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Biochemical analysis of elastic and rigid cuticles of *Cirsium horridulum*

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Abstract The cuticle is a complex structure of soluble lipids, lipid polymers and polysaccharides. In addition to its functions to reduce water loss and provide a protective barrier, its mechanical properties may be significant to plant growth and development. We investigated the cuticle of *Cirsium horridulum* Michx. because of its involvement in the thigmonastic contraction of staminal filaments. The staminal filaments and portions of the style are surrounded by a highly elastic cuticle in contrast to the rigid cuticle of the corolla and leaves. Our aim was to determine if the biochemical composition affected the elasticity of the cuticle. We discovered that the ratio of carbohydrates to lipids is 1:7 in floral parts but 2:1 in leaf cuticle. Esterified cutin components represented about 80% of the cuticle and di-hydroxyhexadecanoic acids were the major monomers of cutin, regardless of origin. The cutin of elastic tissues is characterized by a higher content of tri-hydroxy monomers than the cutin of rigid tissues. The data suggest that hydroxyl groups enhance the hydrophilic character of the cuticle and contribute to cuticular elasticity.

Keywords *Cirsium* (cuticle) · Cuticle · Cutin · Tri-hydroxy fatty acid

Introduction

All aerial parts of vascular plants are covered by a cuticle, a complex membrane synthesized by epidermal cells. One of the functions of the cuticle is as a barrier for the permeation of solutes (Kirkwood 1999) and water

(Holloway 1994). Cuticular resistance to water loss depends on lipids embedded in the polymer matrix (Schreiber et al. 1996). The polymer matrix is composed of cutin and cutan (Nip et al. 1986). Cutin is a high-molecular-weight polyester composed of various esterified C16 and C18 hydroxyalkanoic acids (Walton 1990) and is less stable than cutan, which is defined as the remnant of cuticular material after exhaustive alkali extraction.

The cuticle has to accommodate the plasticity of the cell wall (Carpita and Gibeaut 1993). Therefore, the elasticity of the cuticle impacts the development of fissures (Bally 1999) and consequently influences fruit storage times (Roy et al. 1999). The cuticle also affects the susceptibility to fungal and insect attack (Stoner 1990; Hammer and Evensen 1994) and provides mechanical support (Edwards et al. 1996). Structural and mechanical studies of thistle filaments suggested that the cuticle is vital for the thigmonastic response (Pesacreta et al. 1991; Hasenstein et al. 1993). However, little is known about the parameters that control the mechanical properties of the cuticle. Stark et al. (2000) showed a correlation between the surface elasticity and cutin hydration. Surprisingly, the mechanical properties of the cuticle were independent of the thickness in tomato fruit (Petracek and Bukovac 1995) and leaves (Wiedermann and Neinhuis 1998) but depended most likely on the different cuticular constituents. However, the connection between mechanical properties and biochemical composition has not yet been analyzed.

We investigated the cuticle of *Cirsium horridulum* because of the variability in the mechanical properties of the floral structures. Staminal filaments are surrounded by a highly elastic cuticle in contrast to the rigid cuticle of corolla and leaves (Pesacreta et al. 1991; Hasenstein et al. 1993). In addition, the cuticle of the style is rigid at the base and elastic in the apical part, and the elastic portions of the style and staminal filaments show similar mechanical properties. Our aim was to determine if elastic and rigid cuticles have similar compositions and whether tissue elasticity influenced cuticular composition.

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Materials and methods

Plant material

Mature leaves of *Cirsium horridulum* Michx. were harvested from the greenhouse. Flower heads were collected from the field. Individual flowers were excised and separated according to their degree of maturation. Partially elongated flowers in the center of the head were designated immature, flowers with an open corolla and elongated style mature, and typically peripheral flowers with contracted filaments and elongated style with discharged pollen were specified as post-mature.

Enzymatic isolation of cuticle

Cuticle was prepared after incubation in 10 ml of 1.6% ammonium oxalate and 0.4% oxalic acid solution (pH 4.5, 32 °C; Villena et al. 1999). Floral parts were incubated for 3 h, abaxial leaf cuticle was separated after 1 h and adaxial cuticle required overnight incubation. Cellular residue was removed by incubation in pectinase (20 U ml⁻¹; Sigma P4716; EC 3.2.1.15) and cellulase (24 U ml⁻¹; Sigma C 8546; EC 3.2.1.4) in 10 ml of 1.6% ammonium oxalate and 0.4% oxalic acid (pH 4.5) at 32 °C overnight. The preparation was checked by microscopy. The analyses described below were performed with cuticle isolated from 25 filaments, 8 styles, 2 corollas or 25 mg fresh weight of leaf, which amounted to 10 µg cuticle from each source.

Removal of waxes and cutin

Isolated cuticle was immersed in 1 ml of methanol/chloroform (1:1, v/v) in hydrolyzing tubes to remove cuticular waxes or 1 ml of 1% potassium hydroxide in methanol to saponify cutin. The tubes were closed and kept at 80 °C in a dry bath for 4 h. The residue was rinsed thoroughly with methanol, the liquid fraction was discarded and the solid residue dried under nitrogen and lyophilized before methanolysis.

Biochemical analyses

Methanolysis was carried out using either intact cuticle or the remnants of extracted cuticle using 300 µl of 1 N HCl/methanol (Alltech Associates) for 24 hours at 80 °C. Trimethylsilyl derivatives were prepared by adding 20 µl of N,O-bis(trimethylsilyl)trifluoroacetamide with 1% of trimethylchlorosilane + 80 µl pyridine for 1 h at room temperature (Montreuil et al. 1986).

Gas chromatography-mass spectrometry

Cuticular components were separated and identified by GC-MS (HP6890 gas chromatograph with an HP5973 mass selective detector; column HP-5MS; 30.0 m long, 0.25 mm i.d., 0.25 µm). The carrier gas was helium with a flow rate of 1.5 ml min⁻¹ with the injector and transfer line at 270 and 175 °C, respectively. Samples were injected in pulsed splitless mode. After 2 min at 40 °C, the temperature was ramped to 130 °C at 9 °C min⁻¹ and then to 290 °C at a rate of 2 °C min⁻¹ and maintained at 290 °C for 10 min. Compounds were identified by comparison with mass spectra of the NIST 98 database or those in the literature (Holloway 1981; Bleton et al. 1998).

Results

Identification of trimethylsilyl derivatives

Chromatograms of trimethylsilyl derivatives obtained from enzymatically isolated cuticle from mature fila-

ments showed four different categories of molecules (Fig. 1).

Lipids ($R_t = 35\text{--}75$ min) represented the most abundant compounds (>70%) and comprised alkanes, fatty acids, hydroxy fatty acids and epoxy acids. The second group of components consisted of carbohydrates and eluted between 19 and 33 min. We identified arabinose, rhamnose, xylose, mannose, glucose, galactose and galacturonic acid. These compounds represented about 15% of the filament cuticle. The third group comprised phenolic compounds and eluted at 28 and 37 min. These compounds were identified as cinnamic acid derivatives and corresponded to *p*-coumaric acid and caffeic acid, respectively. The fourth group consisted of triterpenoids that were characterized by retention times greater than 80 min. In filaments we identified α - and β -amyrins and β -sitosterol.

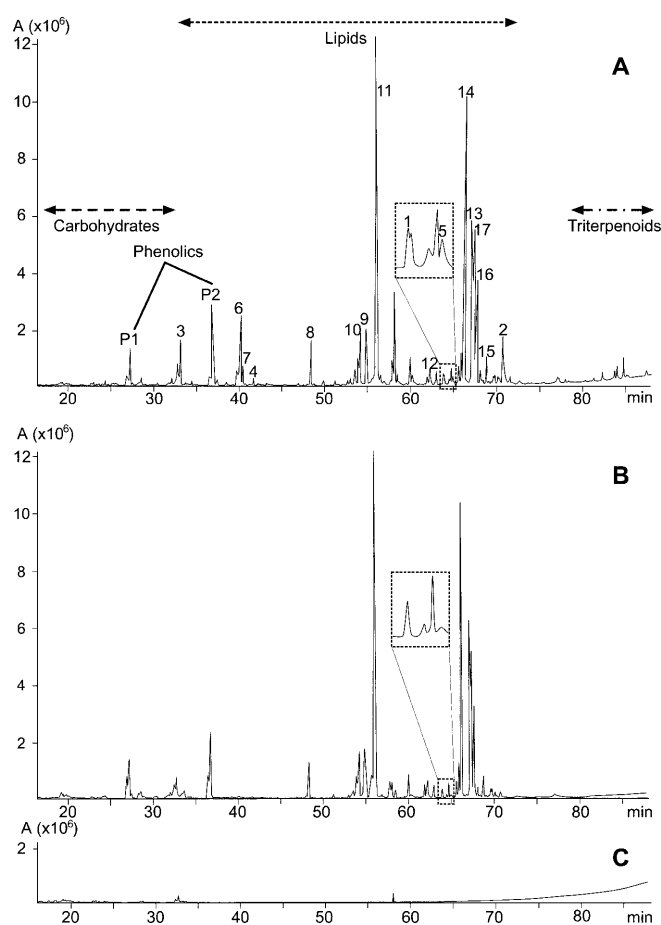


Fig. 1 The distribution of total ionizable compounds (TIC) after chromatography of TMS-derivatized compounds on an HP-5MS column obtained from methanolysis of complete cuticle of thistle (*Cirsium horridulum*) filaments (A), methanol/chloroform-extracted cuticle (B) and the alkali-resistant fraction of cuticle (C). The numbered peaks (1–17) refer to lipid components identified in Table 1. P1 and P2 indicate the peaks of *p*-coumaric acid and caffeic acid, respectively. Non-indexed peaks between 19 and 33 min represent carbohydrates, and peaks with a retention time greater than 80 min correspond to triterpenoids

Cuticle components from floral parts and leaves

The floral cuticles (style, corolla and filament) contained the same components in all examined parts. In contrast, the adaxial and abaxial leaf cuticles differed in their lipid constituents and were characterized by compounds not detected in floral cuticle and the absence of other substances (Table 1). Abaxial and adaxial leaf cuticles showed fewer hydroxy- but more saturated and unsaturated fatty acids than floral parts. 9 Octadecenoic acid was only found in leaf cuticles. C20, C22 and C24 fatty acids and the alkanes tetracosane and hexacosane were exclusively found in abaxial cuticle while tetradecanoic acid was identified only in adaxial cuticle.

In contrast to their dissimilar lipid compositions, the cuticles of leaves and floral parts contained the same carbohydrates (arabinose, rhamnose, xylose, mannose, glucose, galactose and galacturonic acid). Carbohydrates represented about 15% of the floral cuticular compounds but made up 61% and 69% in abaxial and adaxial leaf cuticles, respectively. The phenolic compounds *p*-coumaric and caffeic acids were present in all cuticle prepara-

tions, regardless of origin. The triterpenoids α - and β -amyryns and β -sitosterol were found in all cuticles. The abaxial leaf cuticle contained additional triterpenoids identified as cholest-5-en-24-one and stigmasterol.

Characterization of residue after differential extraction

Chromatograms obtained after increasingly stringent extractions showed discrete patterns. The comparison of chromatograms from enzymatically isolated cuticle (Fig. 1A) with chromatograms obtained after methanol/chloroform extraction (Fig. 1B) and sequential methanol/chloroform and potassium hydroxide extraction of cuticle (Fig. 1C) showed discrete patterns. Methanolysis and extraction with methanol/CHCl₃ resulted in the loss of peaks 1, 2, 4, 5, 6, 7, which were identified as heptane, nonacosane, octadecanoic acid, octacosanoic acid, 9,12 octadecadienoic acid and 9,12,15 octadecatrienoic acid, respectively. The height of peak 3, identified as hexadecanoic acid, decreased (see Fig. 1B). The elimination of these peaks indicates the removal of alkanes

Table 1 Lipid constituents identified by GC/MS after methanolysis and trimethylsilylation of the cuticular extracts from floral organs and leaves of thistle (*Cirsium horridulum*). The numbers and

the retention times (R_t) correspond with Fig. 1. The detection of a compound is indicated by X

Compounds	Peak No.	R_t (min)	Flower ^a	Leaf	
				Abaxial	Adaxial
Alkanes					
Tetracosane		52.7			X
Hexacosane		60.3			X
Heptacosane	1	63.9	X		X
Nonacosane	2	70.6	X	X	
Saturated and unsaturated fatty acids					
Tetradecanoic		24.7			X
Hexadecanoic	3	33.1	X	X	X
Octadecanoic	4	41.6	X	X	X
Eicosanoic		50.0		X	
Docosanoic		50.7		X	
Tetracosanoic		57.6		X	
Octacosanoic	5	64.5	X	X	X
9 Octadecenoic		40.5		X	X
9,12 Octadecadienoic	6	40.1	X	X	X
9,12,15 Octadecatrienoic	7	40.4	X	X	
Hydroxy fatty acids					
Mono-hydroxyles					
16 OH Hexadecanoic	8	48.2	X		X
18 OH Octadecenoic	9	54.8	X	X	
18 OH Octadecadienoic	10	54.1	X		
Di-hydroxyles					
10(9)-16 OH Hexadecanoic	11	56.4	X	X	X
10(9)-16 Octadecanoic	12	63.0	X	X	
Tri-hydroxyles					
9,10,18 OH Octadecanoic	13	67.2	X	X	X
9,10,18 OH Octadecadienoic	14	66.2	X	X	X
Epoxy Acids					
9,10 Epoxy18 OH octadecanoic	15	68.8	X	X	X
9,10 Epoxy18 OH octadecadienoic	16	67.5	X	X	X
9,10 Epoxy octadecadienoic	17	67.3	X	X	X

^aFlower comprises filaments, style, and corolla

and fatty acids by methanol/ CHCl_3 and probably the removal of epicuticular waxes (Pesacreta et al. 1991). The triterpenoids, which are constituents of waxes (Kolattukudy 1996), were also extracted with methanol/ CHCl_3 . The residue after methanolic extraction has been defined as cutin (Kolattukudy 1980). After KOH extraction of cuticle, all peaks detected in previous chromatograms disappeared except a small number of remnants corresponding to carbohydrates. The lack of appreciable signals after saponification identified hydroxy fatty acids, epoxy acids, and phenolics as part of the cutin fraction (Fig. 1C).

Cuticle composition in flowers and leaves

We determined the composition of the cuticle from the filament, the style and the corolla, as well as the adaxial and abaxial leaf cuticles in mature leaves (Fig. 2). The cutin composition of these structures was also determined after methanolic extraction (Fig. 3). The percentage of each lipid was calculated and the result expressed according to the chemical classes described in Table 1, i.e. alkanes, fatty acids, mono-hydroxy, di-hydroxy and tri-hydroxy fatty acids and epoxy acids.

Fig. 2 Composition of the lipids extractable by direct methanalysis of the cuticle isolated from immature (*I*), mature (*M*) and post-mature (*P*) floral organs and mature leaves of thistle. Values are mean \pm SD for five independent analyses. Key to columns: *solid* alkanes, *dots* fatty acids, *down-diagonals* mono-OH fatty acids, *horizontal bars* di-OH fatty acids, *up-diagonals* tri-OH fatty acids, *checker pattern* epoxy acids

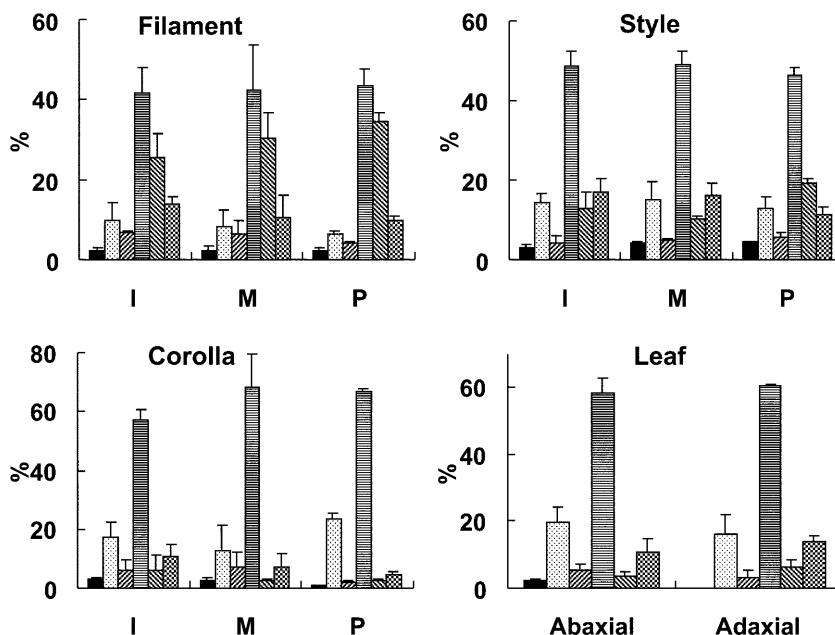
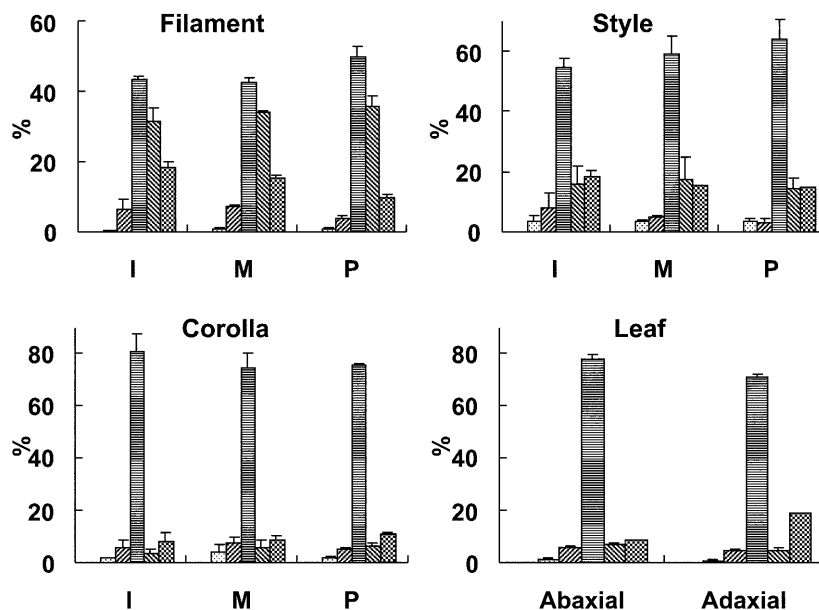


Fig. 3 Composition of the lipids extractable by direct methanalysis of the cutin isolated from immature (*I*), mature (*M*) and post-mature (*P*) floral organs and mature leaves of thistle. Cutin is the residue obtained after extraction of the cuticle by methanol/chloroform (v/v) for 4 h at 80 °C. Values are mean \pm SD of five independent analyses. Key to columns: *dots* fatty acids, *down-diagonals* mono-OH fatty acids, *horizontal bars* di-OH fatty acids, *up-diagonals* tri-OH fatty acids, *checker pattern* epoxy acids



Waxes

The chromatograms of methanol/ CHCl_3 -extracted cuticle showed no alkanes and only minimal amounts of fatty acids. However, the proportion of all other compounds was not affected by methanol/ CHCl_3 extraction (Fig. 3). Thus methanol/ CHCl_3 specifically extracted alkanes and fatty acids, the main components of waxes. Therefore, waxes can be quantified as the sum of alkanes and fatty acids (Fig. 2). Regardless of the age of flowers, waxes made up about 20% of the cuticle from style, corolla and leaf, and 10% of the filament cuticle (Fig. 2). No significant variation occurred during maturation, regardless of the origin of the cuticle.

The triterpenoids found in waxes were identified as α - and β -amyrins and β -sitosterol in the floral organs and adaxial cuticle. The composition of triterpenoids was more complex in the abaxial cuticle where cholest-5-en-24-one and stigmaterol were also found. The triterpenoids associated with the cuticle were extremely variable and depended on tissue age (Fig. 4). The cuticle from the style and the corolla contained up to 35% triterpenoids, as opposed to less than 10% in the filament cuticle. Triterpenoids represented 18% of the abaxial cuticle vs. 10% of the adaxial cuticle.

Cutin

The composition of the dewaxed cuticle, i.e. cutin, showed di-hydroxy fatty acids, mainly 10-16 di-hydroxy hexadecanoic isomers, regardless of cuticle origin and maturation stage (Fig. 3). The di-hydroxy hexadecanoic acids represented 80% of the cutin of corolla and leaf, 60% of style and 45% of filaments. The tri-hydroxy fatty acids made up 5% of the cutin isolated from the corolla and the leaf but reached 15 and 35% in style and filaments, respectively. Epoxy acids occurred in higher proportion in style and adaxial leaf cutin (18%) than in

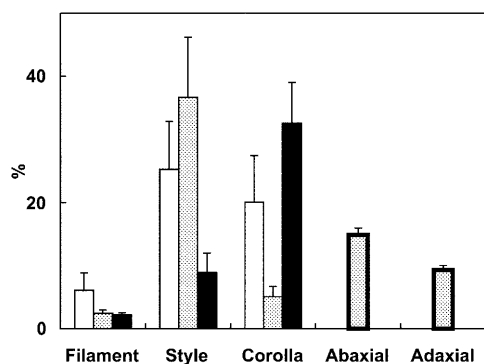


Fig. 4 Proportion of triterpenoids in the cuticle isolated from immature (*open columns*), mature (*dotted columns*), and post-mature floral organs (*solid columns*), and mature leaves (*dotted, rimmed columns*) of thistle. The triterpenoids are quantified in the cuticle analysis and their proportion is expressed by the ratio triterpenoids/lipids + phenolics + terpenoids. Values are mean \pm SD of five independent analyses

corolla and adaxial leaf cutin (8%). In filament cutin, the proportion of epoxy acids decreased from 20% to 10% during maturation. No significant variation was observed for mono-hydroxy fatty acids. The mono-hydroxy fatty acids contributed 5% to the cutin composition in all cuticles, regardless of age.

The mono-hydroxy fatty acids of the floral parts contained one-third hexadecanoic acid and two-thirds octadecanoic acid monomers. In contrast to the mixed distribution in floral parts, the leaf cuticle contained C18 mono-hydroxy monomers on the abaxial surface while the adaxial cuticle contained predominantly C16 mono-hydroxy monomers. Regardless of the origin, at least 90% of the di-hydroxy fatty acids were hexadecanoic acid derivatives. All tri-hydroxy fatty acids and epoxy acids were 18C compounds (data not shown).

The residue after methanol/ CHCl_3 extraction contained phenolic substances. They were covalently linked to the cutin matrix because only stringent hydrolysis generated free *p*-coumaric acid and caffeic acid residues. These phenolic compounds comprised between 5 and 10% of the cutin of floral organs (Fig. 5) and between 1 and 3% of leaf cutin. As the flowers aged, the amount of phenolics decreased in filaments and style but increased in the corolla.

Carbohydrates

The components detectable after cutin saponification and methylation corresponded to the carbohydrates found in the original cuticle and the analysis of cutin, i.e. methanol/ CHCl_3 -extracted cuticle (Fig. 1A–C). The proportion of carbohydrates in cutin amounted to 15% in the floral organs, and 69% and 61% in the cutin fraction of the adaxial and abaxial cuticles, respectively (Table 2).

The hydrolysis of the cuticle (Fig. 6) resulted in the same monosaccharides regardless of the origin of the

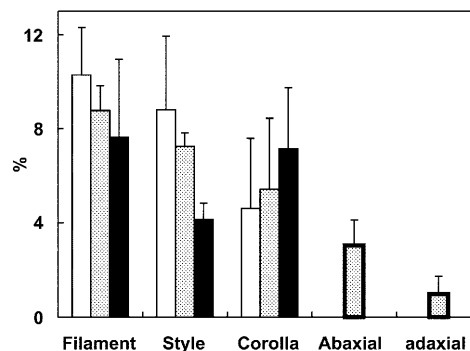


Fig. 5 Proportion of bound-phenolics to cutin of immature (*open columns*), mature (*dotted columns*), and post-mature floral organs (*solid columns*), and mature leaves (*dotted, rimmed columns*) of thistle. The phenolics are quantified in the cutin analysis and their proportion expressed by the ratio phenolics/phenolics + lipids. Shown are mean values \pm SD of five independent analyses

Table 2 The percentage of carbohydrates in thistle cutin was determined by dividing the cumulative area count for all carbohydrate peaks by the cumulative area count for lipids, phenolic compounds and carbohydrates in each of nine analyses for floral material and three analyses of leaf material

	Filament	Style	Corolla	Leaf	
				Abaxial	Adaxial
Carbohydrate, % cutin	14 ± 12	15 ± 6	15 ± 9	62 ± 4	70 ± 6

residue. Glucose was the most abundant monosaccharide. The high variability in monosaccharide composition in all floral parts was probably affected by the small quantity, which was close to the detection limit. In leaves, the larger quantity of monosaccharides allowed a better determination of their composition and indicated hemicellulose polymers, i.e. a high proportion of arabinose, glucose and galactose.

Discussion

Our data indicate that the composition of floral cuticle from elastic tissue is different from the cuticle covering rigid parts, and that the mechanical properties of thistle tissue are correlated with the composition of cutin.

In rigid tissues such as leaves and corolla, 80% of cutin was hexadecanoic acids, which comprise di-hydroxy- and about one-third of all mono-hydroxy fatty acids. The remainder (20%) was octadecanoic acids and included tri-hydroxy fatty acids, epoxy acids, and about two-thirds of mono-hydroxy fatty acids. In elastic tissues, C16 and C18 fatty acids were present in equivalent amounts, varying between 40 and 60%. Thus, in the cuticle of rigid tissues, the quantity of C16 fatty

acids was about 4 times the amount of C18 fatty acids. In contrast, the C16 fatty acids in elastic tissue made up 0.8 (filaments) and 1.6 times (styles) the amount of C18 fatty acids.

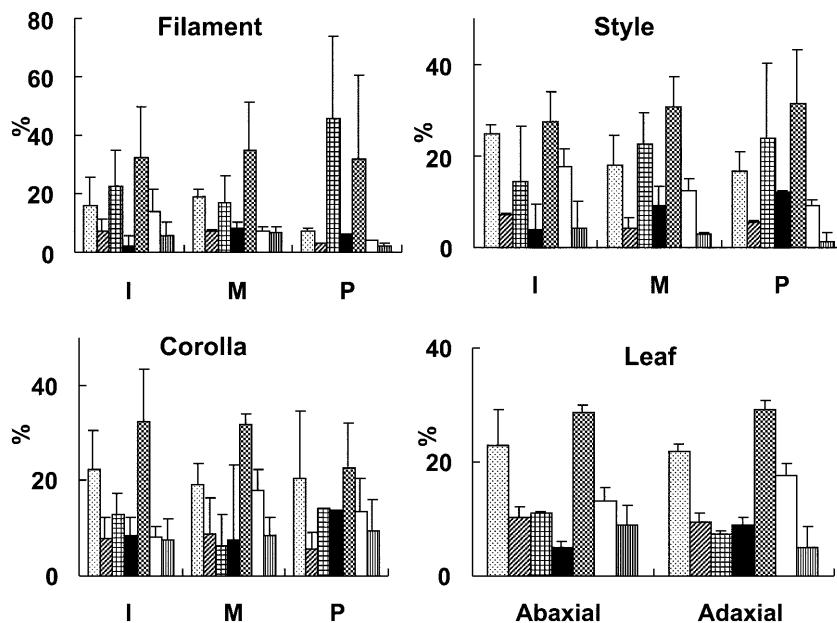
Thus, cutin of rigid tissues can be classified as C16 type, and cutin from elastic tissue corresponds to a mixed C16/C18 type (Holloway 1981). Similar ratios of monomers have also been reported for the cuticle of apple flowers, leaves and fruits (Espelie et al. 1979).

The most remarkable difference between elastic and rigid tissues was the 4- to 8-times-higher content of tri-hydroxy fatty acids of the elastic tissues. Because a similar decrease in the relative amount of the tri-hydroxy C18 monomers occurred during cuticle development in ripening tomatoes (Baker et al. 1981), elasticity appears to be associated with a higher number of hydroxyl groups. The higher amount of trihydroxy monomers in the cuticle of elastic tissue argues for increased hydration of such cuticle and against the potential for increased cross-linking capability (Kolattukudy 1996; Ray et al. 1998), which would reduce flexibility.

To understand if larger numbers of hydroxyl groups increase elasticity, it will be necessary to determine the degree of esterification of the hydroxyl groups and to evaluate the flexibility of the cuticle network in rigid and elastic tissues. Alternatively, the number of free hydroxyl groups can greatly affect the hydrophilic character of the cuticle. Because the extent of hydration is critical for the elastic properties of the filament (Hasenstein et al. 1993), one can speculate that the ability to absorb water and the extent of hydration is an important factor for cuticular elasticity.

The content of phenolics was correlated with cuticular rigidity. Similar to the strengthening of the cell wall by lignins, the cuticle can also be expected to gain mechanical strength by increased cross-linking of phenolic compounds. Cutin-bound phenolics decreased as floral

Fig. 6 Monosaccharide composition (mol%) of polysaccharides remaining after extraction with KOH of cuticle isolated from immature (*I*), mature (*M*) and post-mature (*P*) floral organs and mature leaves of thistle. Shown are mean ± SD of five independent analyses. Key to columns: *dots* arabinose, *down-diagonals* rhamnose, *grid pattern* xylose, *solid* mannose, *checker pattern* glucose, *open* galactose, *vertical bars* galacturonic acid



tissue became more elastic. In filaments the content of phenolics decreased from 10 to 8%, and in maturing, i.e. increasingly elastic, styles the phenolics decreased from 9 to 4%. In contrast, ageing-corolla cutin became enriched in phenolics, similar to the cuticle in expanding tomato fruit (Baker et al. 1981).

However, other components of the cuticle such as waxes, triterpenoids, and carbohydrates, apparently are insignificant for the elasticity of the cuticle.

Waxes comprised about 20% of methanol-soluble lipids in corolla, leaf and style but only 10% from filaments. The proportion of triterpenoids in waxes from the style and corolla (up to 35%) was 2–5 times higher than in filaments or leaves. However, the composition of waxes was not related to the mechanical properties of tissues. Carbohydrates constitute 61–69% of the fairly rigid leaf cuticle but represent only 15% of the cuticle of floral organs. These differences pertain to leaf and floral cuticles and therefore can not be linked to tissue elasticity.

These examples illustrate that although the cuticle from different floral parts contained the same compounds, their proportions apparently determine the mechanical properties of the floral cuticles on the filament, style and corolla.

The composition of the leaf cuticle was different from that of the floral cuticle and also specific as to its abaxial or adaxial origin. Compared with the adaxial cuticle, the abaxial cuticle contained a slightly higher proportion of waxes but, most importantly, exhibited a higher range of fatty acids and triterpenoids. These variations, in conjunction with a lower number of stomata on the abaxial epidermis, could affect solute transport, permeability, and the physicochemical characteristics of the waxes. The origin and consequence of such variations are poorly understood but endogenous and exogenous factors as light, temperature and humidity have been implicated in the composition of waxes (Riederer and Markstadter 1996). We observed quantitative and qualitative differences between the cutin from the adaxial and abaxial cuticle. The adaxial cuticle contains less phenolic compounds than the abaxial cuticle. Finally, a higher amount of carbohydrates is present in the adaxial cuticle than in the abaxial cuticle. The higher content of lipid polymers in the abaxial cuticle may be related to the thicker internal cuticle (Pesacreta and Hasenstein 1999), but we do not know if the internal cuticle has the same composition as the external cuticle.

Aside from the composition of the cuticle, it is important to point out that the classical methods of cuticle analysis required at least several milligrams of isolated cuticle (Ray et al. 1998; Villena et al. 1999). Thus, one of the main challenges was to characterize very small amounts of cuticle. Using exhaustive methanolysis (HCl/methanol extraction at 80 °C), we were able to perform these analyses with only 25 filaments, or about 10 mg fresh weight, which corresponds to about 10 µg cuticle. A second advantage of methanolysis is that identification and quantification of the different cuticle

constituents can be obtained with a single analysis. The relative quantities before and after methanol/CHCl₃ extraction demonstrate that cuticular waxes can be estimated as the sum of alkanes, saturated and unsaturated fatty acids and triterpenoids. The relative composition of cutin can be calculated by subtracting from the complete cuticle analysis the amount of waxes (see Fig. 2).

In conclusion, we have identified that the elasticity of cuticle depends on the amount of hydroxylated fatty acids. Contrary to the notion that more hydroxyl groups can serve as potential sites for cross-linking, the OH groups apparently determine the hydrophilicity of the cuticle and facilitate impregnation with water. As the cuticle ages, natural dehydration, possibly in conjunction with the increase of cross-linkers, most notably phenolic compounds, may lead to the often described hardening of mature cuticle (Kolattukudy 1980; Holloway 1981; Jeffree 1996).

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