Journal of Stored Products and Postharvest Research Vol. 1(1), pp. 1 - 8, May 2010
Available online http://www.academicjournals.org/jsppr

Full Length Research Paper

Climacteric ethylene is not essential for initiating chilling injury in tomato (*Solanum lycopersicum*) cv. Ailsa Craig

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Accepted 8 April, 2010

Chilling-sensitive fruit often produce a burst of ethylene when reconditioned at ambient temperature after cold storage. This has led some authors to propose that chilling injury (CI) may be induced by post-chilling ethylene production. To test this hypothesis we examined two tomato (*Solanum lycopersicum* L.) mutants, non-ripening (*nor*) and ripening-inhibitor (*rin*) that do not produce climacteric ethylene. Fruit were stored at 5°C followed by reconditioning at 20°C, during which time a detailed characterisation of respiration, ethylene production, colour analysis, firmness, total soluble solids, starch content and weight loss was done. The response of the mutants to cold-storage at 5°C differed, and was not as extreme as the parent line cv. Ailsa Craig, still, both mutants showed symptoms of chilling stress on the ripening pathways that are initiated upstream of climacteric ethylene production. When the fruit were stored at 2.5°C for 14 days followed by reconditioning at 20°C for 3 weeks, visual evidence of CI such as water-soaking, non-uniform ripening and minimal colour change was noted in both the control and mutant genotypes. We conclude therefore that 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 lycopersicum* 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 damage 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 inter-relationship between ethylene production and CI has been proposed (Wang, 1989). Many aspects of ripening in climacteric fruit 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 fruit that was previously held in the cold is ripened at warmer temperatures, (Sevillano et al., 2009) and avocadoes, pineapples and persimmons 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 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. Unlike, tomato, melon has clearly defined ethylene-independent ripening pathways, so CI mechanisms may not operate similarly in these crops. This is true even within

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Abbreviations: ***rin*,** Ripening inhibitor; ***nor*,** non-ripening.

closely-related species. Ethylene treatment of persimmons created CI resistance, but in another citrus, the cultivar 'Shamouti,' ethylene hastened CI symptoms (Porat et al., 1999). The overall picture that emerges is 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 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 bio-chemical 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 a primarily influenced by ethylene, injury will be evident due to effect 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 materials

Tomato (*S. lycopersicum* 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, 2009a). 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. Fruit from *nor* and *rin* were harvested when they reached the same chronological age as the parent line that is, at 42 DPA.

Storage conditions

Fruit 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 six - twelve) 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 fruit were then held at 20°C for 8 days or the 'reconditioning' phase.

Another set of fruit (approximately six - twelve) was simultaneously stored at 20°C for 36 (28 + 8) days. This treatment is referred to as the 'temperature control conditions.'

Respiration and ethylene production

A set of 3 fruit per replicate was placed into a sealed 275-ml glass container for an hour. Then, 10 ml of head-space atmosphere was withdrawn using a syringe to measure CO_2 production via an infrared gas analyzer (Model PIR-2000 R. Horiba Instruments, Irvine (CA). Ethylene production was measured on another 10 ml of head-space using a Gas Chromatograph (Model Carle 211, Hach Carle, Loveland (CO) equipped with a flame ionization detector. Samples were taken within 30 s of each other.

Deformation

The deformation of six – twelve fruit from each treatment was measured using a Texture Analyzer Instrument (Texture Technologies Corp., Stable Micro System, NY). The two most convex parts (on opposite cheeks) of each fruit were selected and compressed using a 5.1 mm diameter probe moving at 0.5 mm/sec. The data were recorded manually.

Pericarp colour evaluation

Two opposite surfaces of each fruit were measured using a Minolta Colorimeter (Model CR-200, Minolta Corp., Ramsay, NJ), and expressed as colour space L*, a*, b* mode. L* value indicated lightness, where +a* value was the red direction, -a* was the green direction, +b* value was the yellow direction, and –b* was the blue direction. Chroma, C* = (a*+b*)1/2, indicated the intensity or colour saturation, and hue angle was calculated as h° = arc tangent b*/a*, where 0º = red-purple; 90° = yellow ; 180°=bluish-green and 270° = blue. The colour of 6 - 12 fruit was evaluated for each treatment.

Total soluble solids and starch content

Total soluble solids (TSS) was done as described by (Luengwilai et al., 2007). Juice from a minimum of 3 fruit per replicate was squeezed onto a refractometer (Abb'e Model 10450, American Optical Co. Buffalo. NY) and the values recorded. Starch was measured as described by Luengwilai and Beckles (2009a). 200-mg of pericarp was boiled in 2-ml of 80% (v/v) ethanol for 10 min, the ethanol was discarded, a fresh 2-ml aliquot of ethanol was added and the sample was boiled again. This procedure was repeated twice.

The ethanol-insoluble fraction was then ground to a powder in a mortar and pestle, followed by an all-glass homogenizer. The solids were made up to 2-ml with water, were separated into three 500 µl aliquots and autoclaved for 45 min. Amyloglucosidase (12 U per sample; Roche Applied Science, Indianapolis, IN) and a-amylase (1U per sample; Roche Applied Science, Indianapolis, IN) were added to the samples and allowed to digest the starch overnight at 37°C in a buffer of 200 mM sodium acetate pH 4.5.

The released glucose was used for starch content estimation by high performance liquid chromatography (Model Dionex BioLC system, Dionex, Sunnyvale, CA) equipped with a Hamilton RX-10 anion exchange column (250 × 4.1 mm i.d.; Hamilton, Reno, NV) and pulse amperometric detection. The gradient elution consisted of 15 min of 15% (v/v) of 200 mM NaOH followed by 7 min of 30% (v/v) of 200 mM NaOH and 10 min with 15% (v/v) of 200 mM NaOH. Glucose concentration was calculated from a standard glucose solution (Fluka Bio Chemika Co; Steinheim, Germany). The

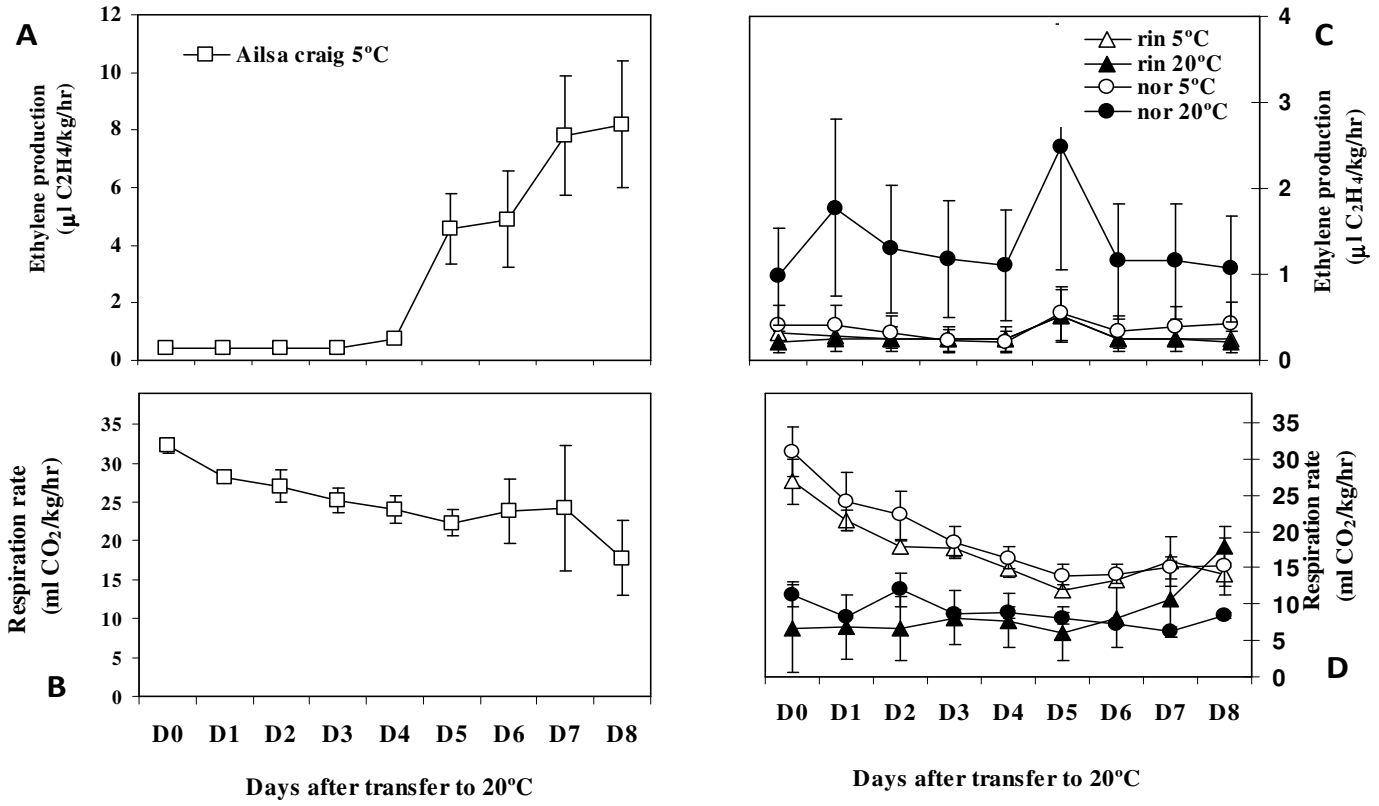

Figure 1. Ethylene production and respiration rates in *rin*, *nor* and Ailsa Craig tomato fruit stored at 20°C for 8 days after 28 days of cold storage. Prior to holding at 20°C fruit were kept either at (i) 5°C for 28 days (described as *rin* 5°C or *nor* 5ºC or Ailsa Craig 5ºC in the legend; light 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. Graphs shown are as follows: ethylene production and respiration rates of Ailsa Craig (A) and (B) and ethylene production and respiration rates of *rin* and *nor* (C) and (D). Values are mean ± SE of 6 fruit for all genotypes. Data for Ailsa Craig under control conditions is not available.

starch content was calculated by multiplying glucose content by (162/180).

Weight loss measurements

A minimum of 3 fruit per replicate were weighed individually and percentage weight loss was calculated as followed:

$(W_i - W_f) / W_i \times 100$

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 fruit at day 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 covering with pitting. The extent of chilling injury damage was expressed as a chilling injury index, which was calculated using the following 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 fruit 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 fruit was restored at 20°C after being held in the cold for 4 weeks. Cold-stored Ailsa Craig fruit showed an increase of CO_2 immediately

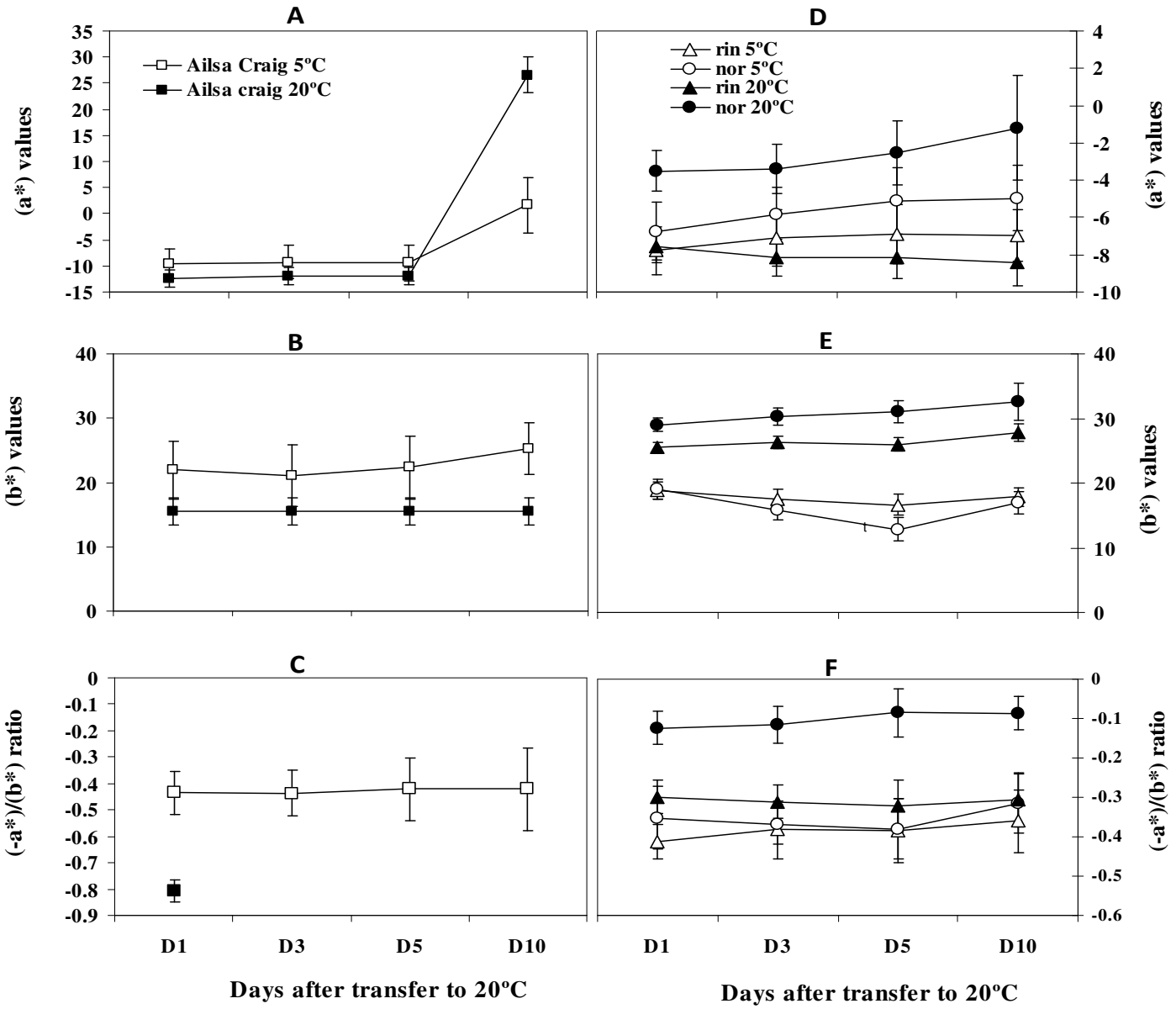

Figure 2. Color development in fruit of Ailsa Craig, *rin* and *nor* tomato fruit after cold storage and transfer to 20°C for 8 days. Graphs (A) and (D) show a* values which represent fruit greenness (negative) and redness (positive) and is strongly influenced by lycopene content. Graphs (B) and (E) show b* values which represent blueness (negative) and yellowness (positive); increased b* values are associated with yellow carotenoids. Graphs (C) and (F) show calculated -a*/b* which indicates changes in chlorophyll content. 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 or Ailsa Craig 5°C in the legend; light symbols) or (ii) were kept in control conditions i.e. 20°C for 28 days described as *rin* 20°C or *nor* 20°C or Ailsa Craig 20°C in the legend; dark symbols. Values are mean ± SE of 6 - 12 fruit for all genotypes and storage conditions.

after reconditioning and this CO_2 remained high up to 8 days at 20°C and the rate of ethylene production increased four-fold (from 2 - 8 µl C_2H_4/kg/hr) during post-chilled ripening (Figure 1 A and B). In this study ethylene production was variable in *rin* and *nor* fruit but did not show any statistical significant increase within the period examined regardless of the storage condition prior to reconditioning (Figure 1C). In both mutants however, chilling caused an increase in respiration immediately upon transferring the fruit to room temperature (Figure 1D). From this data we can make two conclusions. First, that the mutants showed some signs of stress due to cold-storage, as evidenced by 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). Wetherefore paid particular attention

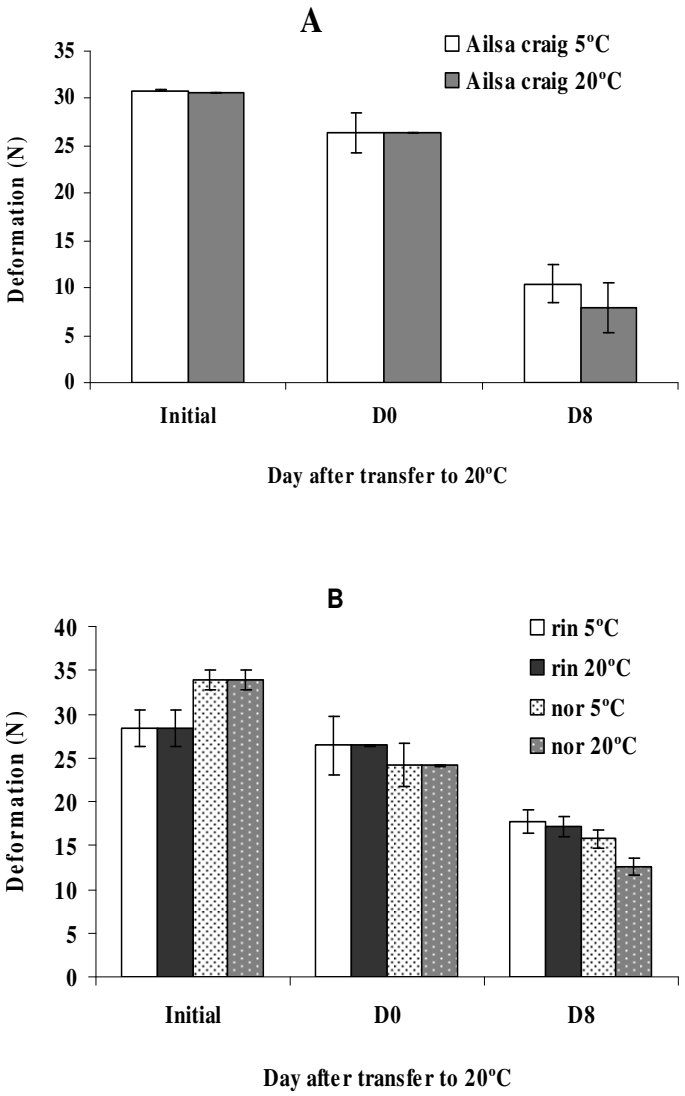

Figure 3. Deformation of Ailsa Craig, *rin*, *nor* tomato fruit after 5ºC storage and reconditioning at 20°C for 8 days. Fruit softening was evaluated using a Texture Analyzer. Fruits were compressed using a 5.1 mm diameter probe moving at 0.5 mm/s. Values are mean ± SE of 6 - 12 fruit for all three genotypes and storage conditions.

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 fruit (Batu, 2004). Chilling delayed colour development in Ailsa Craig and caused abnormal, uneven pigmentation, but after 5 days of reconditioning a* values increased 3-fold (Figure 2A). 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 2D). Evaluation of b* and -a*/b* values suggests that cold treatment simultaneously reduced both values in *nor* but only b* values in *rin* (Figure 2E and F). 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), (ii) that b* values reflect yellow-pigmented carotenoids (Artes et al., 1999; Batu, 2004) and that (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 suggest 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, Ailsa Craig fruit were easier to deform than the mutants but cold-storage did not alter the response (Figure 3A). Fruit from *nor* held at 5°C were firmer during the subsequent room temperature incubation, but there was no effect on *rin*, where the control and treated fruit behaved identically (Figure 3B).

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 fruit were weighed immediately after harvesting. Fruit used for the 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) were also weighed immediately after harvest and following 36 days of storage at 20°C. Ailsa Craig showed the expected pattern of weight loss but cold-storage had no affect on this result (Figure 4A). Only *nor* fruit lost mass over that measured initially (Figure 4B). After reconditioning, fruit mass neither decreased in *rin,* but was attenuated in *nor* (Figure 4B).

Starch degradation is another biochemical process linked to tomato fruit ripening and may contribute to red-fruit TSSs (Luengwilai and Beckles, 2009b). TSS is a cumulative measure of the amount 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

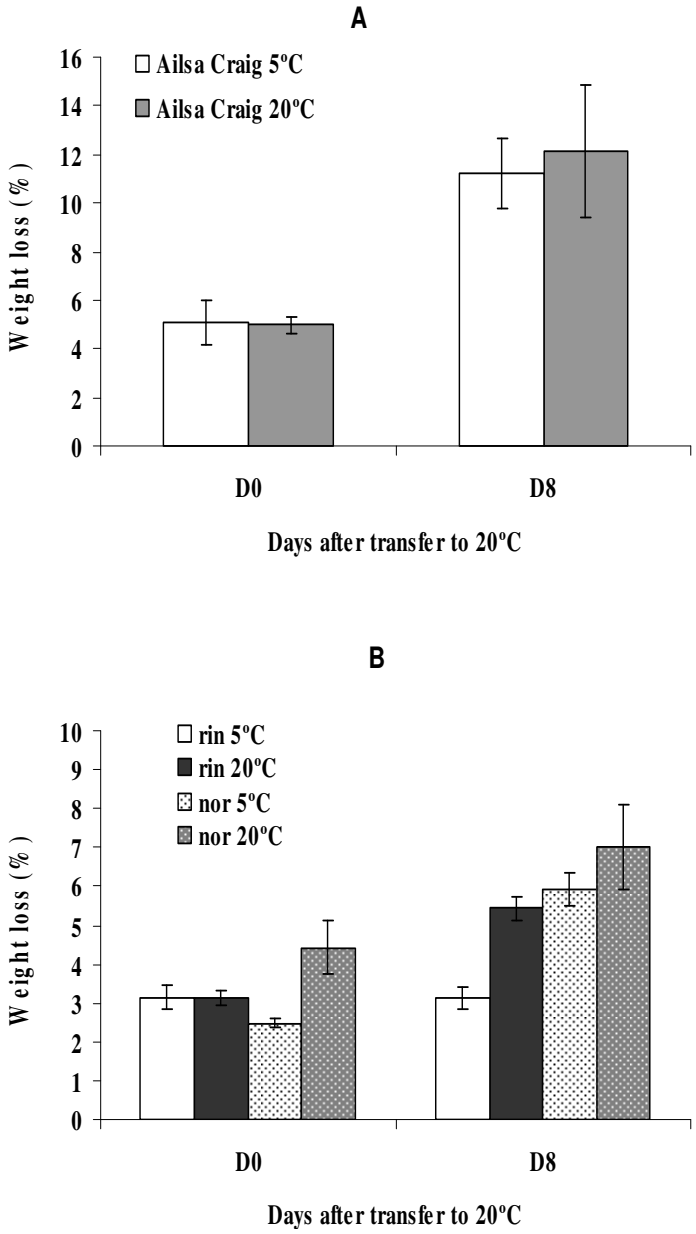

Figure 4. Fruit weight loss in Ailsa Craig, *rin*, *nor* after 5°C storage and reconditioning at 20°C for 8 days. Values shown are the weight loss percentages calculated by subtracting the final weight after storage on the stated days, from the initial weight of freshly harvested fruit. Values are mean ± SE of 6 - 12 fruit.

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 was peaked immediately after the fruit were incubated at 5°C in all the genotypes (Figure 1B and D). In this experiment at least in the mutants, 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 fruits were evaluated for physiological symptoms of CI including fruit decay and surface pitting. There was no evidence of these abnormalities in any of the genotypes. 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 highly context-dependent (Saltveit and Morris, 1990). We therefore incubated 12 fruit for each genotype at 2°C for 21 days followed by storage at 20°C for another 21 days to encourage a response. Lower temperatures usually increase the probability that severe CI symptoms will occur in chilling-sensitive fruit (Saltveit, 1990). After storage at 2.5°C for 21 days, all genotypes showed visible symptoms of CI including a failure to fully ripen and water-soaking (Figure 5). The mutants kept in control conditions were more yellow (*rin*) and yellow-orange (*nor*) in colour while the fruit incubated in the cold retained more chlorophyll and were therefore greener (Figure 5). Surface pitting was visible in Ailsa Craig and *rin* and the calculated CI index was 0.7 and 0.3, respectively (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 suggest that incubation at 5°C altered some 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 fruit 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 2.5°C, clear 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

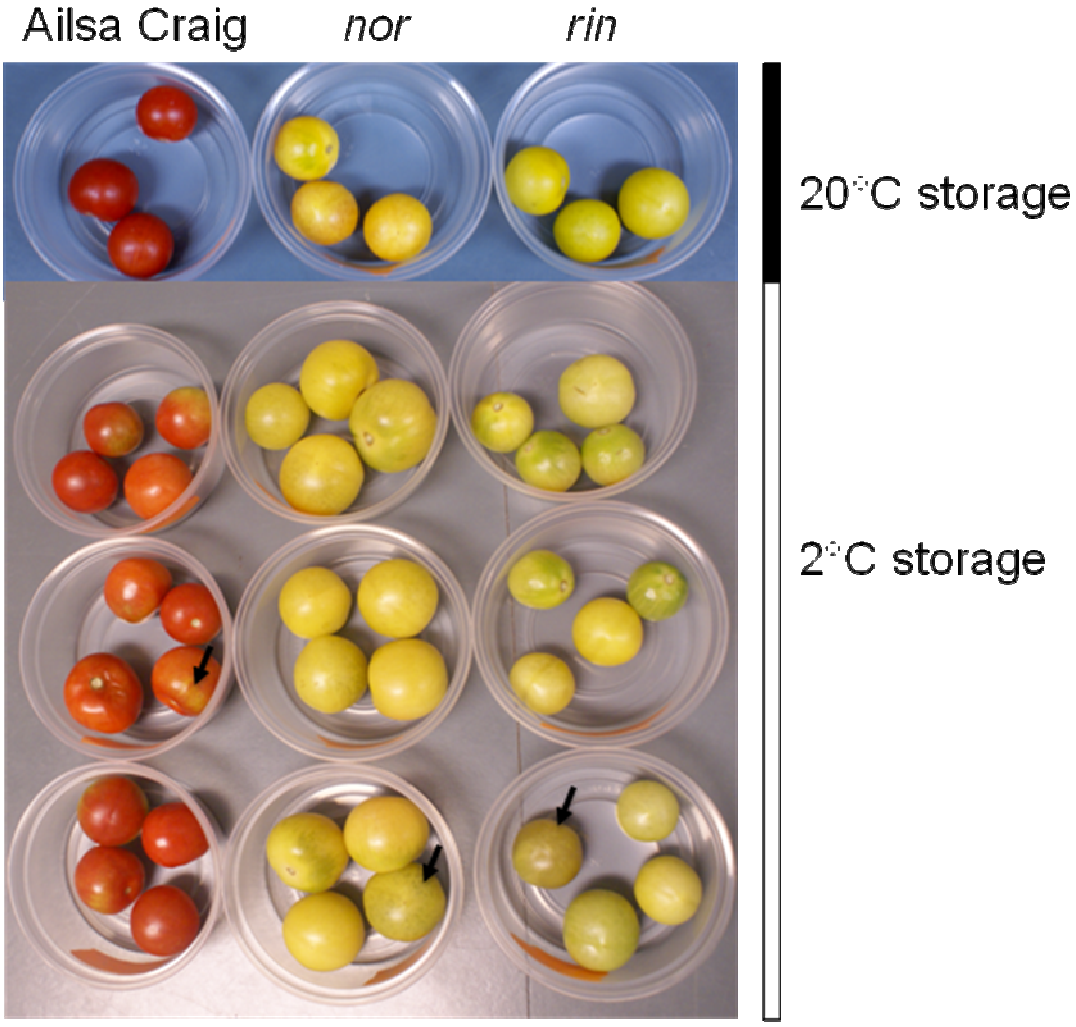

Figure 5. Ailsa Craig, *rin*, and *nor* fruit after storage at 2.5°C or 20°C for 28 days and then, after transfer to 20°C for an additional 28 days. The CI index was 0.75 and 0.3 for Ailsa Craig and *rin* respectively. Fruit of the *nor* genotype did not show any sign of surface pitting injury. Arrows indicate uneven ripening in Ailsa Craig and evidence of water-soaking in *rin* and *nor*.

cultivar.

ACKNOWLEDGEMENTS

We thank Dr. Elizabeth Mitcham for the generous use of controlled temperature rooms and a gas chromatography machine. We 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 the University of California Hatch Projects CA-D*-PLS-7198-H and CA-D*-PLS-7821-H.

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