academicJournals

Vol. 4(3), pp. 35-43, October, 2013
DOI: 10.5897/JSPPR10.012
ISSN 2141-6567 ©2013 Academic Journals
http://www.academicjournals.org/JSPPR

Journal of Stored Products and Postharvest Research

Full Length Research Paper

Effect of low temperature storage on fruit physiology and carbohydrate accumulation in tomato ripening-inhibited mutants

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Accepted 18 May, 2010

Chilling-sensitive fruits often produce a burst of ethylene when reconditioned at ambient temperature after cold storage. This has led some researchers to propose that chilling injury (CI) may be induced by post-chilling ethylene production. To test this hypothesis, we examined two tomato (*Solanum lycopersicon* L.) mutants, *non-ripening* (*nor)* and *ripening-inhibitor* (*rin)*, that do not produce climacteric ethylene, after they were subjected to cold-storage and reconditioning. The response of the mutants differed, and was not as extreme as the parent line cv. Ailsa Craig, but both showed symptoms of chilling stress. Therefore while ethylene production may influence chilling injury, it is not essential for initiating this process in tomato cv. Ailsa Craig.

Key words: Chilling injury, tomato fruit, ripening mutants, rin, nor.

INTRODUCTION

Chilling injury in tomato (*Solanum lycopersicon* L.) is a complex syndrome that is detrimental to fruit quality (Serrano et al., 1996; Sevillano et al., 2009). When tomato is stored at 2-12°C, and is then allowed to ripen at ambient temperature (20°C), a battery of physiological and biochemical responses can be activated that damages the fruit. These responses include a failure to ripen, water-soaking, poor appearance and susceptibility to disease (Morris, 1982). This poses a problem when storing tomato postharvest: low-temperatures are needed to delay senescence but this simultaneously increases the risk of chilling injury (CI).

An interrelationship between ethylene production and CI has been proposed (Wang, 1989). Many aspects of ripening in climacteric fruits like tomato are largely regulated by ethylene. This growth regulator can also hasten senescence, one of the main symptoms of CI (Saltveit, 2003), indicating that extensive cross-talk occurs between the two pathways. Genetic and biochemical evidence also support a connection between the two processes.

In some species, there is a spike in ethylene synthesis when fruits that was previously held in the cold is ripened at warmer temperatures (Sevillano et al., 2009) and avocadoes, pineapples and 'Fortuna' mandarin treated with the ethylene inhibitor 1-MCP each showed enhanced tolerance to CI (reviewed in Pech, 2008). Furthermore, CI is attenuated in transgenic melons with a lesion that severely reduces ethylene biosynthesis in the fruit (Ben Amor et al., 1999). These observations collectively point to a role for ethylene influencing CI in several crops, but this may not be true for all CI-sensitive climacteric fruit. Melon has clearly defined ethylene-independent ripening pathways but this has not been demonstrated unequivocally in tomato, so CI mechanisms may not operate similarly in these crops. This is true even within closely-related species. Ethylene treatment of 'Fortuna' mandarin created CI resistance, but in another citrus, the cultivar 'Shamouti,' ethylene hastened CI symptoms (Porat et al., 1999). The overall picture that emerges is

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that ethylene production does influence CI in some crops but empirical testing is needed.

The aim of this work is to investigate if climacteric ethylene is an important factor in initiating or accelerating CI in tomato. This was addressed by determining if CI is evident in tomato genotypes that have an impaired ethylene-dependent ripening pathway. *Ripening inhibitor (rin)* and *non-ripening* (*nor)* are tomato mutants that produce little or no climacteric ethylene (Tigchelaar, 1977). *Rin* lacks a MADS-box transcription factor (MADS-RIN) while *nor* lacks a transcription factor that may regulate MADS-RIN (Giovannoni, 2007a). Both transcription factors act upstream of the ethylene signaling pathway, so that *rin* and *nor* retain their sensitivity to ethylene (Moore et al., 2002). These mutants show physiological and biochemical changes during maturation that may be associated with ethylene-independent ripening pathways (Jeffery et al., 1984; Lelievre et al., 1997).

We hypothesized that if a burst of ethylene synthesis in post-chilled fruit stimulates the onset of CI, then these ripening impaired mutants may be resistant to CI at an equivalent chronological stage of the parental control. If however, CI is not primarily influenced by ethylene, injury will be evident due to an affect of cold on ethylene autonomous-ripening pathways in the mutants. Such pathways in tomato would include starch degradation, sugar, citric and malic acid production, loss of chlorophyll, and some aspects of fruit softening that is, those initiated before ethylene production in tomato (Jeffrey et al*.,* 1984). Studying CI-sensitivity in *rin* and *nor* may therefore deliver new insight into chilling injury in tomato.

MATERIALS AND METHODS

Plant material

Tomato (*Solanum lycopersicon* L.) cv. Ailsa Craig and two near isogenic lines - *rin* (*ripening inhibitor*) and *nor* (*non-ripening*) mutants were obtained from the Tomato Genetic Resource Centre, University of California- Davis. Plants were grown as described previously (Luengwilai and Beckles, 2009b). Fruit from cv. Ailsa Craig (referred to as the 'control genotype') was harvested at 42 days post anthesis (DPA), which approximates to USDA Mature Green 1. Fruits from *nor* and *rin* were harvested when they reached the same chronological age as the parent line that is, at 42 DPA.

Storage conditions

The fruits were stored at different temperature-time regimes in large controlled-temperature rooms. The length of the experiment was 36 days except where noted. One set of fruit (approximately 6-12) of each mutant genotype was stored at 5°C for 28 days and these conditions are referred to as 'cold storage.' After cold storage the fruits were then held at 20°C for 8 days or the 'reconditioning' phase. Another set of fruits (approximately 6-12) was simultaneously stored at 20°C for 36 (28 + 8) days. This treatment is referred to as the 'temperature control conditions.'

Respiration, ethylene production, firmness, total soluble solids (TSS), color and weight loss measurements

A minimum of 3 fruits per replicate was used for measuring respiration and ethylene production, as well as firmness, total soluble solids and color as described by (Luengwilai et al., 2007). Starch was measured as described by (Luengwilai and Beckles, 2009b). Percentage weight loss was calculated as follows:

$$\frac{(W_i - W_f)}{W_i} \times 100$$

Where W_i = Initial weight (prior to storage), W_f = Final weight (as measured on the stated date)

Chilling injury index

Severe symptoms of chilling injury are manifested by the appearance of surface pitting. These symptoms were evaluated in the fruits at days 1 and 8 of the reconditioning period. Symptom severity was scored as 0 = no pitting; 1 = less than 5% of fruit surface pitting; 2 = pitting covering 5-25% of fruit surface; 3 = pitting covering 25-50% of fruit surface and 4 = more than 50% of fruit surface covered with pitting. The extent of chilling injury damage was expressed as a chilling injury index, which was calculated using the formula:

$$\text{Chilling injury index (score 0-4)} = \frac{\sum(\text{CI level}) \times (\text{Number of fruit at the CI level})}{\text{Total number of fruit in the treatment}}$$

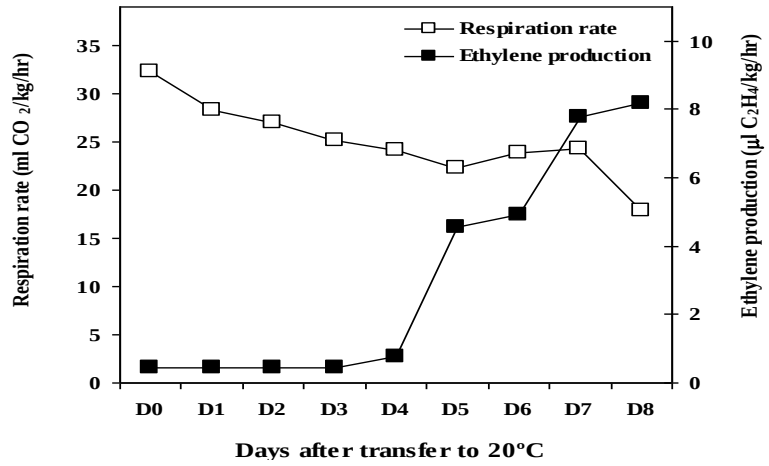
Statistical testing

Values are considered different if *P*-values were less than 0.05 by Student's *t*-test.

RESULTS AND DISCUSSION

We began our investigation by first observing the pattern of ethylene emissions and CO_2 production in the tomato genotypes. Increased production of these gases are characteristic of ripening in climacteric fruit, but *rin* and *nor* do not show this trend (Giovannoni, 2007b). Relevant to our question is the observation that CI in fruits is characterized by increased respiration and ethylene production in the initial phases of reconditioning (Jackman et al., 1989). The evolution of CO_2 and ethylene were measured immediately and up to 8 days after the fruits were restored at 20° C after being held in the cold for 4 weeks. Cold-stored Ailsa Craig fruit showed a climacteric peak of CO_2 and the rate of ethylene production increased four-fold (from 2 to 8 μl C_2H_4/kg/h) during post-chilled ripening (Supplementary Figure 1).

In this study *rin* and *nor* fruits did not show any increase in ethylene production regardless of storage condition prior to reconditioning (Figure 1A). In both mutants however, chilling caused an increase in respiration immediately upon transferring the fruit to room temperature (Figure 1B). From this data we can make two conclusions. First, that the mutants showed some signs of stress due to cold-storage, as evidenced by



Supplementary Figure 1. Ethylene production and respiration in Ailsa Craig fruit. The data showed the characteristic climacteric burst of CO_2 or ethylene during the reconditioning period. Values were mean ± SE of 6 fruit for all genotypes. Error bars are not shown for clarity.

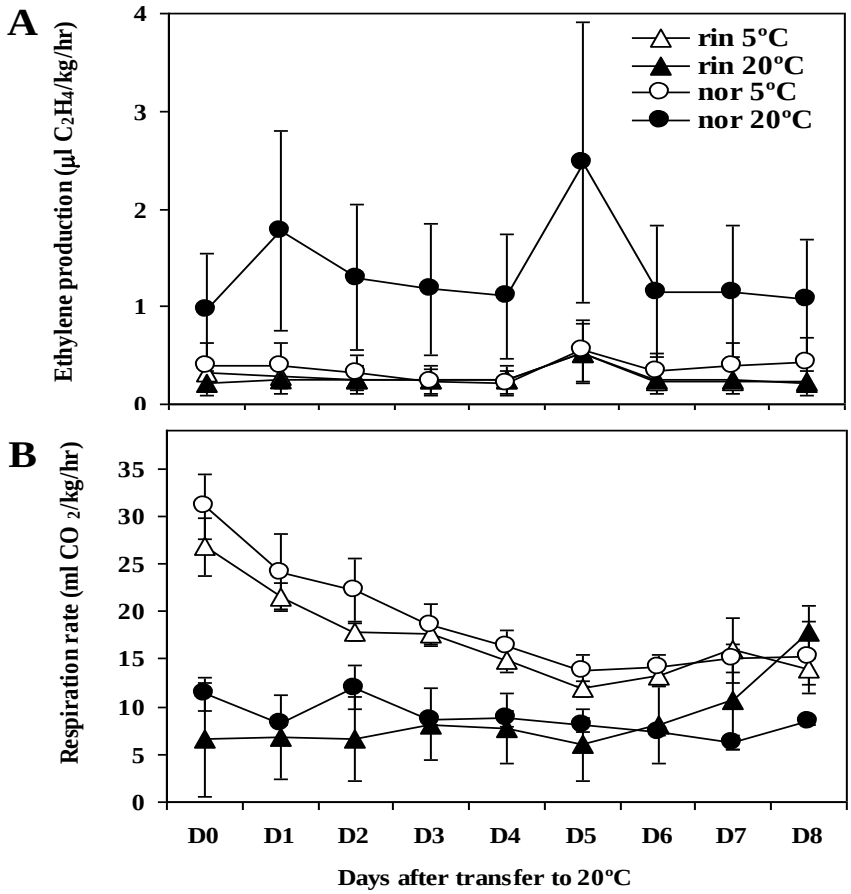
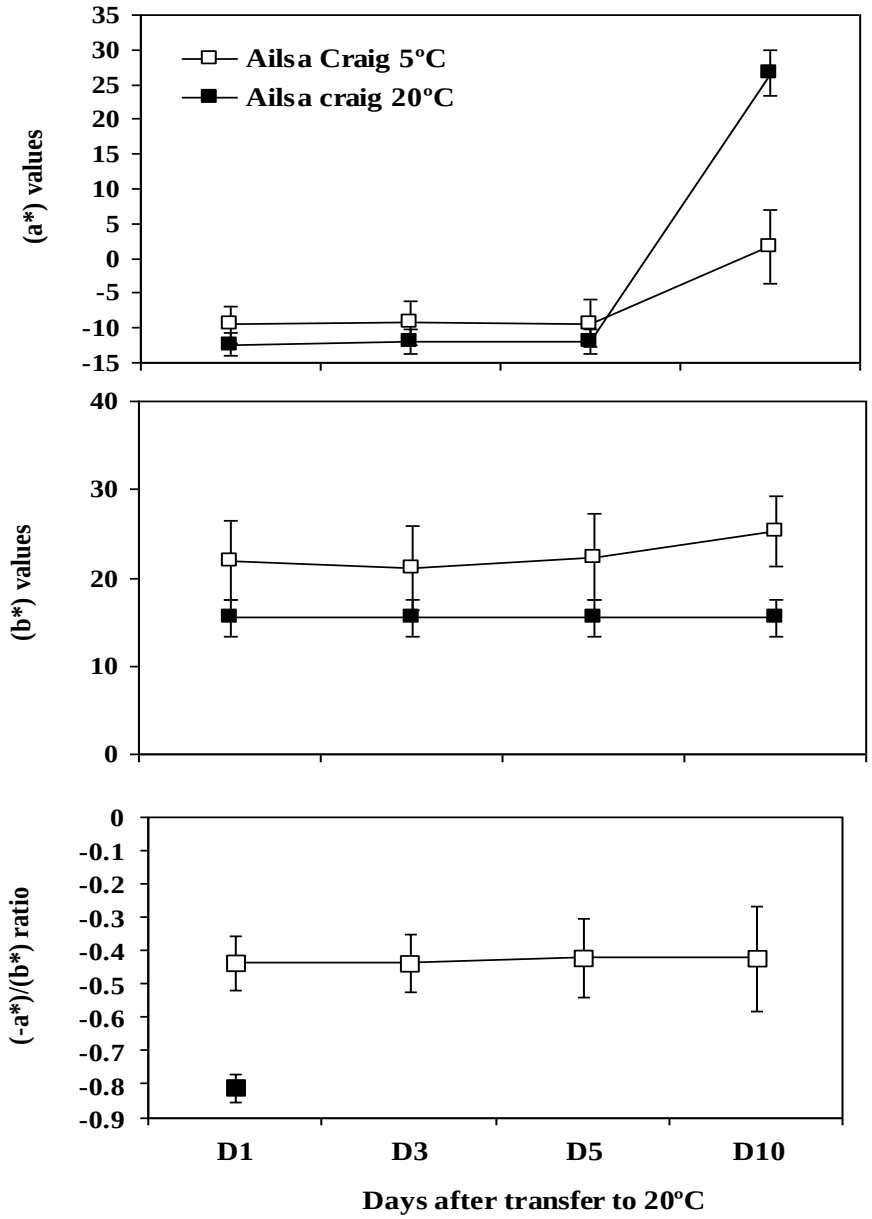


Figure 1(A)**.** Ethylene production in *rin* and *nor* tomato fruit stored at 20ºC for 8 days after 28 days of cold storage. Prior to holding at 20°C fruit were kept either at (i) 5°C for 28 days (described as *rin* 5°C or *nor* 5°C in the legend; light symbols) or (ii) were kept in control conditions, that is 20°C for 28 days described as *rin* 20°C and *nor* 20°C in the legend; dark symbols. (B). Respiration rates of *rin* and *nor* tomato fruit held at 20°C for 8 days after cold storage. Values were mean ± SE of 6 fruit for all genotypes.



Supplementary Figure 2. Color development of Ailsa Craig fruit indicated by a*, b* and -a*/b* values. Fruit was stored at 5°C or at 20°C. For fruit stored at 20°C, a* values were not negative after day one, hence only one point is shown on the graph. Values are mean ± SE of 6 fruits.

increased CO_2 evolution. Second, because no change in ethylene production was recorded in the mutants regardless of storage conditions, any general changes in maturation seen may be attributable to ethylene-independent ripening processes (Jeffrey et al., 1984). We therefore paid particular attention to how these parameters responded to cold treatment in the mutants.

We observed the extent of de-greening and accumulation of lycopene and other carotenoids in ripening fruit using the CIE dimension scores, where a* denotes red-green characteristics and b* denotes yellow-blue. The values and their ratios have been used as important indicators of the levels of various pigments in fruits (Batu, 2004). Chilling delayed color development in Ailsa Craig and caused abnormal and uneven pigmentation, but after 5 days of reconditioning a* values increased 3-fold (Supplementary Figure 2). The mutants did not redden in cold storage or at room temperature and as a result all had negative a* values which remained constant while holding at 20°C (Figure 2A). Evaluation of

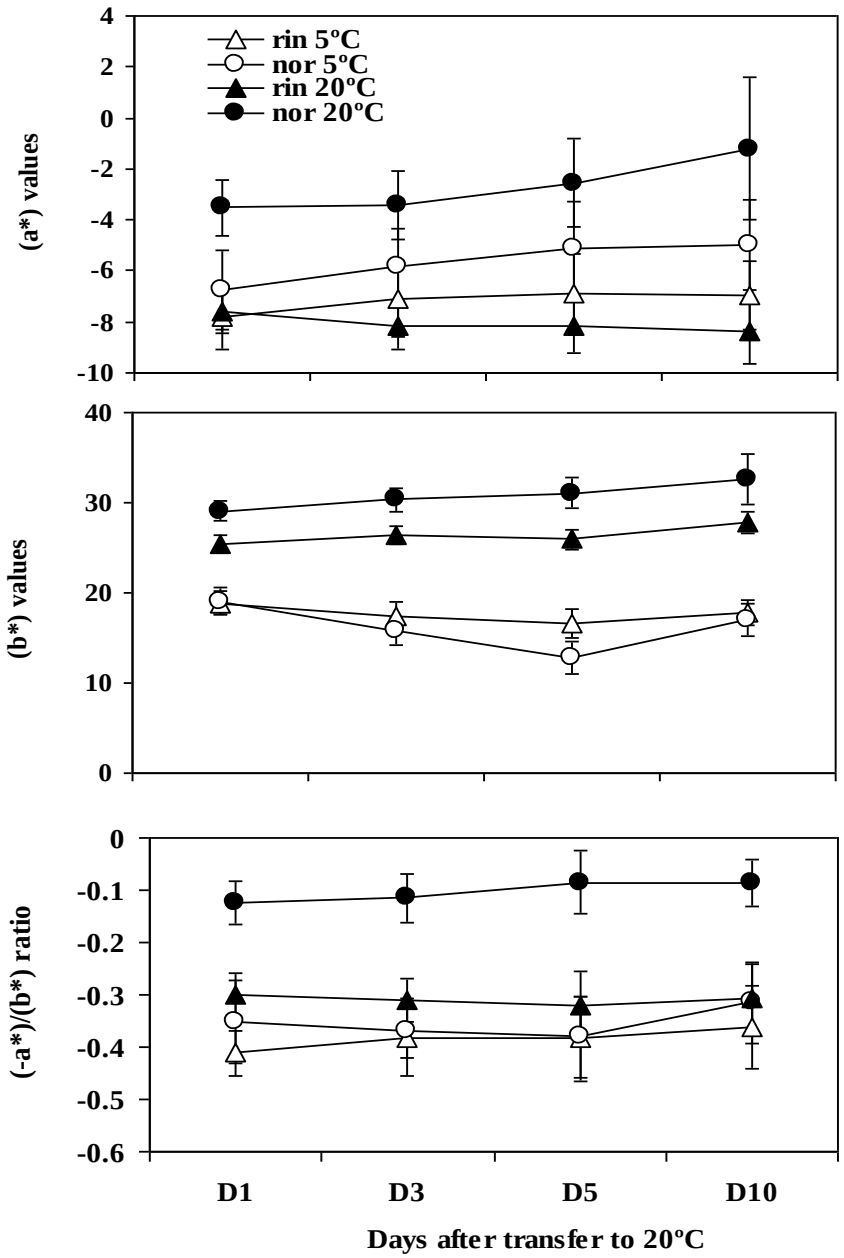
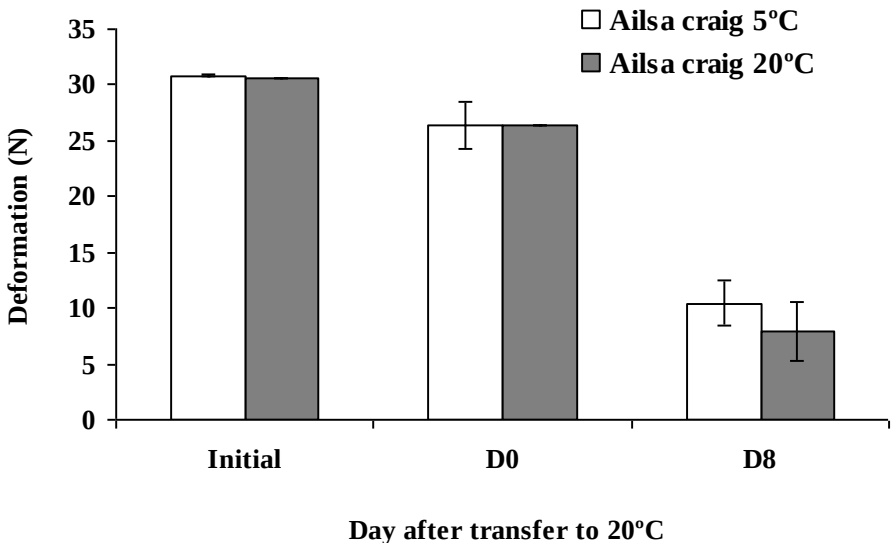


Figure 2. Color development in fruit of *rin*, and *nor* tomato fruit after cold storage and transfer to 20°C for 8 days. (A) a* values represents the greenness (negative) and redness (positive) and is strongly influenced by lycopene content (B) b* values represented the blueness (negative) and yellowness (positive), increased b* values are associated with yellow carotenoids. (C) -a*/b*indicates changes in chlorophyll content. Values were mean ± SE of 12 fruits for all genotypes and storage conditions. Prior to holding at 20°C, fruits were kept either at (i) 5°C for 28 days (described as *rin* 5°C or *nor* 5°C in the legend; light symbols) or (ii) were kept in control conditions that is, 20°C for 28 days (described as *rin* 20°C or *nor* 20°C in the legend; dark symbols).

b* and -a*/b* values suggests that cold treatment simultaneously reduced both values in *nor* but only b* values in *rin* (Figure 2B and 2C). It is possible to use this data to infer an effect of cold on ripening.

This is predicated on the following assumptions:

(i) that -a*/b* scores positively correlate with chlorophyll content (Steet and Tong, 1996; Koca et al., 2007),



Supplementary Figure 3. Deformation of Ailsa Craig fruit. Fruit were stored at 5°C or 20°C and then reconditioned for 8 days at 20°C. Values are mean ± SE of 6 fruits.

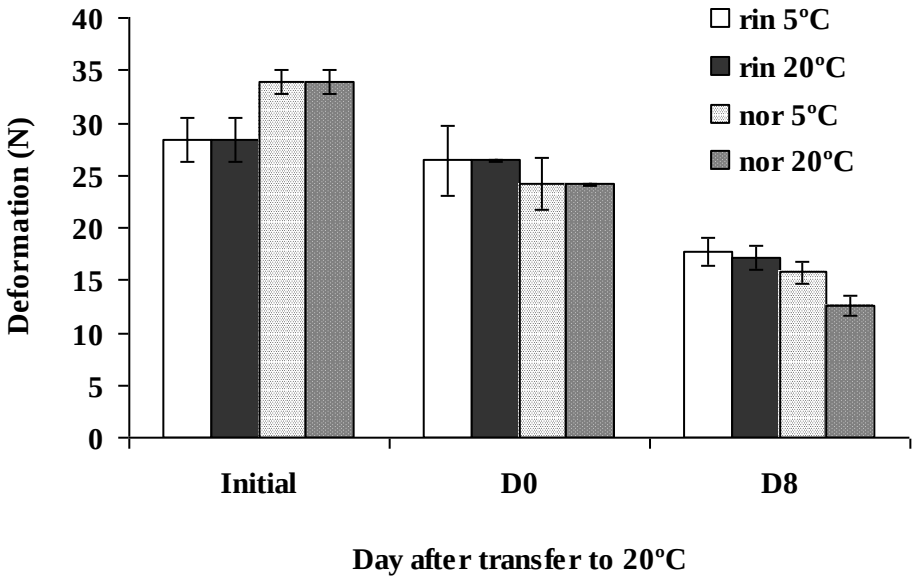
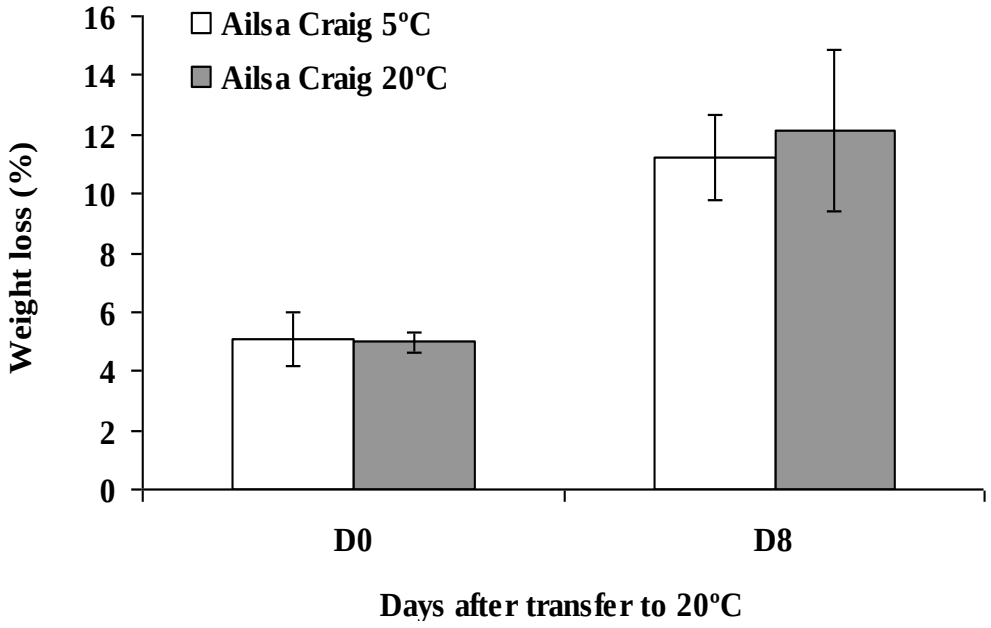


Figure 3. Fruit deformation of *rin*, and *nor* tomato fruit after 5°C storage and transferred to 20°C for additional 8 days. Values were mean ± SE of 12 fruits for both genotypes and storage conditions.

(ii) that b* values reflect yellow-pigmented carotenoids (Artes et al., 1999; Batu, 2004),
(iii) some carotenoids synthesis and loss of chlorophyll are initiated in response to ripening signals that occur before ethylene is produced (Jeffery et al., 1984).

When considered together, this data suggests that the pigment metabolic pathways that are ethylene-independent were affected by chilling in both mutants.

Another signal of ripening includes fruit softening. Softening is stimulated by both the ethylene and non-ethylene ripening pathways in melon (Pech et al., 2008) and this may also be true in tomato. As anticipated, the Ailsa Craig fruits were easier to deform than the mutants but cold-storage did not alter the response (Supplemental Figure 3). Fruits from *nor* held at 5°C were firmer during the subsequent room temperature incubation, but there was no effect on *rin* where the controlled and treated fruit behaved identically (Figure 3).



Supplementary Figure 4 .Weight loss of Ailsa Craig fruit. Fruit were stored in cold or at 20°C as indicated on the graph. Values are mean ± SE of 6 fruits.

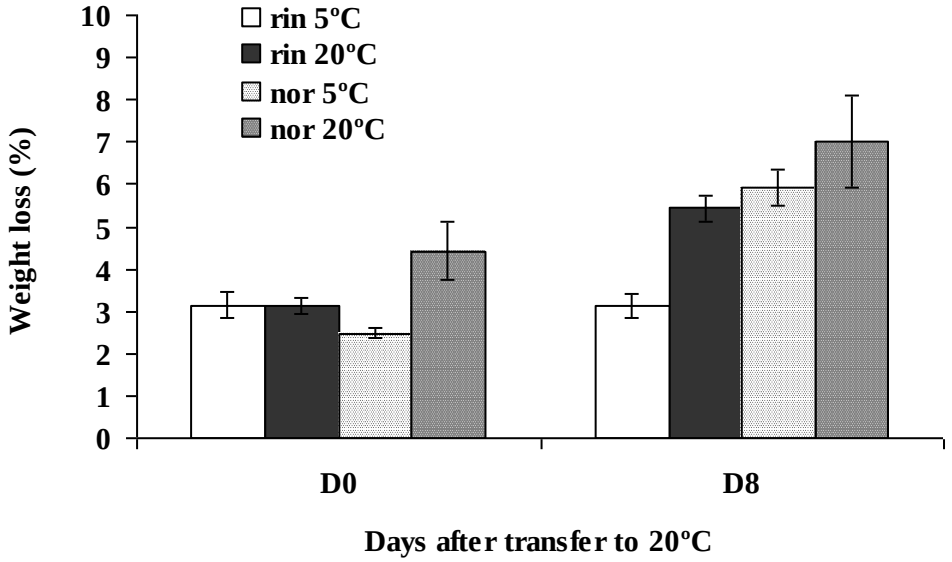
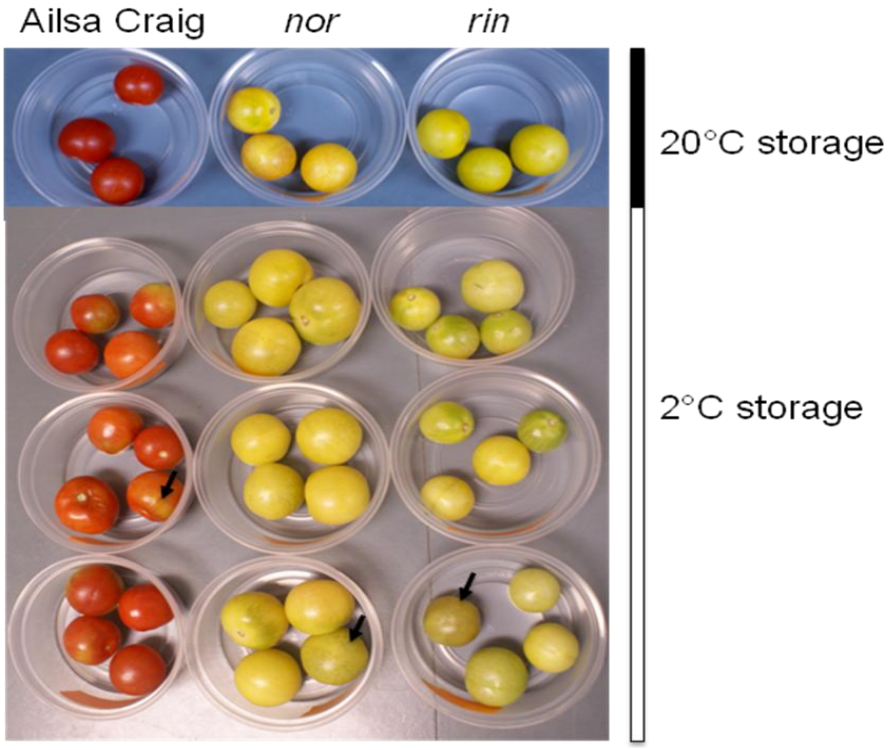


Figure 4. Percentage of fruit weight loss *rin*, and *nor* tomato fruit after 5°C storage and transferred to 20°C for additional 8 days. Values were mean ± SE of 12 fruits.

The results of the weight loss experiment were more complex. A typical manifestation of CI is that fruit will lose mass because of lowered temperature and this will continue when they are allowed to ripen at 20°C (Artes and Escriche, 1994). In our experiment, all fruits were weighed immediately after harvesting. Fruits used for cold treatment were re-weighed 28 days after incubation at 5°C. They were transferred to 20°C, held for 8 days, and were then weighed again. The temperature controls (20°C) was also weighed immediately after harvest and following 36 days of storage at 20°C. During cold-storage only *nor,* lost fruit mass over that initially measured (Supplemental Figure 4). After reconditioning, fruit mass decreased in *rin,* but was attenuated in *nor* (Figure 4).

Starch degradation is another biochemical process linked to tomato fruit ripening and may contribute to red-



Supplementary Figure 5. Ailsa Craig, *rin*, and *nor* fruit after storage at 2°C or 20°C for 28 days and then, after transfer to 20°C for an additional 28 days. The CI index was 0.75 and 0.3 for Ailsa Craig and *rin* respectively. *Nor* fruit did not show any sign of surface pitting injury. Arrows indicate uneven ripening in Ailsa Craig and evidence of water-soaking in *rin* and *nor*.

fruit total soluble solids (TSS) (Luengwilai and Beckles, 2009a). TSS indicates the level of acids and sugars in the fruit; the biochemical pathways that produce these compounds are stimulated by climacteric ethylene but their initiation precedes this event in fruit development (Jeffery et al., 1984). Our measured values of TSS (5.5 ± 0.5%) were identical in all storage time-temperature regimes measured between the mutants, which were 23% lower than Ailsa Craig (7.2% and 5.5% respectively). Starch accumulation was also identical between genotypes in freshly-harvested mature green fruit of *nor, rin* and Ailsa Craig (11 ± 2, 13 ± 3 and 20 ± 5 mg/gFWT respectively). However after cold storage and reconditioning for 7-21 days starch levels were reduced almost 100-fold, with values of 0.20 ± 0.100 mg/gFW in *nor,* 0.40 ± 0.10 mg/gFW in *rin* and 0.46 ± 0.001 mg/g FW in Ailsa Craig. Starch was degraded post-cold-storage presumably as a substrate for respiration, which peaked immediately after the fruit were incubated at 5°C in all the genotypes (Figure 1B).

In this experiment fruit, starch is degraded in the absence of climacteric ethylene, at similar rates to that in the control cultivar. It is possible that in the mutants, respiration had a big influence on starch degradation, or that there was enough ethylene produced in the mutants for near complete starch breakdown. These events may not be mutually exclusive. The fruit was evaluated for physiological symptoms of CI including fruit decay and surface pitting. There was no evidence of these abnormalities in any of the genotypes (data not shown).

Although this seemed unusual, a lack of severe damage to tomatoes held at 5°C was also seen in another study (Chomchalow et al., 2002). This type of data exemplifies the difficulty with studying the CI phenomenon because it is highly context-dependent. (Saltveit and Morris 1990). We therefore incubated 12 fruits for each genotype at 2°C for 21 days followed by storage at 20°C for another 21 days to encourage a response. All genotypes showed CI symptoms - failure to fully ripen and water-soaking. Surface pitting was visible in Ailsa Craig and *rin* and the calculated CI index was 0.7 and 0.3 respectively (Supplementary Figure 5).

Conclusion

Our hypothesis was that if post-chilling climacteric ethylene was important in initiating CI, that the tomato ripening mutants *rin* and *nor* would show enhanced resistance to CI. We tested this by focusing on potential

changes to putative ethylene-independent pathways that is, ripening changes that are initiated before the onset of the climacteric, in response to chilling. The CI response was mild in control and test genotypes at 5°C. In spite of this, our data suggests that this condition altered characteristics associated with non-ethylene ripening in *nor* and *rin.* In all samples, respiration, changes in fruit mass and non-lycopene fruit pigmentation were characteristic of fruits experiencing cold-stress.

There were differences in the pattern and the severity of physiological and biochemical traits assayed between mutants in response to 5°C, however when the fruit was exposed to 2°C, visible evidence of CI phenotypes were found in both mutants. While we cannot rule out the possibility that some basal level of ethylene, that is from wounding or that produced in vegetative tissues was sufficient in the mutants to cause some ripening changes during cold-storage, we were able to effectively show that there was no climacteric burst of ethylene in *rin* and *nor* and infer that this was not responsible for the results observed. We therefore conclude that the discrete pathways that are regulated by the transcription factors encoded by *rin* and *nor* may engender differences in the response by the mutants to cold, but that CI occurs in the absence of climacteric ethylene in the Ailsa Craig cultivar.

ACKNOWLEDGEMENTS

We thank Dr. Elizabeth Mitcham for the generous use of controlled temperature rooms and a Gas Chromatography machine for ethylene and respiration measurement. We also thank Dr. Marita Cantwell for sharing unpublished data on chilling injury in tomato. KL's graduate fellowship is provided by the Anandamahidol Foundation. This work was funded by National Science Foundation Grant MCB-0620001 to DMB and Hatch Projects CA-D*-PLS-7198-H and CA-D*-PLS-7821-H

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