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CELL WALL HYDROLASE ACTIVITY AND CELLULASE GENE EXPRESSION DURING ABSCISSION OF VALENCIA CITRUS FRUIT AND LEAVES

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Abstract. **The physiological and molecular events of ethylene-induced abscission in mature fruit and leaf abscission zones of 'Valencia' were examined. Continuous exposure of fruit explants to 2 or 55 µl/l ethylene for 20 and 40 hr resulted in marked increases in cellulase and polygalacturonase activities. The abscission cellulase had a pI of 7.5 and molecular weight of 51,000 Da. The abscission polygalacturonase had a pI of 5.5 and molecular weight of 66,000 Da. Two distinct abscission cellulase cDNA clones, pCX1 and pCX2, were used to determine cellulase gene expression in ethylene-induced abscission zones. Both abscission cellulase mRNAs of mature fruit were first detectable after 2 (2 µl/l) and 4 hr (55 µl/l) of ethylene treatment, and accumulation continued throughout the 40 hr ethylene incubation period. Both abscission cellulase mRNAs were also expressed in laminar abscission zones after a 40 hr ethylene-induction period. These data will help to elucidate the abscission process at the molecular level, making possible attempts to favorably alter mature fruit abscission.**

Abscission is a process that culminates in the shedding of plant parts. Abscission occurs in defined areas of the plant known as abscission zones. During abscission, cells within the abscission zone may divide and enlarge, but then ultimately undergo a loss of adhesion between adjacent cells. The cell wall hydrolase enzymes, endo-1,4-ß-glucanase (cellulase) and polygalacturonase (PG), are thought to have an important role in abscission. Their action results in the disassembly of cell wall polysaccharides in the abscission zone, and thus directly influences the fracture at the abscission plane and eventual detachment of the organ from the parent plant.

In Florida, fruit abscission has become a process of economic relevance for the citrus industry. With record crops forecast for the next decade, increasing harvesting costs, low fruit prices, and availability of labor uncertain (Whitney, 1995), developing strategies to harvest citrus fruit more economically is of paramount importance. A better understanding of the physiological and molecular events of citrus fruit abscission is a significant step toward the goal of more economical harvesting.

During abscission of mature citrus fruit, increased activities of cellulase and PG have been measured in the abscission zones, and ethylene accelerated the process (Greenberg et al. 1975; Goren and Huberman, 1976; Huberman and Goren, 1979). Although expression of abscission-related genes has not been explored in citrus, research with bean leaves has begun to deduce some of the molecular events associated with abscission. During abscission in bean leaves, *de novo* synthesis of pI 9.5 cellulase protein has been detected, indicating specific activation of cellulase gene expression. In addition, exogenous ethylene hastened the appearance of both cellulase protein, cellulase mRNA, and separation at the abscission zone (Tucker et al., 1988; delCampillo et al., 1988; 1990). In fruit of peach and avocado, specific activation of cellulase gene expression was associated with abscission (Bonghi et al., 1992; Tonutti et al., 1995). Specific activation of PG gene expression has also been demonstrated in abscising peach and tomato fruit (Bonghi et al., 1992; Kalaitzis et al., 1995).

We are interested in characterizing the abscission-related enzymatic and gene expression patterns of citrus abscission zones. Our goals in the abscission research program are to 1) provide physiological information about mature citrus fruit and leaf abscission, 2) determine the expression of key abscission-related genes induced by ethylene, and ultimately utilize the information gained to 3) alter the expression of key genes to achieve the desired abscission response in mature fruit. In this paper, we describe the enzymatic activities of cellulase and PG before and during ethylene treatment in the mature fruit abscission zones of 'Valencia' orange (*Citrus sinensis* [L.] Osbeck) explants. We have concentrated our efforts on the abscission cellulases of citrus fruit because of the importance of cellulase to the overall process of abscission (Sexton and Roberts, 1982). Cellulase gene expression was detected by measuring the accumulation of cellulase mRNA in the abscission zones of mature fruit and leaves of citrus.

Materials and Methods

Plant material and ethylene treatments. 'Valencia' orange fruit were harvested from 7-yr-old trees at the Citrus Research and Education Center, Lake Alfred, FL. Fruit were harvested once a month for 4 months beginning in January and ending in April 1995. Young developing fruit were harvested approximately 8 weeks after bloom, in May 1995. Fruit were removed from the tree by clipping the pedicel 2 to 3 cm above the calyx. Fruit explants were transported to the packinghouse facility, randomized, and either used directly after harvest or placed into chambers for ethylene treatment. For ethylene treatment, fruit explants were placed in temperature and humidity-controlled chambers (approx. 30C, 95% RH) and treated with 2 µl/l or 55 µl/l ethylene for various time peri-

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ods. Leaves were clipped at the base of the petiole and brought to the laboratory. Leaves were rinsed with distilled water, and then made into 3 cm explants by trimming the tissue 1.5 cm on either side of the laminar abscission zone. Leaf explants were placed on trays lined with hydrated absorbent paper and then placed in the ethylene chamber. Ethylene concentration inside the chamber was monitored by gas chromatography. Fruit (200 explants/treatment) to be used for enzymatic determinations were treated for 0, 20, and 40 hr with ethylene. A 40 hr treatment in the chamber without ethylene was included as control. Fruit (50 explants/treatment) or leaves (200 explants/treatment) to be used for nucleic acid extractions were treated for 0, 2, 4, 6, 20, and 40 hr with ethylene.

Abscission zones from control or ethylene-treated fruit were removed from the calyx region of the fruit explants with a cork borer. For enzymatic determinations, calyx abscission zone tissue (4 mm wide × 3 mm above and below the abscission plane) was immediately made into acetone powder by pulverizing the tissue in 20 volumes of ice-cold acetone. After collecting the insoluble material on Whatman filter paper by aspiration, the material was rewashed with the same volume of cold acetone. The recovered acetone powders were dried at room temperature, weighed, and then stored at -20C under desiccant until needed. For nucleic acid extractions, leaf abscission zone explants were further trimmed to 2 mm on either side of the zone, and the abscission zone tissue was removed and immediately frozen in liquid nitrogen and stored at -80C until needed. Fruit abscission zones were removed as described above, frozen in liquid nitrogen, and stored until needed.

Enzyme extraction and determination of cellulase and PG activity. Acetone powders, typically 1 g, were suspended in 10 volumes of solubilization buffer (1 M NaCl and 20 mM K_2HPO_4, pH 7.0) and stirred at 4C. After 1 hr, the suspension was centrifuged at 12,000 × g and the supernatant collected. The supernatant was desalted by passing through a column of Sephadex G-25 (Pharmacia PD-10) pre-equilibrated with 5 mM NaCl. The activities of cellulase and PG in the desalted extract were assayed by the release of reducing groups from the appropriate substrate (Nelson, 1944). The activity of PG was determined by incubation of an aliquot of the extract in 1% polygalacturonic acid (sodium salt), 0.1 M sodium acetate, and 0.15 M NaCl, pH 4.7 for 4 hr at 40°C. Cellulase activity was measured in the same buffer system, except that 1% carboxymethylcellulose (sodium salt, medium viscosity) was used as substrate and the reaction time extended to 24 hr.

Estimation of enzyme pI and molecular weight. An aliquot of desalted supernatant, representing approximately 25 mg protein, was combined with 2% ampholytes (pH range 3-10). Preparative isoelectric focusing was conducted using the Rotophor System (Bio-Rad Laboratories) according to the manufacturer's specifications. Twenty fractions were collected and each fraction was assayed for cellulase and PG activities. An aliquot of each fraction was also electrophoresed on SDS polyacrylamide gels (SDS-PAGE). For PG, active fractions were pooled, concentrated, and run on a Bio-Gel P-60 column equilibrated in 300 mM NaCl and 10 mM Na_2HPO_4, pH 7.5. Active fractions from the column were pooled and subjected to SDS-PAGE. Proteins on SDS-PAGE gels were visualized with silver stain.

mRNA extraction and Northern blot analysis. mRNA was extracted from abscission zones using oligo dT magnetic beads in a direct-from-tissue method adapted from McKendree *et al.* (1995). Twenty abscission zones were pulverized with a mortar and pestle in liquid nitrogen and quickly transferred to a vial containing 2.5 ml lysis/binding buffer (0.1 M Tris, 0.5 M LiCl, 10 mM EDTA, 5 mM DTT, and 1% LiDS, pH 8.0) and further homogenized. After centrifugation, the supernatant was extracted with phenol and then chloroform:isoamyl alcohol. The homogenate was centrifuged, and the supernatant transferred to a tube containing 200 μl of oligo dT magnetic beads (Perceptive Diagnostics) previously equilibrated with 200 μl lysis/binding buffer. Poly(A)$^+$ RNA was hybridized to the oligo dT magnetic beads at 4C for 10 min, the beads collected and washed with wash buffer (10 mM Tris, 0.15 M LiCl, 1 mM EDTA, and 0.2% SDS, pH 7.5), and the poly(A)$^+$RNA eluted from the beads with 30 μl DEPC H_2O at 55°C. The mRNA was concentrated and stored at -80C until needed.

mRNA (500 ng) was denatured with glyoxal/DMSO and separated on agarose gels (Sambrook *et al.*, 1989). In a separate but related study, two distinct abscission-specific cellulase cDNA clones (pCX1 and pCX2) were generated from cellulase genes expressed in ethylene-induced mature citrus fruit abscission zone tissues. DNA probes were made from each cellulase cDNA clone with the NEBlot Phototope Kit (New England Biolabs). Gels were blotted onto Immobilon-S neutral nylon membranes (Millipore) and the membranes probed with pCX1 and pCX2 under conditions of high stringency (6X SSC, 5X Denhardt's solution, 0.5% SDS, and 100 μg/ml Herring sperm DNA at 68C). Lumigraphs were developed using the Phototope Chemiluminescence Detection Kit (New England Biolabs).

Results

Enzymatic assays. Enzymatic assays were used to determine the presence and activities of both cell wall hydrolases during ethylene-induced abscission of mature fruit explants. Total cellulase and PG activity markedly increased in fruit abscission zones treated with ethylene (Table 1). Total enzyme activities were not significantly different in ethylene-induced calyx abscission zones of mature fruit during the 4 month sampling period (data not shown). Therefore data were pooled and averaged to indicate the enzymatic abscission response to ethylene. Levels of both enzyme activities were similar after 20 hr of exposure to either 2 μl/l or 55 μl/l ethylene. However, higher levels of enzyme activities were extracted from mature fruit abscission zones treated for 40 hr with 55 μl/l as compared to 2 μl/l ethylene. After 40 hr of 55 μl/l eth-

Table 1. Cellulase and PG activities extracted from mature fruit abscission zone tissue with or without ethylene exposure. The conditions of both enzymatic assays were as described in Materials and Methods. Data are the means +/- sd.

	μmol Red. grps/abscission zone			
	2 μl/l		55 μl/l	
Time of treatment	PG	Cx	PG	Cx
0 hr	30.5 +/- 3.6	19.7 +/- 3.4	—	—
20 hr	147.3 +/- 7.2	125.1 +/- 6.5	151.8 +/- 4.4	144.7 +/- 6.2
40 hr	219.1 +/- 6.9	151.9 +/- 5.7	375.0 +/- 8.0	193.9 +/- 6.0
40 hr, air	20.4 +/- 1.7	20.2 +/- 7.9	—	—
8 wk fruit, 40 hr	177.0 +/- 4.0	177.7 +/- 8.8	343.6 +/- 7.9	197.8 +/- 8.6

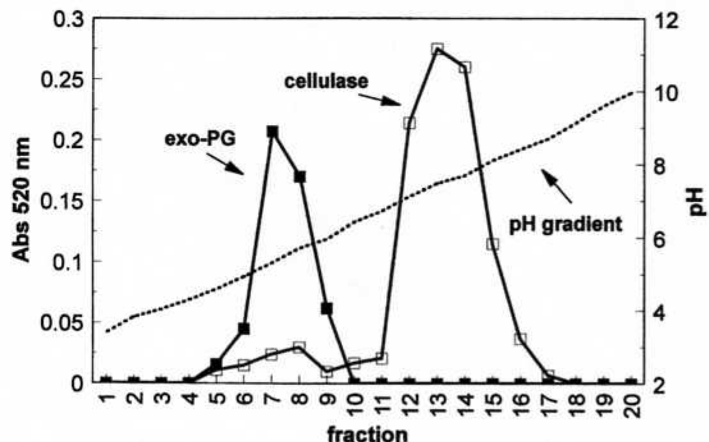

Figure 1. Separation of cellulase and polygalacturonase from extracts of ethylene-induced abscission zones by preparative isoelectric focusing. Mature 'Valencia' fruit abscission zones were treated with 55 µl/l ethylene for 40 hr as described in the text.

ylene treatment, recoverable enzyme activity was approximately ten times greater than the activity of non-ethylene treated control abscission zones. Total enzyme activities were similar in mature and young developing fruit treated with ethylene for 40 hr. Exposure of fruit explants to air under the same temperature and relative humidity conditions for 40 hr resulted in no detectable increases in cellulase or PG activities. Extending ethylene treatment time to 64 hr resulted in an insignificant change in cellulase activity, but PG activity continued to increase in a linear fashion (data not shown).

Protein characterization. Cellulase and PG proteins were characterized using standard techniques. Both enzymes could be separated by isoelectric focusing (Fig. 1). The peaks of cellulase and PG activity corresponded to an estimated pI of 7.5 and 5.5, respectively. Based on SDS-PAGE of the focused fractions, the molecular weight of the mature fruit abscission cellulase(s) was estimated to be 51,000 Da. After size exclusion chromatography followed by SDS-PAGE, the molecular weight of the mature fruit abscission PG was estimated to be 66,000 Da (Fig. 2).

mRNA accumulation. The two abscission zone cellulase cDNA inserts were used to probe Northern blots of mature fruit abscission zone mRNAs to determine the expression of

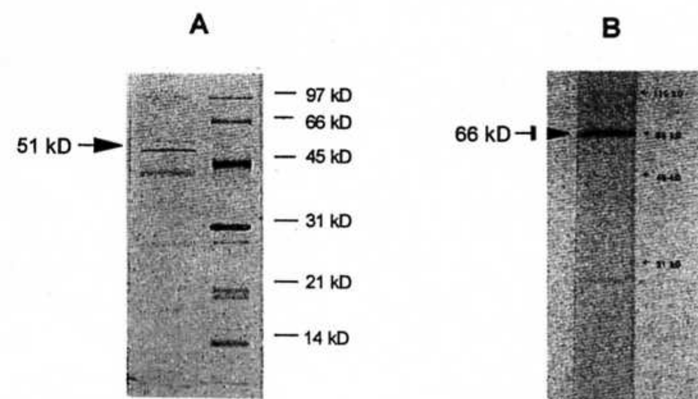

Figure 2. SDS-PAGE of partially purified cellulase (panel A) and polygalacturonase (panel B). Panel A: left lane— 51,000 Da cellulase, right lane— molecular weight stds. Panel B: left lane—66,000 Da polygalacturonase; right lane—molecular weight markers.

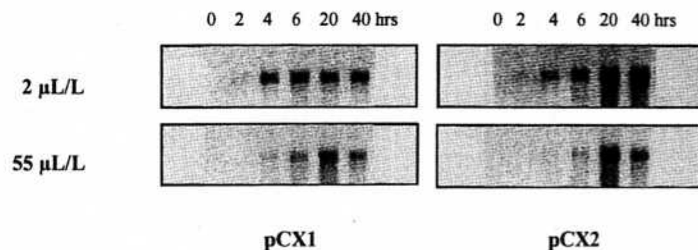

Figure 3. Cellulase mRNA accumulation in mature 'Valencia' fruit abscission zones treated with ethylene. Northern blot analysis of total poly A+ mRNA isolated from abscission zones treated with 2 or 55 µl/l ethylene was hybridized with DNA probes derived from two partial cellulase cDNA clones, pCX1 and pCX2.

both cellulases at harvest and during ethylene-induced abscission. Both pCX1 and pCX2 hybridized to a 2.2 kb mRNA from ethylene treated mature fruit abscission zones (Fig. 3). Cellulase mRNA transcripts could not be detected in mRNA extracted from abscission zones prior to ethylene treatment. Both pCX1 and pCX2-hybridizing transcripts were detected after 2 (2 µl/l) to 4 (55 µl/l) hr of continuous ethylene treatment. After 40 hr of high ethylene treatment, both pCX1- and pCX2-hybridizing fragments appeared to decrease.

Both pCX1- and pCX2-derived probes were used to determine the expression of cellulase in leaf abscission zones. Cellulase mRNA was not detected in laminar abscission zones prior to ethylene treatment (Fig. 4). After 40 hr of ethylene exposure, mRNA hybridizing to both pCX1 and pCX2 were detected in laminar abscission zones. The size of these transcripts were similar to those found in mature fruit abscission zones (i.e. 2.2 kb).

Discussion

The role of the cell wall hydrolases in abscission, and their induction by the hormone ethylene, is well established in plants. In developing and mature 'Shamouti' orange fruit, ethylene increased cellulase and PG activities in calyx abscission zones, and the appearance of these cell wall hydrolases was strongly correlated with cell separation (Greenberg et al., 1975; Goren and Huberman, 1976). We have shown that 'Valencia' fruit calyx abscission zones responded in a similar manner, namely that cellulase and PG were found in the abscission zones during abscission and their activities increased with ethylene exposure.

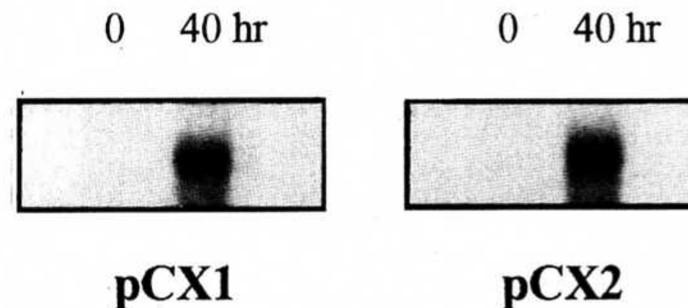

Figure 4. Cellulase mRNA accumulation in 'Valencia' laminar abscission zones treated with 55 µl/l ethylene for 40 hr. Northern blot analysis of poly A+ mRNA hybridized with cellulase DNA probes derived from pCX1 and pCX2.

The maturation stage of the fruit did not affect the response of the calyx abscission zone to a continuous supply of ethylene. Although sampled only at 8 weeks postanthesis, calyx abscission zone cells of young developing fruit responded to ethylene in a similar manner as those of mature fruit. Previous harvesting strategies in Florida sought to apply abscission materials in combination with mechanical harvesting aids to utilize the ethylene-induced abscission response to remove mature fruit more effectively from the tree (Whitney and Harrell, 1989). However, abscission materials caused unpredictable and often significant preharvest drop. During the harvest season, 'Valencia' posed an additional problem because mature fruit and young developing fruit were present on the tree at the same time, and these young fruit would also abscise. The increased cellulase and polygalacturonase activity in ethylene-induced abscission zones of young and mature fruit found in our study may in part explain the difficulties encountered, such as premature fruit drop of young fruit, with abscission materials on 'Valencia' orange.

We have identified and partially characterized the cellulase and PG enzymes from mature 'Valencia' fruit abscission zones. The ethylene-induced cellulase has an estimated pI of 7.5. In contrast, the reported isoelectric point of bean leaf abscission cellulase is 9.5 (Koehler et al., 1981). An acidic nonabscising form of cellulase (pI 4.5) has also been described in bean. No predominant acidic cellulase was found in fruit abscission zones of 'Valencia'; however, we have routinely seen a cellulase of pI 5.5 in extracts of 'Hamlin' fruit abscission zones (unpublished results). The pI 7.5 citrus cellulase has a molecular weight of approximately 51,000 Da. This is in agreement with reported molecular weights of abscission cellulases in avocado fruit (Tonutti et al., 1995), bean leaves (Tucker et al., 1988) and peach leaves, and fruit (Bonghi et al., 1992). The PG found in citrus leaf abscission zones is an exo-form (Riov, 1974), and we tentatively confirmed this in fruit abscission zones by capillary and high pressure LC (Brown and Burns, unpublished results). The citrus abscission zone PG has a pI of approximately 5.5 and a molecular weight of 66,000 Da. A wide range of molecular weights and pI's have been reported for exo-PG from other plant tissues (Burns, 1991), and the citrus abscission zone PG falls within these reported ranges.

Goren and Huberman (1976) reported 7 isoenzymes of cellulase in Shamouti orange abscission zones, with 3 associated with abscission. Kossuth and Biggs (1977) found at least 9 cellulases in abscission zones of 'Valencia' orange fruit. We do not know how many isoenzymes of cellulase are present in abscission zones of citrus. However, the two distinct cDNA cellulase fragments obtained by PCR analysis in our previous unpublished study with 'Valencia' orange strongly suggest that the peak of pI 7.5 cellulase activity represents at least two different cellulase isoenzymes in fruit abscission zones.

Ethylene treatment of mature fruit abscission zones induced transcription of cellulase genes as evidenced by the appearance of a 2.2 kb band that hybridized to probes derived from cellulase cDNA clones. A striking feature of the accumulation of cellulase mRNA is the short time period between the addition of ethylene and the appearance of the cellulase mRNA. Only 2 hr of continuous low ethylene or 4 hr of continuous high ethylene treatment was needed for the both cellulase messages to appear. Tucker et al. (1988) demonstrated that a high concentration of auxin in abscission zones of bean leaves prevented ethylene-induced accumulation of cellulase mRNA and cellulase enzyme. This suggests that concentration of endogenous auxin may have been low in the mature citrus fruit abscission zone area, or that ethylene exposure caused a rapid destruction of the hormone, or both. During abscission of bean and elder leaves, at least 18 hr of continuous exposure at 5 to 10 μl/l ethylene was required for the appearance of cellulase mRNA (Tucker et al., 1988; Taylor et al., 1994). In addition, the accumulation of cellulase mRNA in these leaves was highly correlated with the measured increase in cellulase enzyme activity and leaf detachment. We did not measure cellulase activity in citrus fruit abscission zone extracts treated with 2, 4 or 6 hr of ethylene. However, we did note that the abscission zones did not fracture, as was typical of abscission zones treated with longer ethylene durations. After 20 hr of ethylene treatment, abscission was initiated and a large increase in cellulase activity was measured. If cellulase synthesis and accumulation immediately followed the appearance of cellulase mRNA, then a loosening would be expected in the abscission zone tissue. Since this loosening was not seen by 6 hr of ethylene exposure, it is possible that, although cellulase may have been present, the cell wall may have been inaccessible to cellulase action. The synthesis and action of polygalacturonase may therefore be important for altering the architecture of the cell wall for eventual breakdown by cellulase.

The rapid accumulation of pCX1- and pCX2-hybridizing cellulase mRNAs indicated that response of the abscission zones to ethylene can be detected within 2 to 4 hr of application. This raises the interesting possibility of using either pCX probe to detect an early abscission response in abscission zones of trees treated with various abscission materials. The utility of this approach must be further tested, and experiments are planned to address this issue.

We are interested in characterizing the abscission-related enzyme activities and gene expression of citrus fruit and leaves. This information will be useful in our search for abscission patterns that can be utilized in a molecular approach to favorably alter mature fruit abscission. Because both citrus cellulases are expressed in ethylene-treated abscission zones of mature fruit, young developing fruit, and leaves, a successful strategy might be to search for a strong ethylene-responsive tissue-specific promoter that can be inserted upstream of the cellulase gene(s) to control its expression. We are currently directing our efforts at isolating full-length clones of pCX1 and pCX2, and mapping the promoter regions of cellulase genomic DNA clones isolated from our 'Valencia' genomic DNA library.

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COOLING PERFORMANCE EVALUATION OF STRAWBERRY CONTAINERS

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Abstract. **The decision by Florida strawberry growers to adopt the 40 × 48 inch (100 × 120 cm) MUM (Modularization, Unitization, and Metrification) shipping pallet required that they adopt new flats for these pallets. In order to help select the best packaging system, the growers desired cooling performance evaluations of different corrugated flat designs (several base dimension and vent arrangements), as well as comparisons of strawberries cooled in pint mesh baskets, and pint and quart clamshells. Commercial forced-air cooling tests were conducted to compare various package configurations and the results are presented. Strawberry weight loss and changes in the humidity of the cooling room air during forced-air cooling are also presented.**

During the 1994-1995 season, Florida sold 14 million flats of strawberries with an average price per flat of $8.47 and a total crop value in excess of $118 million (Fla. Dept. Agr. 1996). These figures are based on the standard 13 × 19.5 inch Florida flat packed with square injection-molded plastic mesh pint baskets filled with strawberries with an approximate total weight of 5.4 kg (12 lb). The Florida flats are shipped on 37.5 × 39 inch, 2-way pallets with six flats per layer and 16 flats high. Future reporting will have to account for the remarkable increase in strawberry packaging options that has occurred during the last two years.

Three years ago the national fresh produce industry adopted the 40 × 48 inch (100 × 120 cm MUM) 4-way pallet as the standard shipping pallet. The Florida Strawberry Growers Association, a progressive group of growers who continually explore emerging technologies and techniques to remain competitive, made the commitment to adopt the standard pallet. In response, strawberries are now being packed in new flats because the original Florida flat does not fit squarely onto the 40 × 48 pallet. Two flat options for the 40 × 48 pallet are currently available - the 12 × 20 inch and the 16 × 20 inch (Consumer) flats.

In addition to pallet and flat changes, individual containers are changing from the mesh baskets to clear, thermoformed plastic clamshell containers introduced a few years ago by California shippers. After some initial resistance, the clamshell containers have been received positively all the way along the line from field pickers to produce retailers and final consumers. Berries in clamshells do not need repacking and are handled less than berries in baskets. The containers can also be turned over for easy quality checking.

The past strawberry season was unique for the large number of package configurations used to ship berries or which received serious consideration for use with strawberries. The increasing popularity of clamshell baskets and the change to the 40 × 48 pallet have resulted in a large number of potential combinations of flats and baskets. This situation will inevitably result in a relatively few packaging systems being adopted as "standards", while other systems will fall by the wayside. The sheer number of options can cause aggravation in the field, the packinghouse, and the market. Cady (1995) compared berry capacity and materials costs of nine types of packaging and reported that the most economical packaging choice will vary with the cost of the materials and the market price. In ad-

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