Accumulation of anthocyanins in tomato skin extends shelf life

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Summary

Shelf life is one of the most important traits for the tomato industry. Two key factors, postharvest over-ripening and susceptibility to post-harvest pathogen infection, determine tomato shelf life.

Anthocyanins accumulate in the skin of *Aft/Aft atv/atv* tomatoes, the result of introgressing alleles affecting anthocyanin biosynthesis in fruit from two wild relatives of tomato, which results in extended fruit shelf life. Compared to ordinary, anthocyanin-less tomatoes, the fruits of *Aft/Aft atv/atv* keep longer during storage and are less susceptible to *Botrytis cinerea*, a major tomato pathogen, postharvest.

Using genetically modified tomatoes over-producing anthocyanins, we confirmed that skin-specific accumulation of anthocyanins in tomato is sufficient to reduce the susceptibility of fruit to *B.cinerea*.

Our data indicate that accumulation of anthocyanins in tomato fruit, achieved either by traditional breeding or genetic engineering can be an effective way to extend tomato shelf life.

Key words: anthocyanins, tomato, *Aft/Aft atv/atv*. *Botrytis cinerea*, shelf life,

1. Introduction

Shelf-life is one of the most important agronomic traits for tomato and is determined by two components, fruit softening during over-ripening and susceptibility to opportunistic pathogens. *Botrytis cinerea*, better known as gray mold, is the second most important fungal pathogen of plants, economically (Dean *et al.*, 2012). *B. cinerea* can infect vegetables (cabbage, lettuce and broccoli) and fruit crops (grape, red fruit and tomato), as well as a large number of shrubs, trees, flowers, and weeds (Williamson *et al.*, 2007). Several different strategies have been employed to extend tomato (*Solanum lycopersicum*) shelf life. One major target has been cell-wall modifying enzymes, and different strategies have been developed to decrease their activity (Brummell & Harpster, 2001). Other studies have been directed at increasing the production of antioxidants such as polyamines, because their accumulation is associated with extended shelf life (Valero *et al.*, 2002).The ethylene burst is the key event signaling the onset of ripening in climacteric fruits such as tomato. Manipulation of ethylene biosynthesis and signaling has resulted in varieties with delayed ripening (Vicente *et al.*, 2007). However, all attempts have resulted in only modest delays to the fruit softening processes and are often accompanied by reduced flavour, texture and aroma of tomato fruit (Vicente *et al.*, 2007). Anthocyanins are a group of natural pigments, widely distributed in most vascular plants (Grotewold, 2006). They are stress responsive compounds, used for pollinator and dispersor attraction, but they are also important phytonutrients in a healthy diet, having anti-tumor, pro-apoptotic, anti-oxidative, anti-inflammatory and anti-neurodegenerative properties (Buer *et al.*, 2010; De Pascual-Teresa *et al.*, 2010; Spencer, 2010). Due to their dietary health benefits, anthocyanins are often targets for engineering and plant-breeding programs. Crops having sub-optimal concentration of anthocyanins, like tomato, have been genetically modified to increase their content (Butelli *et al.*, 2008a; Gonzali *et al.*, 2009). Several mutants of tomato, altered in their ability to synthesize anthocyanins have been described (Al-sane *et al.*, 2011). The dominant gene *Aft* (*Anthocyanin fruit*) derived from the interspecific cross of *Solanum lycopersicum* (tomato) to *S. chilense* shows anthocyanin production in the skin of fruit (Jones *et al.*, 2003). *Aft* triggers anthocyanin production and accumulation in fruits upon stimulation by high light (Mes *et al.*, 2008). *Aft* gene has been suggested to encode a MYB-related transcription factor (Sapir *et al.*, 2008). A recessive gene, *atv* (*atroviolacea*), was introgressed into domesticated tomato plants following a cross between *S.lycopersicum* and *S. cheesmaniae* (L. Riley) Fosberg, and influences anthocyanin pigmentation in the entire tomato plant, particularly in stems and leaves (Mes *et al.*, 2008). Tomato plants homozygous for both *Aft* and *atv* alleles show intensely purple-pigmented fruits (Mes *et al.*, 2008). Anthocyanin synthesis in *Aft/Aft atv/atv* is stimulated significantly by high

light and is limited to the epidermis and the pericarp of the fruit, which may have both purple and red regions, depending on exposure of the fruit to light (Fig. S1). Recently, we reported that purple tomatoes, producing anthocyanin throughout the fruit as a result of the ectopic expression of Delila and Rosea1 transcription factors from *Antirrhinum majus*, have double the shelf life of controls (Zhang *et al.*, 2013). In this study, we show that the accumulation of anthocyanins in *Aft/Aft atv/atv* tomatoes, which is predominantly in the skin, is also associated with extended shelf life. Our finding has important agronomic and commercial implications, since *Aft/Aft atv/atv* tomatoes are naturally enriched in anthocyanins and have extended shelf life.

2. Materials and Methods

Storage tests

Near isogenic lines for either *Aft/Aft* or *atv/atv* mutations are not available, so *S. lycopersicum* cv. Ailsa Craig was chosen as a control tomato line for all the analyses. This choice was made because, unlike the *Aft* and *atv* mutant lines, Ailsa Craig does not produce anthocyanins in the skin of fruit although it shows the same vegetative and fruit characteristics (morphology of the plant and its fruit, size of mature tomatoes, and fruit ripening time) compared to *Aft/Aft*, *atv/atv* and *Aft/Aft atv/atv* fruit (Povero *et al.*, 2011).

WT (cv. Ailsa Craig) and *Aft/Aft atv/atv* fruit were tagged at breaker (when the color of WT fruit and the low-anthocyanin regions of *Aft/Aft atv/atv* fruit begin to turn yellow). To induce high anthocyanin production in *Aft/Aft atv/atv* fruit, tomatoes were grown with supplemented light. *Aft/Aft atv/atv* fruit grown under high light have strong, uniform anthocyanin accumulation in the skin (Fig. S1) (Mes *et al.*, 2008; Povero *et al.*, 2011). Fruit were harvested at seven days post breaker (d0=7dpb). All fruits were sterilized in 10% bleach for 10 minutes, followed by rinsing in sterilized water and air-drying. Each fruit was placed in a plastic jar and kept at 17°C or at room temperature (RT) under light. Every week, the fresh weight of each fruit was measured and visual softening and collapse of the fruit were assessed (Nambeesan *et al.*, 2010). After measurement, fruit were transferred to a new jar.

TEAC assay and anthocyanin quantification

TEAC analysis of *Aft/Aft atv/atv* tomatoes was performed at breaker as described by (Pellegrini *et al.*, 2003). Results were expressed as TEAC (Trolox equivalent antioxidant capacity) in mmol of Trolox per kg of fresh weight. Anthocyanin extraction from the skin of *PRD* tomatoes was performed as described by (Butelli *et al.*, 2008b).

Measurements of cuticle thickness

Cuticle thickness measurements were modified from the methods described by Yeats *et al.* (2012). Wild type (WT) Ailsa Craig, *Aft/Aft atv/atv* red regions and *Aft/Aft atv/atv* purple regions were sliced into 10-30 μm thick sections, stained with Sudan red (Fluka) (Buda *et al.* (2009) and thickness was determined using a Leica DM6000 microscope, taking the average of 8-10 measurements. The average and standard error of the mean of three biological replicates are reported.

Botrytis cinerea *infection*

B.cinerea (B05.10) was grown and collected as described by Stefanato *et al.* (2009). WT (Ailsa Craig) and *Aft/Aft atv/atv* tomatoes were harvested 14 days after breaker and surface sterilized. Intact wild type and *Aft/Aft atv/atv* fruits were sprayed thoroughly with spores (2.5×10^5 spores/mL) three times in the flow cabinet and kept at 20°C, in high humidity. Infection symptoms were observed at 4dpi. For wound inoculation, the fungal culture was diluted with medium to 5×10^4 spores/mL (for fruit in the MicroTom genetic background) or 2.5×10^5 spores/mL (for WT Ailsa Craig and *Aft/Aft atv/atv* fruits) and incubated at RT for 1.5 h prior to inoculation. The spore innoculum (5μL) was added to each wound of both red and purple regions of *Aft/Aft atv/atv* fruits grown under natural light. Lesion diameter was measured 72 hours after inoculation. To quantify *Botrytis* growth using qPCR, 1cm samples of infected fruit tissues were harvested three days after inoculation. Seeds were removed and samples were freeze dried. Total DNA was isolated and qPCR was performed as described previously (Zhang *et al.*, 2013).

Plasmid construction and tomato transformation

The light-responsive, PLI promoter which is active predominantly in fruit peel was kindly provided by Dr. Diego Orzaez (Estornell *et al.*, 2009). Using Gateway recombination, the PLI promoter was introduced into pDONR 207 to create pENTR-PLI. The PLI promoter was then inserted into the binary vector pJAM1890 (GATEWAY:Ros1/35S:Del) (Martin *et al.*, 2012) to create pPLI:Ros1/35S:Del (pPRD). pPRD was transferred to *Agrobacterium tumefaciens* strain AGL1 by triparental mating. Tomato variety MicroTom was transformed by dipping cotyledons (Fillatti *et al.*, 1987). More than 40 *PRD* T0 independent transgenic lines were produced. Among these, 12 stable T1 lines accumulating different amounts of anthocyanins were selected for further analysis.

Staining of seed for proanthocyanidins

Tomato seed were stained for proanthocyanidins using 4-dimethylaminocinnamaldehyde (DMACA) as described previously (Abeynayake *et al.*, 2011).

Statistics

Paired or unpaired, two-tailed Student's t-tests were used to compare group differences. p values less than 0.05 were considered significant.

3. Results

Aft/Aft atv/atv *tomato can be stored longer*

To test whether softening is delayed in *Aft/Aft atv/atv* tomatoes, we performed storage tests under different conditions. WT (Ailsa Craig) and *Aft/Aft atv/atv* tomatoes (grown with supplemental light) were harvested one week after breaker. For *Aft/Aft atv/atv* fruit, 70 days of storage at 17°C were required to observe 100% of the fruit softened, equivalent to the level of softening observed in Ailsa Craig fruits at 42 days (Fig. 1a, c) and the proportion of fresh weight loss was higher in Ailsa Craig than in *Aft/Aft atv/atv* fruit (Fig. 1b).We repeated the storage test at RT and observed similar results (Fig. 1e, f). After storage for 42 days at RT, the seed in Ailsa Craig fruits showed viviparous germination, followed by complete fruit collapse while *Aft/Aft atv/atv* tomatoes did not (Fig. 1d). The absence of precocious germination was due to elevated anthocyanin levels in the seed of *Aft/Aft atv/atv* plants, rather than elevated levels of proanthocyanins (Fig. S2). The suppression of precocious germination by anthocyanins in the seed has been observed for *Del/Ros1* purple tomatoes (Butelli *et al.*, 2008b) and has been reported following studies of transparent testa mutants in *Arabidopsis* (Abeynayake *et al.*, 2011) and for red wheat compared to white wheat (Flintham, 2000).

Because tomato is a climacteric fruit, ethylene promotes ripening. However, no difference in ethylene production or signaling were detected between high anthocyanin *Del/Ros1* purple tomatoes and WT tomatoes (Zhang *et al.*, 2013). In addition, due to the light-dependant induction of anthocyanin accumulation of *Aft/Aft atv/atv* fruit, tomatoes grown under natural light have both purple and red skinned regions on the same fruit (Povero *et al.*, 2011). The purple regions have high levels of anthocyanins in the skin, whereas the red regions have very low levels of anthocyanins. The red, low anthocyanin regions underwent normal over-ripening compared to WT Ailsa Craig fruit, and showed more rapid softening than purple regions on the same fruit (Fig. S3). This showed that the rate of fruit softening was a localized function associated with anthocyanin production, and therefore not caused by differences in production of the volatile, ethylene. Taken

together these results suggest that the accumulation of anthocyanins in the peel of tomato fruits is sufficient to delay postharvest over-ripening and extend shelf life, although the extension of shelf life was not as great as the doubling observed between purple *Del/Ros1* tomatoes and their WT controls (Zhang *et al.*, 2013).

Susceptibility to the necrotrophic pathogen **Botrytis cinerea**

The susceptibility of *Aft/Aft atv/atv* fruit to *B. cinerea* was investigated by infecting wounded or intact tomato fruits with fungal spore suspensions. To compare better susceptibility to *B.cinerea* with anthocyanin pigmentation, both purple regions and red regions of fruit grown under natural light were tested. Each *Aft/Aft atv/atv* fruit was sprayed on both purple and red regions or wounded and inoculated with spore cultures of *B. cinerea* strain B05.10. At three days post inoculation (dpi) the proportion of fruits showing symptoms of infection in the purple regions was significantly smaller than for the red regions (Fig. 2a, b). Fungal growth was significantly reduced in *Aft/Aft atv/atv* purple regions compared to growth in the red regions and to growth in the WT line (Ailsa Craig) (Fig. 2c, d). Together, these data demonstrate that resistance was a consequence of anthocyanin accumulation in the purple regions and that anthocyanin pigmentation limited to fruit skin is sufficient to reduce susceptibility to this important necrotrophic pathogen. *Botrytis cinerea* infection induces an oxidative burst by generating reactive oxygen species necessary for pathogen infection (Govrin & Levine, 2000). The reduced susceptibility of anthocyanin-enriched *Aft/Aft atv/atv* fruits could be due to their antioxidant activity, which might counterbalance the oxidative burst induced by the fungus, so limiting pathogen growth (Zhang *et al.*, 2013). Anthocyanin levels are high in *Aft/Aft atv/atv* tomatoes (Mes *et al.*, 2008; Povero *et al.*, 2011) and their prescence in anthocyanin-enriched tomato regions correlates with the antioxidant capacity of those regions. Those *Aft/Aft atv/atv* purple fruits that accumulated the highest concentrations of anthocyanins, as a result of greater exposure to light, had the highest antioxidant capacities (Fig. 3a). Increased cuticle thickness has been reported to be associated with longer shelf life (Yeats *et al.*, 2012), but we observed no significant differences in this trait between *Aft/Aft atv/atv* and Ailsa Craig tomatoes (Fig. 3b). These data suggest that the reduced susceptibility to *B. cinerea* in anthocyanin-enriched fruit is due to their antioxidant content rather than to differences in cuticle thickness.

Accumulation of anthocyanins in tomato fruit skin by genetic modification can extend shelf life.

To confirm that the enhanced pathogen resistance observed in *Aft/Aft atv/atv* fruit was due to anthocyanin accumulation and not to another, unknown, trait linked to or associated with either *Aft* or *atv*, we generated tomato lines which accumulated anthocyanins predominantly in skin by

genetic modification. Because the *Aft* gene is induced by light, anthocyanins accumulate predominantly in the skin of *Aft/Aft atv/atv* fruit (**Fig. S4A**) (Jones *et al.*, 2003; Mes *et al.*, 2008; Povero *et al.*, 2011). We used the promoter of the *PLI* gene, which is induced by light and is active mainly in tomato skin (Estornell *et al.*, 2009), to drive the expression of the MYB transcriptional factor *Rosea 1*. (Martin *et al.*, 2012) We expressed *PLI:Ros1* together with *35S:Del* in tomato using a binary vector that carried both gene constructs (Martin *et al.*, 2012). The new PLI:Ros1/35S:Del (PRD) lines accumulated high levels of anthocyanins in fruit skin, with much less anthocyanin in the fruit flesh compared to previously reported *E8:Del/Ros1* lines (Butelli *et al.*, 2008b). From among more than 40 independent transgenic lines, two lines, *PRD8-2* and *PRD17-2*, which differentially accumulated anthocyanin in the fruit skin, were selected (Fig 4a, b). Although both lines accumulated low levels of anthocyanins in flesh, the anthocyanin contents of the flesh of the *PRD* lines were much lower than in *E8:Del/Ros1* lines (Fig 4a, b and Fig. S4b) When fruit were inoculated with *B.cinerea* culture, both *E8:Del/Ros1* and *PRD* lines showed smaller lesion size at 3dpi compared to WT fruits (Fig 4c). Similar results were observed by spraying intact fruit with *B.cinerea* spores. The proportion of fruit showing severe infection was always lower for the transgenic lines compared to WT fruits (Fig 4d). In both cases, susceptibility was inversely correlated with anthocyanin content; *E8:Del/Ros1* N and *PRD8-2* tomatoes, which had the highest concentration of anthocyanins, were less susceptible to *B.cinerea* than *PRD17-2* and other transgenic lines. These results showed that accumulation of anthocyanins in tomato skin is sufficient to reduce the susceptibility of fruit to *B.cinerea*.

4. Discussion

One of the biggest challenges for the tomato industry is to reduce post-harvest losses resulting from fruit softening and post-harvest infection by several pathogens. So far, biotechnological strategies have been adopted to extend the shelf-life of tomatoes, often at the expense of flavour, aroma, and texture (Baldwin *et al.*, 2011). Anthocyanins, induced by gamma irradiation, have been suggested to prolong the shelf life of grape pomace (Ayed *et al.*, 1999). Recently, we have shown that genetically modified tomatoes accumulating high levels of anthocyanins in fruit have an extended shelf life compared to controls (Zhang *et al.*, 2013). Here, we show that *Aft/Aft atv/atv* tomato fruit accumulating anthocyanins in the skin have an extended shelf life compared to WT tomatoes. The anthocyanin-enriched sectors of *Aft/Aft atv/atv* tomatoes are less susceptible to *B. cinerea* infection in both wound and spray tests (Fig. 2a,b) and this is correlated to the higher antioxidant capacity of purple tomatoes compared to WT (Fig. 3a). Furthermore, the *Aft/Aft atv/atv* tomatoes showed delayed over-ripening (Fig. 1a, b). Fifty percent softening of *Aft/Aft atv/atv* fruits occurred between

1-2 weeks later than for WT (Ailsa Craig) tomatoes (Fig. 1c, d) demonstrating an extended shelf life both at 17°C and at room temperature. Additionally, susceptibility to infection by opportunistic pathogens during storage was higher for red fruit than for purple ones, seeds from *Aft/Aft atv/atv* fruit are not viviparous compared to seed from wild type fruit (due to anthocyanin accumulation in *Aft/Aft atv/atv* seed), and *Aft/Aft atv/atv* tomatoes can be stored longer at RT which could reduce the cost of shipping and storage. Taken together, these data show that *Aft/Aft atv/atv* tomatoes have enhanced shelf life due to delayed over-ripening and reduced susceptibility to *Botrytis*. The increase in shelf life correlated with the prescence of anthocyanins and the antioxidant activity of these anthocyanins could also explain the lower susceptibility to *B. cinerea* (Fig. 3b) (Zhang *et al.*, 2013). To confirm that skin-specific accumulation of anthocyanins in tomato is sufficient to reduce the susceptibility to *B.cinerea* and extend shelf life, we also produced tomatoes which accumulated anthocyanins predominantly in their skin (*PDR* lines). *PDR* fruits, either sprayed or wounded, showed a reduced susceptibility to *B. cinerea* infection (Fig. 4) and susceptibility was inversely correlated with anthocyanin levels. These data strongly support our observations of the extended shelf life of *Aft/Aft atv/atv* tomatoes. This study demonstrates clearly that anthocyanin accumulation in skin is sufficient to reduce susceptibility to *B. cinerea* in tomato fruit. The ability to synthesize anthocyanins in the fruit skin in *Aft/Aft atv/atv* tomatoes could be exploited by breeders to obtain new tomato varieties with both extended shelf life and reduced susceptibility to *B.cinerea.*

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Figure Legends

Figure 1. Accumulation of anthocyanins in *Aft/Aft atv/atv* tomatoes delays late ripening

Ailsa Craig red, and *Aft/Aft atv/atv*, purple tomato fruits were stored at 17°C (a, b, c) and at room temperature (d, e, f). At 42 days of storage the WT fruit showed severe over-ripening symptoms while the *Aft/Aft atv/atv* fruit were still firm (a, d). *Aft/Aft atv/atv* fruits showed slower decrease in fresh weight (FW) compared to red, Ailsa Craig tomatoes (b, e) and slower over-ripening as determined by the percentage of firm fruit (c, f). Fruits were harvested at 7 days post breaker (d0=7dpb). Fresh weight reduction is presented using the percentage of the initial weight. Error bars show the standard error of the mean ($n \geq 8$). Percentages of fruit showing over ripening symptoms (softening and shriveling) were assessed visually every week during storage tests.

Figure 2. Accumulation of anthocyanins in *Aft/Aft atv/atv* tomatoes reduces susceptibility to *B. cinerea*

(a) and (b) Symptoms of either wounded or sprayed purple and red regions of *Aft/Aft atv/atv* tomatoes fruits after inoculation with *B. cinerea* B05.10. (c) Quantitative PCR revealed more *Botrytis* growing on the red regions of *Aft/Aft atv/atv* fruits than on purple regions at 3dpi. *Botrytis* growth was calculated by comparing the ratio of *Botrytis* DNA to tomato DNA. Error bars show the standard error of the mean (n=3). * ($p<0.05$) compared to control red regions. (d) The ripening-related increase in susceptibility to *Botrytis* did not occur in *Aft/Aft atv/atv* purple regions. Lesion diameter was measured 3dpi. Error bars show the standard error of the mean ($n \geq 3$). * ($p<0.05$) and ** ($p<0.01$) for values for purple regions compared to red regions of *Aft/Aft atv/atv* fruits grown under natural light at the same stage of ripening. Ailsa Craig, which does not synthesize anthocyanins in its fruit, was used as control for *B. cinerea* infection.

Figure 3. Delayed over-ripening and reduced pathogen susceptibility are associated with the increased antioxidant capacity due to increased anthocyanin levels in *Aft/Aft atv/atv* tomatoes.

(a) Trolox equivalent total antioxidant capacity (TEAC) of water and acetone extracts from purple, medium and red regions of *Aft/Aft atv/atv* tomatoes during ripening. Error bars show the standard error of the mean (n=3). * ($p<0.05$) values for purple regions compared to red regions at the same stage. (b) Cuticle thickness of purple and red regions. Measurements were made above the centre of each epidermal cell. Error bars show the standard error of the mean ($n \geq 3$).

Figure 4. *PRD* tomatoes show reduced *B. cinerea* susceptibility

(a) Pictures of different anthocyanin enriched lines: *E8:Del/Ros1* N and C, *PRD* 8-2 and 17-2 tomatoes were taken at the red stage and whole fruit, peeled fruit and skin are shown compared to the wild type Microtom. (b) Anthocyanin levels for all the transgenic tomato lines, error bars show SEM (n=3). (c) All transgenic lines showed less susceptibility to *B.cinerea* in wound infection tests. Lesion diameter was measured 3dpi. Error bars show SEM ($n \geq 3$). * ($p<0.05$) and ** ($p<0.01$) for values of anthocyanin-enriched tomatoes compared to red Microtom fruit at the same stage. (d) In spray tests the proportion of susceptible tomatoes was low in the anthocyanin-enriched lines, in particular in *E8:Del/Ros1* N and *PRD* 8-2, and was inversely correlated with anthocyanin concentration.

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