

Benzyladenine and gibberellin treatment of developing “Pink Lady” apples results in mature fruits with a thicker cuticle comprising clusters of epidermal cells

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Abstract A mixture of 6-benzyladenine (BA) and gibberellins GA₄ plus GA₇ applied to “Pink Lady” apple at early phenological stages was previously shown to result in an immediate increase in epidermal cell density and associated reduction in calyx-end cracking disorder in the mature fruit, implying a long-term effect of the BA+GA₄₊₇ mixture. Here, we analyzed the anatomical changes in the mature peel at the calyx end 210 days after full bloom (DAFB), following application of the plant growth regulators (PGRs) at the cell-division phase of fruit development, 21–50 DAFB. Experiments were conducted in northern Israel, and the PGRs were applied as the commercial formulation Superlon™ (Fine Agrochemicals Ltd.), composed of 19 g l⁻¹ BA and 19 g l⁻¹ GA₄₊₇. Trees were sprayed with 0.025, 0.1, or 0.2 % (v/v) Superlon™. The most obvious phenomenon was the presence of epidermal cell clusters within the cuticular matrix that were detached from the native epidermal layer located at the bottom of the cuticle and which could not be detected in the untreated control fruits. Treatment with 20 mg l⁻¹ BA+GA₄₊₇ (0.1 % Superlon™) resulted in a markedly thicker cuticle, a higher percentage of detached epidermal cells within the cuticular membrane and a significant reduction

in calyx-end cracking at harvest. The presence of cuticle-embedded epidermal cell clusters may have contributed to strengthening the peel by adding more cell-wall components, thickening the cuticle layer and possibly enhancing crack repair.

Keywords Calyx-end cracking · Fruit cracking · *Malus x domestica* · Peel anatomy · Plant growth regulator

Introduction

Calyx-end cracking in “Pink Lady” apples (*Malus x domestica* Borkh.) appears toward the end of the growth period and develops as concentric cracks located around the styler end of the fruit. The disorder is characterized by cracks that traverse the cuticle, and although corky periderm develops at their margins, it is not sufficient to prevent pathogen invasion of the inner fruit tissues. Calyx-end cracking differs from the well-studied skin-russeting disorder. The latter results from microscopic cracks in the cuticle that appear on the surface of fruits at an early stage of apple development, probably due to high air humidity, and subsequently induce the formation of brown-colored healing periderm tissue (Faust and Shear 1972; Fogelman et al. 2009; Khanal et al. 2013). The factors that promote calyx-end cracking have not been identified, but an association with high growth temperatures has been suggested (Ginzberg et al. 2014).

Experiments conducted by us during the growth seasons of 2008 to 2012 indicated that spraying “Pink Lady” fruits with a mixture of the cytokinin 6-benzyladenine (BA) and gibberellins A4+A7 (GA₄₊₇) significantly reduces the incidence of calyx-end cracking at harvest, while treatment with each of the plant growth regulators (PGRs) alone was ineffective (Stern et al. 2013). The PGRs were applied at two phenological stages of fruit development: the cell-division phase that occurs at 7 to 35 days after full bloom (DAFB) and the cell-expansion phase at 60 to 90 DAFB. To reduce cracking incidence, two or

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three sprayings of the BA+GA₄₊₇ mixture were required. Histological studies performed 1 week after the sprayings showed an increase in epidermal cell density compared to untreated controls; however, there was no monitored change in cuticle thickness (Ginzberg et al. 2014).

Our results were in accordance with previous reports showing that treatments with gibberellins reduce cuticular cracking in apple, probably by affecting the epidermal tissue. Eccher (1975) showed that treatment with GA₄₊₇ results in less irregularity among epidermal cells and reduced cuticle cracking compared to untreated fruit. Knoche et al. (2011) showed that water-induced russeting and micro-cracking in “Golden Delicious” apples could be reduced by multiple applications of GA₄₊₇. The treatment had no effect on the amount or rate of cutin or wax deposition, suggesting that the reductions in russeting and micro-cracking were due to the effects of GA₄₊₇ on the epidermal and hypodermal layers of the cell. Fogelman et al. (2009) reported a similar result of less russeting on “Smoothie Golden Delicious” apple after multiple applications of BA+GA₄₊₇ during the first month of fruit development.

Accumulating data from ours and others’ laboratories thus indicate that the epidermal layer of the fruit is the target of the BA+GA₄₊₇ mixture. The PGRs probably increase peel flexibility due to enhanced cell division and increased cell density at the fruit surface, thus reducing its susceptibility to cracking at the developmental stage of dramatic fruit expansion (Stern et al. 2013). Notably, the PGR treatments and subsequent histological studies of the peel were performed at an early stage of fruit development, whereas the positive effect of reduced calyx-end cracking was monitored at the end of the growing season, 4 to 5 months later (180–210 DAFB). This implies a long-term effect of the BA+GA₄₊₇ mixture on peel anatomy. Accordingly, in the present work, PGR treatments were applied at an early stage of fruit development (21–80 DAFB), similar to previous experimental years. However, the anatomy of the calyx end of “Pink Lady” fruit was studied at harvest. We used light and confocal microscopies to study the anatomy of the peel at the calyx end of “Pink Lady” following the PGR treatment. Results showed that the BA+GA₄₊₇-induced increase in epidermal cell density observed at the early stage of fruit development translates to clusters of epidermal cells that are detached from the basal epidermal layer and suspended within the cuticular matrix in the mature fruit. A significant increase in cuticle thickness was also monitored. The contribution of these alterations to reduced peel cracking is discussed.

Materials and methods

Experimental orchards and conditions

Experiments were conducted in the 2013 growth season on 10- to 12-year-old “Pink Lady” apple trees grafted on MM

106 rootstock at Matityahu farm, located 700 m asl in the Upper Galilee, where all trees were 3.5 m high, with 4.5 m × 2.5 m spacing (900 trees ha⁻¹). The orchard is located in a semi-arid region with high temperatures (ca. 35 °C max.) and low humidity (<40 % RH) during the summer (May to October). Annual precipitation (November–April) is approx. 700 mm. The soil layer is 0.8 m thick and classified as well-drained protogromosol (68 % clay) on terra rossa soil. The soil pH is 7.7 with CaCO₃ content of approx. 7 % (w/w).

Application of PGRs

The commercial product Superlon™, containing synthetic cytokinins and gibberellins (GAs), was used as the solution of plant growth regulators (PGRs). This liquid formulation contains 1.9 % (v/v) BA and 1.9 % (v/v) GA₄₊₇ (Fine Agrochemicals, Whittington, UK). Superlon™ was applied three times during the fruit developmental stage of cell divisions—21, 35, and 50 DAFB—at 0.025 % (v/v) (BA plus GA₄₊₇, 5 mg l⁻¹ each), 0.1 % (20 mg l⁻¹ each), or 0.2 % (40 mg l⁻¹ each). The latter concentration was also applied at the fruit-expansion phase—50, 65, and 80 DAFB. Commercially mature fruits were harvested at 210 DAFB for analysis. An additional set of trees served as nontreated controls.

The PGRs were applied as a foliar spray at 3 to 5 l per tree, using a high-pressure handgun (Kibbutz Degania, Israel) until runoff. Triton X-100, at 0.025 % (v/v), was included in all sprays as the nonionic surfactant.

The experiments were conducted on whole trees bearing a similar crop load (ca. 240 fruits tree⁻¹, ca. 35 kg tree⁻¹). Control trees with the same crop load were not sprayed. The experimental design was randomized complete blocks, with six blocks of one tree per treatment. One buffer tree was always used between two treatment trees to avoid drift.

Assessment of fruit cracking

Incidence of fruit-cracking disorder was assessed at harvest. Twenty random fruits were collected from each tree—five to six individual trees per treatment and the control. Fruits affected by calyx-end cracking were counted, and values were expressed as their percentage of total fruits.

Anatomical studies of the peel at the calyx end of the apple fruit

Histological studies were conducted on fruit harvested at the end of the growing season, 210 DAFB. Four to five fruits were collected for each treatment. Samples of apple surface tissue (blocks of 5 mm × 3 mm × 3 mm) were collected from the calyx end of each fruit. Samples were fixed in FAA (50 % ethanol, 5 % acetic acid, and 3.7 % formaldehyde in H₂O), dehydrated in an ethanol/Histoclear series (Finkelman Chemicals, Petach

Tikva, Israel) and embedded in Paraplast™ (Paraplast Plus; McCormick Scientific, St. Louis, MO, USA) according to standard methods (Ruzin 1999).

Tissue sections (15 µm thick) were stained with toluidine blue (Sigma Chemicals, Rehovot, Israel) for examination of tissue morphology (Johansen 1940). Sections were observed under a light microscope (Leica DMLB; Wetzlar, Germany), and images were displayed on a monitor through a CCD camera (Leica DC2000) using the Leica IM1000 program.

To measure cuticle thickness, one cross section was selected from each fruit. Cuticle thickness was determined by measuring the lengths (µm) of arbitrary lines from the base of the epidermal cell layer to the outer edge of the cuticle at five locations along the section (Fig. S1). An average value was calculated for the section. To determine epidermal cell density, cells were counted along an artificial line of 1400 µm tangential to fruit surface. An average value of cuticle thickness and epidermal cell number was calculated for each treatment.

For confocal studies, thin hand cuts were taken from calyx-end tissue blocks that were kept in FAA and washed lightly in ethanol. Microscopic observation and image acquisition were performed with an Olympus IX-81 laser scanning confocal microscope (FV 500, Olympus Optical Co., Tokyo, Japan) equipped with a 405-nm diode laser and 488-nm argon-ion laser with a UPlanSApo ×40 0.9N.A objective. To observe UV autofluorescence, a 405-nm laser was used for excitation with a BA430–460-nm barrier filter. For FM4-64 stain, a 488-nm excitation line was used with a BA610 IF emission filter. Transmitted light images were obtained using Nomarski differential interference contrast (DIC) microscopy.

Statistical analysis

Data on cuticle thickness, number of epidermal cells, and fruit-cracking incidence were analyzed for statistical significance using the JMP software (SAS Institute Inc., Cary, NC, USA).

Results

Anatomical studies of the fruit surface

At harvest, fruits from treatments and a control were taken for anatomical studies. The treatments were 5, 20, and 40 mg l⁻¹ PGR solution. The 40 mg l⁻¹ PGR solution was applied at two fruit developmental stages: an early stage characterized by cell-division activity and a later stage characterized mainly by cell expansion. Because harvesting was performed at 210 DAFB, the anatomical study was performed at around 5 months after the last treatment given at the cell-division phase (50 DAFB) and around 4 months after the last treatment given at the early cell-expansion phase (80 DAFB).

Cross sections of the fruit surface were stained with toluidine blue and observed under a visible light microscope. In the control sample, the peel consisted of a single layer of triangle-shaped epidermis cells that were separated from each other and covered with cuticle (Fig. 1a, arrows with rounded tail). Below the epidermal cells, there was a darker double-cell

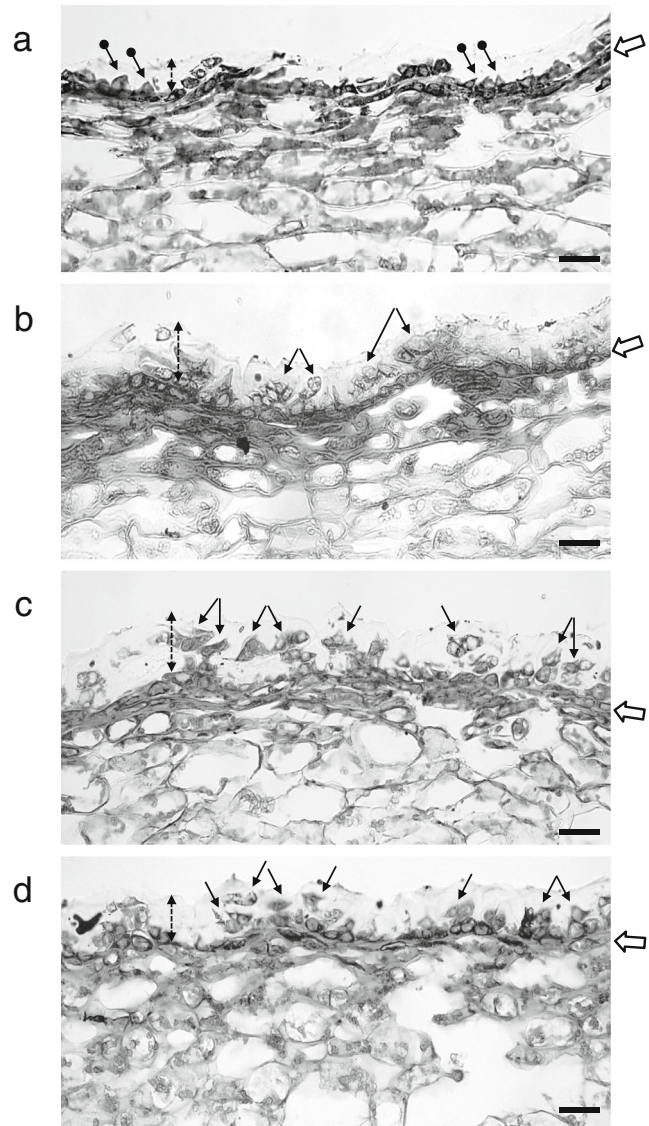


Fig. 1 Peel morphology of “Pink Lady” apples at the end of the growing season (210 days after full bloom (DAFB)) following PGR treatments. Cross sections were made through the surface of untreated control fruits (a) and fruits sprayed three times—at 21, 35, and 50 DAFB—with 0.025 % (v/v) Superlon™ (BA plus GA₄₊₇, 5 mg l⁻¹ each) (b), 0.1 % (v/v) Superlon™ (20 mg l⁻¹ each) (c), and 0.2 % (v/v) Superlon™ (40 mg l⁻¹ each) (d). Double-headed dashed arrows indicate cuticle thickness. In the control (a), triangle-shaped epidermis cells (rounded-tail arrows) are situated at the base of the cuticle. A darkened layer of elongated and tangentially flattened hypodermis cells is located below the epidermis, labeled with a block arrow on the right-hand side of each panel. PGR-treated samples (b–d) contain clusters of epidermal cells (regular arrows) that are detached from the native epidermis and hypodermis layers. The cuticular matrix of untreated control fruits is devoid of these cell clusters. Bar=50 µm

layer of tangentially flattened hypodermis cells (Fig. 1a, block arrows on the right).

The cuticular membrane of the PGR-treated fruit seemed to be thicker and included many more epidermal cells than in the control; these epidermal cells were arranged in cell aggregates that seemed to be detached from the native epidermal layer (Fig. 1b–d, arrows). Examination of these sections also suggested that the detached epidermal cells secrete cutin or wax around them, and accordingly, the cuticular membrane of the PGR-treated fruits seemed less homogeneous, with micro-cracks that did not extend to the epidermal cell walls, compared to the homogeneous and clear cuticle of the control (Fig. 1).

The epidermal cells in the cuticular matrix were further examined by confocal microscopy (Fig. 2). Whereas the peel sample from control fruit contained triangular epidermal cells positioned at the base of the cuticular membrane with heavy cuticular deposits above them, the peels of the PGR-treated fruit showed a different pattern: the cuticular membrane contained multiple cells, some in duplicate or triplicate, supporting the results from the visible light microscopy.

Epidermal cell organization

To study the spatial arrangement of the epidermal cell aggregates within the cuticular matrix, cross sections were made tangential to the surface of the fruit from tissue blocks with known orientation respective to the calyx end (Fig. 3). The first sections removed 15–30 μm of the outermost cuticle layer and exposed the epidermal cells that were embedded within the cuticular matrix (Fig. 3a). Since the apple surface is curved, sections also exposed some of the fruit flesh parenchyma cells (Fig. 3b); however, the cuticular matrix still remained at their margins (Fig. 3b–c). The upper view of the section under the microscope revealed the epidermis cells embedded in the semi-transparent cuticular matrix at the margins of the section (Fig. 3c).

Microscopic observations showed that the epidermal cells embedded in the cuticular matrix are oriented parallel to the longitudinal axis of the fruit, i.e., the cell aggregates were facing the calyx end (Fig. 3d–f). In sections taken from the PGR-treated fruits, the aggregates of the epidermal cells could be easily identified, arranged in elongated clusters (Fig. 3d–e). In the section taken from the control treatment, the epidermal cells seemed to be fewer, shrunken, and less dense (Fig. 3f), supporting the previous data.

Measurements of cuticular membrane thickness and epidermal cell density

The anatomical studies with the visible light microscope were used to determine the thickness of the cuticular membrane of the mature fruits (Fig. S1). This included samples from three

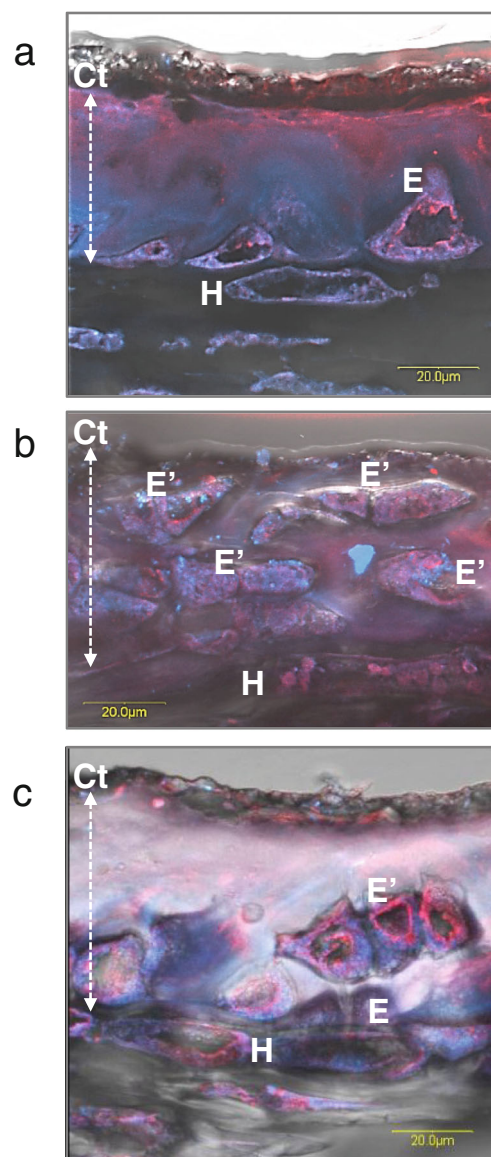


Fig. 2 Anatomy of the cuticular membrane of mature “Pink Lady” apples as viewed by confocal microscopy. Fresh cuts were made through the surface of untreated control fruits (a) and fruits sprayed three times during the growth phase of fruit cell division (21, 35, and 50 days after full bloom (DAFB)) with 0.1 % (v/v) Superlon™ (BA plus GA₄₊₇, 20 mg l⁻¹ each) (b) or 0.2 % (v/v) Superlon™ (40 mg l⁻¹ each) (c). Confocal studies were performed on fruit harvested at the end of the growing season (210 DAFB). Sections were stained with FM-64 to enhance visualization. Figures represent superposition of frames taken with three filters: visible light and autofluorescent and FM-64 staining. Bar=20 μm . White dashed arrows indicate cuticle (Ct) thickness. Triangle-shaped epidermis cells (E) are situated at the base of the cuticle. Elongated and tangentially flattened hypodermis cells (H) are located below the epidermis. PGR-treated samples (b, c) contain clusters of epidermal cells (E') that are detached from the native epidermis and hypodermis layers and seem to be “floating” in the cuticular membrane

to six fruits from each treatment and the control. Results indicated significantly thicker cuticle in fruits treated with 20 mg l⁻¹ PGR solution compared to the control (Table 1). Intermediate value was obtained for the 40 mg l⁻¹ PGR

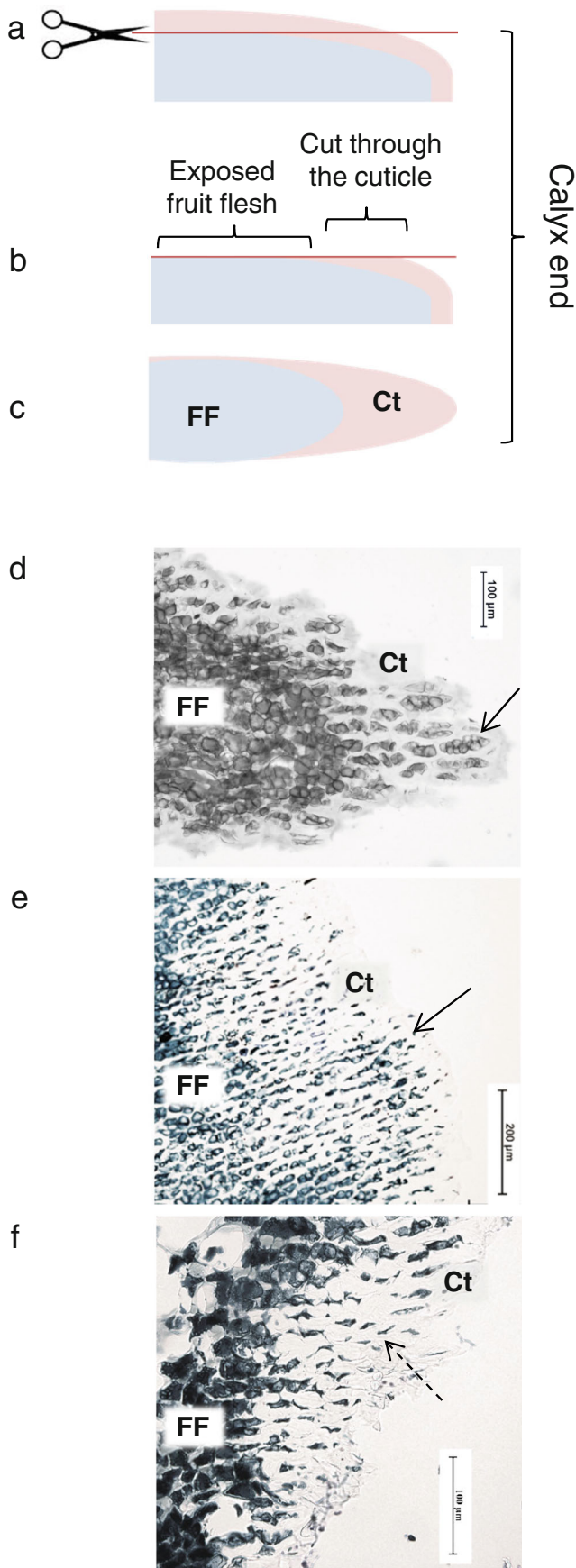


Fig. 3 Orientation of the epidermal cell clusters within the cuticular matrix with respect to the calyx end of mature “Pink Lady” fruit (210 days after full bloom (DAFB)). **a–c** Sample preparation. **d–f** Respective fruit sections. Tissue blocks were taken from the calyx end, and sections (15 µm) were made tangential to the surface of the fruit (**a**). Since the fruit sample is curved, some of the fruit flesh (FF, blue region) was exposed with margins of cuticular matrix (Ct, pink region) (**b**). **a, b** Side views of the sample. **c** Resultant view from above. The respective sections are of fruits treated with 0.025 % (v/v) Superlon™ (BA plus GA₄₊₇, 5 mg l⁻¹ each) (**d**) and 0.1 % (v/v) Superlon™ (20 mg l⁻¹ each) (**e**) at 21, 35, and 50 DAFB and of untreated control fruit (**f**). **d–f** Dark region on the left is the fruit flesh (FF) and corresponds to the blue region in **c**. The semi-transparent region on its right is cut through the cuticular matrix (Ct) with clusters of epidermal cells embedded in it (indicated by arrows). The cell clusters are oriented toward the calyx end, parallel to the longitudinal axis of the fruit. Epidermal cells of control fruit seem shrunken (broken arrow) relative to the epidermal clusters of the treated fruits (unbroken arrow). Bars=100–200 µm

treatment that was applied at the cell-expansion phase of fruit growth. Cuticle thickness following the 5 mg l⁻¹ PGR treatment and the 40 mg l⁻¹ PGR treatment that was applied at cell-division phase did not differ from the untreated control (Table 1).

The same histological sections were used to determine epidermal cell density in fruit following the PGR treatments. Counting included epidermal cells that were attached to the hypodermal layers and those that were detached from it (Table 1). Total number of epidermal cells was higher in the PGR-treated fruits except for the lower concentration of 5 mg l⁻¹ solution, which was similar to the untreated control. Of these, the number of cells that remained attached to the hypodermal layer was similar for all samples, but the number of detached cells and their fraction out of the total epidermal cells in the peel was higher in the PGR-treated fruits compared to control (Table 1). These results supported the above-described anatomical studies.

Furthermore, a positive correlation ($R^2=0.7$) was found between cuticle thickness and the percentage of detached epidermal cells of PGR-treated fruits (Fig. S2), implying that the fraction of PGR-induced detached cells contribute to the thickness of the cuticle membrane.

Rate of calyx-end cracking in apple fruit

To evaluate the association between peel morphology at the calyx end and fruit susceptibility to calyx-end cracking, apple fruit from the PGR treatments and control were collected for determination of cracking rate at harvest. Results indicated lowest rate of calyx-end cracking in the 20 mg l⁻¹ PGR treatment and highest rates in the control (Fig. 4). Intermediate values were obtained following application of 5 mg l⁻¹ PGR solution and the 40 mg l⁻¹ treatment that was applied at the cell-division phase of fruit growth (Fig. 4). Application of 40 mg l⁻¹ PGR solution at cell-expansion phase of fruit development was less effective in reducing cracking incidence.

Table 1 Fruit cuticle thickness and epidermal cell density at the end of the growth season

BA+GA ₄₊₇ treatment ^a	Ct ^b	Number of EC ^c			EC (%) ^g	
		Total ^d	Attached ^e	Detached ^f	Attached	Detached
Control	39.1 B	23.5 BC	20.5 A	3 B	87.3 A	12.7 B
5 mg l ⁻¹ ×3	38.5 B	21.6 C	15.4 A	6.2 B	67.3 B	32.7 A
20 mg l ⁻¹ ×3	48.3 A	32 AB	16.8 A	15.2 A	53.8 B	46.2 A
40 mg l ⁻¹ ×3	39.2 B	34.8 A	20.8 A	14 A	59 B	41 A
40 mg l ⁻¹ ×3; II ^h	44.3 AB	36.5 A	20.5 A	16 A	56.8 B	43.3 A

Values are average of three to six replicates. Data was analyzed by Student's *t* test using the JMP software. Mean values followed by different uppercase letters differ significantly at $P \leq 0.05$

^a Concentration (mg l⁻¹)

^b Cuticle thickness in micrometer

^c Number of epidermal cells (EC) along an artificial line of 1400 µm tangential to fruit surface

^d Total number of EC

^e Number of EC attached to the hypodermal layers (out of total EC)

^f Number of EC that were suspended in the cuticular membrane (out of total EC)

^g Percentage of attached and detached EC out of total EC

^h Second growth phase

A weak but negative association was found between the incidence of calyx-end cracking and the thickness of the cuticle ($R^2=0.3$; Fig. S3) in fruits that were treated with the

PGR solutions at cell-division phase of fruit growth and control. However, a stronger negative correlation was found between the incidence of calyx-end cracking and the fraction of the detached epidermal cells in the respective fruits ($R^2=0.9$; Fig. S4), suggesting that epidermal cell clusters that were embedded in the cuticular membrane contributed to the reduction of calyx-end cracking.

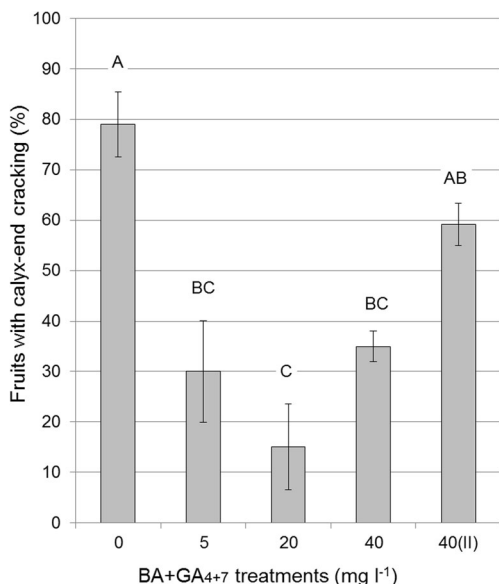


Fig. 4 Effect of PGR treatments on the incidence of “Pink Lady” fruit with calyx-end cracking at harvest. Fruits were sprayed three times—at 21, 35, and 50 days after full bloom (DAFB)—with 0.025 % (v/v) Superlon™ (BA plus GA₄₊₇, 5 mg l⁻¹ each), 0.1 % (v/v) Superlon™ (20 mg l⁻¹ each), and 0.2 % (v/v) Superlon™ (40 mg l⁻¹ each). The latter concentration was also applied at the fruit cell-expansion phase [40(II)]—at 50, 65, and 80 DAFB. Fruits were harvested at 210 DAFB for estimation of cracking incidence. Each data point is the mean of five to six trees per treatment ($n=20$ fruit per tree). Data was analyzed by one-way ANOVA followed by Tukey-Kramer HSD comparison for all pairs. Different uppercase letters denote significant differences between means ($P \leq 0.05$)

Discussion

Calyx-end cracking disorder of “Pink Lady” fruits develops toward the end of the growing season, around 1 month prior to harvest. We previously showed that multiple applications of BA+GA₄₊₇ at early stages of fruit development reduce cracking incidence (Stern et al. 2013). We further showed that applications of BA+GA₄₊₇ to the developing fruit up to 75 DAFB increase the density of the epidermis cells at the calyx end of the apple (Ginzberg et al. 2014). At that time, we monitored the PGR-induced alterations in the peel 1 week after treatment; however, the resistance to calyx-end cracking was maintained until harvest—at 180–210 DAFB—implying a long-term change in the peel. In the present work, epidermal cell density and cuticle thickness were monitored at the end of the growing season, 4 to 5 months after the PGR treatments were done.

The most pronounced phenomenon observed in fruit treated with the PGRs (at all concentrations tested) was the presence of cell clusters in the cuticular matrix. These clusters

were detached from the native epidermal layer that is located at the bottom of the cuticle and from the hypodermal layer below it (Figs. 1 and 2). Similar epidermal cell clusters could not be detected in the untreated control fruit where the native epidermal cells seemed more shrunken (Fig. 3). Accordingly, PGR-treated fruits had increased number of epidermal cells and higher percentage of the detached cells compared to control (Table 1), supporting the microscopic observation.

The percentage of the detached epidermal cells that were suspended in the cuticular membrane was positively correlated with the thickness of the cuticular membrane ($R^2=0.7$)—this could be due to enhanced accumulation of cuticle and wax by greater number of epidermal cells, or that the space occupied by these cells contributed to its thickness.

The PGR treatment of 20 mg l⁻¹ BA+GA₄₊₇ applied at the cell-division phase of fruit growth produced a significantly thicker cuticle and greater fraction of detached epidermal cells (Table 1) with a significant reduction in calyx-end cracking at harvest (Fig. 4). The treatments of 5 and 40 mg l⁻¹ BA+GA₄₊₇ also resulted in high fraction of detached epidermal cells and reduced cracking incidence; however, their cuticle thickness did not differ significantly from that of the control (Table 1). Respectively, cracking incidence was negatively correlated to the fraction of the detached cell ($R^2=0.9$) while showing weaker negative correlation to cuticle thickness ($R^2=0.3$). It is suggested that both components—the epidermis and the cuticular matrix—are involved in the reduction of cracking incidence, as epidermal characteristics may play a major role. The increase in cell density may produce more cell walls per unit surface area, further providing strong structural support of the peel (Knoche et al. 2011).

A link between epidermal development and cuticle formation has been previously reported. SHINE clade transcription factors were found to regulate the transcriptional network that acts in epidermal patterning and fruit-cuticle formation and links cutin metabolism with the more global program of epidermal cell patterning and organ formation in tomato fruit (Shi et al. 2011, 2013). Similarly, MIXTA-like MYB transcription factors, which regulate epidermal cell morphology, also regulate cuticle development coordinately with WAX INDUCER1/SHINE1 transcription factor that regulates the biosynthesis of waxy substances in *Arabidopsis thaliana* (Oshima et al. 2013). Furthermore, studies of cuticular mutants suggest that the process of epidermal cell patterning is tightly associated with the metabolism of cuticle constituents, as mutations in cuticle genes often result in altered trichome, epidermal pavement cell, and stomatal development (Bird and Gray 2003; Kurdyukov et al. 2006; Yephremov et al. 1999). The chemical constituents of the cuticle may differ in the untreated control vs. PGR-treated “Pink Lady” fruits, as well as between different stages of fruit development (Martin and Rose 2014); the composition of the cuticle and its contribution to reduced calyx-end cracking remains to be elucidated.

The cuticle is mainly composed of a three-dimensional network of cutin and integrated and superimposed lipids called “waxes”; superimposed waxes are also called “epicuticular waxes” (Koch and Ensikat 2008). A confocal laser scanning microscopy study of the surface of intact apple fruits identified a thin wax layer of crystalline character with spots of amorphous wax deposits (Veraverbeke et al. 2001). Below it was the cutin layer and at its bottom, the epidermal cell walls. The outer wax surface was smooth, but covered with a network of micro-cracks; the latter delineated a group of three to five epidermal cells, suggesting that the cracks do not correspond to the anticlinal walls of the epidermal cells (Curry 2009; Veraverbeke et al. 2001) and implying that shallow micro-cracks are expected in the apple peel and may have no impact on fruit quality. However, the cracks may deepen and extend toward the epidermal walls as a result of splitting of the wax platelets (Roy et al. 1999).

Cuticular wax biosynthesis is known to occur predominantly in epidermal cells. Its components are generated in the endoplasmic reticulum and are deposited on the plant surface via Golgi-mediated secretory vesicles, ATP-binding cassette transporters (McFarlane et al. 2010), and lipid transfer proteins (Kim et al. 2012). This suggests that epidermal cells may fix deeper fissures by continuous secretion of cuticular components—in the present context, the epidermal cell clusters in the cuticular matrix may also play a role in curing cracked cuticle and preventing further damage to the fruit. Filling of the cracks in apples may occur via a tear-and-repair mechanism, where extension of wax microtubules and microtubular aggregates from both sides of the separated surface fill in the crack (Curry 2009). Complementary to this, a positive effect of gibberellin treatments has also been shown in other systems. It was suggested that gibberellin delays peel senescence and strengthens the peel due to increased cellulose content and extends epidermal cell viability, even if applied shortly before harvest (Ben-Arie et al. 1996; Biton et al. 2014; Lewis et al. 1967).

Konarska (2013) suggested that cell-division activity may extend to later stages of apple fruit development and also showed an increase in the thickness of the wax layer during storage, which again indicates activity in the epidermal/hypodermal layers of the fruit after harvest. This is in accordance with other reports showing that synthesis of cuticular waxes proceeds throughout the fruit’s life and continues in storage until the substrates in the epidermal cells are exhausted or tissue necrosis occurs (Belding et al. 1998; Morice and Shorland 1973). Our observations suggest extended viability following the BA+GA₄₊₇ treatments while epidermal cells from untreated controls seemed fewer and shrunken (Figs. 2 and 3).

Viewing the “Pink Lady” fruit surface from above showed that cell clusters and, accordingly, the wax platelets that surround them are organized in elongated lineages that form

parallel rows pointing toward the calyx end (Fig. 3). Konarska (2013) also reported the arrangement of wax platelets in parallel rows in the peel of other apple cultivars. The size and orientation of the epidermal cells might also contribute to the resistance of the peel to tension stresses during the fruit-expansion phase. Using onion epidermis peels, Vanstreels et al. (2005) suggested that tissue consisting of many small cells is stiffer than that consisting of larger cells due to the increase in cell-wall material. However, when tensile forces are applied in a direction perpendicular to the main elongation axis of the cells, tissue stiffness and strength are significantly reduced. In “Pink Lady”, cracks at the calyx end appear vertical to the direction of the epidermal cells and the wax platelets, meaning that growth-induced tensile forces are applied in the direction of the longitudinal axis of the cells. The implication for susceptibility of the calyx end to cracking requires further clarification.

In summary, this work and our previous studies, which overall cover six experimental years in different orchards and under different annual climatic conditions, show that spraying of BA+GA₄₊₇ modifies fruit-peel anatomy and reduces calyx-end cracking incidence. The PGR treatments at early stages of fruit development induced an increase in epidermal cell density that at later stages of fruit maturity resulted in detached clusters of these cells suspended in the cuticular matrix. This might have contributed to strengthening the peel by adding more cell-wall components, thickening the cuticle layer, and possibly by enhancing crack repair.

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Conflict of interest The authors declare that they have no conflict of interest.

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