition; Tocopherol; Hydrophobicity

1. Introduction

For all terrestrial higher plants, the cuticle forms a protective coating of aerial parts preventing the plant from desiccation due to uncontrolled non-stomatal water loss and the loss of organic and inorganic compounds by leaching. Moreover, the cuticle protects the plant against the infiltration of xenobiotics from the environment as well as from potentially harmful irradiance, like UV-B radiation. This interface between plant and environment, in particular the waxes forming its outermost surface, is relevant for the colonization by epiphytic microorganisms and the host recognition by pathogenic fungi as well as insects (Kolattukudy, 1985; Carver et al., 1990; Van Loon et al., 1992; Podila et al., 1993; Eigenbrode and Espelie, 1995; Flaishman et al., 1995; Schoonhoven et al., 1998).

The highly lipophilic cuticle, varying from 0.1 to 20 μm in thickness depending on plant species and organ, consists of two main components, cutin and wax. The amount of

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cuticular wax ranges from 1% to 10% of the total cuticle (Baker, 1982; Walton, 1990). Regarding the micromorphology of waxes, twenty three types have been classified in total (Barthlott et al., 1998). Thin wax films appear to be ubiquitous, while thick layers or crusts are rare.

The cuticular wax fraction can be differentiated into intra- and epicuticular waxes (EW). The chemical composition of cuticular waxes significantly varies among plant species and among cultivars within a species (Post-Beittenmiller, 1996). For apple fruits, six classes of EW compounds have been described, including: triterpenoids; primary alcohols: ketones; aldehydes and secondary alcohols and acids (Holloway, 1982; Belding et al., 1998). Among apple cultivars 'Golden B', 'Golden Delicious', and 'Ozark B' the chemistry of cuticular waxes differed, e.g., in the nonacosane content (Belding et al., 1998; Verardo et al., 2003). The chemical composition of surface waxes largely determines the morphology of the surface. Studies by Jeffree et al. (1976) as well as Jetter and Riederer (1994) demonstrated that nonacosan-10-ol is essential for the formation of wax tubules.

In addition to the dominant constituents, cuticular wax may include also some minor or unusual constituents like sterols and phenolic compounds (Baker, 1982). Phlorizidin, phloretin, quercetin and quercetrin – phenols known for their antimicrobial properties – have been detected in the leaf waxes of *Malus* spp. (Richmond and Martin, 1959). The triterpenoid pathway and tocopherol synthesis are closely linked. Derived from isoprene, tocopherol is synthesized only by photosynthetic organisms in the envelope of plastids and consists of a polar chromanol ring and a 15-carbon lipophilic prenyl chain derived from homogentisic acid and phytyl diphosphate (Collakova and DellaPenna, 2003). The lipid-soluble vitamin E is known to be an important intracellular antioxidant and scavenger of lipid peroxy radicals in plant tissue crucial for membrane stability (Munné-Bosch and Alegre, 2002). Tocopherols are not usually found in cuticular waxes of Rosaceae. They have been, however, detected in the wax of *Gingko* (Gülz et al., 1992) and *Rubus* (Robertson et al., 1991). The biological activity of tocopherol – the prevention of plants from damage induced by a great number of abiotic and biotic stress factors – has been well described (Schmitz and Noga, 2000a,b; Schmitz-Eiberger and Noga, 2001; Förschler et al., 2003).

In addition to genetic (Wissemann, 2000) and environmental factors, e.g., light (Von Wettstein-Knowles et al., 1980; Letchamo and Gosselin, 1996), relative humidity and water stress (Sutter, 1984; Prior et al., 1997), chilling (Nordby and McDonald, 1991), plant nutrition (Schwab et al., 1994) and seasonal variation (Gülz and Müller, 1992), the ontogenetic development of plants and plant parts, e.g. leaves, is reported to affect the composition of epicuticular waxes (Rhee et al., 1998; Riederer and Markstädter, 1996). In *Fagus sylvatica*, the bimodal distribution of aliphatic wax constituents with maxima in the range of C_{28} and C_{52} shifted within 20 days after bud stage to a large single maximum of C_{28} when the leaf reached final size (Riederer and Markstädter, 1996). During leaf expansion of *Prunus laurocerasus*, the average chain length of alcohols and fatty acids of epicuticular waxes increased from C_{24} to approximately C_{32} (Jetter and Schäffer, 2001). Information on changes in the composition of EW during ontogenesis is rare amongst the species of Rosaceae as reported by Shepherd et al. (1999).

The objective of this study was to characterize the dynamics in chemical composition and physical characteristics of the adaxial surfaces of apple leaves during early stages of ontogenesis. Under controlled conditions, the influence of environmental factors were minimized in order to focus on the effect of ontogenetic leaf age. Major physical characteristics of the adaxial epidermal cuticle were assessed microscopically and goniometrically, the amount of apolar waxes and their chemical composition were investigated using GC techniques. The α-tocopherol content of the wax layer was quantified as this antioxidant is reported to be involved in the reaction of plants to abiotic stress.

2. Results

2.1. Growth rate of apple leaves depending on leaf insertion and developmental stage

The development of *Malus domestica* leaf surfaces was monitored for apple plants which had been grown under greenhouse conditions for 40 days after planting. The area of all leaves – with leaf 1 being the youngest leaf at the end of the experiments – was calculated from measurements carried out on day 0, 4, 8, 12 and 16, respectively (Table 1). During the experiment, apple seedlings produced a new leaf every four to five days. For young leaves, the leaf area almost doubled within four days. Subsequently, the growth rate decreased rapidly and approached 0 about 20 days after first leaf appearance. Low leaf insertions, i.e. the oldest leaves produced by the seedlings, remained smaller than higher leaf insertions formed at later stages of leaf development. The latter's fully expanded leaves reached a leaf area of almost 18 cm^2.

2.2. Structure of leaf surfaces

Microscopic investigations using SEM displayed the typical pattern of puzzle-like epidermal cells forming the leaf surface of dicots. The adaxial leaf side did not show any stomata. Leaf age had an influence on the surface structures. Epidermal cells of the upper side of young leaves (leaf insertion 1) showed a distinctive curvature of the periclinal cell walls resulting in a noticeably undulated surface of the leaf cuticle (Fig. 1, left side). Pronounced lamellae of the cuticle showed highest density at the centre of the epidermal cells (on the top). Crystals of epicuticular waxes were detected neither by scanning electron microscopy nor by the ESEM technique without preparation of

Table 1
Growth rate of apple leaves depending on leaf insertion and leaf age

Day	Leaf area ± SE (cm^2) and *relative growth rate per day* ($cm^2\ day^{-1}$), respectively											
	Leaf insertion 1	Leaf insertion 2	Leaf insertion 3	Leaf insertion 4	Leaf insertion 5	Leaf insertion 6	Leaf insertion 7	Leaf insertion 8	Leaf insertion 9	Leaf insertion 10	Leaf insertion 11	Leaf insertion 12
0						3 ± 0.5	11 ± 1.9	17 ± 0.7	16 ± 2.9	17 ± 1.8	14 ± 0.8	13 ± 2.3
4				2 ± 0.1	4 ± 1.2	*0.38*	*0.11*	*0.04*	*0.01*	*0.00*	*0.00*	*0.00*
8			3 ± 0.3	*0.41*	*0.25*	*0.11*	*0.03*	*0.01*	*0.01*	*0.00*	*0.00*	*0.00*
12		3 ± 0.4	*0.3*	*0.16*	*0.12*	*0.03*	*0.01*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*
16	4 ± 1.1	*0.2*	*0.1*	*0.04*	*0.04*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*	*0.00*
Final size	4 ± 1.1	7 ± 2.9	16 ± 4.3	18 ± 4.5	20 ± 2.4	23 ± 1.9	20 ± 2.4	21 ± 1.2	17 ± 3.0	17 ± 1.8	14 ± 0.7	13 ± 2.3

Data indicate absolute leaf area (cm^2) for the first and the final measurement and relative growth rate per day for all subsequent measurements. Leaf insertion 1 was the youngest, leaf insertion 12 the oldest leaf of apple seedlings (cv. Golden Delicious, means ± SE, $n = 6$).

cuticles. During ontogenesis, the epidermal surface of leaves became more even, showing only minor surface sculpturing. The number of cuticular lamellae per cell was lower and lamellae were less pronounced (Fig. 1, right side).

The leveling of the leaf surface probably resulted from the expansion of epidermal cells (Table 2). The surface area of cells within the epidermal layer increased from about 630 μm^2 (leaf insertion 1) to approximately 2220 μm^2 (leaf insertion 5). For the oldest leaves investigated (leaf insertion 7) the cell area was smaller (about 1975 μm^2) largely corresponding to the smaller total leaf area. The number of epidermal cells per leaf calculated from these data varied from 1.4×10^6 (leaf insertion 7) to 1.9×10^6 (leaf insertion 3, +32%), with the three upper leaf insertions investigated showing a variation of only 7%. The height of epidermal cells decreased during ontogenetic development, with differences of up to almost 7 μm between the youngest and the oldest leaf insertion measured (Table 2). Therefore, the oldest leaf insertion had a lower number of epidermal cells, and also a smaller volume, than mature younger leaves.

2.3. Hydrophobicity of leaf surfaces

The contact angle of water on the adaxial surface of young apple leaves (leaf insertion 1) was almost 110°, indicating a pronounced hydrophobicity of the cuticle (Fig. 2). The wettability of artificial surfaces such Parafilm® and glass slides was measured as references for a hydrophobic and a hydrophilic surface, respectively. The hydrophobicity of apple leaf surfaces decreased with increasing leaf age. The largest increase in wettability was identified between leaf insertion 3 and 5 (contact angle >100° and 80°, respectively), with the wettability showing higher variability for older leaves. The contact angle for leaves of insertion 7 averaged 76.4° – well above the level of the hydrophilic glass surface.

2.4. Characterization of the wax layer

The chemical composition of apolar wax compounds, extracted from the upper leaf surface layer of apple seedlings cultivar 'Golden Delicious' were analyzed in relation to the leaf insertion (Fig. 3). The amount of wax per unit of area ranged from 1.5 $\mu g\ cm^{-2}$ for leaf insertion 1 to 0.9 $\mu g\ cm^{-2}$ for leaf insertion 7. The area of young leaves increased during ontogenesis, while the wax amount per unit of leaf area decreased; the correlation coefficient between wax mass per cm^2 and the area of total leaf surface was −0.833 ($p \leqslant 0.05$). Differences in the total amount of waxes per leaf were not significant among leaf insertions 3 to 7.

Wax components were identified as primary alcohols, fatty acids, esters, triterpenes and alkanes. For young leaves, triterpenes (ursolic acid and oleanolic acid), esters and alcohols were the main wax components (Fig. 4).

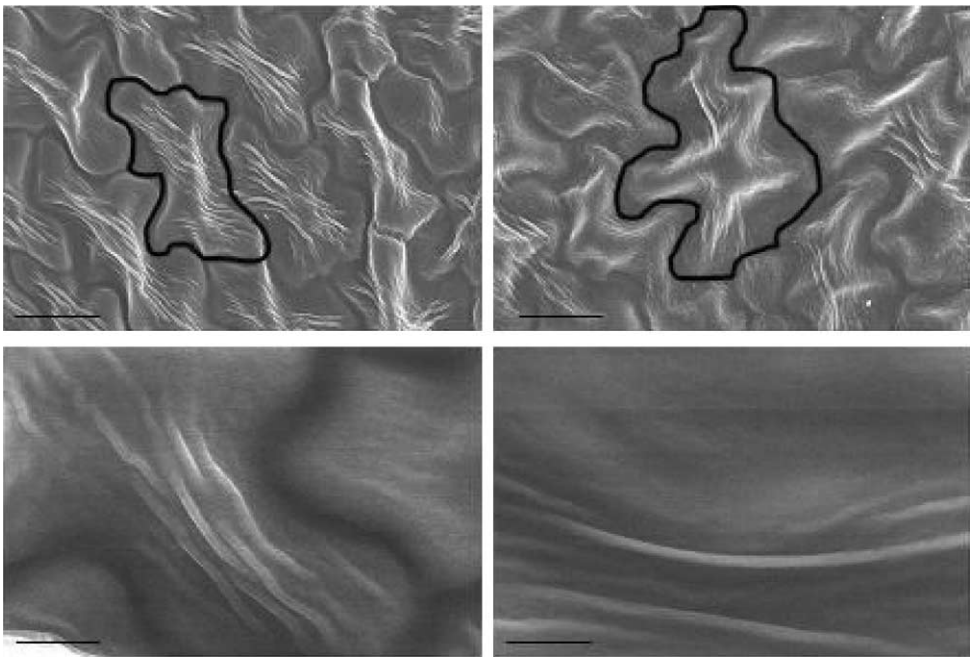

Fig. 1. Adaxial surface of the epidermal cell layer of apple leaves, cv. Golden Delicious. Left: youngest, completely unfolded leaf; right: seventh leaf from the top, (top: SEM, 1000x, bars represent 20 μm; bottom: ESEM, 4000×, bars

Table 2
Area and height of epidermal cells from the upper side of apple leaves depending on the leaf insertion (cv. Golden Delicious, means ± SE, $n = 30$)

	Leaf insertion			
	Leaf 1	Leaf 3	Leaf 5	Leaf 7
Cell area (μm^2)	634 ± 20^d	1566 ± 52^c	2224 ± 82^a	1976 ± 51^b
Cell height (μm)	27 ± 1^a	26 ± 1^a	23 ± 0^b	20 ± 1^c

Figures with different letters are significantly different, Tukey test, $p \leqslant 0.05$.

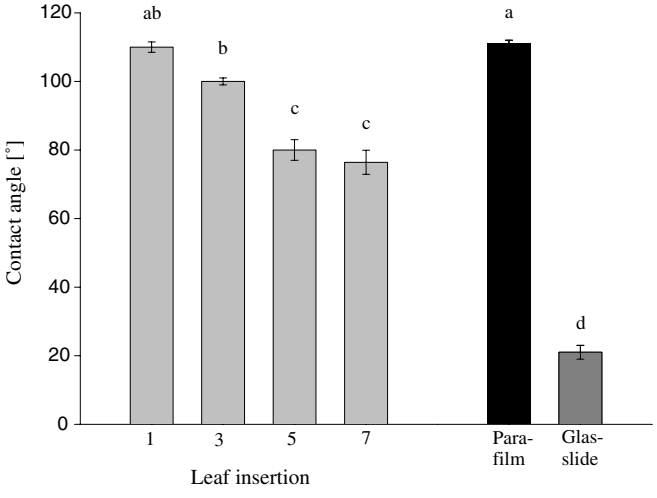

Fig. 2. Effect of ontogenesis of apple leaves (cv. Golden Delicious) on the wettability of cuticular surfaces as measured by goniometry; artificial surfaces were measured for comparison ($n = 18$, Parafilm® $n = 12$, glass slides $n = 6$, mean ± SE). Columns with different letters are significantly different, Tukey test, $p \leqslant 0.05$.

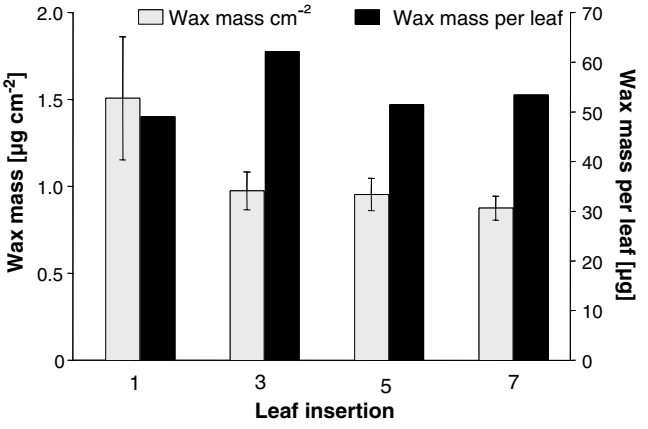

Fig. 3. Effect of ontogenesis of apple leaves on the apolar wax mass per leaf area and total apolar wax mass per leaf (cv. Golden Delicious, $n = 5$).

The triterpenes represented up to 32.3% (leaf insertion 1) of the total amount of apolar waxes and decreased during ontogenetic development to 22.6% (leaf insertion 7). Esters slightly increased from 23.2% for leaf insertion 1 to about 25% for the oldest leaves. The content of wax alcohols increased considerably during ontogenetic development from 19% to 28.8%. The amount of C_{22} up to C_{30} acids was always below 10%; it was halved during the expansion of leaf area and did not alter in later stages of development. The amount of alkanes remained largely constant during leaf ontogenesis varying only from 16.6% to 19%.

Data on the main constituents of the chemical classes detected are summarized in Table 3. In young leaves, C_{29} alkane represented almost 5% of total waxes. It decreased noticeably to less than 3% in older leaves. C_{31} alkane was

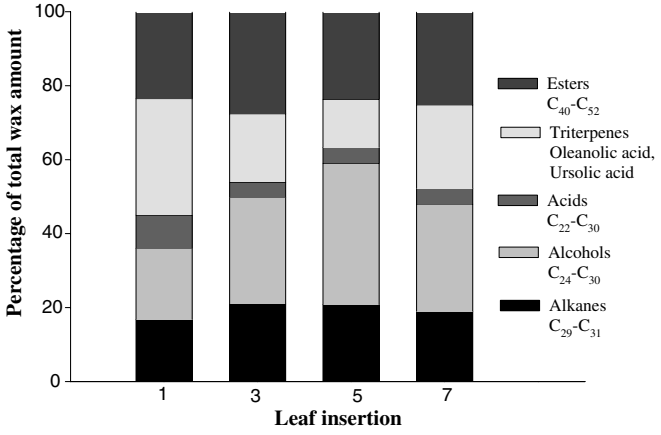

Fig. 4. Effect of ontogenesis of apple leaves on the percentage of apolar wax compound (% of total apolar waxes, $n = 5$).

the main constituent of this chemical class representing about 9.0% and 13.5% in young (leaf insertion 1) and old (leaf insertion 7) leaves, respectively. In absolute terms, however, the content of C_{31} alkane as well as of C_{33} alkane did not alter. The absolute amount of C_{24} alcohol significantly decreased during leaf development. The amount of the other alcohols largely remained constant among leaf insertions. At a very low range, long-chain acids formed a fraction of about 0.3% (C_{22} acid) to 4.7% (C_{26} acid) in young leaves. With a decline in absolute amounts – especially for C_{24}, C_{26}, and C_{30} acids – of more than 70% the percentage of these acids in the total amount of apolar wax constituents decreased by about 50% during leaf ontogenesis. The chain length for the C_{28} acid remained at a level of about 0.3% during the ontogenetic development.

In young apple leaves, the main triterpene compound was ursolic acid, which represented up to 25.9% of the total wax mass (leaf insertion 1). Oleanolic acid amounted to 6.4% for leaf insertion 1. During the ontogenetic development of leaves the amount of both triterpenes decreased, whereas the percentage of ursolic acid and oleanolic acid in the total wax amount was reduced to 20.3% and 2.3%, respectively. The total content of esters with chain length of $C_{40–48}$ and C_{50} in the cuticular wax did not change. The C_{40} ester decreased significantly, whereas the concentration of the others remained largely constant. In contrast, the amount of C_{46} and C_{52} esters increased in both absolute terms and in the percentage of total wax amount from 1.0% to 4.4% for C_{46} esters, and from 2.4% to 6.5% for C_{52} esters) with increased leaf age.

2.5. Content of α-tocopherol in the surface wax layer

In the epicuticular wax, fraction prepared with the freeze-embedding method, α-tocopherol was detected by HPLC measurements during all developmental stages. The amount of α-tocopherol increased significantly from 0.38 ng cm^{-2} for leaf insertion 1 to 1.5 ng cm^{-2} for leaf insertion 5 (Fig. 5). The content in the oldest leaves remained at an increased level. Neither γ- nor δ-tocopherol was found in the epicuticular wax layer.

3. Discussion

The dynamic process of the leaf development in relation to the surface chemical and physical characteristics was

Table 3
Effect of ontogenesis of apple leaves on the composition of apolar wax components; bold numbers indicate the percentage of the total extracted and identified wax compounds (cv. Golden Delicious, $n = 5$)

Compound	Leaf 1 (ng cm^{-2})		Leaf 3 (ng cm^{-2})		Leaf 5 (ng cm^{-2})		Leaf 7 (ng cm^{-2})	
	Mean ± SE	%	Mean ± SE	%	Mean ± SE	%	Mean ± SE	%
C_{29} alkane	71 ± 28	**4.7**	26 ± 3	**2.7**	23 ± 4	**2.4**	22 ± 3	**2.5**
C_{31} alkane	135 ± 23	**9.0**	133 ± 45	**13.9**	125 ± 40	**13.1**	118 ± 34	**13.5**
C_{33} alkane	44 ± 19	**2.9**	33 ± 13	**3.4**	32 ± 8	**3.3**	24 ± 8	**2.7**
C_{24} alcohol *	35 ± 15	**2.3**	5 ± 1	**0.6**	4 ± 1	**0.4**	4 ± 0	**0.4**
C_{26} alcohol	88 ± 15	**5.9**	110 ± 33	**11.4**	166 ± 26	**17.4**	100 ± 12	**11.5**
C_{28} alcohol	86 ± 16	**5.7**	69 ± 7	**7.1**	85 ± 7	**8.9**	79 ± 7	**9.0**
C_{30} alcohol	77 ± 16	**5.1**	72 ± 12	**7.5**	76 ± 13	**8.0**	69 ± 9	**7.9**
C_{22} acid	5 ± 2	**0.3**	3 ± 1	**0.3**	2 ± 0	**0.2**	2 ± 1	**0.2**
C_{24} acid	31 ± 22	**2.1**	5 ± 1	**0.5**	4 ± 0	**0.5**	3 ± 1	**0.4**
C_{26} acid	70 ± 36	**4.7**	21 ± 4	**2.1**	22 ± 6	**2.3**	22 ± 6	**2.5**
C_{28} acid	3 ± 2	**0.2**	2 ± 1	**0.2**	4 ± 1	**0.4**	3 ± 1	**0.3**
C_{30} acid	24 ± 15	**1.6**	7 ± 1	**0.7**	5 ± 1	**0.6**	6 ± 1	**0.7**
Oleanolic acid*	97 ± 37	**6.4**	30 ± 5	**3.1**	21 ± 1	**2.2**	20 ± 1	**2.3**
Ursolic acid	390 ± 184	**25.9**	192 ± 40	**20.0**	179 ± 24	**18.8**	177 ± 29	**20.3**
C_{40} ester *	40 ± 13	**2.6**	19 ± 3	**1.9**	9 ± 2	**1.0**	9 ± 1	**1.0**
C_{42} ester**	44 ± 10	**2.9**	26 ± 5	**2.7**	20 ± 6	**2.1**	18 ± 4	**1.0**
C_{44} ester	96 ± 24	**6.4**	58 ± 9	**6.0**	42 ± 6	**4.4**	48 ± 18	**5.5**
C_{46} ester**	15 ± 4	**1.0**	27 ± 4	**2.8**	42 ± 4	**4.5**	37 ± 5	**4.4**
C_{48} ester	118 ± 33	**7.9**	89 ± 18	**9.2**	54 ± 12	**5.7**	59 ± 11	**6.5**
C_{52} ester	37 ± 9	**2.4**	34 ± 3	**3.6**	38 ± 10	**4.0**	57 ± 8	**6.5**

* Significant difference according to Tukey test, $p \leqslant 0.05$.
** Significant difference according to Kruskal-Wallis test, $p \leqslant 0.05$.

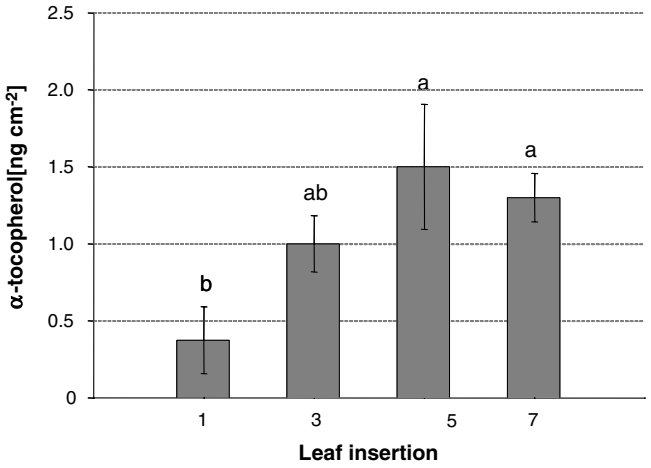

Fig. 5. Effect of ontogenesis of apple leaves (cv. Golden Delicious) on the α-tocopherol content in the epicuticular waxes of adaxial apple leaves (means ± SE, $n = 5$). Columns with different letters are significantly different according to Duncan's multiple range test, $p \leqslant 0.05$.

studied by means of apple leaves cultivar 'Golden Delicious'. The plants were grown under controlled and particle-free conditions in order to exclude any environmental factors, which may have an effect on physical and chemical properties of the cuticle; the investigations focused on the effect of ontogenetic development and leaf age.

The adaxial side of apple leaves had an amorphous surface detected at the SEM level. The arrangement of wax in platelets or as an amorphous structure has an impact on the micromorphology of leaves and fruits, e.g., wettability and water repellence. Wettability of cuticles differs depending on the presence of epicuticular waxes, their chemical composition and the micro-structure of waxes. The cuticle of young apple leaves exhibited at the upper side cuticular ridges or wrinkles about 0.8–1.0 μm in height, especially above the lumen of epidermal cells. The flattening out and partial disappearance of these wrinkles on older leaves may be attributed to the expansion of cell area. The increase in epidermal leaf area largely depended on the expansion of cells present. The cuticle of organs which undergo rapid area expansion may form pronounced folds in juvenile stages as described for grape ovaries (Considine and Knox, 1979; Rosenquist and Morrison, 1988). The folding may increase the surface area of cuticles by a factor of two to three (Jeffree, 1996) and could largely accommodate the expansion of epidermal area during later stages of leaf development. Wrinkles are also supposed to be the sites of transportation and incorporation of new material – waxes and cutin – for cuticle growth.

For about 20 days, the area of epidermal cells increased with leaf age, resulting in a flattening of cuticular lamellae as well as of total cells. The cuticle forms only a thin film above the cutin matrix known to increase during leaf expansion and the ontogenetic development of leaves, respectively (Hellmann and Stösser, 1992; Rhee et al., 1998). Hellmann and Stösser (1992) reported that the development of cuticle thickness was faster than leaf expansion and enlargement of epidermal cells reaching 80% of final thickness within one week as compared to two weeks for final leaf size. All results point to a change in the wax arrangement and not to an increase in the wax amount. During all stages of leaf development, the wax mass of adaxial cuticles remained at a low level (10–15 μg cm^{-2}), as compared to about 280 μg cm^{-2} (total wax mass) and 76 μg cm^{-2} (epicuticular waxes) for apple leaves in the field (Hellmann, 1992), and only 0.1–0.4% of the amount of apple fruits as reported by Belding et al. (1998). According to calculations by Jetter and Schäffer (2001), this wax yield corresponds to a layer thickness of 10–15 nm. For *P. laurocerasus*, the total amount of chloroform-extracted cuticular waxes differed for the adaxial (280 μg cm^{-2}) and the abaxial (830 μg cm^{-2}) leaf surface (Jetter and Schäffer, 2001), well above the amounts detected for *M. domestica*. In addition to differences between plant parts, the growth under high relative humidity at ambient temperature without UV light, conditions preventing the induction of cuticular wax production described for various environmental stress conditions (Maier and Post-Beittenmiller, 1998; Jenks et al., 2001; Gordon et al., 1998) are likely to contribute to the overall low wax mass per unit of area.

During ontogenetic development of apple leaves, the leaf area increased and the wax mass per unit of area tended to decrease. This was especially true during early leaf ontogenesis. During later stages of development the expansion of epidermal cell areas converged to zero and the amount of wax per unit of area remained constant. Examining leaves and fruits of citrus, Freeman et al. (1979) described the same phenomenon of expanding leaf area and a corresponding decline in wax concentration per unit of leaf area. They discussed a rapid leaf expansion, which exceeded the rate of wax production. Under experimental conditions, the formation of a new leaf position took about 4–5 days and according to leaf area development and changes in chemical composition of cuticle's waxes the maturation of leaves took about 20–25 days. For older leaves with growth rates approaching zero, both the wax mass per unit of area and the contact angle, remained largely constant. Changes in the total wax mass and the chemical composition of the surface wax layer have been well documented. During ontogenesis of peach leaves the individual wax mass as well as the composition of major components – e.g. triterpenes and alkanes – varied (Bukovac et al., 1979). Changes in *Fagus sylvatica* over a three years period demonstrated that the appearance (= synthesis and secretion) and disappearance (= loss) of wax constituents results from dynamic processes and every developmental stage has a specific pattern in the composition of wax compounds (Markstädter, 1994).

This could also apply to cuticles of apple leaves, as the triterpene content amounted to 30% (leaf insertion 1). Ursolic acid is especially prominent in the leaf and fruit waxes of *Malus* and *Prunus* species (Baker, 1982). However, the role and localization of the triterpenoid acids in the wax layer of

cuticles is not clear. In grape berry cuticle, oleanolic acid can constitute up to 60% of total wax mass and seems to be present also in epicuticular waxes to a high percentage, but may be not present in leaf waxes (Comménil et al., 1997; Casado and Heredia, 1999). In *P. laurocerasus*, triterpenoids accounted for a high percentage of intracuticular waxes, but were absent in EWs (Jetter and Schäffer, 2001). In apple leaves from the field, triterpenoids were the prominent constituent of intracuticular waxes, whereas the content in EWs was minor (Hellmann, 1992). The thin wax layer of apple leaves grown under protected conditions is likely to favour the extraction not only of epicuticular but also of intracuticular wax constituents resulting in the presence of oleanolic acid and ursolic acid in lipid extracts.

The hydrophobic character of the adaxial side of leaf surfaces is caused by the chemical composition of the epicuticular waxes; this correlation is supported by the results demonstrating a high apolarity of alkanes and primary alcohols resulting from long and very long alkyl chains of hydrocarbons in the cuticular waxes. C_{31} alkane was the most prevalent homologue. C_{26} and C_{28} were the predominant chain lengths for alcohols, whereas C_{26} was predominant amongst acid for juvenile leaves. Amongst alcohols, the average chain lengths increased with leaf ontogenesis, while absolute amounts of alcohols largely remained constant with a tendency to increase. The diversification in chain length is a modification of cuticular waxes often reported for the process of leaf maturation (Hellmann, 1992). For apple leaves, the chain length of alkanes increased with leaf ontogenesis. A same effect was detected for the ester fraction; the C_{40}:C_{52} ratio was approximately 1:1.1 for the youngest leaf, and changed to 1:5 for the oldest one. C_{48} ester was the predominant compound.

Hydrophobicity of upper leaf surfaces decreased during the ontogenetic development of apple leaves. The increase in hydrophilic was associated with a decrease in the total amount of extractable surface waxes as well as with modifications in the composition of wax compounds. Non-regarding differences in chain length, the content of alcohols significantly increased with leaf age, while triterpenes decreased. The accumulation of the OH-functional group plays an important role concerning the wettability; during the ontogenesis the leaf surface becomes more wettable. Hellmann (1992) studied the surface wax of different varieties of *M. domestica* depending on age and variety in field experiments. Leaf age had no effect on total wax mass, the proportion of alkanes and esters decreased during leaf ontogenesis, while primary alcohols increased. For grape berry, cuticular waxes became progressively enriched in wax esters and hydrocarbons from bloom to veraison, while the proportion of primary alcohols steadily decreased during fruit development (Comménil et al., 1997). These results point to diametrically opposed developments in the modification of cuticles from leaves and fruits, respectively, during ontogenesis.

The α-tocopherol concentration of adaxial epicuticular waxes of apple leaves was 0.5–1.5 ng cm^{-2}, equivalent to about 130–400 μg g^{-1} leaf dry weight, compared to <1 μg g^{-1} to >1 mg g^{-1} dry weight for the tocopherol content of plant tissue according to Munné-Bosch and Alegre (2002). The α-tocopherol content increased during ontogenesis of leaves, very similar to the significant increase in the intracellular α-tocopherol content associated with aging of plants reported for other species (Rise et al., 1989; Molina-Torres and Martinez, 1991; Tramontano et al., 1992). Glz et al. (1992) reported on high amounts of γ-tocopherol in the cuticle of *Gingko biloba* leaves. Shepherd et al. (1999) detected high levels of δ- and γ-tocopherol, and additionally low levels of α-tocopherol in the wax layer of red raspberry (*Rubus idaeus* L.). While γ-tocopherol is predominantly found in seeds, α-tocopherol is the main tocopherol in leaves (Bramley et al., 2000; Franzen and Haas, 1991; Shintani and DellaPenna, 1998; Sircelj et al., 2005). The role of extracellular α-tocopherol in the epicuticular wax of apple leaves, described here for the first time, may be the protection of biomolecules from peroxidation processes incited by singlet oxygen radicals, thus maintaining the chemical composition and the physical function of epicuticular wax films. Herbicide stress of apple leaves induced by paraquat causing the formation of radicals, was significantly reduced by a treatment with α-tocopherol (Schmitz-Eiberger and Noga, 2001). In apple leaves subjected to drought stress, increased levels of α-tocopherol were reported to be involved in the adaptation to oxidative stress (Sircelj et al., 2005).

According to Holloway (1970) damages in superficial waxes are the cause of the increased wettability of older leaves, however, this factor, which may be of great impact under field conditions, could not be held responsible in our experiments. In addition to modifications in chemical composition due to tissue age, changes in hydrophobicity of plant surfaces in the field may result from environmental conditions altering wax composition, mechanical strain or injury, and the colonization of surfaces by saprophytic and pathogenic bacteria and fungi which alter physical properties of the surface towards hydrophily (Knoll and Schreiber, 2000; Schreiber et al., 2004). All these factors were largely excluded in our experiments demonstrating that changes in the physical properties during ontogenetic development were associated with modifications in the chemical composition of cuticular waxes. Nevertheless, it seems very likely, that ontogenetic modifications are superimposed by modifications due to environmental factors. The impact of individual compounds and chemical groups for structure and function of the cuticle have to be elucidated in further experiments in more detail.

4. Experimental

4.1. Plant material

Seeds of *M. domestica*, cv. 'Golden Delicious', were treated with 0.1% Euparen® M WG 50 (Tolylfluanid, Bayer

CropScience, Monheim, Germany) for five minutes and than stored at 4 °C in the dark for two weeks. After sowings in substrate for salt-sensitive plants (pH 5–6, salt content: 0.8 g/l, Klasmann-Deilmann GmbH, Geeste, Germany) they grew in a greenhouse at 18–20 °C and 18 h daylight. Two weeks later the seedlings were singularized and grown in plastic pots (8.5 cm × 8.5 cm × 7.5 cm) filled with standard potting mixture (special mixture, Klasmann-Deilmann GmbH, Geeste, Germany), irrigated and fertilized (liquid fertilizer Flory 2 special, 16 + 9 + 22, 4 were protected from dust, chemical substances and fungal infection by growing the plants in a specific cabinet covered with cellophane in order to allow for the exchange of air and humidity. After 8 to 10 weeks apple seedlings were used for the wax analysis and the other studies. The experiments were carried out using the adaxial side of the youngest completely unfolded leaf (leaf insertion 1) and leaves of insertion 3, 5 and 7, respectively.

4.2. Wax extraction by chloroform and analyses

The adaxial side of the leaf was immersed twice in chloroform (purity >99%) for 10 s in a glass Petri dish. Previous tests had shown that cuticular waxes from leaf samples were extracted almost completely after 10 s and that longer extraction periods were associated with tissue damages from $CHCl_3$. It was assured that during extraction only the adaxial surface had contact with chloroform. After adding an internal standard (C_{24} alkane, tetracosan) the samples were evaporated under nitrogen. The internal standard was ca. 10% of the estimated total wax amount. By adding 20 µl pyridine (Merck, Darmstadt, Germany) and 20 µl of BSTFA (*N*,*O*-bis (trimethylsilyl) trifluoroacetamide, Macherey-Nagel, Dueren, Germany) the samples were derivatized for 40 min at 70 °C according to Hauke and Schreiber (1998).

The samples were diluted with 100 µl of chloroform before GC–MS analysis (5890 series II, Hewlett-Packard, Avondale, PA, with on-column injection and applying a high resolution gas chromatography column, Agilent Technologies, 30 m × 0.321 mm DB-1, phase thickness 0.1 µm, J&W, Folsom, CA). The temperature program was as followed: start at 50 °C, 2 min at 50 °C, 40 °C min^{-1} to 200 °C, 2 min at 200 °C, 3 °C min^{-1} to 320 °C, then 30 min at 320 °C. The carrier gas was hydrogen. The pressure program was: injection at 50 kPa, 5 min at 50 kPa, 3 kPa min^{-1} to 150 kPa, 39 min at 150 kPa.

For qualitative GC–MS analysis, the same method was used but instead of hydrogen, helium was used as carrier gas; injection volume was 1 µl.

4.3. Wax extraction by the freeze-embedding method

For the extraction of α-tocopherol from the epicuticular wax layer of apple leaves, another method for the isolation of epicuticular waxes was used, a freeze-embedding method according to Ensikat et al. (2000). A disk (∅ 16 mm) was punched from apple leaves, cv. 'Golden Delicious', avoiding any leaf vein. The adaxial surface of the leaf segment was placed onto a drop of glycerol applied on a spoon and than immersed into liquid nitrogen until frozen. With the help of tweezers, the sample was lifted accurately off the spoon. After defrosting of the sample, the wax was extracted with chloroform and filtered through a special metal filter (Frintrup, Bonn, Germany) with 200 holes/mm^2 to hold back the glycerol. In order to have a clean sample of extracted wax, the chloroform procedure was repeated twice. The samples were evaporated using nitrogen and re-dissolved in 1000 µl of *n*-hexane. The tocopherol content was determined by HPLC (Schmitz and Noga, 2000b). Following equipment was used: HPLC model 6000A (Waters Associated Chromatography, Langenfeld, Germany) with a pump Waters 510, Sunchrom Marathon autosampler, Waters Millenium software, precolumn: 5 cm × 3 mm i.d. Lichrospher 100 diole, 5 µm (CS Chromatography service, Langerwehe, Germany); the applied eluent was a mixture of *n*-hexane 96.4%, ethylacetate 3.6%; the flow rate was 1.2 ml/min. The fluorescence detector model RF 551 was used with an extinction of 285 nm and emission: 320 nm. The external standard was α, β, γ, δ-tocopherol with 100 µl injection volume. The analyses were carried out at 22 °C and every sample was kept at −80 °C until 15 min before injection to prevent degradation of α-tocopherol. The sample injection volume was 100 µl.

4.4. Determination of apple leaf area of the adaxial leaf side

The investigations were conducted with six apple seedlings which had been grown for 40 days after planting. The areas of all leaves per plant were measured five times with an interval of four days. At the end of these investigations, the seedlings were 56 days (8 weeks) old. This age corresponds to those plants used in other investigations of this study. Leaf area of the adiaxal leaf side was calculated from length and width of leaves using an additional factor of 0.71 which had been derived in preliminary destructive measurements of leaf area. The relative growth rate (RGR) of leaf area was used for the assessment of the growth depending on the insertion (= leaf age) of apple leaves. RGR was calculated according to the formula $RGR = (\ln A_{L2} - \ln A_{L1})/(t_2 - t_1)$, where A_{L2} is the leaf area at time t_2 and A_{L1} the leaf area at time t_1, respectively, modified from Hunt (1982).

4.5. Goniometry

The hydrophobicity of apple leaves was assessed quantitatively by a drop shape analysis system (Contact Angle System OCA 30-2, Software SCA 202, DataPhysics Instruments GmbH, Filderstadt, Germany). Samples cut from the central area of the leaf lamina were affixed to glass slides by double-sided adhesive tape (TesaFix, Beiersdorf, Hamburg, Germany) to ensure an even surface. The sessile

drop method was used. Contact angles were calculated by using the Laplace–Young-Fitting method. The volume of water drops (distilled water) amounted to 10 μl. Measurements were made on the adaxial side of four leaf insertions, representing different leaf ages. Six seedlings with up to 10 leaves were investigated. For every developmental stage of leaves three measurements were made resulting in 18 values per leaf insertion.

4.6. Microscopy

For examination of the surface relief, samples of about 1 cm^2 cut from the middle of leaf lamina were prepared. For high-resolution SEM (Leo 440, Leica, Bensheim, Germany) fresh leaf samples were affixed to aluminum stubs and air dried. All samples were sputtered with gold (65 mA for 30 s, Sputter SCD 040, Balzers Union), and were examined at 15 kV and a beam diameter of 20 nm under high vacuum conditions. For ESEM (XL 30 ESEM, Philips Electron Optics, Eindhoven, The Netherlands) fresh samples were affixed on a polycarbonate adhesive tape and examined uncoated at 4 °C with a gaseous secondary electron detector (GSED) within a water vaporous environment under low vacuum conditions (4.5 Torr) in the chamber.

The area and height of epidermal cells of the adaxial surface of apple leaves were assessed with a CLSM (LSM 310, Zeiss, Oberkochem, Germany) equipped with a neon–helium laser (543 nm). Samples totally cleared in chloral hydrate (Sigma–Aldrich, Steinheim, Germany) were observed with a 40× oil immersion objective lens. The topography of periclinal cell walls was analyzed over series of optical *xy* slices in the reflection mode. Five measurements on leaves of six different plants were made resulting in 30 values per leaf insertion.

4.7. Statistical analysis

Experimental data were analyzed with the statistic program SPSS 11.0 for Windows (SPSS Inc., Chicago, IL, USA). The data were tested for normal distribution and variance homogeneity and compared by Tukey-HSD multiple range test or Duncan's multiple range test. Modifications in the amount of wax components were tested for significance using the non-parametric Kruskal–Wallis test. A 5% probability level was accepted to indicate significant differences. All experiments were carried out at least twice.

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