

## ULTRASTRUCTURAL ASPECTS OF PROGRAMMED CELL DEATH IN THE EXOCARP OIL GLANDS OF MANDARIN (*CITRUS DELICIOSA* TEN.)

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**Abstract:** In the exocarp of mandarin fruit (*Citrus deliciosa* Ten.), numerous globular/ovoid oil glands occur. In the centre of each gland, an essential oil-accumulating cavity is formed by a process of cell lysis. This process is induced by PCD which becomes ultrastructurally evident by the presence of a large number of fragmented ER-elements with a dark content. They appear only at the stage of PCD initiation and they disappear afterwards. ER-elements are scattered over the entire cytoplasmic area and do not locally aggregate or associate with other cell organelles and particularly the vacuoles. TEM observations favour the interpretation that ER involves in PCD of oil gland cells by releasing hydrolytic enzymes directly to the cytosol.

**Keywords:** *Citrus deliciosa* Ten., endoplasmic reticulum (ER), hydrolytic enzymes, oil cavities, programmed cell death (PCD)

### Introduction

Programmed cell death (PCD) is a catabolic process which occurs during late development of specific tissues and results in their necrosis. It is genetically controlled and is associated with important physiological activities of the plant. PCD functionally deviates from deadly damages of tissues accidentally induced by biotic or abiotic agents. Plant tissues that undergo PCD are the following [BELL, 1996; RAVEN & al. 1999; EGOROVA & al. 2010; BOSABALIDIS, 2012]:

*Xylem.* Vessel elements of vascular bundles ultimately undergo disorganization of their protoplasts to constitute open tubular structures involved in water conduction.

*Aerenchyma.* Parenchyma cells in specific tissues disintegrate to create large intercellular spaces facilitating movement of gases during respiration, transpiration, and photosynthesis.

*Sclerenchyma.* Elongated cells with thick walls (fibers) undergo degeneration of their protoplasts contributing to the support of organs.

*Cotyledons.* During seed germination, cotyledon cells degrade after their content has been mobilized for growth of the seedling.

*Pith.* The central cells of some primary stems lyse resulting in the formation of an axial cavity which makes stems flexible.

*Capsule.* In the wall of the poppy capsule below the star-like stigma, groups of cells disintegrate to locally create pores through which seeds pass out and become dispersed.

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*Cork.* Dead cells with water-impermeable walls constitute the outer layer of the periderm in woody stems. They substitute the epidermis when herbaceous plants turn into woody plants.

*Tapetum*, embryo suspensor, nucellus. Their cells degrade after they have completed their nutritional mission.

*Megasporogenesis.* In the ovule of angiosperm flower, three out of four meiospores disintegrate to allow embryosac to develop.

*Salt glands.* Glands on leaves of halophytes differentiate, secrete NaCl, age, and ultimately die becoming replaced by other active glands. In this way the salt is continuously eliminated from the plant and does not accumulate in the tissues to create hyperosmotic phenomena.

*Lysigenous oil glands.* In the exocarp oil glands of citrus fruit, the central cells disorganize to create an internal cavity in which the secreted essential oil is accumulated.

In the present work, the manner of formation of the central cavity in the oil glands of the fruit peel of mandarin was studied as being associated with a process of programmed cell death.

### Materials and methods

The present study was conducted at the mandarin orchard of the Agricultural School Farm of the Aristotle University, Thessaloniki. Small segments of ovaries (3-4 mm in diameter) randomly taken from ten open flowers of different plants of *Citrus deliciosa* Ten. (Rutaceae), were used. Ovary segments were initially fixed for 3h at room temperature with 5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2). After washing in buffer, the specimens were post-fixed for 4h with 2% osmium tetroxide, similarly buffered. Samples were then dehydrated in an ethanol series (50-100%) and finally embedded in Spurr's resin. Semi-thin sections (1  $\mu$ m thick) for light microscopy were obtained with a Reichert Om U<sub>2</sub> microtome (Reichert Optische Werke AG, Wien, Austria), stained with 1% toluidine blue in 1% borax solution, and observed in a Zeiss Axiostar Plus light microscope (Zeiss Microimaging GmbH, Göttingen, Germany). Ultrathin sections (80 nm thick) for electron microscopy were cut using a Reichert-Jung Ultracut E ultramicrotome, stained with uranyl acetate and lead citrate, and examined with a JEM 2000 FX II transmission electron microscope (Jeol Ltd, Tokyo, Japan).

### Results

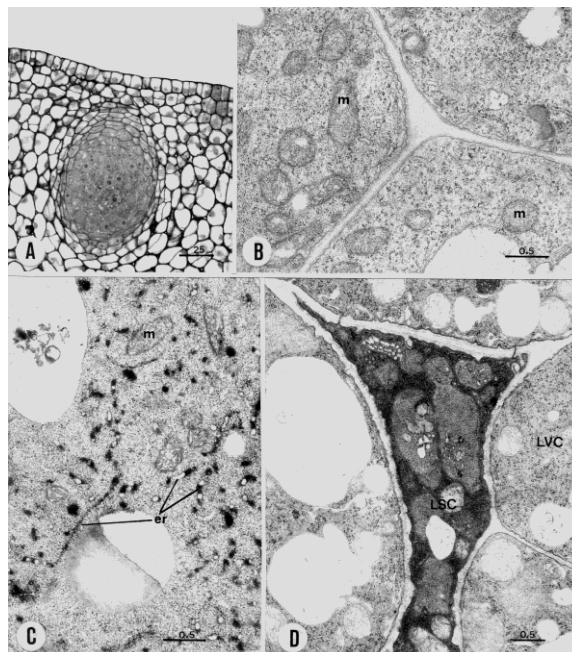
The oil glands in the exocarp of *Citrus deliciosa* exhibit, after conclusion of cell divisions, a globular/ovoid shape (Fig. 1A). The gland cells appear under the light microscope plasma-rich with small scattered vacuoles and they greatly differ from the surrounding parenchyma cells which contain large central vacuoles (Fig. 1A). Under the electron microscope, gland cells appear densely occupied by ribosomes and bear numerous mitochondria (Fig. 1B). The endoplasmic reticulum is represented by a few profiles of rough elements.

At late development of the gland, the cells of the central region enter PCD, i.e. they start to disintegrate and ultimately lyse creating a cavity (Fig. 2A, B). The cavity opens always prior to the stage of essential oil secretion to facilitate accumulation of the secreted oil. Opening of the cavity appears to initiate from a single cell in the centre of the gland which becomes disorganized (the degenerated cell organelles like plastids, dictyosomes,

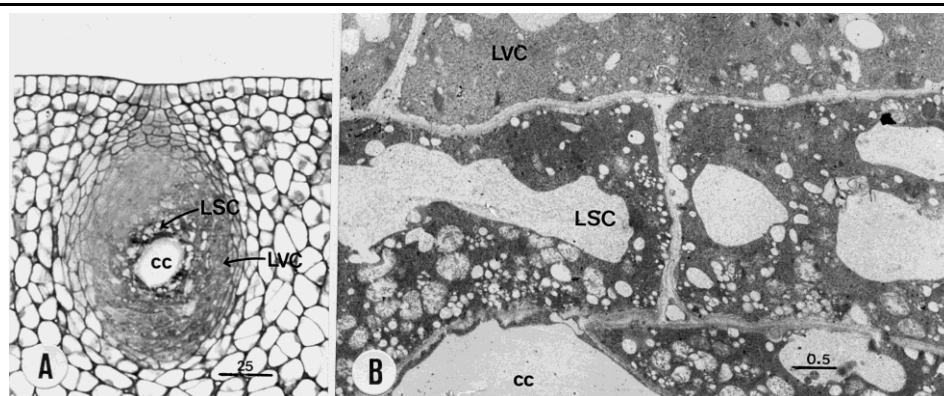
mitochondria, etc. can be still discerned in Fig. 1D). Cell disorganization later extends to the surrounding gland cells, thus increasing the diameter of the cavity (Fig. 2A, B).

At early disorganization of the initial central cell of the gland, the endoplasmic reticulum (ER) greatly develops into many short elements having a dark content (Fig. 1C). Fragmented ER-elements have a normal thickness (66 nm) and do not aggregate at certain areas of the cytoplasm, but they are uniformly scattered all over the cytoplasm. Close associations of the ER-elements with other cell organelles or the vacuoles, were not observed. Vacuoles have a normal outline and do not form engulfments enclosing cytoplasmic portions.

At advanced disorganization of the gland central cell, the ground plasm becomes highly electron dense, the organelles deform, and the cell walls undergo an internal bending (Fig. 1D). Wall bending is probably due to the reduction of the osmotic pressure of the central degenerated cell, so that the surrounding turgid cells make protrusions into its lumen. Presence of dilated or deteriorated ER-elements was not observed. Finally, the central cell dies and lyses. These signs progressively extend to its bordering cells, ultimately leading to the formation of an open cavity (Fig. 2A, B). The gland central cavity is initially small and by PCD gradually increases in diameter (at the expense of the secretory cells) until it finally meets the peripheral sheath cells of the gland. After the gland cavity is fully-formed, secretory cells start secreting the essential oil which becomes released into it.



**Fig. 1.** *Citrus deliciosa*. A. Light-microscopical view of an exocarp oil gland after completion of cell divisions. B. Ultrastructural appearance of active gland cells. Mitochondria (m) are numerous and endoplasmic reticulum elements scanty. C. The central cell of an oil gland at early PCD. The ER is highly developed and consists of many short elements with a dark content (er). D. The central cell at advanced PCD. The ground plasm is electron dense and the organelles degenerated. LVC = living gland cell, LSC = lysed gland cell. Scale bars in μm.



**Fig. 2.** *Citrus deliciosa*. A. Semi-thin section of an oil gland with an essential oil-accumulating cavity in the centre (cc). The gland cells facing the cavity (LSC) have undergone PCD, whereas those beyond the former (LVC) are still living. B. Ultrathin section illustrating at high magnification the difference in appearance between LSC cells and LVC cells, respectively. Scale bars in  $\mu\text{m}$ .

### Discussions

Secretory oil cavities have been reported so far to initially open either lysigenously by disintegration of one or more cells [HEINRICH, 1969; BOSABALIDIS, 1982] or schizogenously by separation of two or more cells [BUVAT, 1989; TURNER & al. 1998]. Regardless whether the initial stage of cavity formation proceeds lysigenously or schizogenously, the important fact is that the whole cavity later develops (increases in volume) exclusively by disintegration of the secretory cells (lysigenously). In this process, PCD has a decisive participation. In the oil cavities of *C. deliciosa*, the central space appears to initially open by disintegration of a single cell. A prominent feature of advanced senescence of this cell is the high electron density of the ground plasm. The degenerated protoplasm remains closely attached to the cell wall and does not exhibit signs of plasmolysis [BOSABALIDIS, 2012]. At the stage just prior to senescence of the gland central cell (PCD initiation), the ER characteristically undergoes pronounced development. An analogous feature has been reported in the senescent cells of the abscission zone of apple flower and fruit [PANDITA & JINDAL, 2004]. The fact that ER greatly develops at the stage just prior to gland cell senescence (and not at any previous stage) and disappears after this stage, strongly indicates that this organelle has a crucial role in PCD initiation. A role of the ER as a PCD initiator has been also expressed by EICHMANN & SCHAEFER (2012).

Of interest is the observation that ER cisternae at early PCD of the gland central cell become fragmented into many short elements distributed all over the cytoplasmic area. ER fragmentation may be mediated by ER stress which in turn induces PCD [WOJTYLA & al. 2013]. ER stress is activated by misfolded or unfolded proteins that accumulate in the ER lumen [DENG & al. 2013]. Relevant to the above observation is that fragmented ER elements do not develop associations with other cell organelles and particularly with the vacuoles. The fusion of ER membranes with the tonoplast and the release into the vacuoles of lytic enzymes (autophagic vacuoles) has been well established [OUFATTOLE & al. 2005; YAMADA & al. 2005; MUENTZ, 2007]. The lack of association of the ER with the vacuoles in the senescing oil gland cell of mandarin, and also the lack of cytoplasm-

engulfing vacuoles, favour the interpretation that PCD in these cells is induced not by autophagic vacuoles, but by the autonomous operation of the ER (release of hydrolytic enzymes directly from ER to cytosol).

Presence of various hydrolytic enzymes in the ER has been reported in a number of studies. Thus, MOOR & WALKER (1981) identified in the cytosol of *Sedum* cells a high activity of acid phosphatase associated with cellular autolysis and cell death. They considered that the hydrolytic enzyme is biosynthesized in the ER (cytochemical localization) from where it becomes release into the cytosol leading finally to the necrosis of the cells. LAMPL & al. (2013) further reported that PCD in plants is promoted by the release into the cytosol of ER-compartmentalized proteases. Similarly, MULISCH & al. (2013) identified by immunogold labeling, ER-associated cysteine proteases in the tracheary elements and considered that they involve in PCD. Activity of the hydrolytic enzymes acetylsterase and nuclease was cytochemically localized also in the ER [DEJONG & al. 1967; FARAGE-BARHOME & al. 2011].

### Conclusions

Conclusively, in the present study, anatomical and ultrastructural results indicated that ER might have a decisive role in PCD of mandarin oil glands as an independent organelle.

### References

- BELL P. R. 1996. Megaspore abortion: A consequence of selective apoptosis? *Int. J. Plant Sci.* **157**: 1-7.
- BOSABALIDIS A. M. 2012. Programmed cell death in salt glands of *Tamarix aphylla* L.: an electron microscope analysis. *Cent. Eur. J. Biol.* **7**: 927-930.
- BOSABALIDIS A. M. & TSEKOS I. 1982. Ultrastructural studies on the secretory cavities of *Citrus deliciosa* Ten. II. Development of the essential oil-accumulating central space of the gland and process of active secretion. *Protoplasma*. **112**(1-2): 63-70.
- BUVAT R. 1989. *Ontogeny. Cell differentiation and structure of vascular plants*. Springer Verlag, Berlin: 504-506.
- DE JONG D. W., JANSEN E. F. & OLSON A. C. 1967. Oxidoreductive and hydrolytic enzyme patterns in plant suspension culture cells. Local and time relationships. *Exp. Cell Res.* **47**(1): 139-156.
- DENG Y., SRIVASTAVA R. & HOWELL S. H. 2013. Endoplasmic reticulum (ER) stress response and its physiological roles in plants. *Int. J. Mol. Sci.* **14**: 8188-8212.
- EGOROVA V. P., ZHAO Q., LO Y. S., JANE W. N., CHENG N., HOU S. Y. & DAI H. 2010. Programmed cell death of the mung bean cotyledon during seed germination. *Bot. Stud.* **51**: 439-449.
- EICHMANN P. & SCHAEFER P. 2012. The endoplasmic reticulum in plant immunity and cell death. *Front. Plant Sci.* 2012; 3: 200. Published online Aug 22, 2012. doi: 10.3389/fpls.2012.00200
- FARAGE-BARHOME S., BURD S., SONEGO L., METT A., BELAUSON E., GIDONI D. & LERS A. 2011. Localization of the *Arabidopsis* senescence - and cell death-associated BFN 1 nuclease: From the ER to fragmented nuclei. *Mol. Plant.* **4**(6): 1062-1073.
- HEINRICH G. 1969. Elektronenmikroskopische Beobachtungen zur Entstehungsweise der Exkretbehälter von *Ruta graveolens*, *Citrus limon* und *Poncirus trifoliata*. *Oesterr. Bot. Z.* **117**: 397-403.
- LAMPL N., ALKAN N., DAVYDOV O. & FLUHR R. 2013. Set-point control of RD21 protease activity by AtSerp1 controls cell death in *Arabidopsis*. *Plant J.* **74**(3): 498-510.
- MOORE R. & WALKER B. D. 1981. Studies of vegetative compatibility-incompatibility in higher plants. *Protoplasma*. **109**: 317-334.
- MÜNTZ K. 2007. Protein dynamics and proteolysis in plant vacuoles. *J. Exp. Bot.* **58**(10): 2391-2407.
- MULISCH M., ASP T., KRUPINSKA K., HOLLMANN J. & HOLM P. B. 2013. The Tr-cp 14 cysteine protease in white clover (*Trifolium repens*) is localized in the endoplasmic reticulum and is associated with programmed cell death during development of tracheary elements. *Protoplasma*. **250**(2): 623-629.

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- OUFATTOLE M., PARK J. H., POXLEITNER M., JIANG L. & ROGERS J. C. 2005. Selective membrane protein internalization accompanies movement from the endoplasmic reticulum to the protein storage vacuole pathway in *Arabidopsis*. *Plant Cell*. **17**(11): 3066-3080.
- PANDITA V. K. & JINDAL K. K. 2004. Physiological and anatomical aspects of abscission and chemical control of flower and fruit drop in apple. *Acta Horticult.* **662**: 333-339.
- RAVEN P. H., EVERT R. F. & EICHHORN S. E. 1999. *Biology of plants*. 6<sup>th</sup> Edition, Freeman and Co., New York, pp. 944.
- TURNER G. W., BERRY A. M. & GIFFORT E. M. 1998. Schizogenous secretory cavities of *Citrus limon* (L.) Burm. f. and reevaluation of the lysigenous gland concept. *Int. J. Plant Sci.* **159**(1): 75-88.
- WOJTYLA L., RUCINSKA-SOBKOWIAK R., KUBALA S. & GARNCZARSKA M. 2013. Lupine embryo axes under salinity stress. I. Ultrastructural response. *Acta Physiol. Plant.* **35**(7): 2219-2228.
- YAMADA K., SHIMADA T., NISHIMURA M. & HARA-NISHIMURA I. 2005. A VPE family supporting various vacuolar functions in plants. *Physiol. Plant.* **123**(4): 369-375.

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