Rapid report

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 (Solanum lycopersicum) industry. Two key factors, post-harvest 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 with 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, post-harvest.
- 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 Botrytis 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.

Introduction

Shelf-life is one of the most important agronomic traits for tomato (Solanum lycopersicum) 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). Botrytis 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 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 flavor, 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., 2008; 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). The Aft gene has been suggested to encode a MYB-related transcription factor (Sapir...
et al., 2008). A recessive gene, atv (atrosiulacca), 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 (Supporting Information, Fig. S1). Recently, we reported that purple tomatoes, producing anthocyanin throughout the fruit as a result of the ectopic expression of Delila and Rose1 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.

Materials and Methods

Storage tests

Near isogenic lines for either Aft/Aft or atv/atv mutations are not available, so Solanum lycopersicum L. 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 with Aft/Aft, atv/atv and Aft/Aft atv/atv fruit (Povero et al., 2011).

Wild-type (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 (Supporting Information, Fig. S1) (Mes et al., 2008; Povero et al., 2011). Fruit were harvested at 7 d post-breaker (d0 = 7 dbp). All fruits were sterilized in 10% bleach for 10 min, 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 (Trolox equivalent antioxidant capacity) analysis of Aft/Aft atv/atv tomatoes was performed at breaker as described by (Pellegrini et al., 2003). Results were expressed as TEAC 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. (2008).

Measurements of cuticle thickness

Cuticle thickness measurements were modified from the methods described by Yeats et al. (2012). 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 to 10 measurements. The average and standard error of the mean of three biological replicates are reported.

Botrytis cinerea infection

Botrytis 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 d after breaker and surface sterilized. Intact WT and Aft/Aft atv/atv fruits were sprayed thoroughly with spores (2.5 × 10^7 spores ml^-1) three times in the flow cabinet and kept at 20°C, in high humidity. Infection symptoms were observed at 4 d post-inoculation (dpi). For wound inoculation, the fungal culture was diluted with medium to 5 × 10^8 spores ml^-1 (for fruit in the MicroTom genetic background) or 2.5 × 10^8 spores ml^-1 (for WT Ailsa Craig and Aft/Aft atv/atv) fruits) and incubated at RT for 1.5 h before inoculation. The spore inoculum (5 μl) 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 h after inoculation. To quantify B. cinerea growth using quantitative polymerase chain reaction (qPCR), 1 cm samples of infected fruit tissues were harvested 3 d 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 < 0.05 were considered significant.

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 1 wk after breaker. For Aft/Aft atv/atv fruit, 70 d 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 d (Fig. 1a,c) and the proportion of fresh weight loss was softer, equivalent to the level of softening observed in Ailsa Craig the seed of

precocious germination was due to elevated anthocyanin levels in
tomatoes did not (Fig. 1d). The absence of
Aft/Aft atv/atv
f). After storage for 42 d at RT, the seed in Ailsa Craig fruits showed
severe over-ripening symptoms while the
(a, d).

Fig. 1 Accumulation of anthocyanins in Aft/Aft atv/atv tomatoes (Solanum lycopersicum) delays late ripening. Ailsa Craig red (open circles), and Aft/Aft atv/atv, purple (closed circles) tomato fruits were stored at 17°C (a–c) and at room temperature (d–f). At 42 d of storage the wild type (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 with 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 d post-breaker (d0 = 7 dbb). Fresh weight reduction is presented using the percentage of the initial weight. Error bars, ± standard error of the mean (SEM) (n ≥ 8). Percentages of fruit showing over ripening symptoms (softening and shriveling) were assessed visually every week during storage tests.

proanthocyanins (Supplementary Information, Fig. S2). The suppression of precocious germination by anthocyanins in the seed has been observed for Del/Ros1 purple tomatoes (Butelli et al., 2008) and has been reported following studies of transparent testa mutants in Arabidopsis (Abeynayake et al., 2011) and for red wheat compared with 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 with WT Ailsa Craig fruit, and showed more rapid softening than purple regions on the same fruit (Supplementary Information, Fig. S3). This showed that the rate of fruit softening is 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 post-harvest 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 Botrytis 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 3 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 with growth in the red regions and to growth in the WT line (Ailsa Craig) (Fig. 2c,d). Together, these data demonstrate that resistance is 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 presence 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 (Supplementary Information, Fig. S4A) (Jones et al., 2003; Mes et al., 2008; Povero et al., 2011).

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 flavor, 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 with 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 with WT tomatoes. The anthocyanin-enriched sectors of Aft/Aft atv/atv tomatoes are less susceptible to Botrytis cinerea infection in both wound and spray tests (Fig. 2a,b) and this is correlated to the higher antioxidant capacity of purple tomatoes compared with 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 and 2 wk later than for WT (Ailsa Craig) tomatoes (Fig. 1c,d) demonstrating an extended shelf life both at 17°C and at RT. 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 with seed from WT 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 B. cinerea. The increase in shelf life correlated with the presence 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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References


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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 High light induces anthocyanin accumulation in the skin of Aft/Aft atv/ atv fruit.

Fig. S2 Seeds of Aft/ Aft atv / atv fruit accumulate anthocyanins.

Fig. S3 Delayed over-ripening is directly associated with anthocyanin production.

Fig. S4 Both Aft/ Aft atv / atv and PRD fruit predominantly accumulate anthocyanins in the skin.

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