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Changes in ultrastructure, protease and caspase-like activities during flower senescence in *Lilium longiflorum*

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ABSTRACT

The last phase of flower development is senescence during which nutrients are recycled to developing tissues. The ultimate fate of petal cells is cell death. In this study we used the ethylene-insensitive *Lilium longiflorum* as a model system to characterize Lily flower senescence from the physiological, biochemical and ultrastructural point of view. Lily flower senescence is highly predictable: it starts three days after flower opening, before visible signs of wilting, and ends with the complete wilting of the corolla within 10 days. The earliest events in *L. longiflorum* senescence include a fall in fresh and dry weight, fragmentation of nuclear DNA and cellular disruption. Mesophyll cell degradation is associated with vacuole permeabilization and rupture. Protein degradation starts later, coincident with the first visible signs of tepal senescence. A fall in total protein is accompanied by a rise in total proteases, and also by a rise of three classes of caspase-like activity with activities against YVAD, DEVD and VEID. The timing of the appearance of these caspase-like activities argues against their involvement in the regulation of the early stages of senescence, but their possible role in the regulation of the final stages of senescence and cell death is discussed.

1. Introduction

Flowers play a critical role in angiosperm reproduction and are often pigmented and or scented to attract pollinators. However, despite its irreplaceable ecological role, the flower is energetically expensive to maintain beyond its useful life, it therefore has a limited life-span and is usually removed after pollination [1]. The length of time a flower remains open and functional varies among species from one day to several weeks. After this period it undergoes senescence, showing petal wilting, withering or petal abscission [2].

The main events associated with the progression of flower degeneration have been at least partially elucidated at the cytological, biochemical and molecular level [3]. Loss of cellular organization, increase of membrane permeability and degradation of cytoplasm and organelles are common features [4–6]. Ultrastructural studies of senescing petals revealed the presence of vesicles, cytoplasm, and organelles within the vacuole, leading to the conclusion that the autophagic machinery is implicated [7,8]. At the same time, nucleic acids are degraded, seen in some species as the appearance of a DNA ladder, due to the internucleosomal fragmentation of nuclear DNA [6,9]. The protein content of petals also decreases during senescence [10–13] and an increase in different proteolytic activities, has been reported in senescing flowers of many species including species from the Liliaceae [10,11,14], leading to the conclusion that different classes of proteolytic enzymes play an important role during flower senescence. In animal cells a class of cysteine proteases, caspases, participate in a cascade of proteolytic activation orchestrating different elements of programmed cell death [15]. Direct homologues of caspases are not found in plants, however caspase-like activities have been associated with developmental cell death [16–18] and a few other systems (for review see [19,20]), though not to date in petal or leaf senescence. Activities have been detected using synthetic substrates with different target sequences corresponding to the specific targets of different animal caspases although it is unclear whether a direct

Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethyl ammonium]-1-propanesulfonate; CTAB, cetyl trimethyl ammonium bromide; DAPI, 4,6-diamidino-2,25-phenylindole; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; HEPES, 4,2-idroxyethil-1-piperazinil-ethanesulfonic acid; TCA, trichloroacetic acid; TdT, terminal deoxynucleotidyl transferase; TUNEL, TdT-mediated dUTP nick end labelling.

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equivalence can be made between caspase classes in animal cells and substrate cleavage specificities in plant cells [19].

The signals responsible for the initiation of flower senescence are complex and not yet entirely known. Ethylene has been shown to regulate senescence of many flowers, which therefore are classified as ethylene-sensitive. However in many other species floral senescence is not associated with the production of this hormone and ethylene sensitivity is absent or very low [2,21]. Flowers of *Lilium longiflorum* are generally considered to be ethylene-insensitive due to their low sensitivity to applied ethylene and ethylene inhibitors. Moreover, ethylene production of *L. longiflorum* flowers is very low and often below the detectable level [22–24]. *L. longiflorum* is characterized by large flowers and a long vase life, which is not affected by removing the flower from the stem. Moreover, the process is highly predictable leading to complete flower wilting within 10 days making it a good model to study ethylene-insensitive petal senescence.

How petal senescence is coordinated in ethylene-insensitive species remains to be elucidated. However the timing of some of the events associated with ethylene-independent senescence has been charted [6,25,26]. In *Alstroemeria* some processes such as increased transcript levels of a cysteine protease gene and changes in cell structure started very early and changed gradually throughout senescence, whereas other events such as increases in electrolyte leakage, DNA laddering and total protease activity changed suddenly coinciding with the first visible signs of petal deterioration [6].

In this study the petals of *L. longiflorum* were used to investigate the biochemical and ultrastructural events associated with ethylene-independent flower senescence. We analysed typical senescence and PCD hallmarks to investigate the mechanisms underlying the progression of flower wilting. We report here that lily flower senescence is accompanied by many of the widely accepted PCD hallmarks. Moreover, we report a rise in total proteases, and also a rise of three classes of caspase-like activity as later events during senescence. Their possible role in the regulation of the final stages of senescence and cell death is discussed.

2. Materials and methods

2.1. Plant material and vase life analysis

Eight stages of *L. longiflorum* cv. "White Heaven" were used. Plants were grown at CRA-VIV, Pescia or in a commercial greenhouse and flowers were harvested by cutting above the last leaf, placed in 50 ml tubes filled with distilled water and kept in a growth chamber at 22 °C and 50% relative humidity. Flowers were harvested at closed bud stage (Day-2, indicated with D2) and the time by which the tepal tips begin to separate and anthers began dehiscence was called D0 and considered as a reference stage. Under the conditions used, flower development and senescence progressed at a uniform rate and samples were collected from flowers at harvest (D2), D0, and 2, 3, 4, 5, 7, and 10 days after the reference stage (stages D2–D10). Vase life in water was evaluated both in isolated flowers cut above the last leaf and in flowers with a stem of 80 cm. No differences were found in flower longevity, and flowers cut above the last leaf were used for all the experiments. For fresh and dry weight measurements, both inner and outer tepals were used while for all other analyses only outer tepals were sampled.

2.2. Microscopy

Outer tepals from flowers at stages D0, D3 and D5 were sampled and cut into 1 mm sections with a scalpel. Samples were incubated for 1.5 h at room temperature in 3% (v/v) formaldehyde, 3% (v/v) glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. After two washes in the same buffer, sections were post-fixed in 2% (w/v) OsO_4 for 1.5 h at room temperature. Samples were washed twice with distilled water and dehydrated in an ethanol series at 4 °C. Samples were then infiltrated in LR White resin (Agar Scientific Ltd., Stansted, UK), embedded in gelatine capsules and polymerised for 48 h at 4 °C. For transmission electron microscopy, thin sections were cut with a diamond knife and transferred to pioloform-coated 200 square mesh copper grids. Grids were stained with 2% (w/v) uranyl acetate and lead citrate before being examined in a Philips EM 208 electron microscope at 80 kV accelerating voltage. For light microscopy, semi-thin sections were cut and mounted on poly-lysine coated slides, stained with 0.05% toluidine blue and examined with a Leica DMLB microscope and a Leica DC 300F CCD camera.

2.3. Ion leakage

Discs (8 mm in diameter) were cut from each side of the central vein of the outer tepals about half-way from the tip (20 discs per tepal) and placed in 10 ml distilled water in Petri dishes (80 discs per dish). After a 2 h wash to remove ions from cut surfaces, the water was aspirated and fresh distilled water was added. Following incubation, conductivity of the bathing solution (sample conductivity) was measured with a conductivity meter (HI-8733, HANNA Instruments). Fresh distilled water (10 ml) was then added to the tepal discs and boiled for 15 min. After cooling to room temperature, conductivity was measured again to obtain the subtotal conductivity. Ion leakage was expressed as relative conductivity, which was calculated as sample conductivity divided by total conductivity (the sum of sample conductivity and subtotal conductivity).

2.4. DNA extraction and analysis

For the isolation of intact, high molecular weight DNA, the CTAB method was used [27]. Briefly, frozen tepals were ground in N_2 to a fine powder, and extracted with two volumes of CTAB buffer (2% (w/v) CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris–HCl pH 8, and 0.2% (w/v) β-mercaptoethanol) for 45 min at 65 °C. The DNA was extracted with one volume of chloroform/isoamyl alcohol (24:1) and the aqueous phase was recovered and precipitated with one volume of isopropanol. After centrifugation for 20 min at 12,000 × *g*, the supernatant was discarded and the pellet washed, dried, and dissolved in TE buffer (10 mM Tris–HCl pH 8, 1 mM EDTA) supplemented with RNase A (100 mg/ml). DNA (10 μg) was subjected to electrophoresis on a 1.5% (w/v) agarose gel, stained with 0.5 mg/ml ethidium bromide for 30 min, washed in water, and observed on a UV light box. A 100 bp ladder was used as the standard.

2.5. In situ detection of DNA fragmentation (TUNEL assay)

Tepals were fixed over night at 4 °C in 4% (w/v) paraformaldehyde in phosphate buffer saline pH 7.4. After dehydration through ethanol series, the samples were embedded in Paraplast Plus (Sigma, St. Louis, MO, USA). Sections of 12 μm were cut and stretched on poly-lysine coated slides. The sections were then dewaxed in xylene and re-hydrated before examination. TUNEL assay was performed using an "In Situ Cell Death Detection Kit" (Promega, Madison, WI, USA) according to the manufacturer's instructions. To facilitate the entry of TdT enzyme into the tissue sections, the slides were treated with proteinase K (20 mg ml^{-1}) for 20 min. The labelling reaction was performed at 37 °C in a humidified chamber in the dark for 1 h. A negative control was included in each experiment by omitting TdT from the reaction mixture. As a positive control, sections were incubated with DNase I (10 U ml^{-1})