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Expansin protein levels decline with the development of mealiness in peaches

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Abstract

Expansin mRNA and protein expression in peaches was examined to investigate the possibility that expansins may be involved in the development of mealy flesh texture. Immunoblot analysis, using an expansin antibody, detected a 27-kDa protein corresponding to the predicted molecular mass of expansins in the later stages of ripening but not in full-size green fruit and indicated that expansin is associated with the progression of ripening in peaches. Peach cultivars 'O'Henry' and 'Summer Lady' were stored at 5 °C to induce the development of mealiness and individual fruit samples collected periodically to measure mealiness (free water) and provide samples for expansin quantification. Initially, stored fruit were juicy (50–60% free water), but as storage progressed they became visibly mealy at free water percentages of 30% for 'O'Henry' and 46% for 'Summer Lady'. In both cultivars expansin protein abundance decreased as the fruit became mealy. Development of mealiness within individual fruit was often not uniform and lead to the existence of distinct juicy and mealy regions. Immunoblot analysis indicated that mealy regions contained substantially less expansin than juicy regions. Analysis of expansin mRNA abundance using a ripening-related expansin cDNA probe from peach indicated that expansin mRNA expression was also markedly reduced in mealy tissues. The relationship observed in this study between expansin expression and mealiness suggests a possible role for expansin in the development of the disorder.
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1. Introduction

Mealiness, also termed woolliness (Von Mollendorf et al., 1989, 1992) is a disorder of stone fruit in which the flesh develops a lack of juiciness during and following cold storage. A number of other quality disorders such as browning, gel

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formation, loss of flavor and failure to ripen also can be induced in stone fruit by chilling and, in addition to mealiness, are often collectively referred to as internal breakdown. Mealiness has a genetic basis and cultivars differ greatly in the length of time that the fruit can be stored prior to the development of the disorder (Crisosto et al., 1999). This difference in susceptibility is a primary factor that determines how long the fruit remain marketable following harvest.

Research into the biochemical cause of mealiness has primarily focused on relative differences in pectic substances that are observed in cell wall extracts from juicy or mealy fruit. Mealy fruit have been found to contain a large proportion of insoluble pectic material with higher molecular weight and a lower degree of esterification than juicy fruit (Ben-Arie and Lavee, 1971), and it is believed that pectin in this form binds free water in the tissue and produces the characteristic dry texture. Cold storage of stone fruit also results in a reduction in the activity of polygalacturonase (PG) (Buescher and Furmanski, 1978; Von Mollendorf and De Villiers, 1988), a key enzyme involved in pectin depolymerization and solubilization. It is believed that this loss of PG activity combined with continued pectin de-esterification as a result of the activity of pectin esterase (PE) leads to accumulation of the gel-forming pectic compounds (Ben-Arie and Sonego, 1980). In support of this idea, it has been shown that delayed storage and controlled atmosphere storage, two means of inhibiting the development of mealiness, also lead to an increased PG/PE activity ratio (Zhou et al., 2000c). Moreover, it has been demonstrated that soluble pectic fractions extracted from non-ripened peaches are capable of forming gels in vitro in the presence of either commercial PE or PE extracted from ripe peaches and that addition of PG decreased the rate of gel formation (Zhou et al., 2000a).

Although most evidence indicates a relationship between extractable PG activity assayed in vitro and the development of mealiness, it is possible that the reduction in polyuronide degradation observed in mealy fruit may not be solely due to in vivo changes in PG activity per se. The depolymerization of pectin is known to be a cooperative process, a good example of this being the de-esterification of pectin by PE that enhances susceptibility to PG degradation (Fischer and Bennett, 1991). In tomato it has been shown that polyuronide depolymerization occurs much more extensively in vitro than in vivo (Rushing and Huber, 1984; Huber and O'Donoghue, 1993) and that the action of PG is believed to be restricted by either physical or chemical means in vivo (Brummell and Labavitch, 1997; Seymour et al., 1987). While PE has been extensively studied in relationship to the development of mealiness, little attention has been paid to other proteins whose actions could modulate PG activity, and ultimately influence the degree of pectic degradation. Recently, it was shown that reduced expression of the ripening-related cell wall modifying protein expansin (LeExp1) in transgenic tomato plants resulted in marked decreases in polyuronide degradation, even though LeExp1 appears not to cause polyuronide depolymerization directly (Brummell et al., 1999). This observation lead the authors to speculate that LeExp1 influences the degradation of pectin by altering the manner in which PG interacts with its pectic substrates. In this study we examined the expression of peach expansins cross-reacting with antibody to LeExp1 in two different peach cultivars during the development of mealiness to address the possibility that expansins might also be involved in the development of mealiness in stone fruit.

2. Materials and methods

2.1. Fruit samples

Ripening 'Autumn Red' peaches (*Prunus persica*) were harvested on a weekly basis for a period of 6 weeks from an orchard in Fresno, CA beginning at the mature green stage and ending when the fruit had ripened to 16 N of firmness. After picking, the fruit were measured for firmness using a University of California Firmness Tester with a 7.9-mm diameter tip and samples of the fruit taken from the equatorial region, frozen in liquid nitrogen and stored at −80 °C. A total of five fruit were measured individually for firmness

and samples from each of the five were pooled for expansin quantification.

Additional peaches were harvested from a field near Parlier, CA and immediately placed into storage at 5 °C to facilitate the development of mealiness. The two cultivars used, 'Summer Lady' and 'O'Henry,' had been previously classified as being yellow-flesh cultivars susceptible to internal breakdown (Crisosto et al., 1999). At 3- or 4-day intervals fruit samples were removed from cold storage and ripened at 20 °C for approximately three days to ⩽18 N firmness at which time the fruit were visually evaluated for mealiness and subjected to free water determination. Occasionally, striking differences in the amount of mealiness present were visually apparent in different regions of an individual fruit. To enable comparisons of juicy or mealy sectors within individual fruit, samples were taken from each sector, frozen in liquid nitrogen and stored at −80 °C.

2.2. Evaluation of mealiness

Visual determination of mealiness consisted of lightly squeezing a section of each fruit by hand and noting the texture, as well as whether or not juice freely flowed from the fruit. Fruit with a coarse, dry appearance with little or no juice expression were classified as mealy, all other fruit were classified as juicy. To allow a more quantitative classification of mealiness, free water was measured in individual fruit following the protocol described in Crisosto and Labavich (2002). Approximately 5 g of tissue was cut in a longitudinal wedge from each peach with the remainder being peeled, frozen in liquid nitrogen and stored at −80 °C for use in later analyses. The 5-g sample was wrapped with four layers of cheesecloth and then squeezed using a homemade press at a constant 667 N force for a period of 1 min. The sample was pressed three more times in this manner after repositioning the sample prior to each application of force. The total weight of the juice that was expressed from the tissue sample was determined and divided by the weight of remaining tissue to give the percentage of free water present. Free water was measured in three or four fruit for each storage duration.

2.3. Protein gel blot analysis

A protein extraction procedure similar to that of Brummell et al. (1999) was used. Peach mesocarp was powdered in liquid nitrogen and homogenized in 2 volumes of buffer consisting of 40 mM sodium phosphate (pH 7.0), 3 mM EDTA and 1 mM DTT. The resulting mixture was centrifuged at $12\,000 \times g$ for 10 min and the supernatant discarded. The pellet was resuspended in 0.75 ml of SDS loading buffer containing 62.5 mM Tris (pH 6.8), 2% SDS, 10% glycerol and 1 mM DTT and then heated in a boiling water bath for 10 min. The sample was mixed, heated for a further 10 min and centrifuged at $12\,000 \times g$ for 10 min to produce a pellet of insoluble material. The supernatant was removed and utilized for subsequent immunoblot analysis. Protein was determined in the extracts by the Bio-Rad DC Protein assay (Bio-Rad Laboratories, Richmond, CA) using BSA as a standard.

Proteins were separated using SDS-PAGE on a 10% polyacrylamide gel and electrotransfered to a nitrocellulose membrane (Bio-Rad). Protein blots were blocked for 1 h using a solution of 5% non-fat dry milk and incubated overnight in PBS-Tween containing 0.4% non-fat dry milk and a 1:2000 dilution of an antibody raised to the tomato fruit ripening-related expansin LeExp1, as described in Rose et al. (2000). Following washing steps in PBS-Tween, the blot was incubated for 1 h in PBS-Tween with 0.4% non-fat dry milk and horseradish peroxidase-conjugated secondary antibody at a 1:75 000 dilution. The blot was again extensively washed with PBS-Tween and immunoreactive protein detected using the Pierce SuperSignal West Dura Chemiluminescent system (Pierce, Rockford, IL) and CL-XPosure X-ray film (Pierce). Use of the chemiluminescent substrate was as specified in the manufacturer's protocol.

2.4. Gene expression analysis

RNA was extracted from frozen peach pericarp by the method of Wan and Wilkins (1994). Total RNA (15 μg per lane) was subjected to electrophoresis on 1.2% agarose (w/v) and 10% (w/v) formaldehyde gels, visualized with ethidium bro-

mide to confirm equal loading, and transferred to Hybond-N membrane (Amersham). The blot was hybridized with a cDNA corresponding to the full-length 1193 bp sequence of the peach expansin *PpExp1* (accession number AB029083) that was radiolabeled by random hexamer priming using [α-32P] dATP (3000 Ci mmol, DuPont-NEN, Boston) and Klenow DNA polymerase (New England Biolabs, Beverly, MA). Hybridization was performed at 42 °C in 50% (w/v) formamide, 6 × SSPE, 0.5% (w/v) SDS, 5% Denhardt's solution, and 100 g l^{-1} of sheared salmon sperm DNA. The blot was washed three times in 5 × SSC and 1% (w/v) SDS at 42 °C for 15 min, followed by three washes in 0.5 × SSC at 65 °C for 20 min, and finally exposed to film.

3. Results and discussion

Ripening of the fruit during the 6-week course of the experiment was evident as firmness decreased gradually until week 4 and thereafter declined rapidly during the next 2 weeks (Fig. 1A). The change in firmness was also accompanied by changes in coloration as the fruit matured from its initial unripe green stage to a yellow ground color with dark red blush (data not shown). Proteins were extracted from fruit across the ripening stages and subjected to immunoblot analysis with an antibody raised to the tomato fruit ripening-related expansin LeExp1 (Rose et al., 2000). A cross-reacting protein of 27 kDa, corresponding to the predicted molecular mass of the ripening-related peach expansin *PpExp1* (Hayama et al., 2000), was detected in fruit at the 2 week stage, corresponding to the onset of softening, and in all subsequent stages (Fig. 1B). This protein was not, however, detectable in the initial sample (week 0) or after 1 week of tree ripening, even upon lengthening the exposure times of the immunoblots. This association between expansin protein accumulation and ripening agreed with previous studies showing an increase in LeExp1 mRNA expression (Rose et al., 1997) and expansin protein abundance (Rose et al., 2000) during tomato ripening. Enhanced expansin gene expression during ripening has also been

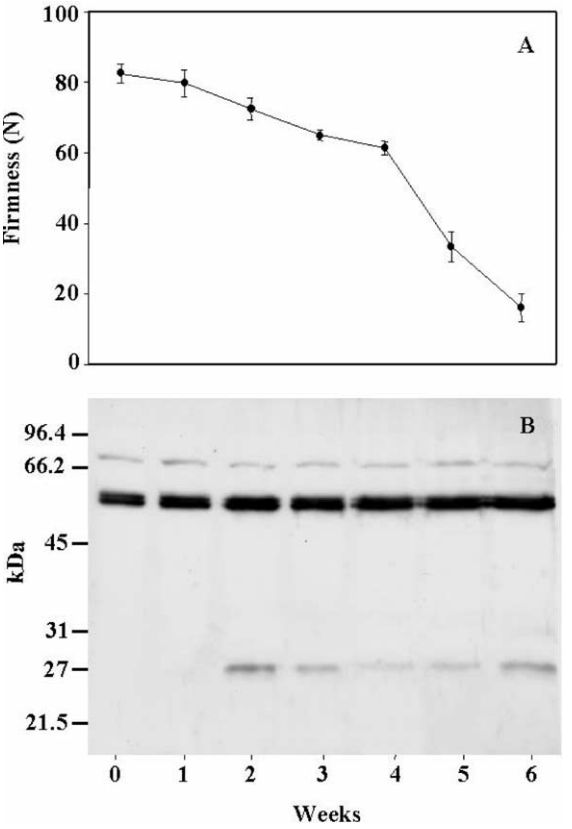

Fig. 1. Fruit firmness (A) and expansin protein accumulation (B) in ripening 'Autumn Red' peaches. Sampling was initiated (week 0) when fruit were full-size, yet green (green mature) and was completed (week 6) when the fruit had fully ripened to a firmness of ⩽18 N. Firmness measured at each sampling date represents the mean of five fruit ±S.E. Mesocarp tissue was pooled from each of the five fruit to extract proteins for the immunoblot analysis. Forty microgram of protein was added per lane. Immunoblots were probed with antibody raised to the tomato expansin LeExp1. Molecular weight markers are indicated to the left.

reported in strawberry (Civello et al., 1999) and peach (Hayama et al., 2000). Three cross-reactive bands of higher mass, including two of approximately 55-kDa, were also present in the immunoblots, however, unlike the cross-reacting 27-kDa protein, their abundance remained constant at all fruit maturities. The intensities of these bands, unlike that of the 27-kDa, remained constant at all fruit maturities. It was previously reported that the LeExp1 antibody recognized proteins of approximately 55-kDa from tomato and a number of

other fruit species (Rose et al., 2000). The antiserum is polyclonal and the identities of the higher molecular weight cross-reacting proteins are not known.

Visually evaluating a given peach sample for mealiness is subjective, inaccurate, and provides only a crude indication of the extent to which the disorder has developed. To enable a firmer basis for evaluation, a number of methods have been developed and utilized to quantify mealiness (Harker et al., 1997; Lill and Van Der Mespel, 1988; Von Mollendorf et al., 1989). Measurement of the percentage of free water, as used in this study, has been found to be an effective method for assessing mealiness (Crisosto and Labavich, 2002). Fig. 2 shows the percentage of free water for the peach cultivars 'O'Henry' and 'Summer Lady' following different durations of storage at 5 °C. 'O'Henry' displayed a range of free water values from 65.4 to 13.3% and 'Summer Lady' from 66.3 to 15.6%. Both of the cultivars exhibited a high degree of variability between individual fruit stored for the same length of time. Mealiness became visually apparent when free water percentages reached 38% for 'O'Henry' and 46% for 'Summer Lady' and it was clear that a loss of free water occurred prior to the onset of visible symptoms. Sometimes, as in the case of 'Summer Lady' fruit number 6, it was observed that fruit had a low free water content but had not been judged to be mealy by visual means. This further highlights the inadequacy of visual evaluation as a means of detecting mealy fruit.

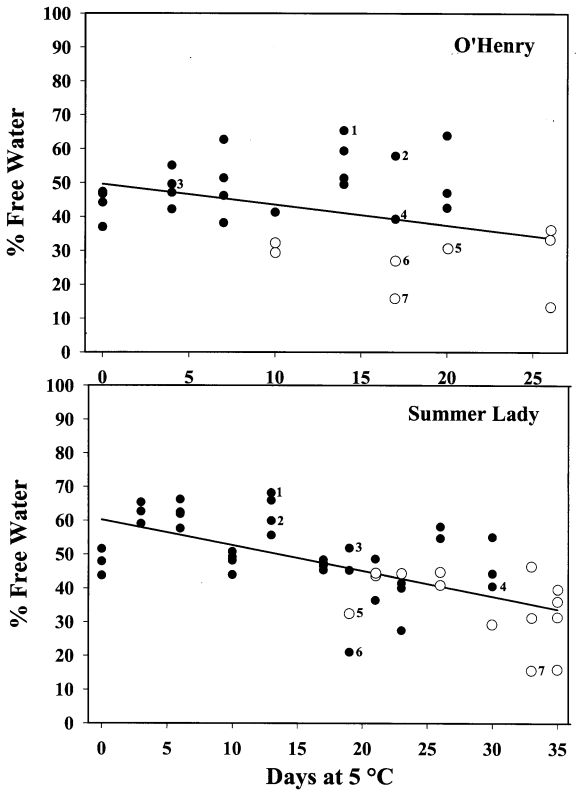

Fig. 2. Percentages of free water in 'O'Henry' and 'Summer Lady' peaches determined following different durations of storage at 5 °C and subsequent ripening. Each circle represents an individual fruit, closed circles being fruit that were visually judged to be juicy and open circles fruit that were mealy. Numbers to the right of some of the circles indicated fruit that were selected for expansin quantification.

Fig. 3 shows immunoblots, using the tomato expansin antibody, that were made from individual fruit samples from each cultivar. These samples, indicated by numbered circles in Fig. 2, differed substantially in free water content. Expansin protein, as determined by the presence of the 27-kDa band, was abundant in fruit from both cultivars that possessed a high free water content, but absent in low free water samples. Although both cultivars showed a similar rapid decline in expansin expression with decreasing free water content, the levels in 'Summer Lady' decreased more dramatically than in 'O'Henry' as the tissue became progressively more mealy. In both instances, the reduction in expansin abundance preceded the appearance of visual symptoms of mealiness. As noted in previous samples, 55-kDa cross-reacting bands of a similar intensity were also present in samples of both cultivars.

Occasionally, within individual fruit, sharply defined mealy regions were observed immediately adjacent to regions that were juicy in appearance. Comparison of these regions offered an opportunity to examine the relative abundance of expansin in juicy and mealy tissues within single fruit, eliminating possible effects of differences due to storage times and potential fruit-to-fruit variability. Immunoblot analysis of extracts from juicy/mealy regions of three fruit indicated that while the juicy areas contained high levels of expansin

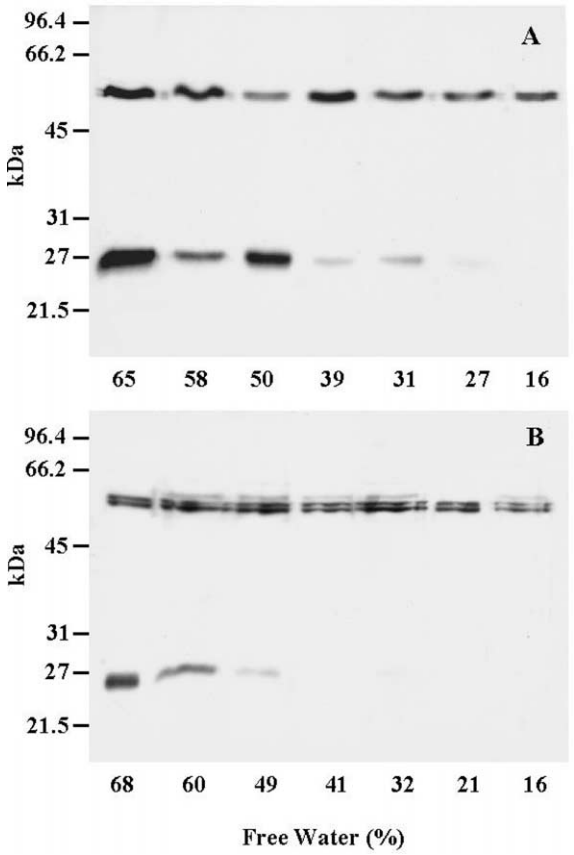

Fig. 3. Quantification of expansin protein present in 'O'Henry' (A) and 'Summer Lady' (B) peaches containing differing percentages of free water. Extracts were made from individual peaches that had been selected to provide a wide range of free water percentages. The peaches sampled correspond to numbered circles in Fig. 2. Forty microgram ('O'Henry') or 26 μg ('Summer Lady') of protein was added per lane. Immunoblots made using the extracts were probed with LeExp1 antibodies. Molecular weight markers are indicated to the left.

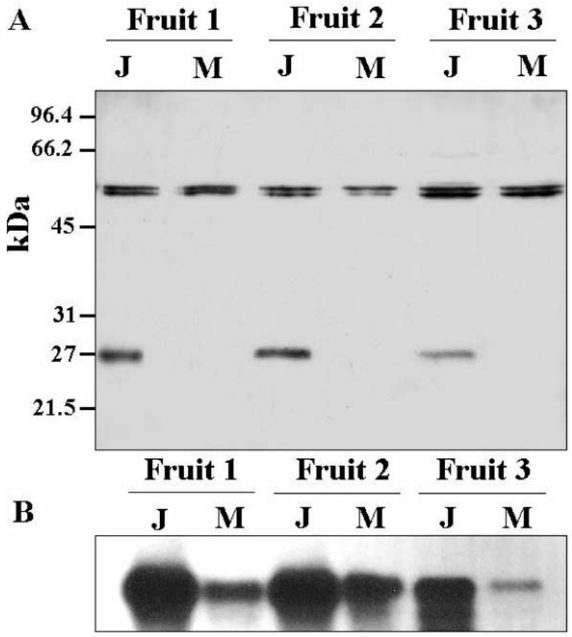

Fig. 4. Expansin protein and mRNA expression in juicy or mealy sectors within individual 'O'Henry' peach fruit following storage for 3 weeks at 5 °C and ripening of 3 d at 20 °C. Juicy (J) or mealy (M) sectors were identified by visual evaluation, sampled and subjected to immunoblot analysis using LeExp1 antibodies (A) and Northern blot analysis using PpExp1 cDNA (1193 bp) as a probe (B). Thirty microgram of protein was added per lane. Molecular weight markers are indicated to the left.

protein, expansin was essentially absent in the adjacent mealy areas (Fig. 4A). Additional immunoblots using different fruit sometimes detected a small amount of expansin protein in the mealy tissue, but the abundance was always far higher in juicy tissue than mealy (data not shown). Immunoreactive bands at approximately 55 kDa were again noted in both juicy and mealy tissues and these bands were once more of an equivalent intensity in both tissue types (Fig. 4A).

To determine whether transcriptional regulation contributed to the overall down-regulation of expansin protein abundance in the mealy fruit, an RNA gel blot analysis was performed using a cDNA probe corresponding to the peach ripening-related expansin PpExp1 (Hayama et al., 2000). Northern analysis of RNA extracted from the same juicy and mealy fruit samples used for the immunoblot experiment indicated that PpExp1 mRNA expression was markedly reduced in mealy areas of the fruit compared with the juicy areas.

Expansin protein was undetectable in unripe peaches, but was detectable at the onset and throughout fruit ripening (Fig. 1), suggesting a role for expansin in fruit softening. Mealiness in peaches, and other stone fruit, is believed to be caused by a ripening disorder, most commonly thought to be due to an imbalance in pectin

degradation (Ben-Arie and Sonego, 1980). In this study we have observed that the expression of expansin mRNA and protein is strongly suppressed in mealy tissue and that the suppression begins to occur very early in the development of the disorder, prior to the onset of visible symptoms. It is possible that the lack of expansin protein is related to the inhibition of ethylene production that has been observed during the development of mealiness (Zhou et al., 2000b) as expansin expression has been shown to be ethylene-regulated (Hayama et al., 2000; Rose et al., 1997). Although much work has been done showing a link between the activities of PG and PE and the development of mealiness, the relationship shown in this study between expansin and mealiness indicates that PG and PE may not be sole proteins involved. This conclusion may also help to explain results that we have obtained (Obenland and Carroll, 2000) showing a lack of complete correspondence between PG and PE activity and mealiness development. Since expansin has been postulated to be involved in controlling polyuronide depolymerization (Brummell et al., 1999) it is possible that an inhibition of expansin accumulation in the fruit could influence the expression of the disorder by limiting the ability of PG to degrade pectin. This possible synergy between PG and expansin warrants further experimentation.

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