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Investigating postharvest chilling injury in tomato (*Solanum lycopersicum* L.) fruit using magnetic resonance imaging and 5-azacytidine, a hypomethylation agent

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Abstract

Tomato, like most species from tropical and subtropical regions, exhibits postharvest chilling injury (PCI) when stored at low temperatures. Because of its economic importance and the functional genomics tools available, we used tomato to investigate aspects of fruit PCI development. We asked two questions: first, are there spatial-temporal differences in the development of PCI that can be detected by magnetic resonance imaging (MRI)? Here, the aim was to use a non-invasive method to study PCI progression in vivo. At mature green and breaker, the pericarp, locular tissue and columella produced distinct *D*-values while in contrast, there was no such differentiation in riper fruit. Although the pericarp is where most PCI symptoms are visible, this tissue showed less dynamism upon cold exposure, compared to the inner tissues as detected by MRI. This suggests the occurrence of distinct, independently modulated mechanisms contributing to the development of PCI-symptomatology. Collectively our data showed that the MRI could detect fruit ripening, its attenuation by cold, and fruit tissue-specific responses to chilling stress. The second question we asked was if epigenetic modification of the tomato genome or transcriptome influences PCI response. We examined PCI severity in fruit injected with a demethylating agent, 5-azacytidine (AZA). Two tomato genotypes exposed to varying severities of cold-stress were studied. Results suggested that AZA was able to moderate PCI in 'Micro-Tom' after 3 weeks at 2.5°C, while different patterns were observed in 'Sun Cherry' across various cold treatments. The effects of AZA on PCI were complex, multilayered and highly context-dependent.

Keywords: fruit respiration, spatial-temporal changes, magnetic resonance imaging, methylation, 5'-azacytidine

INTRODUCTION

The tomato (*Solanum lycopersicum* L.) is a functional genomics model for fleshy-fruited species (Azzi et al., 2015) and is one of the most popular and economically important crops globally (Beckles, 2012). However, storage at temperatures below 12.5°C followed by rewarming to room temperature, compromises fruit quality, hampering the postharvest handling of this commodity (Lyons, 1973). This cold-induced damage to the fruit called postharvest chilling injury (PCI) may only be detectable as a loss of flavor, or in severe cases, as fruit spoilage, the extent of which depends on the storage temperature, length of exposure, genotype and fruit developmental stage (Raison and Lyons, 1986).

The progression of PCI in fruit tissues is complex. It is marked by a loss of selective membrane permeability, increased solute leakage, reactive oxygen species accumulation and metabolic dysfunction (Biswas et al., 2012). After the fruit is transferred to room temperature for rewarming or reconditioning, higher respiration ensues within days (Farneti et al., 2015), and within a week, secondary symptoms such as uneven color formation, surface pitting,

water soaking and decay are visible (Cheng and Shewfelt, 1988). Symptoms are more intense in green compared to riper fruit, since maturation processes are disrupted by chilling (Saltveit and Morris, 1990). Because of the negative effect on tomato quality and shelf-life, our goal is to better understand PCI development and regulation in this species. Some aspects of the disorder or approaches used here may be relevant for other PCI-sensitive species.

First, we investigated the spatial and temporal evolution of PCI in the whole tomato fruit using MRI. Most studies of tomato PCI have focused on the pericarp, ignoring the internal tissues, which can account for 30% (Heuvelink, 2005) and 70% (unpublished observations) of the fresh mass of round and cherry tomatoes, respectively. Tao et al. (2014), investigated changes in chilled 'Micro-Tom' fruit using non-invasive MRI. They showed that the columella and locular region differed from the pericarp in their response to cold, which has implication for understanding the underlying causes of PCI. The fruit in that study were subjected to a severe cold stress (0°C), since this genotype is not as sensitive to chilling temperatures as many commercial varieties (Gonzalez et al., 2015). Further, only one developmental stage was chosen (Tao et al., 2014). It is not known if their findings are applicable to other cultivars, storage conditions or maturation stages.

Second, we investigated if 5-azacytidine (AZA) could alter PCI. This chemical inhibits DNA and RNA methylation (Christman, 2002), epigenetic modifications that regulate gene expression, in response to developmental and environmental stimuli in a tissue-specific manner (Liang et al., 2011). DNA methylation is a key regulatory process for tomato fruit ripening (Zhong et al., 2013; Cao et al., 2014; Liu et al., 2015); injecting AZA in round tomato fruit accelerated ripening (Zhong et al., 2013). It was shown that chilling-induced reductions in red fruit volatiles correlated with methylation of key ripening genes. Co-regulation of the ripening and cold response regulatory networks in fruit undergoing chilling stress seems likely (Zhou et al., 2001; Rugkong et al., 2011; Shan et al., 2014). Since differential methylation is essential to both processes, we wanted to determine if AZA could influence PCI symptoms in tomato fruit.

In this study, two questions were asked: 1) is it possible to detect spatio-temporal differences in chilled tomato fruit differing in maturation stage, and temperature × time of storage by low-resolution MRI?, and 2) would AZA influence PCI response? For the former, we used commercial cherry tomatoes and mild to moderate chilling stress. For the latter, fruit were injected with AZA weekly in order to detect changes in PCI by methylation (Zhong et al., 2013), specifically on respiratory activity. Fruit from a commercial cherry cultivar and the functional genomics model 'Micro-Tom' (Meissner et al., 1997) were used in this study.

MATERIALS AND METHODS

Materials

All cherry tomatoes (*S. lycopersicum* var. c*erasiforme*) were harvested from Capay Organic (Capay, California). The 'Micro-Tom' cultivar was grown according to Luengwilai et al. (2012), and all fruit in this study were handled as described by Luengwilai et al. (2012). The control or non-chilling temperature used throughout was 12.5°C, and the reconditioning or ripening temperature was 20°C for the spatio-temporal study, and 25°C for the AZA experiments.

Magnetic resonance imaging (MRI) experiment

'Sweet 100' was harvested in July 2014, and was used to study the effect of different chilling parameters on PCI development. Fruit was picked at mature green and stored at 0, 2.5, 5 or 12.5°C for 7 days, followed by transfer at 20°C for another 7 days. The chilling injury index or CII was assessed on 32 fruit as previously described (Vega-García et al., 2010).

'Sunsugar' was harvested in July 2015, and was used to investigate the effect and interaction of ripening and chilling on PCI. Fruit was picked at breaker, pink and red stages. Pink and red fruit were stored at 2.5 or 12.5°C for 5 days, but only pink fruit were transferred at 20°C for 3 additional days. Breaker fruit was only analyzed at day zero.

The MRI captured and mapped the apparent diffusion coefficient (ADC) of the whole

fruit, and this was used to determine the *D*-values as previously reported by Tao et al. (2014). Data were obtained from a single equatorial slice of each fruit. Fruit were removed from storage 3 h before analysis on the MRI. Two and four replicates per treatment were used for 'Sweet 100' and 'Sunsugar', respectively, each one containing three fruit, in a single scan (Figure 1).

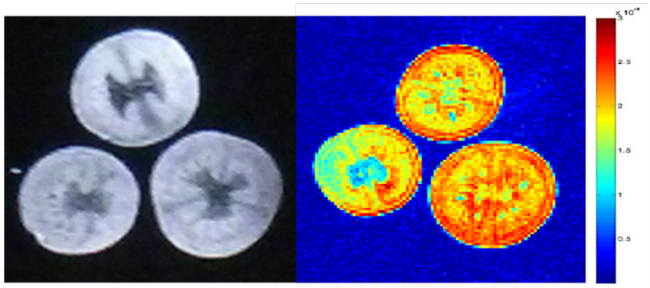

Figure 1. Equatorial slice of three cherry tomato fruit. Left: MRI scan. Right: ADC map. The color scale on the right represents the *D*-values: voxels in red and blue have high and low values, respectively.

The effect of AZA on fruit respiratory activity post-chilling

Mature green 'Micro-Tom' fruit were stored at 2.5 or 12.5°C for 21 days, followed by 7 days at 25°C. 'Sun Cherry' was harvested at mature green and stored at 2.5, 5 or 12.5°C for 7, 14, 21 or 28 days, followed by 7 days at 25°C. Fruit were treated with either AZA or water (control) with 3 biological replicates of 18 and 20 fruits each, for 'Micro-Tom' and 'Sun Cherry', respectively. Starting at day 0 of cold storage, 50 µL of 1 mM AZA or water was injected into the columella once a week (Zhong et al., 2013) before transfer to 25°C. Fruit respiration rate was measured as previously described (Cantwell et al., 1992).

RESULTS AND DISCUSSION

Using MRI to examine the spatio-temporal development of PCI in tomato fruit

If PCI induces loss of cell membrane integrity and increases water mobility within tissues, then higher MRI *D*-values would be observed when chilled fruit are rewarmed (Tao et al., 2014). Chilled fruit at different developmental stages, i.e., mature green, pink and red, and storage conditions were scanned by MRI, and examined for any emerging patterns. The resulting observations were grouped considering the maturation stage of the fruit.

1. Mature green fruit.

At this developmental stage in 'Sweet 100', the pericarp, columella and locular tissue showed a differentiated pattern in terms of their *D*-values after 7 days of chilling (Figure 2). Values were highest in the pericarp followed by the locular tissue and columella. Similar patterns were seen in freshly-harvested breaker fruit (Figure 3A). These three tissues have heterogeneous transcriptional (Lemaire-Chamley et al., 2005; Mintz-Oron et al., 2008) and metabolic profiles (Obiadalla-Ali et al., 2004; Van de Poel et al., 2014; Schouten et al., 2016; Wang et al., 2016) due to their distinct origin (ovary wall, carpels septa, placenta) and functionality (Gillaspy et al., 1993). This likely contributed to the distinct *D*-values observed.

When *D*-values for each region were compared as over each chilling period, no changes were observed except for the columella in fruit held at 5°C. Unchanged *D*-values may be due to cold-induced reductions in free water movement within tissues, and pectin solubilization (Almeida and Huber, 2008). Fruit exposed to warmer temperatures, i.e., after storage at the control temperature (12.5°C) for 7 days, or after transfer from the cold to 20°C, showed more dynamism in *D*-values. The different tissue fractions, which had distinct *D*-values during chilling, changed and became more similar when exposed to warmer temperatures (Figure 2). These non-chilling temperatures may have allowed ripening and other physiological events

to take place, leading to these changes.

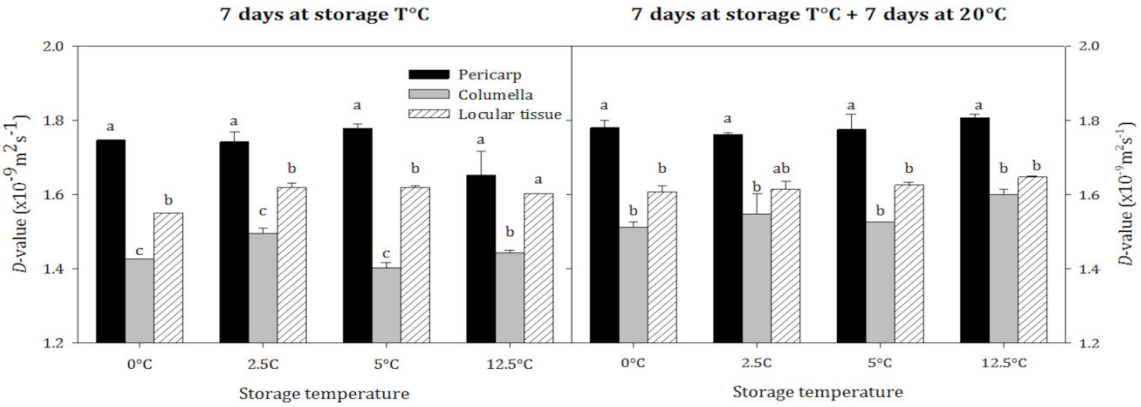

Figure 2. Changes in *D*-values of 3 types of tissues of mature green cherry tomato fruit 'Sweet 100' stored at 0, 2.5, 5 and 12.5°C for 7 days, followed by storage at 20°C for 7 days. Each bar is the average of 6 fruit per treatment. Different letters represent significant difference ($P \leq 0.05$) among tissues within each temperature and time point by Tukey's test. Vertical bars represent standard error and are contained in the symbols when not shown.

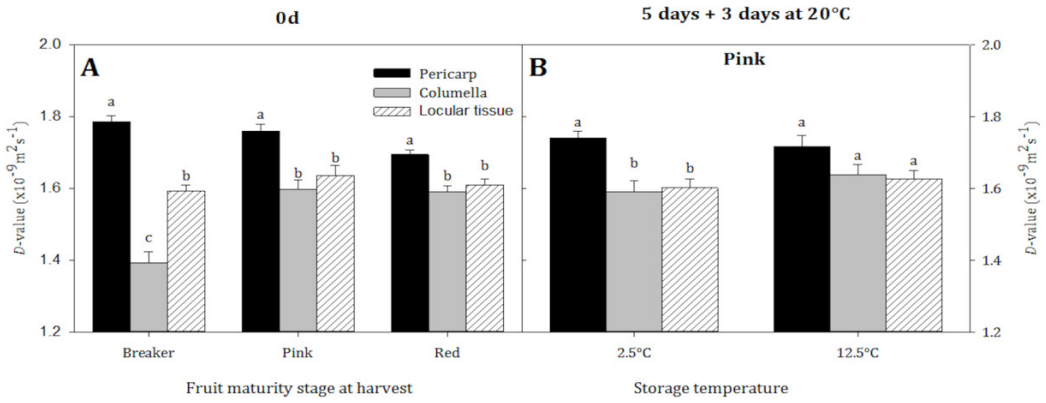

Figure 3. Changes in *D*-values of 3 types of tissues of cherry tomato 'Sunsugar'. Each bar is the average of 12 fruit per treatment. Vertical bars represent the standard error. A) Fruit from 3 maturity stages at harvest. Different letters represent significant difference ($P \leq 0.05$) among tissues within each maturity stage by Tukey's test. Only fruit from the 'pink' stage were used in subsequent storage tests. B) Pink fruit stored at 2.5 and 12.5°C for 5 days, followed by 3 days at 20°C. Different letters represent significant difference ($P \leq 0.05$) among tissues within each temperature by Tukey's test.

2. Pink and red fruit.

Figure 3A shows the *D*-values of 'Sunsugar' ripened fruit. These data, gathered from breaker, pink, and red fruit immediately after harvest, suggest that as ripening progresses, the *D*-values of the columella and locular region become more similar (Figure 3A). Ripening increases the proportion of free water and metabolites within tissues, due to liquefaction of the locules and breakdown of the structural components of the cell (Carrington et al., 1993; Watson et al., 1994; Clark et al., 1997). These changes may have underscored the increased *D*-values seen here, and in other studies (Chen et al., 1989; Saltveit, 1991). A similar occurrence was seen when red fruit was stored at 2.5°C for 5 days (data not shown). When *D*-values for each region were compared over time, there was no significant difference. Tissue liquefaction

in red fruit was so extended as a consequence of ripening, that cold did not generate any detectable increase by the MRI, or did not increase membrane leakage since it was already fluid.

The observations of pink fruit stored in the cold and then rewarmed are less clear. Both chilling-induced damage during low-temperature storage, and ripening-related tissue deconstruction during rewarming would lead to increased membrane permeability and *D*-values (Sharom et al., 1994; Biswas et al., 2012), thus making it difficult to attribute higher *D*-values to one or the other biological phenomenon.

There are some points to emphasize with respect to the data when analyzed across cultivars and conditions. First, pericarp *D*-values did not vary as much as those in the columella and locular regions (Figure 2). Second, there was a weak correlation between MRI-derived values for the pericarp and the physical changes caused by cold, visible on the pericarp e.g., poor color development, pitting and decay as reported by the CII data (r^2=0.1; Figure 4). In contrast, there was more synchrony for the columella and CII (r^2=0.5) which is similar to the data published by Tao et al. (2014). Surprisingly, the locular fraction showed a similar *r*-value to the pericarp (r^2=0.1) when CII was considered. Therefore, other mechanisms besides the increased water mobility we were able to detect under the experimental conditions used, may have a higher contribution to the development of chilling-induced external symptomatology. Third, different *D*-values were recorded in the three tissues as ripening progressed: they decreased in the pericarp, increased in the columella and were unchanged in the locular tissue (data not shown), exemplifying the unique response of each tissue-type. Fourth, MRI could only detect changes after transfer of chilled fruit to room temperature. Loss of membrane selective permeability due to a cold-induced membrane phase transition was not sufficiently advanced to produce detectable increases in free water mobility during cold storage. This supports the view that, increased membrane permeability is unlikely to be one of the earliest events in PCI response, but occurs at a significant rate during rewarming (Sevillano et al., 2009; Aghdam et al., 2015).

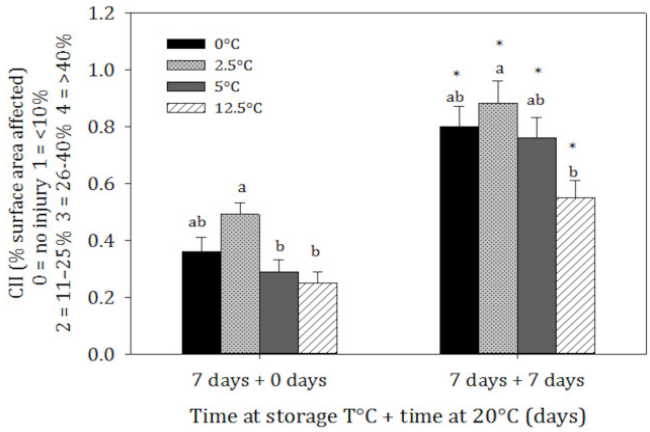

Figure 4. Changes in CII of mature-green cherry tomato 'Sweet 100' stored at 0, 2.5, 5 and 12.5°C for 7 days, followed by storage at 20°C for 7 days. Each bar is the average of 32 fruit per treatment. Different letters represent significant difference (P≤0.05) among temperatures within each time point by Tukey's test. Asterisks indicate significant difference between time points within each temperature. Vertical bars represent standard error of the mean.

Interfering with epigenetic methylation to examine cold response in tomato fruit

Fruit undergoing PCI normally exhibit a transitory burst of CO_2 when transferred from chilling to room temperature, which acts as a reliable marker for the early stages of cold-injured tissue (Lyons and Breidenbach, 1990). If AZA-treated fruit show differences in respiratory activity after cold stress compared to the water-treated fruit, this could be

indicative of an effect of methylation on PCI. Different responses were observed across varying cold stress in 'Micro-Tom' and 'Sun Cherry' and are described in turn.

1. 'Micro-Tom'.

For this experiment, fruit was stored for 21 days only. After fruit were transferred to 25°C, cold-treated (2.5°C) and control fruit (12.5°C; Figure 5), both increased in respiration, with the rate in the former twice as high as that of the latter (the control) (Figure 5). After 7 days at 25°C, the rate in control fruit decreased to the same level (~50 mg CO_2 kg^{-1} h^{-1}) as fruit that was continuously held at 20°C (Luengwilai et al., 2012), while cold-stored fruit maintained a two-fold increase in respiration rate (Figure 5). The latter is indicative of tissue that underwent irreversible chilling injury (Eaks and Morris, 1956; Lyons, 1973). Respiratory values here are a robust marker for 'Micro-Tom' undergoing chilling stress.

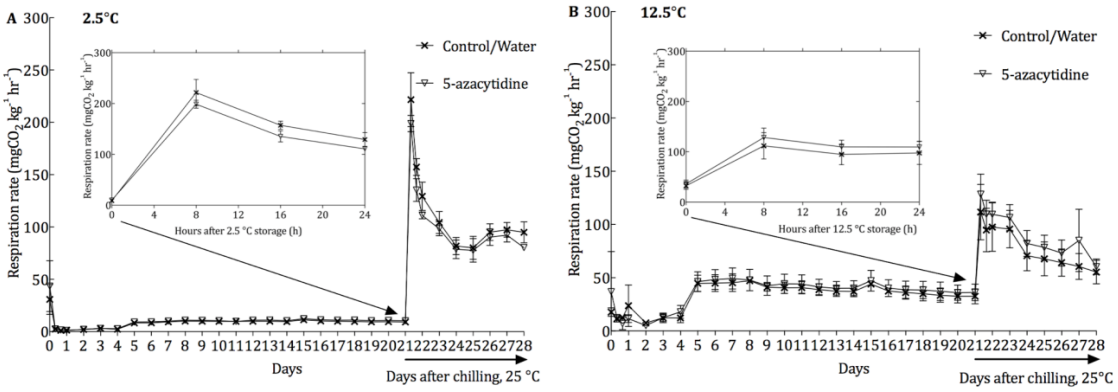

Figure 5. Changes in respiration rate of 'Micro-Tom' stored at 2.5 (A) and 12.5°C (B) for 21 days only. After 7 days, fruits were transferred to 25°C for 7 days (indicated with arrows). Each bar is the average of 18 fruit per treatment. The inserted graphs show respiration rate within 24 h after 2.5 (A) or 12.5°C (B) storage.

AZA affected respiration in fruit stored at both cold and control temperatures across the rewarming period. After 21-days at 2.5°C, AZA led to a lower respiratory rate compared to the water-control fruit during reconditioning (P<0.001), suggesting that AZA might moderate chilling injury in 'Micro-Tom'. In contrast, at 12.5°C, AZA-treated fruit had higher respiratory rates compared to the water-treated fruit after storage (P<0.01). This effect was likely a result of accelerated climacteric respiration caused by AZA-accelerated ripening (Zhong et al., 2013). AZA may have varying effects in different conditions, which is consistent with the fact that methylation patterns are diverse across developmental stages with various environmental stimuli (Sheldon et al., 2000; Jaenisch and Bird, 2003).

2. 'Sun Cherry'.

This cultivar was more susceptible to PCI than 'Micro-Tom,' and may show a different response to AZA-treatment. In all experiments, an increase in respiration was observed after transfer of fruit to 25°C following cold storage (Figure 6). Effect of AZA was evaluated across reconditioning period (Figure 7).

To understand the effect of AZA on respiratory rates in the absence of chilling stress, fruit held at 12.5°C were examined over the entire storage period. AZA treatment led to higher respiration after 14 days (Figure 6C), likely due to accelerated ripening. In contrast, water-injected fruit showed increases in respiration later – after 21 and 28-day storage (Figure 6C). This may be due to a 'delayed' climacteric response relative to that in the AZA-fruit.

AZA affected the respiratory activity of postharvest chilled fruit after reconditioning. As expected, fruit stored at 2.5°C exhibited a higher respiratory burst than those held at 5°C, while it was minor in fruit at the control temperature (Figure 6), indicating severe chilling

injury at lower temperatures (Lyons, 1973). Unlike 'Micro-Tom', AZA had no effect on 'Sun Cherry' fruit exposed at 2.5°C for 21 days or less, nor fruit held at 5°C for 7 days (Figure 7). AZA did influence fruit respiration after storage at 2.5 or 5°C for 28 days (Figure 7). Extreme fungal growth on fruit upon rewarming made it hard to further evaluate effect of AZA on respiration in 'Sun Cherry' (data not shown).

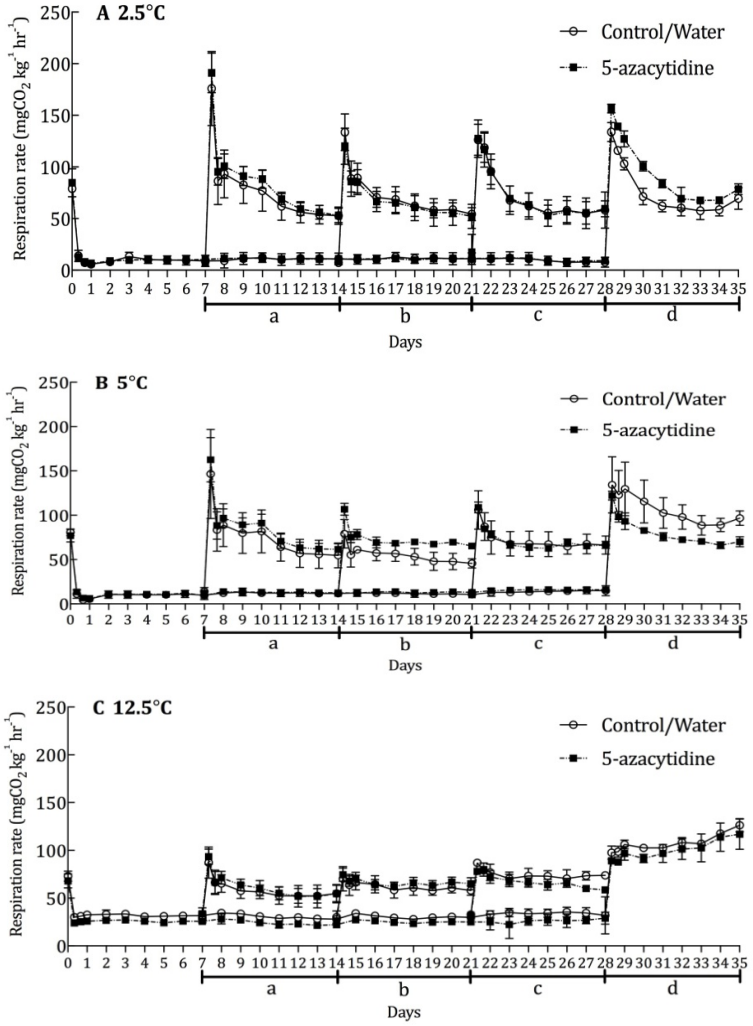

Figure 6. Changes in respiration rate of 'Sun Cherry' stored at 2.5 (A), 5 (B) and 12.5°C (C) for 7, 14, 21 and 28 days, followed by transferred to 25°C for 1 week. Segment a, b, c, d indicate 7 days at 25°C after 7, 14, 21 and 28 days, respectively. Each bar is the average of 20 fruit per treatment. To integrate data, respiration rate at 2.5, 5°C and 12.5°C for 28 days was used to represent data of 7-, 14- and 21-days treatment at corresponding temperature, since no significant differences of respiration at each temperature were detected among the four time lengths (ANOVA, all $P<0.05$).

One anomaly observed was in AZA-injected 'Sun Cherry' fruit held at 5°C for 14 days with subsequent warming (Figures 6B and 7). These fruit showed a similar respiration rate compared to those held at ripening temperature (12.5°C) for 7 days (Figure 6B, C). It is speculated that the higher rate in the 5°C-held fruit resulted from accelerated ripening, which was nullified when fruit were held for an additional 7 days (21 days) in the cold. After 21 days at this same temperature (5°C), AZA-treated and water-control fruit exhibited similar respiration rate; no AZA effect on ripening nor PCI was detected (Figures 6B and 7).

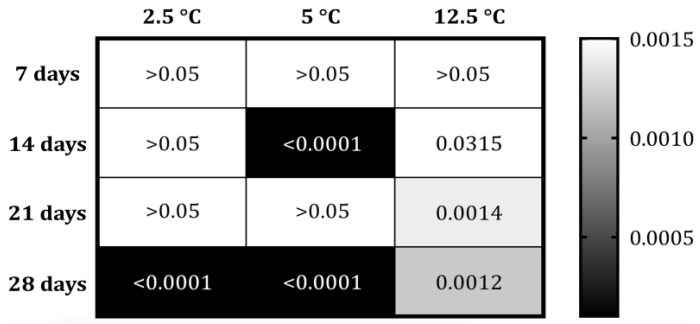

Figure 7. The effect of AZA (indicated by *P*-values derived from ANOVA testing) on 'Sun Cherry' fruit respiration rate. Data are the result of comparisons between AZA-treated and water-treated fruit across rewarming for 7 days at 25°C, after storage at the temperatures and days indicated. Values in each box indicate if, and the extent to which there was a significant effect of AZA on respiration rate. Greater significance levels are indicated with a darker color. The figure does not illustrate the directionality of the effect, i.e., higher or lower.

CONCLUSIONS

MRI was useful for detecting fruit ripening, its attenuation by cold, and fruit tissue-specificity in the cold response. MRI non-invasively differentiated among tomato pericarp, columella and locular fractions. However, when fruit were scanned after reconditioning, or when at an advanced stage of ripening, these distinctions were lost. Chilling-induced damage was detected by the MRI in the columella but not in the pericarp and locular tissue. MRI scans in the columella throughout the experiment better reflected the CII. Cold stress likely repressed the mechanisms leading to fruit free water production or increased water mobility.

AZA was used to determine if demethylation could modulate the effect of PCI in chilled tomato fruit. The effect of AZA on PCI was determined by many multilayered factors, e.g., genotype, severity of stress and how it influences the underlying ripening pathways. This complexity is probably a consequence of the ubiquity of epigenetic methylation on the genome and transcriptome and the multitude of factors that influence its status. Even though in some conditions, the effect of AZA was not detected, it does not mean that methylation is not important to PCI, since the phenotypes assessed probably do not reflect all methylated-regulation of fruit gene expression.

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