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Vesicles between plasma membrane and cell wall prior to visible senescence of *Iris* and *Dendrobium* flowers

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ABSTRACT

Cut *Iris* flowers (*Iris x hollandica*, cv. Blue Magic) show visible senescence about two days after full opening. Epidermal cells of the outer tepals collapse due to programmed cell death (PCD). Transmission electron microscopy (TEM) showed irregular swelling of the cell walls, starting prior to cell collapse. Compared to cells in flowers that had just opened, wall thickness increased up to tenfold prior to cell death. Fibrils were visible in the swollen walls. After cell death very little of the cell wall remained. Prior to and during visible wall swelling, vesicles (paramural bodies) were observed between the plasma membrane and the cell walls. The vesicles were also found in groups and were accompanied by amorphous substance. They usually showed a single membrane, and had a variety of diameters and electron densities. Cut *Dendrobium* hybrid cv. Lucky Duan flowers exhibited visible senescence about 14 days after full flower opening. Paramural bodies were also found in *Dendrobium* tepal epidermis and mesophyll cells, related to wall swelling and degradation. Although alternative explanations are well possible, it is hypothesized that paramural bodies carry enzymes involved in cell wall breakdown. The literature has not yet reported such bodies in association with senescence/PCD.

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Introduction

Programmed cell death (PCD) is the regulated demise of cells. PCD is often accompanied by degradation of cell constituents (Kuriyama et al., 2007). During petal PCD the degradation products are transferred to other parts of the plant. Nitrogen from proteins, for example, is transported out of the dying or dead cells as amines and amides, and carbon is mainly transported as sugars (van Doorn and Woltering, 2008). Cell wall degradation is also found, depending on the system studied. During aerenchyma formation very little remains of the walls of dead cells (Gunawardena et al., 2001, 2007) but during PCD in xylem elements the walls largely remain (Fukuda, 2000; Kuriyama et al., 2007).

Senescence, typical for leaves and flowers, is as a type of PCD. It can be defined as the outward symptoms, observed at the organ level, of the underlying PCD program. In carnation petals visible senescence symptoms coincided with a decrease in cell wall hemicellulose levels (de Vetten and Huber, 1990). Cell wall degradation accompanied PCD during petal senescence. In senescing *Ipomoea* flowers both cellulose and hemicellulose concentrations decreased. Degradation started when the mesophyll cells were still alive (Winkenbach, 1970; Wiemken-Gehrig et al., 1974). By contrast, in *Sandersonia* flowers similar wall degradation occurred only after cell death (O'Donoghue et al., 2002, 2005).

Vesicles have been reported between the plasma membrane and the cell walls. This was associated with cell wall synthesis (Newcomb, 1963; Marchant, 1968; Harris, 1981), with fruit ripening (Hallett et al., 1992), abscission (Bar-Dror et al., 2011) and responses to plant pathogens (An et al., 2006). The extracellular vesicles have been called paramural bodies (Marchant, 1968). Robards and Kidwai (1969) suggested that they derive from the Golgi/ER and they suggested some ways of transport to the cell walls. Nonetheless, Liljegren (2012) concluded that their biogenesis and function is currently still a mystery.

We previously noted swelling of the cell walls by the time of cell collapse in senescent *Iris* flowers (van Doorn et al., 2003). No reports

Abbreviations: PCD, programmed cell death; TEM, transmission electron microscopy.

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have apparently been published on the ultrastructure of cell walls prior to visible flower senescence. We here report on the presence, prior to and during cell wall swelling related to senescence, of vesicles between the plasma membrane and the cell walls, both in *Iris* and *Dendrobium* flowers.

Materials and methods

Plants

Work with *Iris x hollandica* cv. Blue Magic was carried out at Wageningen University and Research Centre, The Netherlands. Flowers were obtained from commercial growers. They were harvested at the commercial stage (blue tip of the floral bud just visible) and immediately placed in water. After harvest, the flowers were transported to the laboratory in a non-refrigerated car. The time between harvest and arrival in the laboratory was less than 2 h. Upon arrival, the flowers were immediately used for experimentation or were placed at 5 °C for at most 4 h. Flowers were selected for uniformity, and part of the stem ends (2–6 cm) was cut in air, resulting in stems of 45 cm length.

Flowers were individually placed in glass bottles of 20 cm height. The bottles contained approximately 250 mL of demineralised water. Bottles were standing in a climate-controlled room at 20 °C and 60% RH. The photosynthetic quantum flux at the flower was 15 $\mu mol\, m^{-2}\, s^{-1}$. Light was provided by fluorescent tubes which were on 12 h per day (07.00–19.00).

Experiments with *Dendrobium* hybrid cv. Lucky Duan were carried out at the Agricultural University of Thailand (Kasetsart University). Inflorescences were freshly harvested at a commercial grower, and were brought to laboratory with the stem ends in water, within 2 h after harvest. Inflorescences were selected for lack of infection and uniformity. They had 5–7 open flowers and 5–7 flower buds. Stem ends were recut in air at 12 cm below the lowermost flower, removing about 10–15 cm. The inflorescences were placed in glass bottles containing demineralised water. Bottles were standing in a room at 25 °C and about 60–70% RH. The photosynthetic quantum flux at the top of the inflorescence was about 15 $\mu mol\, m^{-2}\, s^{-1}$. Light was provided by fluorescent tubes which were on 12 h per day (07.00–19.00).

Chemicals and transmission electron microscopy (TEM)

In contrast to flowers with green sepals and variously coloured petals, some flowers, such as *Iris* and *Dendrobium*, contain floral leaves that are all non-green. These leaves are called tepals. In *Iris*, segments from the distal margins of the three outer flag tepals were isolated and trimmed back in fixative to a few square millimetres. The fixative was a mixture of 3% glutaraldehyde and 2% paraformaldehyde, in 100 mM phosphate/citrate buffer pH 7.2, with 1.5 mM $CaCl_2$. Fresh fixative containing tannic acid (1%, w/v) was vacuum-infiltrated at room temperature for 15 min and the samples were kept overnight at 4 °C under vacuum. Samples were washed five times for 5 min with phosphate/citrate buffer, and post-fixed with 1% (w/v) OsO_4 for 1 h. They were washed five times in distilled water, gradually dehydrated in an alcohol series, and vacuum-embedded in LR White resin (Agar Scientific, Stansted, Essex, U.K.) for 1 h at room temperature. LR White resin was replaced twice with fresh LR White resin for two additional 1 h embedding periods. The samples were incubated overnight at 60 °C. Sections (90 nm) were cut, double-stained with uranyl acetate and lead citrate, and studied under a Philips 201 transmission electron microscope (Eindhoven, The Netherlands).

In *Dendrobium* the samples were taken from the lowermost flowers on the inflorescence, which had opened about 3 days prior to harvest. The distal edges of two lateral tepals from the outer whorl were used. Segments from the distal tepal edges were removed using a razor blade. The tissue was fixed in 5% glutaraldehyde in 25 mM sodium phosphate buffer, pH 6.8, for 0.5 h at room temperature. Segments were transferred to 3% glutaraldehyde in buffer, for 2 h, in ice. Samples were rinsed with buffer every h for 12 h, post-fixed overnight with 1% osmium tetroxide in water, then rinsed with distilled water before dehydration (ethanol series) and gradual substitution of ethanol by propylene oxide. Samples were embedded in Spurr's resin at 60 °C for 24 h. Ultrathin sections were mounted onto copper grids and air-dried before staining in 2% uranyl acetate and lead citrate. Sections were examined using a JEOL TEM-1230 transmission electron microscope (Tokyo, Japan).

Cell wall swelling

The maximum diameter of the walls of epidermal and mesophyll cells was determined by measuring the structure on micrographs, using a ruler. Sampling occurred at the time of harvest (day 0) and just prior to cell death, i.e. day 3 after harvest (day 1 after full flower opening) in *Iris*, and day 14–19 after harvest in *Dendrobium*. One cell was used per micrograph, and 20 micrographs per sampling time. Data were analyzed using ANOVA at $P \geq 0.05$.

Results

Tepals of Iris *flowers*

Iris flowers have two whorls of three tepals each. *Iris* flowers (cv. Blue Magic) that are cut at the commercial harvest stage (tips of the single floral bud just visible) and placed in water at 20 °C open mainly during day 0–1 and slightly further during day 1–2. An increase in tepal length accompanies flower opening. The tepals showed visible senescence symptoms (discoloration and wilting) first at the distal edges, by day 2 after full opening. The visible senescence symptoms were due to collapse of the epidermal cells. This collapse started at the distal petal edge. About two days earlier, the underlying mesophyll cells, except those at the vascular bundles, collapsed (results not shown). In order to distinguish between processes relating to elongation growth and those relating to PCD, we did not study mesophyll cells, as these grew until they collapsed. Using transmission electron microscopy (TEM), we studied epidermal cells on day 0 (controls) and just after cessation of elongation growth (day 3–4), i.e. just prior to and during cell collapse.

On day 0 no vesicles were observed between the plasma membrane and the cell wall of *Iris* petal epidermal cells (data not shown). Fig. 1 shows epidermal cells on day 1 after full flower opening. Fig. 1A exhibits vesicles (black arrows), one larger than the two others, outside the plasma membrane. Note that the plasma membrane (white arrows) is folded inward, around the vesicles. Other vesicles of various shapes, located outside the plasma membrane, are depicted in Fig. 1B–G. Fig. 1B shows some vesicles of similar shape outside the plasma membrane (black arrows). In Fig. 1C three vesicles of dissimilar size are found (white arrows). Most of these paramural bodies contained an outer membrane, but in some no such membrane was visible (Fig. 1D; black arrows). Vesicles that varied considerably in diameter and in electron-density were also found in clusters (Fig. 1E–G, black arrows). Some of these clusters contained electron dense amorphous material (white arrows). The plasma membrane in Fig. 1G is indicated with arrows.

Just after full flower opening, some walls of epidermal cells, such as those in Fig. 1A and E, were still thin, similar to the walls at the beginning of flower opening (data not shown). The wall in Fig. 1C seems slightly thickened. Relatively low electron density in the centre of the wall, possibly the middle lamella, was observed in

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