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Altered Tomato (*Lycopersicon esculentum* Mill.) Fruit Cuticle Biomechanics of a Pleiotropic Non Ripening Mutant

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Abstract

Tomato (*Lycopersicon esculentum* Mill.) fruit ripening involves multiple metabolic changes resulting in softening and pigmentation. We investigated the mechanics and morphology of the enzymatically isolated cuticular membrane (CM) of cv. Ailsa Craig wild-type (*wt*) and nonripening mutant (*nor*) at three developmental stages. Cuticle thickness and degree of cutinization increased significantly from immature to fully ripe fruits for both *wt* and *nor* without differences between them. Mechanical characterization was carried out on dry and fully hydrated samples in uni-axial tension to determine their modulus of elasticity, stress, and strain at failure. Corresponding stress-strain diagrams were biphasic and showed yield for virtually all dry CM samples, while that of hydrated CM displayed considerable differences between *wt* and *nor* fruits. Concerning the mechanical properties, the CM of *wt* fruits was characterized by increasing stiffness and strength during fruit growth and maturation in both dry and hydrated states, whereas the CM of *nor* fruits was significantly less stiff and weaker at full maturity. Hydration generally caused lower moduli of elasticity and strength, while breaking strain was significantly affected only for the CM of ripe *nor* fruits. This plasticizing effect of water increased towards full maturity for both *wt* and *nor*, and may be related to fiber content in the CM matrix and hydration state of the cuticle. Comparative analysis of two additional wild-type tomato cultivars supported the ripening-related stiffening of the CM of Ailsa Craig *wt* and the altered mechanical properties of the *nor* mutant, as well as the plasticizing effect of water.

Key words: Tomato (*Lycopersicon esculentum* Mill.); Fruit ripening; Nonripening mutant (*nor*); Cuticle; Biomechanics; Cutin; Flavonoids; Plasticizer

Introduction

Fruit ripening is a complex developmental event that involves multiple metabolic changes. During ripening, a large variety of fruits undergo modifications of texture, cell wall ultrastructure and composition, sugar content, coloration and biosyn-

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thesis of pigments, as well as flavor (Brady 1987). Two major classifications of fruits, climacteric and nonclimacteric, have been established to distinguish fruits on the basis of ethylene biosynthesis and control of their ripening process. Whereas nonclimacteric fruits do not require ethylene, climacteric fruits show increased respiration and ethylene biosynthesis rates necessary for complete ripening (for example, Lelievre and others 1997). The climacteric berry fruit type of tomato (*Lycopersicon esculentum* Mill.) is the most intensively studied model species for fruit ripening, and large genomic libraries are available (Van der Hoeven and others 2002). Changes in pigmentation towards the final stages of ripening of tomato fruit involve, amongst others, the biosynthesis and accumulation of carotenoids, in particular lycopene, and to a lesser extent its orange cyclization product beta-carotene (pro-vitamin A) (Giuliano and others 1993; Rosati and others 2000). Their biosynthesis depends on climacteric ethylene production (Oeller and others 1991; Lelievre and others 1997). Like coloration, the texture is typically altered during the ripening process, leading to softened fruits. Owing to the cell wall-modifying activity of several enzymes, including polygalacturonase, pectin-methyl-esterase, endo-β-mannase, α- and β-galactosidases, expansins, and β-glucanases, structural components necessary to reinforce the cell wall and the adhesion of cells are degraded (for example, Fischer and Bennett 1991; Rose and others 1997; Seymour and others 2002).

Several nonripening mutants have been described for tomato fruit, among them Never-ripe (*Nr*), Colourless nonripening (*Cnr*), ripening-inhibitor (*rin*), and nonripening (*nor*), as reviewed by Giovannoni (2001). Although non-allelic, these pleiotropic mutants appear to have a set of common features related to incomplete ripening, among them reduced fruit softening resulting in firmer fruits, and altered pigmentation. As for the latter, both *rin* and *nor* ripening mutants do not completely change color to red at full maturity; ripe fruits of *rin* remain green to yellowish, whereas *nor* may get yellowish-orange (Giovannoni and others 1989; Bewley and others 2000). It has been proposed that each of the mentioned nonripening mutants has different genetic defects resulting in individual impacts on the ripening cascade, either directly affecting ethylene biosynthesis and signaling (Wilkinson and others 1995; Thompson and others 1999), or higher order regulatory developmental mechanisms independent of ethylene signaling, as has been proposed for *rin* and *nor* (Giovannoni and others 1995; Giovannoni 2004). These mutations are believed to involve genes encoding for developmental transcription factors. For *rin*, a MADS-box gene (LeMADS-RIN) with a proposed function in the regulation of fruit ripening has been located by positional cloning strategies (Vrebalov and others 2002).

During the ripening process, the mechanical performance of the tomato fruit skin is of considerable physiological and economical significance. It affects not only fruit appearance, handling and storage (Chu and Thompson 1972), but also plays a prominent role in the resistance to cracking caused by water uptake shortly before harvest, potentially resulting in large economic deficits for the fruit industry (Emmons and Scott 1997). The growth rate of plant tissues is regulated by the interaction of cell wall stress and cell wall mechanical properties (Cosgrove 1993), and the tomato fruit epidermis is thought to be important in determining the rate of fruit expansion (Thompson and others 1998). The fruit skin (FS) combines cuticle, epidermis and a variable number of hypodermal cell layers as the outer boundary of the fruit. Several studies have analyzed the mechanical properties of the tomato fruit skin, but mostly without distinguishing its components (Batal and others 1970; Voisey and others 1970; Thompson 2001; Andrews and others 2002). It has been proposed that the tomato fruit cuticle is of increasing importance for the structural integrity of the fruit during ripening, because the cuticle mirrors the mechanical properties of the epidermis to a large extent (Bargel and Neinhuis, in print). Matas and others (2004a) have drawn a similar conclusion for ripe tomato fruits, and stated that the cuticle is a mechanically important component of the tomato fruit. The plant cuticle is a multifunctional interface that primarily prevents the plant from uncontrolled water loss (Schönherr 1982), but also serves as a protective layer against biotic and abiotic environmental influences (Bargel and others 2004a). It is an extracellular natural composite structure deposited on the outer epidermal cells that consists of two main components, the insoluble biopolymer cutin and soluble lipids, collectively called ''waxes'' (Kolattukudy 1980). During development, cell wall polysaccharides, in particular cellulose, are gradually incorporated and are believed to link the cuticle to the epidermis (Jeffree 1996). In tomato fruit, dynamic changes of both cutin monomer (C_{16}/C_{18}) and cuticular wax composition have been analyzed in relation to fruit ripening (Baker and others 1982). The same holds true for phenolic compounds, among them p-coumaric acid, as well as the flavonoids naringenin and chalconaringenin (Hunt and Baker 1980; Laguna and others 1999).

Literature on tomato fruit ripening mutants generally lacks information on the cuticle. Recently, Moctezuma and others (2003a) have described a thicker cuticle for immature and mature-green fruits in an antisense suppression study of β-galactosidase gene (TBG6). However, in contrast to the analysis of gene expression related to tissue texture and ripening, virtually nothing is known about the impact of the ripening mutants on the mechanical behavior or the chemical composition of the cuticle. In this study, the mechanical properties related to fruit growth and ripening of the enzymatically isolated cuticular membrane (CM) of the tomato fruit nonripening mutant *nor* and the corresponding wild-type (*wt*) were analyzed. For this purpose, morphological analysis and one-dimensional tensile testing were performed on air-dried and fully hydrated isolated CM at three distinct ripening stages.

Material and Methods

Plant Material

Tomato plants (*Lycopersicon esculentum* Mill.) of cv. Ailsa Craig wild-type (*wt*) and the nearly isogenic nonripening mutant (*nor*) homozygous for NOR (provided by J. Giovannoni, Boyce Thompson Institute for Plant Research, Cornell University, USA) were grown in parallel rows on a 1 × 5 m patch under standard greenhouse conditions. Fruits with diameters greater than 30 mm were harvested at three distinct ripening stages, that is, immature-green (*ig*), mature-green (*mg*) and mature-red (*mr*), following the nomenclature of Emmons and Scott (1997). Because *nor* is deficient rather than delayed in ripening, the faint yellow to yellowish-orange ''ripe'' fruits were harvested at the same time as fruits at the *mr* stage of *wt*. For comparison, two additional wild-type cultivars, cvs. Vanessa F1 and Roma, were analyzed at comparable ripening stages, but from an earlier growing season. Ripening stages were classified based on the relative parameters size, weight, and color change after the ''breaker'' stage instead of days from anthesis. This may have introduced inaccuracies due to cross-cultivar variation of fruit maturation, however, with the cultivars used, the classification system matched sufficiently uniform ripening stages, as shown in another study (Bargel and Neinhuis, in print).

Parallel cut samples (sample size: min. 45 × 3 mm) were obtained with a custom-built razorblade device, with the cutting direction from the peduncle end to the tip. After levering the strips carefully from the fruit, cuticular membranes (CM) were isolated enzymatically using a cellulase/pectinase solution (Schreiber and others 2001) containing cellulase (1.75% [w/v], Celluclast; Novo Nordisk, Bagsvaerd, Denmark), pectinase (3.5% [w/v], Fluka, Buchs, Switzerland), sodium azide (NaN_3, 2 mM; Merck Schuchardt, Hohenheim, Germany), polyvinyl pyrrolidone (0.2% [w/v]) and citric acid monohydrate (20 mM; Merck, Darmstadt, Germany) at 30°C. A pH of 5.0 was established for optimum enzyme activity. Completely isolated CM were rinsed twice in distilled water, air dried on Teflon meshes, and stored in Petri dishes at room temperature before testing.

Scanning Electron Microscopy

Scanning electron microscopy (SEM) and digital image analysis of cross-sections were used for morphological characterization and determination of sample thickness, from which the corresponding cross-sectional area A (thickness × width of the sample) for the calculation of tensile stress was derived. Prior to SEM, air-dried CM samples were coated with gold (~25 nm) in a sputter coater (108auto; Cressington, Watford, UK; and SCD 040; Balzers Union, Wiesbaden, Germany), and subsequently examined on a modified aluminum stub at 15 kV (430i; LEO, Oberkochen, Germany; and Cambridge Stereoscan 200; Leica, Bensheim, Germany). Digital image analysis was carried out with the free NIH Image software (http://rsb.info.nih.gov/nih-image/). Thickness measurements covered all cutinized layers of enzymatically isolated cuticular material, excluding the areas of the former cells. Measurements were perfomed with at least eight double replicates per ripening stage of cv. Ailsa Craig *wt* and *nor* with the number of fruits varying between $n = 9$ and 20. Results are given as mean ± confidence interval (C.I.). Measurements of cvs. Vanessa F1 and Roma were performed with at least six double replicates per ripening stage with $n = 3$ to 5 fruits, and results presented as mean ± standard error (S.E.).

Mechanical Tests

Mechanical characterization of air-dried and fully hydrated samples of cv. Ailsa Craig *wt* and *nor* was carried out by means of one-dimensional tension testing with a custom-built computer-controlled device featuring vice-type clamps as used by Bargel and others (2004b). See Figure 1 for a schematic view of the testing device. The force was measured with a force transducer at 0.1 mN resolution (K300K60N; Megatron, Putzbrunn, Germany), and displacement was obtained at 10 µm resolution by a

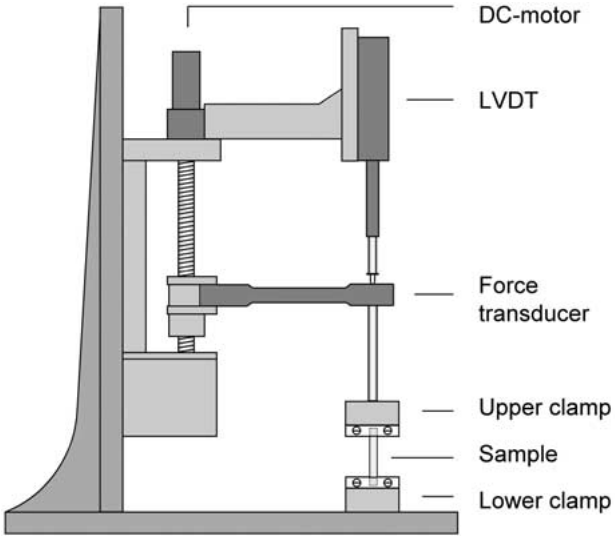

Figure 1. Schematic drawing of the custom-built uniaxial tension testing device. The upper clamp is free to rotate horizontally. The effective sample length between the upper and the lower clamp was 32 mm, and the testing speed was 2 mm min^{-1}. External PC control and power supply are not shown. *LVDT*-Linear Variable Differential Transformer.

LVDT (Linear Variable Differential Transformer; MS50-G-S-TTL-10; Megatron, Putzbrunn, Germany) situated on top of the force transducer. Data were recorded to file with acquisition software (DasyLab; DasyTec, Monchengladbach, Germany) and subsequently analyzed. The effective free testing length of the CM samples was 32 mm, and the crosshead speed was set to 2 mm min^{-1} during testing. Hydrated samples were kept moist during the course of testing by adding small amounts of distilled water with a hand pipette to mimic the full hydration state. From the tensile tests, the modulus of elasticity E (stress σ/strain ϵ), conventional breaking stress σ (force/cross-sectional area), and conventional breaking strain ϵ (change of length/initial length) were derived from stress-strain curves. The majority of these stress-strain curves displayed an initial and a second linear slope, resulting in two different moduli of elasticity, as well as a transition point between the first and the second slope. In addition, modulus, stress, and strain values were also calculated as true stress σ_t, and strain ϵ_t, because plant polymers can change their form to a great extent (>10%) over a relatively short period of time (Vincent 1990a). Hence, conventional stress and strain could resemble underestimated and overestimated values, respectively. Calculations of true stress and strain were related to the conventional numbers for the starting conditions as stated by Biewener (1992), assuming isovolumetric deformation of the CM (Shadwick 1992)

$$\sigma_t = (1 + \varepsilon)\sigma \quad (1)$$

and

$$\varepsilon_t = ln(l/l_0) \quad (2)$$

Means ± confidence interval (C.I.) are given under Results for the modulus of elasticity and the transition point. Measurements were carried out with at least eight replicates per ripening stage of each *wt* and *nor* with a fruit number varying between $n = 9$ and 20. For strength and breaking strain, trend values are given instead, because a large number of samples fractured close to the clamps, which made reproducible results difficult (Wiedemann and Neinhuis 1998). This phenomenon is commonly observed in tensile tests and is caused by shear stresses perpendicular to the tensile stresses near the clamping site. Although stiffness is not affected, fracturing close to the clamps may occur at lower stress and strain at failure compared to fracturing distant from the clamping site. Where possible, measurements of samples that fractured in the region of free sample length are preferred for the calculation of mechanical properties, but trend values reported here do account for this problem.

CM samples of cvs. Vanessa F1 and Roma were tested analogously with a similar testing device described by Wiedemann and Neinhuis (1998). Here, only values for E_1 are given, and results are given as mean ± standard error (S.E.) of at least six replicates per ripening stage, with a fruit number ranging between $n = 3$ and 5.

Statistical Analysis

Unbalanced two-way ANOVA with Bonferroni post-test (Prism 4; GraphPad Software, San Diego, California, USA) at α equal to 0.05 as the level of significance was performed on thickness and E_1 of dry and hydrated CM of cv. Ailsa Craig *wt* and *nor* separately to evaluate significant changes related to fruit ripening. Outlier tests after Nalimov (Lozan and Kausch 1998), as well as normality tests were carried out prior to data analysis.

Results

Morphology

Fruits of *nor* showed no considerable differences in size or gross morphology, but remained faint yellow to yellowish-orange at the time when *wt* fruits were

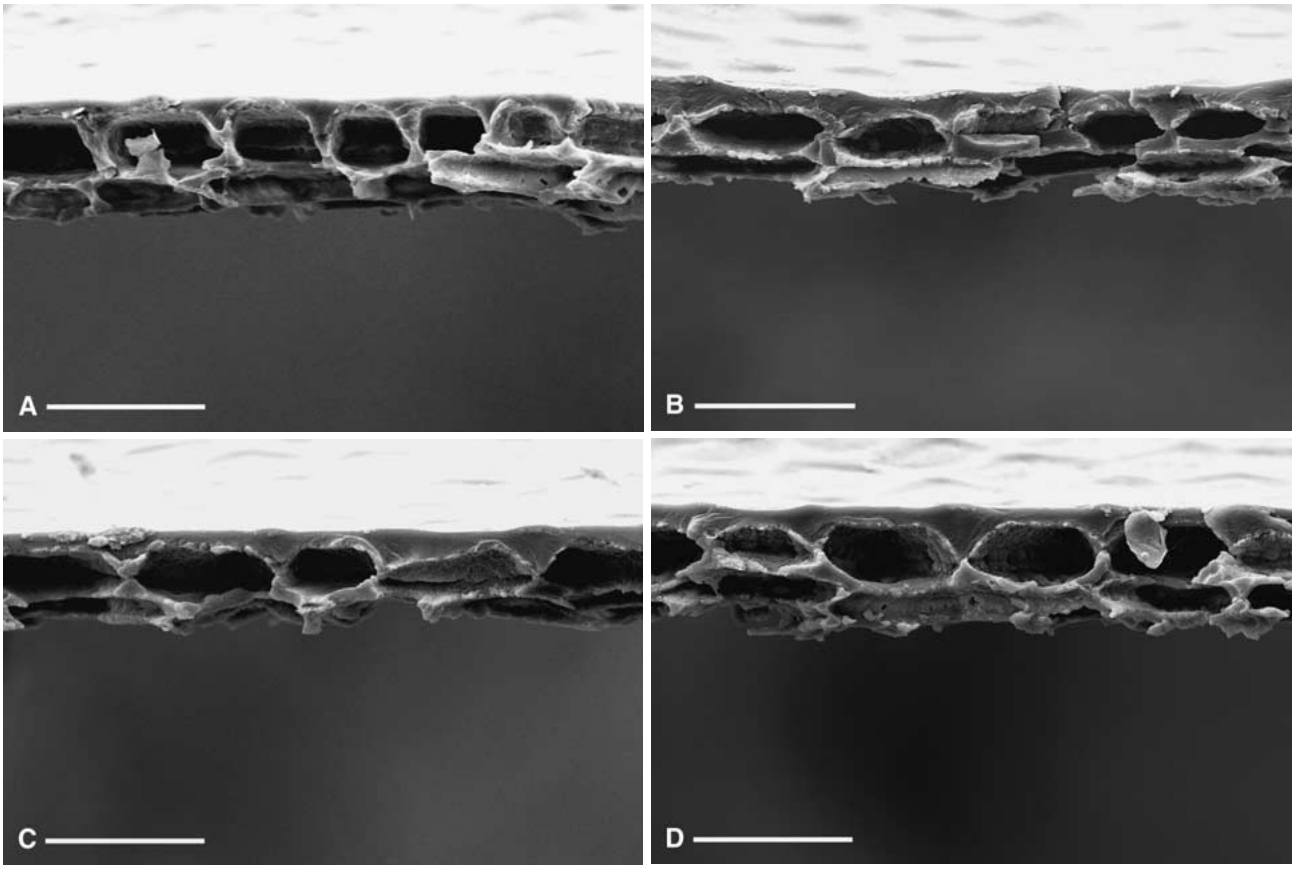

Figure 2. SEM micrographs of cv. Ailsa Craig CM cross-sections as related to fruit development and ripening. (**A, C**) CM at immature stage of (**A**) *wt* fruits and (**C**) *nor* fruits. **(B, D)** CM at mature-ripe stage of (**B**) *wt* fruits and (**D**) *nor* fruits. Scale bars: 50 μm.

fully ripe. Isolated cuticles of *wt* fruits were bright yellow-orange at the final stages of ripening, whereas that of *nor* fruits displayed a reduced degree of pigmentation ranging from light green to faint yellow. SEM analysis of the CM cross-sections generally revealed an excessive encasement of the epidermal cells at *mg* and *mr* ripening stages of comparable magnitude for both *wt* and *nor* (Figure 2). In addition, cuticle thickness increased from immature to fully ripe fruits in a similar way, although a trend towards thinner CM for *nor* fruits at earlier developmental stages appeared to be visible (Figure 3). On the other hand, at full maturity *nor* CM thickness was slightly larger than that of *wt*, but overall thickness was not significantly different between wild-type and mutant except for the ripening stage *mg*. In detail, the mean thickness of *wt* CM increased very significantly ($p < 0.01$) from 8.2 ± 0.4 μm at stage *ig* to 9.5 ± 0.7 μm at stage *mr*. Mean thicknesses of the mutant *nor* also increased from 7.4 ± 0.8 μm at stage *ig* to 9.9 ± 0.9 μm at stage *mr* and were highly significantly ($p < 0.001$) different as related to ripening. Thus, no major variation in cuticle morphology could be detected between *wt* and *nor* during fruit growth and ripening.

Cuticle thickness and cutinization also increased for cvs. Vanessa F1 and Roma, but with cultivar-specific differences. Although thickness of cv. Vanessa increased from 7 ± 0.5 μm at stage *ig* to 8.2 ± 0.3 μm at stage *mr*, that of cv. Roma increased from 5.8 ± 0.3 μm to 12.2 ± 0.4 μm (Figure 4).

Biomechanical Properties

From the uniaxial tensile tests, stress-strain curves were derived and subsequently analyzed. The stress-strain curves were biphasic (lower stress with increasing strain) for virtually all dry CM samples of *wt* and *nor*, whereas for the hydrated samples a concave form (increase in modulus) as well as an almost linear form could be detected at any ripening stage. Representative stress-strain curves of *wt* are shown as examples in Figure 5. Considerable differences between *wt* and *nor* could be found for

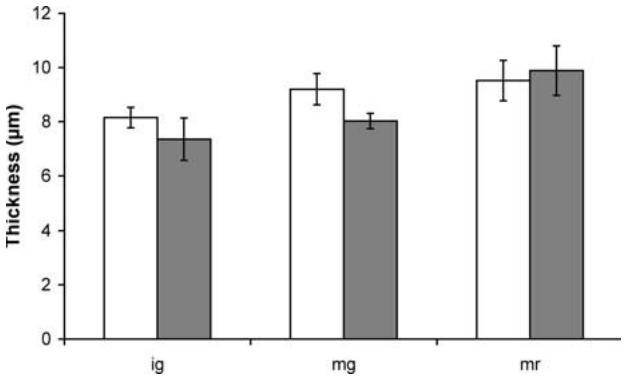

Figure 3. Mean thickness ± C.I. of the CM of cv. Ailsa Craig *wt* (open bars) and *nor* (closed bars) fruits as related to development and ripening. Thickness increased significantly from immature to fully mature fruits for both *wt* ($p < 0.01$) and *nor* ($p < 0.001$) fruits. Measurements were derived from SEM and digital image analysis. *ig*-immature-green; *mg*-mature-green; *mr*-mature-red.

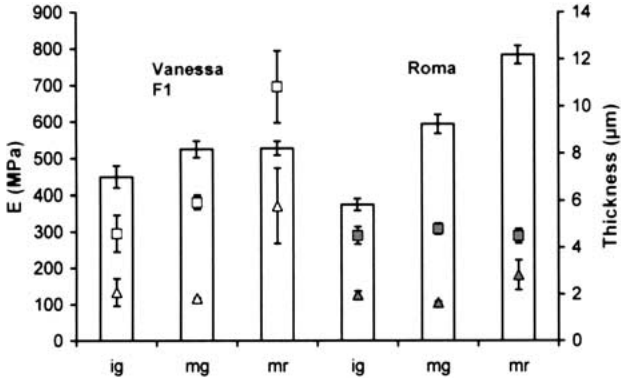

Figure 4. Mean modulus of elasticity E ± S. E. (left scale) and mean thickness ± S. E. (columns, right scale) of isolated cuticle from cvs. Vanessa F1 and Roma as related to development and ripening. Mechanical characterization was carried out with dry (squares) and hydrated (triangles) samples. *ig*-immature-green; *mg*-mature-green; *mr*-mature-red.

immature and fully ripe fruits. Most of the *wt* fruit samples tend to increase in modulus, whereas the CM of *nor* fruits generally showed an almost linear form. CM in ripening stage *mg* of both *wt* and *nor* were intermediate between strain hardening and linear. From the stress-strain curves, the modulus of elasticity E, strength and breaking strain were derived as conventional and true values. Two moduli of elasticity, denoted E_1 and E_2, were calculated where appropriate. As a consequence of the different material behavior, a second modulus could only be calculated for the *nor* mutant at ripening stage *mg* (Table 1). The conventional E_1 of dry CM of cv.

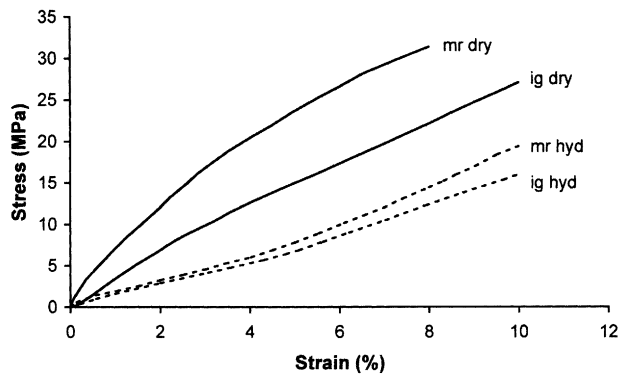

Figure 5. Representative stress-strain curves of CM of cv. Ailsa Craig *wt* related to fruit growth as examples. Although dry CM (bold lines) displayed yield independent of age, hydrated (hyd) CM samples (dashed lines) of immature and fully ripe fruits showed an increase in modulus. *ig*-immature-green; *mr*-mature-red.

Ailsa Craig *wt* displayed a highly significant ($p < 0.001$) ripening-related increase. Hydrated CM also showed a significant ($p < 0.05$) ripening-related increase of stiffness, but with markedly lower values (Figure 6). Stiffness of fully ripe dry CM was 37% higher compared to that of immature fruits, and 17% higher for hydrated CM. In contrast, dry *nor* CM displayed a nonsignificant tendency to decrease in stiffness during ripening. Moreover, stiffness of the hydrated *nor* CM decreased significantly ($p < 0.05$) (Figure 6). Here, stiffness of dry *mr* CM declined about 9% compared to that of *ig*, whereas it was about 29% lower for hydrated CM.

Values of the second modulus E_2 of dry *wt* CM were about 54% lower on average (56% at stage *ig* to 51% at stage *mr*, Table 1) than E_1. For the hydrated CM of the wild-type, E_2 increased by about 36%, 33%, and 13% during fruit growth and ripening. A similar trend could also be detected for the dry and hydrated CM of the *nor* mutant. Dry measurements revealed a lower E_2 of the CM of about 78% at *ig*, 63% at *mg*, and 54% at *mr*. Hydrated CM of *nor* fruits had a 35% higher E_2 compared to E_1 at *mg*. No second modulus could be calculated for the other two ripening stages due to almost linear stress-strain curves. In strength, dry *wt* CM increased from 22 MPa to 28 MPa during maturation. In contrast, the strength of *nor* CM markedly decreased from 23 MPa at *ig* to 9 MPa at full maturity (Figure 7A). When measured in the hydrated state, the strength of the CM of both *wt* and *nor* fruits generally decreased analogously to E_1 to 30%-65% of the corresponding dry measurements. In addition, CM of both cultivars displayed a trend to lowest values at full maturity, with slightly lower

Table 1. Moduli of Elasticity E_1 and E_2 of Dry and Hydrated (hyd) CM from cv. Ailsa Craig *wt* and *nor* Fruits as Related to Development and Ripening. Values are given as mean ± C.I. and were calculated from conventional stress and strain (E_{conv}) and the corresponding true numbers (E_{true}) after equations (1) and (2). *ig*-immature-green; *mg*-mature-green; *mr*-mature-red

Stage		$E_{1\ conv}$		$E_{1\ true}$		$E_{2\ conv}$		$E_{2\ true}$	
		Dry	Hyd	Dry	Hyd	Dry	Hyd	Dry	Hyd
wt	*ig*	335 ± 36	113 ± 11	344 ± 37	135 ± 17	189 ± 33	176 ± 15	225 ± 40	219 ± 23
	mg	318 ± 36	118 ± 11	329 ± 37	129 ± 12	172 ± 32	175 ± 27	202 ± 38	222 ± 33
	mr	460 ± 39	132 ± 7	477 ± 39	144 ± 8	236 ± 39	152 ± 19	295 ± 50	188 ± 22
nor	*ig*	290 ± 53	137 ± 12	306 ± 55	148 ± 14	225 ± 60	–	276 ± 76	–
	mg	299 ± 38	113 ± 13	319 ± 41	127 ± 13	187 ± 15	175 ± 23	215 ± 17	217 ± 29
	mr	263 ± 53	97 ± 20	273 ± 53	106 ± 22	142 ± 37	–	172 ± 19	–

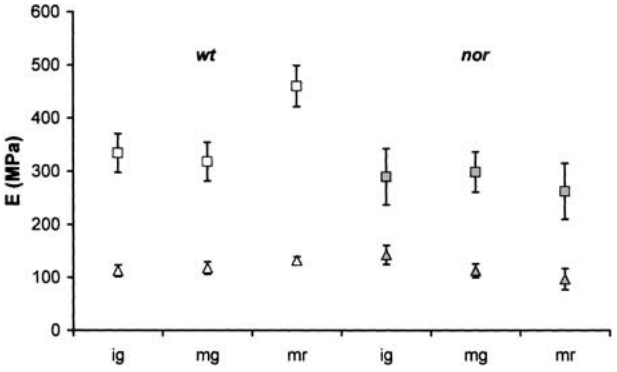

Figure 6. Mean modulus of elasticity E_1 ± C.I. of the CM from cv. Ailsa Craig *wt* and *nor* fruits as related to development and ripening. Samples were tested dry (squares) and fully hydrated (triangles). Stiffness of CM from *wt* fruits increased from immature to fully mature fruits for both dry ($p < 0.001$) and hydrated ($p < 0.05$) measurements, whereas stiffness of dry and hydrated ($p < 0.05$) CM from *nor* fruits decreased during ripening. *ig*-immature-green; *mg*-mature-green; *mr*-mature-red.

values for the *nor* mutant at any ripening stage. This trend could also be found for the breaking strain of the CM of both wild-type and *nor* mutant. The ripening-related decline in breaking strain from 10.1% to 7.4% was more pronounced for hydrated CM of *wt* fruits, but the contrary was measured for dry *nor* CM, where the breaking strain declined from 9.9 to 4.7% (Figure 7b). Hydration caused a decrease in total extensibility for the CM of *nor* fruits only at stage *ig* and *mg*. In hydrated *wt* CM, the breaking strain increased by about 1% at the same ripening stages. For both cultivars and testing conditions, breaking strain was highest at stage *mg*.

Comparing the conventional and true stress and strain, the magnitude of differences depended on the considered mechanical property. The true modulus E_1 was about 10-21 MPa higher for dry CM and 9-23 MPa higher for hydrated CM compared to the conventional equivalent, whereas the true modulus E_2 of dry CM was 28-60 MPa higher, and 36-46 MPa for hydrated CM (Table 1). True stress and strain for dry and hydrated CM were about 1-3 MPa higher and 0.5-1.5% lower, respectively (results not shown).

Analogously to cv. Ailsa Craig *wt* and *nor*, the stress-strain behavior of dry CM of cvs. Vanessa F1 and Roma showed strong yield throughout fruit development and ripening, whereas hydrated samples showed a transition from a concave form at stage *ig*, a mostly linear form at stage *mg*, to yield at full maturity similarly for both cultivars (results not shown). Stiffness generally increased and was also ripening-related as observed for cv. Ailsa Craig *wt*, but with cultivar-specific differences. Although *E* of dry CM of cv. Vanessa F1 increased from 295.2 ± 50.4 MPa at stage *ig* to 695.9 ± 98.4 MPa at stage *mr*, the corresponding measurements for cv. Roma revealed no clear ripening-related change of *E*, which remained at about 290 MPa (Figure 4). On the other hand, hydrated CM samples of both cultivars displayed a ripening-related increase in stiffness, and values of *E* ranged from 133.6 ± 37.8 MPa to 370.7 ± 102.7 MPa for cv. Vanessa F1, and from 126.0 ± 10.9 to 181.2 ± 40.9 MPa for cv. Roma.

Discussion

Morphology as Related to Fruit Growth and Ripening

As revealed by the SEM analysis, the CM of cv. Ailsa Craig showed excessive encasement of the underlying epidermal cells up to two cell rows in both *wt* and *nor*. This appears to be typical of a number of tomato cultivars, for example cv. Pik Red (Petracek and Bukovac 1995) or cvs. Vanessa F1, Harzfeuer,

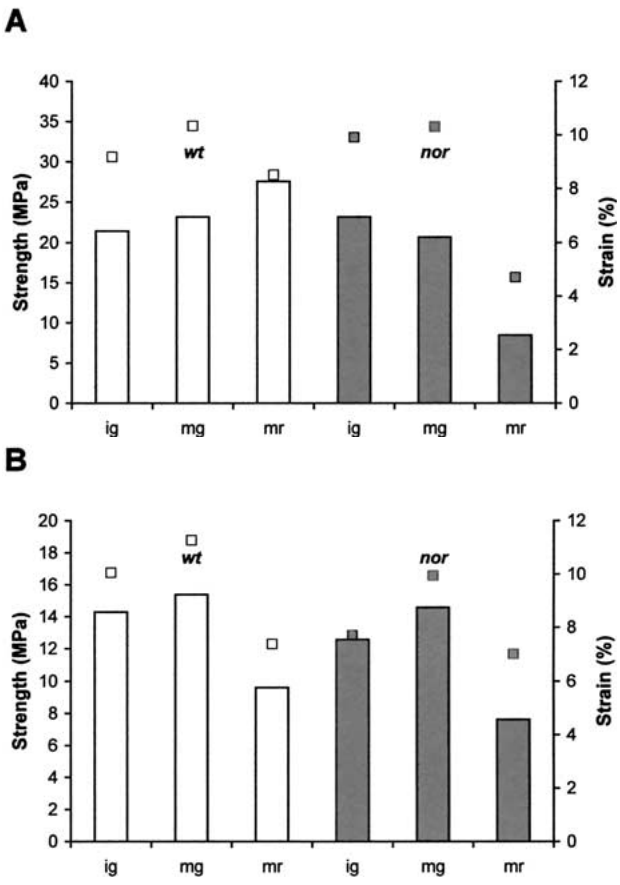

Figure 7. Mean breaking stress (columns, left scale) and breaking strain (squares, right scale) of the CM from cv. Ailsa Craig *wt* and *nor* fruits as related to development and ripening. (**A**) CM at dry conditions. (**B**) CM at fully hydrated conditions. Breaking strain decreased at the onset of ripening for both dry and hydrated conditions and tomato lines. Strength of dry CM increased in *wt* fruits, but decreased for *nor* fruit and hydrated CM regardless of origin. *ig*-immature-green; *mg*-mature-green; *mr*-mature-red.

and Roma (Bargel and Neinhuis, in print). However, there are also some cultivars where encasement of epidermal cells is absent and only cuticular pegs are present (Chu and Thompson 1972; Matas and others 2004a). Increasing overall thickness of the CM of cv. Ailsa Craig (*wt* and *nor*), Vanessa F1, and cv. Roma, from immature to fully ripe fruits, as well as cutinization of underlying cells requires biosynthesis and incorporation of cuticular material throughout fruit growth and ripening, as was shown by Baker and others (1982). The reported CM thicknesses were well within the range of cv. Pik Red, ranging between 6.7 and 15.3 μm (Petracek and Bukovac 1995). Because there have been no distinct differences in cuticle micromorphology between cv. Ailsa Craig *wt* and the ripening-impaired mutant, fruit cutin monomer and wax biosynthesis rates are supposed to be unaffected in *nor*. Fatty acid metabolism therefore appears to be independent of the ethylene-mediated ripening cascade which is deficient in *nor* (Giovannoni and others 1995).

In an antisense suppression study of the β-galactosidase gene (TBG6), Moctezuma and others (2003a) described a softened fruit texture at the mature-green stage and a concurrent doubled cuticle thickness compared to the cv. UC82B *wt*. The authors proposed enhanced cuticle deposition being structurally antagonistic to the modified galactan metabolism as a possible explanation. Thus, a thicker cuticle might also be anticipated to counteract the tomato fruit softening at the red-ripe stage, as is the case during the normal ripening process, as shown in cvs. Ailsa Craig *wt*, Vanessa F1, and Roma. However, CM thickness of the *nor* mutant increased to a similar amount as that of cv. Ailsa Craig *wt*, although fruits of *nor* have been described as being firmer compared to the corresponding *wt* (Giovannoni and others 1989).

Interestingly, cuticle thickness at red-ripe stage did not vary significantly between control and transgenic fruit in the above-mentioned study (Moctezuma and others 2003a), indicating a reduced biosynthesis of cuticular material in the transgenic lines during the ripening process. At this point, discussion can only be speculative: 1) it is possible that the degree of tissue softening is still high enough in the *nor* mutant to induce sufficient cuticular material biosynthesis and incorporation during ripening, and 2) the increase in cuticle thickness in the transgenic β-galactosidase fruits at mature-green stage may reflect an advanced activity of a constitutive metabolic program that normally takes place after the onset of ripening, as seen in the control fruits as well as in cv. Ailsa Craig *wt* in this study. Similar CM thicknesses of transgenic and control fruits at the fully ripe stage may then be due to a reduced synthesis of cuticular material, as shown for ripe and over-ripe tomato fruits cv. Michigan Ohio (Baker and others 1982).

Mechanical Properties as Related to Fruit Growth and Ripening

Similar to morphology, the mechanical properties of the CM of cv. Ailsa Craig *wt* and *nor* fruits changed during fruit development and ripening, but with significant differences. Interestingly, a typical feature of the dry CM, regardless of age and origin, was biphasic stress-strain behavior, differing only in stiffness. Analysis of stress-strain curves of hydrated

Table 2. Modulus of Elasticity E, Stress σ, and Strain ϵ at Failure of Tomato Fruit Cuticle and Fruit Skin Collected from Various References.

Cultivar	E (MPa)	σ (MPa)	ϵ (%)	Reference
Inbred 10[a]	70.3	1.2	—	Matas and others (2004a)
Pik Red[a]	—	4.6	48.8	Petracek and Bukovac (1995)
Sweet 100[a]	53.1	1	—	Matas and others (2004a)
Unknown[a]	60	≥5	3.0	Wiedemann and Neinhuis (1998)
Beefsteak[b]	600	2.5	23.5	Vincent (1990b)
Espero[b,c]	30	5	24	Andrews and others (2002)
Espero[b,d]	110	10	10	Andrews and others (2002)
Inbred 10[b]	70.3	1.3	—	Matas and others (2004a)
Mercheast 55[b]	18.4	1.8	13.7	Batal and others (1970)
Moreton hyb.[b]	—	9.5	23.5	Voisey and others (1970)
Scout[b]	—	15.3	27.9	Voisey and others (1970)
Sun Up[b]	14.1	1.0	7.8	Batal and others (1970)
Sweet 100[b]	53.1	1	—	Matas and others (2004a)

All tests performed in one-dimensional tension with hydrated samples of ripe fruits except where stated.
[a]*Isolated cuticle*
[b]*fruit skin*
[c]*10 dpa*
[d]*60 dpa*

tomato fruit CM as related to development and ripening was carried out extensively elsewhere (Bargel and Neinhuis, in print). In brief, a concave form of CM from cv. Ailsa Craig *wt* fruits was also observed for most of the hydrated cuticles of cv. Vanessa at ripening stage *mr*. At stage *ig*, a mixture of convex and linear form could be depicted for most of the CM, which differs from the concave form predominant for cvs. Ailsa Craig *wt*, Vanessa F1, and Roma in the results presented here. On the other hand, stress-strain curves of CM from immature and fully mature *nor* fruit showed mostly a linear form, which is different from that of the *wt*, particularly at the ripe stage, indicating a change in the material behavior of the *nor* fruit cuticle during the ripening process. Different mechanical properties were also characteristic of the CM of *nor* fruits. Whereas E_I of both dry and hydrated CM of *wt* fruits significantly increased during maturation, it tended to decline for the CM of *nor* fruits for the dry condition, and decreased significantly for the hydrated CM at the fully ripe stage. In addition, dry CM of *wt* fruits displayed increasing strength related to growth and ripening, while that of the ripening-impaired *nor* mutant was weaker at the final stages of ripening. Ripening-related changes in breaking strain generally followed a similar pattern for the hydrated CM of both *wt* and *nor* fruits without pronounced variations between them, but dry CM of *nor* fruits showed significantly less extensibility than that of *wt* fruits. The magnitudes of the mechanical properties of the CM of cv. Ailsa Craig *wt* and *nor* lie within the range of other studies on tomato fruit cuticle and fruit skin. In Table 2, data collected from various references are given.

Compared to studies available, stiffness of the Ailsa Craig CM was generally higher, particularly under dry conditions. Similar values for E ranging between 96 and 258 MPa have been measured for hydrated CM of the cvs. Harzfeuer, Vanessa F1 and Roma (Bargel and Neinhuis, in print), and a comparably high modulus ranging from 290 MPa up to about 700 MPa was calculated for dry CM of cvs. Vanessa and Roma, whereas values for hydrated CM ranged between 134 and 371 MPa (Figure 4). These data confirm the ripening-related increase of the modulus of elasticity during fruit development reported for cv. Ailsa Craig *wt*, as well as a water-induced decrease in membrane stiffness. The modulus of CM of cvs. Vanessa F1 and Roma decreased about 40-70% upon hydration, with the highest impact of hydration at the *mg* stage. The lowest influence of hydration occurred at full maturity. As far as strength and breaking strain are concerned, values in a similar range have been measured for the hydrated tomato fruit CM and fruit skin by Bargel and Neinhuis (in print), reporting strength values of the CM between 5.1–16 MPa at stage *ig* and 4.9–15 MPa at stage *mr*, and breaking stresses between 5.2–10% and 2.7–5.2%, respectively.

In summary, the results of this study as well as the cited investigations provide evidence that the mechanical properties of the tomato fruit CM appear to be cultivar-specific, and can neither be

canonically generalized across cultivars of a single species nor for different species (Wiedemann and Neinhuis 1998; Bargel and others 2004a).

Chemical Constituents of the CM Affecting Mechanical Properties

Because the morphological variation between the CM of cv. Ailsa Craig *wt* and *nor* fruits appeared to be insignificant in this study, the changes of and differences in the mechanical properties of the cuticle during tomato fruit growth and ripening could be assigned mainly to modifications of its chemical composition and molecular organization. For instance, Baker and others (1982) showed that the amount of C_{18} trihydroxy-fatty acids decreased from immature to mature fruits of cv. Michigan Ohio, which would give rise to a relative gain of C_{16} cutin monomers in the cuticle of fully ripe tomato fruits. Indeed, in their study, the proportions of the dihydroxy-C_{16} monomers increased from 74% of the cutin from young fruits to about 92% of the cutin from ripe fruits. More recently, Graca and others (2002) have characterized isolated tomato fruit cutin from ripe fruits as being dominated by C_{16} monomers, and reported small amounts of glycerol as an esterified constituent in the cutin-matrix that possibly reinforces the polymeric structure. Marga and others (2001) undertook chemical analysis of cuticles from various organs of a thistle (*Cirsium horridulum* Michx.), each having different mechanical properties, and classified rigid cuticles by predominant C_{16} cutin monomers, whereas more extensible cuticles correspond to mixed C_{16}/C_{18}.

Thus, hydroxyl groups appear to enhance the hydrophilic character and possibly the hydration state of the tomato fruit cutin matrix, which in turn would result in a higher extensibility for immature fruits (Bargel and others 2004a). This scenario might reasonably apply for the ripening-related increase of the stiffness and strength of the CM of cv. Ailsa Craig *wt* fruits, as well as for cvs. Vanessa F1 and Roma. Another feature of the cutin composition that could explain the ripening-related changes of the cuticle from *wt* fruits is the molecular organization of the biopolyester matrix. The cutin monomers of the CM isolated from immature and mature-green ripening stages have a low degree of cross-linking (Matas and others 2004b), which could give the weaker cuticles reported for early ripening stages of both cv. Ailsa Craig *wt* and *nor*. It is largely accepted that the cutin monomers form a linear polyester by esterified primary hydroxyl groups, while only 50% of the secondary hydroxyl groups are involved with cross-linking and three-dimensional branching (Deas and Holloway 1977; Kolattukudy 1980; Ray and others 1998). As the cuticle ages in the course of ripening, increased cross-linking, for example, at the site of secondary hydroxyl groups, and a reduced hydration state may lead to the described stiffening at full maturity (Vincent 1990a; Marga and others 2001). Concerning the altered mechanical properties of the CM isolated from fully ripe *nor* fruits, the question arises as to whether assumed dynamic changes of cutin monomer composition during fruit development and ripening in *wt* fruits, as observed in cv. Michigan Ohio (Baker and others 1982), might be affected by the pleiotropic mutation at the NOR locus. NOR is also very likely to encode for a developmental transcription factor (Giovannoni 2004), as was described for the RIN locus (Giovannoni and others 1995; Vrebalov and others 2002). Both *nor* and *rin* mutants fail to elevate ethylene production at the onset of ripening, so that ripening is inhibited to a large extend (Giovannoni 2001). RIN regulation may play an integral role within the transition from system-1 to system-2 ethylene synthesis, that is, a ripening-dependent switch from auto-inhibitory towards auto-stimulatory ethylene regulation (Nakatsuka and others 1998; Barry and others 2000). Lelievre and others (1997) have stated that aminocyclopropane-1-carboxylic acid (ACC) and its conjugated derivative malonyl-ACC (MACC) do not accumulate in fruits of the *nor* mutant due to the lack of autocatalytic ethylene production. These and other findings indicate that NOR appears to affect at least the developmental regulated expression of one ACC oxydase (ACO; presumably ACO1) of the ACO gene family, that finally produces ethylene from the ACC precursor (McKeon and Yang 1990; Lelievre and others 1997).

So far, no information is available on either the molecular control of cuticle formation and biosynthesis of cutin monomers during tomato fruit development and ripening, or the role of ethylene in this process. In addition, molecular mapping of genes involved in tomato fruit ripening so far lacks information on fatty acid biosynthesis (Giovannoni and others 1999; Zegzouti and others 1999). Although speculative, it is possible that the lesion at the NOR locus also bears some consequences for the cutin matrix composition of the CM of the cv. Ailsa Craig *nor* fruits, for example, by hampering cutin biosynthesis and thus the transition from C_{16}/C_{18} mixed type towards a C_{16} dominated cutin type in ripe fruits. Such interference would not necessarily alter the morphology of the cuticle during fruit

ripening, but its mechanical properties. On the other hand, the *nor* mutation affects several aspects of fruit ripening, among them coloration due to the biosynthesis of the red carotenoid lycopene, and probably also phenolic compounds. The red lycopene is derived from the colorless phytoene in the carotenoid pathway, and it has been shown that mRNA of the first dedicated enzyme catalyzing phytoene, phytoene synthase, does not accumulate, or only accumulates at reduced levels, in the ripening mutants *rin* and *nor* (Guiliano and others 1993), resulting in a reduced lycopene accumulation and the faint yellow to yellow-orange colour of *nor* fruits.

The phenolic compounds could be target candidates in determining the mechanical behavior of the outer fruit membrane. It has been shown that p-coumaric acid typically bound to glucosides increases in the pulp of the fruits of the cvs. Ailsa Craig and Pik-Red during ripening, indicating a shift in hydroxycinnamic acid conjugate metabolism with the onset of ripening (Buta and Spaulding 1997). Increasing levels of p-coumaric acid and both the flavonoids naringenin and the yellow chalconaringenin in the tomato fruit cuticle have also been reported by several authors (Hunt and Baker 1980; Baker and others 1982; Laguna and others 1999). Phenolics and flavonoids provide important protection against damage from UV irradiation (Holton and Cornish 1995; Krauss and others 1997). These phenolic constituents may form molecular clusters and/or bind covalently to cutin (Laguna and others 1999), most reasonably via hydrogen bonds and free available secondary hydroxyl groups (Deas and Holloway 1977; Heredia 2003).

In their study, Matas and others (2004b) reported that cutin isolated from ripe fruits did not display a glass transition in contrast to immature and mature green stages, a consequence of the accumulation of flavonoids as well as polysaccharides into the plant polymer contributing to a higher stiffness. Generally, the glass transition marks the temperature at which segmental mobility becomes thermally activated in macromolecules, and increases with chain rigidity and restricted internal rotation (Courtney 1990). Thus, an increase of cutin-bound phenolic compounds during tomato fruit ripening could well be responsible for the increasing stiffness of the CM of the *wt*. Chalcones and flavanols, to which chalconaringenin and the isomeric naringenin belong, are synthesized in the flavonoid pathway by the enzymes chalcone synthase (CHS) and chalcone isomerase (CHI) (Holton and Cornish 1995). Several studies on a variety of plant species indicated that at least the enzyme chalcone synthase is regulated by ethylene, for example, in climacteric apple (Awad and de Jager 2002) and non-climacteric grapes (El-Kereamy and others 2003). Because the CM of tomato *nor* fruits displayed a lesser degree of pigmentation at the red-ripe stage than the *wt*, evidence is high that the biosynthesis and incorporation of flavonoids into the tomato fruit cuticle, in particular the yellow chalconaringenin, seemed to be altered in the ripening-impaired *nor* mutant due to reduced ethylene production during ripening, resulting in the reported changes in the mechanical properties. Quantitative and qualitative chemical analysis of phenolic compounds of the cuticle of ripening-impaired mutants could help to clarify this topic.

As for cell wall polysaccharides, it has been generally established that the fiber content in the CM increases during development of the cuticle, and in the tomato fruit the quantity of cell wall constituents is higher at full maturity compared to immature fruits (Baker and others 1982; Jeffree 1996). Moreover, Matas and others (2004a) have proposed a model based on diverse degrees of cutinization and hence of polysaccharide content to explain cultivar-specific mechanical properties of tomato. It is therefore plausible that cutinized cellulose fibers originating from epidermal cell walls within the CM contribute to the ripening-related stiffening according to the theory of fiber-reinforced composite materials (Courtney 1990). This could hold true for the CM of *wt* fruits, while in the *nor* mutant the lack of elevated ethylene production at the onset of ripening may result in decreased transcription and expression patterns of cell wall-degrading enzymes as has been described for β-galactosidases (Moctezuma and others 2003b), hemicellulose-degrading endo-β-mannanase (Bewley and others 2000), and expansins (Rose and others 2000), as well as in altered solubility of cell wall-bound homogalacturan-rich pectins, as has been proposed for the *Cnr* mutant (Orfila and others 2001).

One could then speculate that these changes in expression patterns in ripening-inhibited tomato mutants such as *nor* may lead to altered incorporation of cell wall material into the cuticle layer, either quantitative or qualitative, which would in turn result in changed mechanical properties. However, the strongest argument against this consideration is that the highest amount of epidermal cell wall constituents was reported at the mature-green ripening stage of cv. Michigan Ohio (Baker and others 1982). The most significant changes in the mechanical properties occurred during fruit ripen-

ing and not during development for the CM of both *wt* and *nor* fruit. Thus, neither the presence of the polysaccharide fibers *per se* at stage *mr* nor a ripening-related differential enzyme activity in the nonripening tomato fruit mutant could account for the lower stiffness and strength of the *nor* CM at full maturity.

Thus, one should be cautious not to exaggerate the mechanical role of the polysaccharides in the cuticle. They are very likely orientated randomly within the tomato fruit CM, and further investigation of cellulose fiber length and volume fraction in the cutin matrix is expected to shed light on the actual contribution of cutinized cell wall material to the mechanical properties of the CM. This topic is discussed further in the context of hydration.

Effect of Water on Mechanical Properties

Hydration of the CM generally lowered stiffness by 50-70% compared to the dry state, thus decreasing the tensile stress in the polymer during testing. Moreover, the strength of the CM of both cv. Ailsa Craig *wt* and *nor* fruits declined to 30%-65% of the corresponding measurements at dry conditions, while the total extensibility was slightly higher at earlier developmental stages of *wt* fruits. As a consequence, water acts as a plasticizer on the cuticle. This has also been described by other authors (Petracek and Bukovac 1995; Wiedemann and Neinhuis 1998), and is further supported by nanomechanical studies that revealed the decreasing effect of water on the surface elastic modulus of isolated tomato fruit cutin (Round and others 2000). It is tempting to assume that water causes the cuticle to swell if the dipole molecules bind to polar functional groups, for example, hydroxyl groups of the cutin monomers, or even disrupt hydrogen-bonded cross-links in the cutin-matrix, and thus alters its rheological properties (Petracek and Bukovac 1995; Heredia 2003). For example, it has been shown that water is sorbed to 61% in the polymer of *Agave americana* L. as bound water, while the rest appears to be involved in intermolecular hydrogen bonds with mainly hydroxyl groups (Dominguez and Heredia 1999). Moreover, water molecules can be incorporated in very narrow macromolecular holes in the cutin matrix (Matas and Heredia 1999), or embedded between carboxyl groups of the polyester and hydroxyl groups of polysaccharides in the matrix via hydrogen bonds (Marechal and Chamel 1996). In fact, the polysaccharides incorporated into the polymer have been reported to contribute significantly to the water sorption in the plant cuticle, depending on the polysaccharide content (Chamel and others 1991; Dominguez and Heredia 1999).

In the present study, fully hydrated CMs have been mechanically characterized for all developmental stages. Interestingly, the CM isolated from both *wt* and *nor* fruits displayed the highest plasticizing effect of water at the ripening stage *mr* that is, the highest difference between dry and fully hydrated measurements. This may be caused by the increase of cellulose fibers in the cutin matrix during tomato fruit development owing to an overall higher water binding capacity, although again limited by the fact that the cuticle of mature-green fruit of cv. Michigan Ohio has been reported to have the highest polysaccharide content (Baker and others 1982). The results concerning the CM of the cvs. Vanessa F1 and Roma, which show that the plasticizing effect of water was highest at the mature-green ripening stage and stiffness of the CM was less reduced at full maturity, may even better illustrate the outlined relation among polysaccharides, water content and mechanical behavior of the CM. Here, a pronounced cultivar-specific inhomogeneity of the tomato fruit cuticle, as was stated earlier, is again indicated. From these results, the conclusion may be drawn that higher water content promotes weakening of the membrane, resulting in a reduced modulus of elasticity and strength compared to the dry material. Concerning the latter, cellulose fibers could be supplemental to the ripening-related stiffening of the CM caused by the dynamic change of chemical composition of the cutin matrix itself, since crystalline cellulose is very stiff (Vincent 1990a).

Thus, the influence of water on the mechanics of the tomato fruit cuticle *in vivo* strongly depends on the amount of water actually present in the membrane. Although several studies on the water sorption capacity of isolated cuticles from various species including tomato fruit have been performed (Chamel and others 1991; Coret and Chamel 1993), the natural state of hydration still remains to be determined. Matas and others (2004b) have reported that water sorption shifted the glass transition temperature of tomato fruit cutin isolated from immature fruits of cv. Cascade from around 23°C to 16.3°C. This would promote the flexural motion and segmental mobility inside the macromolecular network of the cutin matrix if the CM is in the hydrated state, resulting in reduced stiffness and breaking stress, as reported in the present study for both *wt* and *nor* fruit CM.

Conclusions

1) The CM of cv. Ailsa Craig *nor* has altered mechanical properties related to fruit development and ripening compared to the corresponding *wt*. Although the outer fruit membrane of the wild-type fruits was characterized by an increasing stiffness and strength during ripening in the dry and hydrated state, as was also shown for two additional cvs, Vanessa F1 and Roma, the CM of *nor* fruits was significantly weaker at the fully ripe stage. Hydration generally caused lower moduli of elasticity and strength for the CM of both lines, while breaking strain was significantly affected only for the CM of ripe *nor* fruits. 2) Several nonripening tomato mutants, among them *nor*, remain firmer than the corresponding wild-type lines. It has been claimed that this feature bears several advantages for the crop industry, including a prolonged shelf life (Giovannoni 2001). However, the results of this study indicate that the homozygous *nor* mutation strongly affects the mechanical properties of the cuticle at full maturity, counteracting the positive effect of firmer tissues. Thus, commercial use of tomato fruit mutants may be limited by the mechanical properties of their cuticle. 3) Ripening-impaired mutants provide fundamental insights in the multifaceted physiology of fruit development and ripening, and help to expand our knowledge of key regulative factors. The NOR locus has been proposed to encode a genetic regulatory component required to initiate ethylene biosynthesis at the onset of ripening in addition to requisite factors whose regulation is not influenced by ethylene, but its precise regulatory nature still must be clarified. It is evident that a careful analysis of the complex chemical composition of the cuticles of both *wt* and the nonripening mutant *nor* fruits will be helpful to further elucidate the observed biomechanical differences. The results of this study yield a clear impetus to expand the research on ripening-impaired fruit mutants and the plant cuticle using molecular genetics and biochemical and mechanical approaches.

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