Scanning Electron Microscopy, Video-image Analysis, and Confocal Imaging of Changes Occuring during Peach Fruit Abscission

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Abstract. 'Redhaven' peach (*Prunus persica* L. Batsch) fruit abscission has been investigated using scanning electron microscopy, computer-assisted video-image analysis, and confocal laser scanning microscopy in conjunction with chlorotetracycline and ethidium bromide as fluorescent probes for membrane Ca^{2+} and nuclear DNA. This enabled us to document the morphological changes of the cells, distribution patterns of membrane Ca^{2+} in the constituent cells of the abscission zone, and the nuclear morphology with accompanying changes in nuclear DNA. The digitized images of CTC-fluorescence emissions revealed that the membrane Ca^{2+} levels in the pre-abscission zone (control) is uniform and similar to that present in the cells of the spongy proximal region of the peduncle and that of the fruit parenchyma. However, with the induction of abscission, 2 days after embryotomy, there was a significant increase in membrane Ca^{2+} in the cells of the abscission zone compared to the neighboring cells of the fruit and the peduncle. Thereafter, with the gradual separation of the cells and the concomitant vacuolation, the membrane Ca^{2+} level decreased substantially. Confocal imaging of EB labeled cells of the abscission zone before induction invariably revealed a well-organized nucleus. However, during cell separation, significant changes in the cellular and nuclear morphology occurred, including 1) rounding of cells, 2) reduction in the nuclear volume, and 3) concomitant fragmentation of nuclear DNA. The possible role of Ca^{2+} during the process of peach fruit abscission and nuclear DNA fragmentation leading to cell death is discussed. Chemical names used: chlorotetracycline (CTC), ethidium bromide (EB).

Studies on fruit abscission in peaches (Bukovac, 1971; Daniell and Wilkinson, 1972; Dermen and Stewart, 1973; Martin and Nishijima, 1972; Jerie, 1976; Schneider, 1977) have mainly dealt with chemically induced fruit drop (Arnold et al., 1981; Nelson et al., 1984). Following the detailed anatomical studies of Nelson et al. (1984), Rascio et al. (1985) investigated some of the ultrastructural and biochemical aspects related to peach fruit abscission.

Recently, Ca^{2+} has been considered as an important intracellular messenger in plants (Poovaiah, 1988) and has been implicated in senescence-related biochemical processes. Calcium is essential for the maintenance of structural integrity of biomembranes and cell walls (Hanson, 1983; Jones and Lunt, 1967; Marinos, 1962; Paliyath et al., 1984; Poovaiah and Leopold, 1973; Poovaiah and Rasmussen, 1973).

Stösser et al. (1969a, 1969b) histochemically investigated abscission layer formation in *Prunus cerasus* L. These authors reported that the cells of the abscission layer contained less total polysaccharides than the adjacent cells. They also found that the pectins were degraded and that cellulose associated with the walls was broken down, resulting in cell separation. Interestingly, they also detected a decrease in Ca and Mg in the cell walls during

abscission. A striking observation made by these authors was the relatively lower ^{45}Ca binding capacity of the cells of the abscission layer than that of the distal or proximal layer of cells. In a subsequent detailed histoautoradiographic study Stösser (1969a, 1969b) reported that, in sweet and sour cherries, ^{45}Ca was uniformly distributed in the abscission zone and in the tissue distal and proximal to it. Furthermore, the level of ^{45}Ca in the abscission zone decreased and was lost from these cells after initiation of abscission.

It is known from earlier studies (Nelson et al., 1984; Rascio et al., 1985) that fruit drop in peaches occurs by the activation of three abscission zones (AZs), namely, AZ1 (present between the peduncle and the twig), AZ2 (situated between the flower receptacle and the peduncle), and AZ3 (situated between the fruit and the receptacle). However, there is no information pertaining to the changes in Ca^{2+} levels or in the nuclear DNA of the constituent cells of the AZ3 zone. In the present study, we have investigated structural aspects of the AZ3 cells, the spatial and temporal changes in membrane Ca^{2+} (Wolniak et al., 1980), and the changes in nuclear DNA in cells of the abscission zone in peach

Materials and Methods

Plant material and experimental treatments. All experiments were carried out on peaches from 5-year-old 'Redhaven' trees grown in the orchards of the Univ. of Udine (Italy) during May to June 1993. Abscission was experimentally induced in the fruits by embryotomy at the end of stage I of fruit development (Connors, 1919). Embryotomy was carried out as described by Rascio et al. (1985). Briefly, 6 to 10 fruit (with a transversal diameter of 37 mm) were cut and the seeds were scooped out with a sterilized scalpel,

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after which the kinetics of fruit drop was monitored.

Scanning electron microscopy. Samples of $\approx 3 \text{ mm}^2$ inclusive of the AZ3 zone were fixed in 3% glutaraldehyde prepared in 0.06 M cacodylate buffer at pH 7.2 for 30 min at room temperature, rinsed with the same buffer, and immediately dehydrated through ethanol series. For critical-point drying, ethyl alcohol was replaced by

freon 113 and then by liquid CO_2 , which was brought to critical point in Balzers chamber. Subsequently, samples were coated with gold in Edwards sputter coater S 150 A and observed with Philips SEM 501 B. Black and white photographs were taken with Ilford PAN F ISO 50 film.

Labeling with fluorescent probes. Fresh sections or isolated

Fig. 1. (a) Vertical macroscopical view of the abscising region (after 4 day\ of embryotomy) between the peduncle (p) and the fruit (f). (b) Higher magnification of a part of the abscising region showing the differences in the texture of the tissue in the fruit (f) and the peduncle (p). (c) Close-up view of the mucilaginous secretion (arrows) present in the abscising region.

cells of the abscission zone were labelled with 20 μM CTC prepared in 4.4% glucose plus 2.5% mannitol for 5 min, rinsed in the same solution without CTC, and examined. The control included post-treatment of CTC-labeled sections with 5 mM EGTA. For labeling of the nuclei, cells were initially fixed in 2.5% glutaraldehyde (in

phosphate buffer, pH 7.4) for 15 min at room temperature, rinsed in the buffer, and incubated in 2 μM ethidium bromide. Cells were then rinsed in three changes of the buffer for 5 min each and observed under a CLSM as described below.

Video recording and image analysis of CTC-fluorescence.

Fig. 2. (a) Scanning electron micrograph of the longitudinal profile of tissues of the abscission zone (AZ3), the fruit parenchyma and that of the peduncle. Scale bar = 130 μm . (b) Scanning electron micrograph of the spongy fruit parenchyma and the epidermal hair. Scale bar = 30 μm . (c) Scanning electron micrograph of the proximal band of spongy cells with large intracellular space (n = nucleus, w = cell wall). Scale bar = 30 μm . (d) Scanning electron micrograph of the surface view of the small distal band of cells adjoining the fruit parenchyma. Note the presence of relatively fewer intracellular spaces. Scale bar = 30 μm . (e) Longitudinal view of an intact xylem vessel (xy) present in the vascular strand of the control material. Scale bar = 60 μm .

Cells loaded with CTC were examined with a Leitz Orthoplan epifluorescence microscope. Ultraviolet excitation was obtained from a 50-W mercury-vapor lamp and the dye was excited using a combined filter set: BP365 (excitation filter), FT395 (dichroic mirror), and LP397 (barrier filter). Fluorescence emanating from the cells was observed with either 20×/0.50 Pol or 40×/0.75 Pol Plan-Neofluar objectives combined with 1 Ox eyepieces. For image acquisition, fluorescence signals emanating from the sections were recorded with a black and white video camera (Sony DXC-101/101P; at low light setting) mounted on the microscope tube (Tirlapur and Cresti, 1992; Tirlapur et al., 1992). Individual video images were subsequently transferred to the hard disk of the host computer.

For image analysis, images were retrieved from the hard disk and the hardware and software system supplied with the BioRad MRC 500 confocal laser scanning microscope were used to manipulate and enhance fluorescence microscope images. Digitization and pseudocolor representation (wherein variations in pixel intensity profiles are demonstrated using color coding from 0 to 255 gray values) of the images was done as described by Tirlapur and Cresti (1992).

Confocal laser scanning microscopy of EB-labeled cells. The nuclear EB fluorescence was excited using a GHS filter set interfaced with an Argon-ion laser scanner of a BioRad MRC 500 laser scan confocal microscope. A Nikon oil immersion objective (600×, PlanApo) was used for noninvasive optical sectioning with zoom setting at 3 and scan speed of 3 sec per frame while at least nine frames were averaged with the kalman filter. The z-series images were acquired every 0.5 or 1.0 µm spacing steps by the computer-controlled motor drive. A total of 15 or 30 optical images was collected and stored in the hard disk memory of the host computer for further image analysis. Photobleaching of EB fluorescence was minimized by scanning the samples during the acquisition of the images, and the laser intensity was attenuated by interfacing a variable neutral density filter into the illumination path.

Image analysis including pseudocolour representation was carried out using the standard software supplied with BioRad MRC 500 confocal laser scanning microscope (Bacon et al., 1991; Tirlapur and Cresti, 1992; Tirlapur et al., 1992, 1993a, 1994b).

Results

Observation of the AZ3 zone 4 days following embryoctomy (Fig. 1 a and b) reveals that these cells have an amorphous appearance unlike that of the neighboring cells of the fruit or the receptacle. Following separation of the cells, mucilaginous matrix (Fig. 1c) is often seen in the space separating the fruit and the receptacle.

Scanning electron microscopy. The scanning image (Fig. 2a) of thick median section of the AZ3 (before induction) and a part of the pedicel reveals the presence of proximal spongy region and a distal compact region. The outer surface of the fruit parenchyma (Fig. 2b) is covered by numerous trichomes that are absent on the surface of the pedicel. The abscission zone constitutently appears to be formed by two different set of bands of cells consisting of 8 to 10 rows. The proximal band of cells has a spongy appearance but with relatively more intercellular spaces (Fig. 2c) than distally located small cells that have very little intercellular spaces (Fig. 2d). The zone also contains well-organized vascular bundles consisting of phloem and xylem elements (Fig. 2e) traversing between the receptacle and the fruit. In the spongy fruit parenchyma, some of the cells contain highly organized crystalline

inclusions (Fig. 3a). The first morphological evidence of abscission by way of cell separation was apparent 4 days after embryoctomy (Fig. 3b). During this period, the vascular strands between the receptacle and the fruit were severed (Fig. 3c).

Video imaging of changes in CTC fluorescence in the AZ3 zone. In the longitudinal optical view, relatively high CTC fluorescence was observed in the central part of the vascular tissue between the

Fig. 3. (a) Scanning image of (a) a highly organized crystal (possibly calcium-oxalate) in one of the fruit parenchyma cell. Scale bar = 7.5 µm. (b) Scanning electron micrograph of the abscised zone with a few separated cells. Scale bar = 30 µm. (c) Scanning electron micrograph of the abscised zone in the area of the vascular strands. Note the separation of the xylem vessels. Scale bar = 30 µm.

receptacle and the fruit, while in the pre-abscission AZ3 and in the tissue distal and proximal to the pre-abscission AZ3, CTC fluorescence was uniformly low (Fig. 4a). However, 2 days after embryotomy, a significant increase in CTC fluorescence in the cells of AZ3 could be clearly visualized (Fig. 4b). A slight increase in the CTC fluorescence in the tissue distal and proximal to the AZ3 could be seen. Control samples posttreated with 5 mM EGTA did not reveal any fluorescence above the background level (data not shown).

With the progression of abscission, the cells began to separate and clusters of 20 to 30 club-shaped or spheroidal cells (Fig. 4c) could often be found in AZ3. The rounding of these cells was accompanied by the development of small vacuoles that gradually coalesced into one large vacuole (Fig. 4d). During the later stages of cell separation and rounding, CTC fluorescence in >80% of the cells decreased, while there was an increase in the CTC fluorescence in the intercellular spaces (Fig. 4e).

Confocal laser scanning microscopy of nuclear DNA in abscising cells. The nuclei in the club-shaped abscising cells were generally found in the narrow end of the cell (Fig. 5a). With further vacuolation of this cell type, the originally spherical nuclear

morphology was obliterated with a concomitant loss in the EB stainability (Fig. 5b). Similarly, the cells before separation contain a large nuclei showing significantly high EB stainability (Fig. 5c). Following cell separation and rounding (Fig. 5 d and e), which is accompanied by vacuolation, there is gradual change in nuclear morphology and a decrease in the EB stainability. Eventually, in highly vacuolated cells (Fig. 5 f and g) with peripherally regionalized cytoplasm, there is further decrease in nuclear EB stainability. The dead cells in the population are characterized by fragmentary remains of the nuclei that show weak EB fluorescence (Fig. 5h).

Discussion

The morphological features of the abscission region in peaches are in several ways comparable to those reported earlier by Rascio et al. (1985). They are distinguishable before activation and are thus consistent with the target-cells hypothesis proposed by Osborne (1979). The abscission zone consists of two discrete proximal and distal bands of cells, the former composed of large cells with numerous intercellular spaces and the latter with relatively smaller cells with few intracellular spaces. This situation is analogous to

Fig. 4. (a) Video Image of the pre-abscission zone showing low level of CTC fluorescence. Scale bar= 120 μ m. (b) Video image of the AZ3 showing the increase in the CTC fluorescence 2 days after embryotomy. Scale bar = 90 μ m. (c) Bright-field video image of cluster of abscised round cells 4 days after embryotomy. Scale bar = 60 μ m. (d) Higher magnification of abscised round cells showing the presence of large vacuole (v) 4 days after embryotomy. Scale bar = 15 μ m. (e) Video image of CTC fluorescence of the cluster of cells comparable to that shown in Fig 4c. Note the loss of cellular CTC fluorescence in most of these cells and the presence of high CTC fluorescence in the intracellular spaces. Scale bar = 30 μ m.

Fig. 5. Pseudocolor coded confocal images of EB-labeled cells of AZ3 zone. The inset wedge represents pixel-intensity values between 0 to 255. (a) Confocal image of a isolated club-shaped cell of the AZ3 with a spherical nuclei (n) at the base. Pseudocolor coded to show the intensity of EB fluorescence of nuclear DNA. Scale bar = 10 μ m. (b) Confocal image a vacuolate club-shaped cell in the population of the AZ3 with obliterated nuclei (n). (c) Pseudocolor coded EB fluorescence image showing a large nuclei (n) in the unseparated cell of the AZ3 with distally located vacuole (v). (d-f) Pseudocolor coded confocal images of completely separated AZ3 cells which began to round-off. Note the increase in the size of the vacuole (v) and gradual change in the size, loss in the EB stainability and the shape of the nuclei (n). (g) Pseudocolor coded confocal image of a AZ3 cell showing peripherally regionalized cytoplasm and loss in the nuclear (n) DNA-associated EB fluorescence. (h) Pseudocolor code image of a highly vacuolate dead cell of the AZ3 showing very weak EB fluorescence allocated with the fragmented nuclei (n).

that observed in abscission regions of other taxa (Sexton and Roberts, 1982). However, we were not able to detect the presence of any cluster of thick-walled cells in the abscission zone before or after induction either in the scanning electron microscope images or in semi-thin sections as observed by Ruscio et al. (1985). This discrepancy might be due to the differences in the age of the plants under investigation or the differences in the climate. However, the possible role of such a group of cells in the induction of abscission is still unclear.

The scanning images of the completely developed abscission zone reveal that the abscission layer is also formed across the vascular bundles leading in part to the separation of the vascular strands. Such a situation has so far not been reported in *Prunus persica*. Based on our data, it is not possible to determine the mechanism for rupture of xylem vessels. However, it seems logical to consider that it is similar to that observed in *P. cerasus* (Stösser et al., 1969b), wherein the final separation is apparently achieved by mechanical fracturing.

The observed features of CTC fluorescence in the cells of the AZ3 indicate that it is mainly reporting Ca^{2+} distribution because: 1) unlabeled cells hardly show any fluorescence beyond the background levels, 2) posttreatment of CTC-labeled sections with 5 mM EGTA (Tirlapur and Willemse, 1992; Wolniak et al., 1980) eliminates the fluorescence, 3) the CTC has higher affinity to Ca^{2+} in apolar environments by a factor of 3 than that for Mg^{2+} , and 4) CTC fluoresces in the cellular locations that contain high levels of free Ca^{2+} adjacent to hydrophobic sites, particularly membranes (Tsien, 1989). More recently, Timmers et al. (1991) used confocal laser scanning microscopy to demonstrate that distribution of Ca^{2+} visualized by Fluo-3 (a fluorescent indicator for free Ca^{2+}) and that detected with CTC in plant cells are identical.

Following the activation of the AZ3, we found an increase in the CTC fluorescence, which by implication reflects an increase in the level of membrane-associated Ca^{2+} of these cells. Such a transitory increase in Ca^{2+} in the cells of the abscission zone has also been reported during abscission in bean (Rasmussen and Bukovac, 1969). However, Stösser (1969) was unable to detect such an initial increase in ^{45}Ca in the cells of the abscission zone in *P. cerasus*. This might be due to the differences in the sampling procedure adopted (Stösser, 1969). Nevertheless, our finding of subsequent depletion of Ca^{2+} from the cells of the AZ3 concomitant with the process of cell separation are consistent with those of Stösser (1969) and Stösser et al. (1969a, 1969b) reported for cherries.

The observed transitory increase in Ca^{2+} in the cells of the AZ3 following embryoctomy could be linked to C^{2-} -dependent activation of polygalacturonase (Pressey and Reger, 1989) implicated in degradation of cell walls (Rascio et al., 1985). In fact, it has been demonstrated that, with the activation of the AZ3 zone, there is a significant increase in endocellulase and exopolygalacturonase activity (Rascio et al., 1985). Moreover, the lysis of the middle lamella is observed throughout the abscission zone (Ruscio et al., 1985) during this period (i.e., 2 days after the treatment). It may well be that the initial increase in Ca^{2+} in the cells of the AZ3 is involved in activation of Ca^{2+} -dependent nucleases involved in fragmentation of the nuclear DNA leading to death of these cells. This view is consistent with the gradual decrease in the size and morphology of the nucleus and also the loss of DNA. Interestingly, Nii et al. (1988) have demonstrated that, during senescence of leaves in *P. persica*, changes in the DNA content are accompanied by changes in the levels of nuclease activity and that cell nucleus degradation is mediated by activation of a nuclease system as proposed in the present study.

These changes in nuclear morphology and loss of nuclear DNA

are analogous to the situation during apoptosis or programmed cell death seen in animal systems and that recently reported in *Euglena gracilis* (Tirlapur et al., 1992, 1993b). It is therefore conceivable that the death of abscising cells in peach is, in part, a physiological event for elimination of these cell types.

Our finding of high CTC fluorescence in the intercellular spaces is consistent with the suggestion (Stösser, 1969) that Ca from the cells of the abscission zone is lost. It was also proposed by Stösser et al. (1969) that there is a loss of Ca^{2+} from the cell walls, particularly that complexed with the pectic bridges. Thus, the major consequence of induction is the selective permeability of the membrane that results in leakage of ions as observed in senescing tissues (Ferguson, 1984; Ferguson and Drobak, 1988).

Using the modified antimonate precipitation technique (Poenie and Epel, 1987), we have recently observed unloading of cell-wall-associated Ca^{2+} into the intracellular spaces during later stages of cell separation (U.K. Tirlapur, unpublished data). Brady et al. (1985) investigated the interaction of the amount and molecular forms of polygalacturonase and Ca^{2+} in tomatoes and found that the differences in the digestion of the walls by polygalacturonase were dependent upon differences in Ca^{2+} content or the distribution of these ions. In a subsequent investigation, Burns and Pressey (1987) studied the Ca^{2+} content in the cell walls of ripening peaches and reported that the Ca^{2+} content remains constant in peach mesocarp. These authors suggested that pectin deesterification and wall softening during ripening may in part be factors that control the presence and amount of cell wall-middle lamella-associated Ca^{2+} .

The observed initial increase in Ca^{2+} might be potentially involved in activation of calmodulin mediated senescence of the cells as observed in pea (Leshem et al., 1984). This view is consistent with our recent confocal imaging of immunocytochemically detected changes in calmodulin levels in the cells of AZ3 (U.K. Tirlapur, unpublished data).

It has been demonstrated that newly synthesized protein may play an important role during induction of abscission (Rascio et al., 1985; Sexton and Roberts, 1982; Stösser, 1971); hence, it seems quite unlikely that abscission is directly mediated by ubiquitin-like protein (Hershko and Ciechanover, 1992; Jentsch et al., 1990; Vierstra, 1987) that has hitherto been implicated in proteolysis in different cell types.

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