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Expression and localisation of a senescence-associated KDEL-cysteine protease from *Lilium longiflorum* tepals

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ABSTRACT

Senescence is a tightly regulated process and both compartmentalisation and regulated activation of degradative enzymes is critical to avoid premature cellular destruction. Proteolysis is a key process in senescent tissues, linked to disassembly of cellular contents and nutrient remobilisation. Cysteine proteases are responsible for most proteolytic activity in senescent petals, encoded by a gene family comprising both senescence-specific and senescence up-regulated genes. KDEL cysteine proteases are present in senescent petals of several species. Isoforms from endosperm tissue localise to ricinosomes: cytosol acidification following vacuole rupture results in ricinosome rupture and activation of the KDEL proteases from an inactive proform. Here data show that a *Lilium longiflorum* KDEL protease gene (*LlCYP*) is transcriptionally up-regulated, and a KDEL cysteine protease antibody reveals post-translational processing in senescent petals. Plants over-expressing LlCYP lacking the KDEL sequence show reduced growth and early senescence. Immunogold staining and confocal analyses indicate that in young tissues the protein is retained in the ER, while during floral senescence it is localised to the vacuole. Our data therefore suggest that the vacuole may be the site of action for at least this KDEL cysteine protease during tepal senescence.

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1. Introduction

Petal senescence is a tightly regulated process involving, in most species, nutrient remobilisation and terminating in cell death. In many species this is accompanied by organ abscission [1,2]. In some species this process is coordinated by the growth regulator ethylene, while in others, including lilies, ethylene does not appear to play a major role in petal senescence [2]. At a cellular level, petal cell death is found to resemble most closely an autophagic pattern [3]. In several species, vesicles accumulate in the cytosol followed by enlargement of the central vacuole and ultimately vacuolar rupture (e.g. *Dianthus* [4], *Iris* [5], *Lilium longiflorum* [6]).

Nutrient remobilisation from senescent organs such as leaves and petals requires the action of a suite of degradative enzymes including nucleases, lipases, and proteases [1,2]. The synthesis and activation of these enzymes needs to be under tight temporal and spatial control to ensure the ordered breakdown of cellular macromolecules. Total protease activity generally increases with petal senescence while protein content falls (e.g. in *Alstroemeria* [7], *Hemerocallis* [8], *Sandersonia* [9]) and the pH optimum of protease activity in senescent petals is often relatively acidic (e.g. pH 5.5–6 in *L. longiflorum* [6]). This suggests that these enzymes are either active in an acidic sub-cellular compartment such as the vacuole, or that they are activated in an acidified cytosol following vacuole rupture.

Transcriptomic studies have revealed the expression of genes encoding both cysteine proteases (EC 3.4.22), and aspartic proteases (EC 3.4.23) during floral senescence [5,10,11]. However using inhibitors for specific protease classes, it was shown that cysteine proteases are those primarily responsible for protease activity in senescent petals [7,9,12]. Cysteine proteases comprise a large gene family divided into several classes but those associated with senescence are mainly of the papain class [13]. In petals, multiple cysteine protease genes are expressed with varying temporal patterns [9,12,14]. For example in petunia only four out of nine

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cysteine protease genes expressed in petals were up-regulated in the later stages of petal senescence, three were down-regulated, two peaked in expression in early senescence after which their expression fell, and of the nine genes, expression of only one was senescence specific [12].

KDEL cysteine proteases form an important group of papain class cysteine proteases that are unique to plants and characterised by a C-terminal KDEL sequence that directs retention in the endoplasmic reticulum (ER) [13,15]. These proteases were initially identified in association with PCD in the castor bean (*Ricinus communis*) endosperm [16]. However they are also found in senescing petals of several species including *Hemerocallis* [17], *Sandersonia aurantica* [9] and *Dendrobium* [18]. Although the in vivo substrates of PCD-associated KDEL proteases are unknown, Helm et al. [15] showed that the castor bean enzyme has activity against some types of extension proteins.

The castor bean KDEL cysteine protease was located to ricinosomes [16]. Ricinosomes are small organelles, first discovered in the castor bean endosperm [19,20], that derive from the ER [21]. They have subsequently also been found during castor bean nucellar programmed cell death (PCD; [22]), in tomato anthers, associated with anther dehiscence [23], and in senescent *Hemerocallis* petal cells [17]. A 45 kDa KDEL cysteine protease was localised to ricinosomes in *Hemerocallis* petal cells, however was not further investigated. During castor bean endosperm PCD, the ricinosomes appear at the same time as other PCD markers and then rupture, releasing their protease cargo into the cytosol. This is accompanied by autocatalytic processing of the KDEL protease from 45 kDa to 35 kDa mature form [16,21]. Acidification of isolated ricinosomes also results in KDEL protease processing and activation [21] supporting the hypothesis that cytosol acidification triggers ricinosome rupture and KDEL protein maturation. Thus it would seem that ricinosomes are distinct from autophagic-type vesicles that deliver their cargo to the vacuole prior to tonoplast rupture [1]. However, in *Vigna mungo* seeds, the SH-EP KDEL protease is transported to the vacuole via KDEL vesicles (KV) independently of the Golgi [24] a process dependent on the C-terminal KDEL sequence. In fact if the KDEL sequence is removed and the SH-EP protein overexpressed in transgenic Arabidopsis, the SH-EPΔKDEL is secreted into the extracellular spaces and plants die prematurely.

L. longiflorum is an important commercial cut flower with a well-characterised senescence programme [6] making it a useful model for studying mechanisms of floral senescence and PCD in an ethylene-insensitive species. Here data are presented on a *L. longiflorum* KDEL cysteine protease whose expression is strongly up-regulated during petal senescence. RFP fusions confirm it is translocated into the ER, however immunogold staining indicates localisation of this protease to the vacuole rather than to ricinosomes during floral senescence. This is important in the context of understanding the role for KDEL cysteine proteases during petal senescence. Although a number of these proteins have been studied in different species [7,9,17,18] and are clearly highly expressed during the later stages of petal senescence, their mechanism of action in relation to the timing of cell death events remains uncertain. Here evidence is provided for localisation of these enzymes to the vacuole prior to tonoplast rupture.

2. Materials and methods

2.1. Plant material

Plant material was as described in [6]. *L. longiflorum* cv. "White Heaven" was grown in a commercial greenhouse and individual flowers harvested by cutting above the last leaf. Flowers were placed in distilled water and kept in a growth chamber at 22 °C and 50% relative humidity. Flowers were harvested at stage D-2 (closed bud) and, under the conditions used, flower development and senescence progressed uniformly from stage D0 (loose bud, tepal tips beginning to separate, dehiscence begins, used as a reference stage) to stage D10 (full senescence, 10 days after the reference stage) [6]. At D2 flowers were fully open, D3 is full bloom, at D4 first signs of senescence were visible (tepal translucence) which was more marked at D5. By D7 tepals were wilting and browning and by D10 the corolla had completely collapsed (though it does not abscise in this species).

2.2. RNA extraction and cDNA preparation

RNA was extracted with TRI reagent (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. RNA was subjected to DNase treatment using a TURBO DNA-free kit (Ambion Inc., Austin, TX, USA) to remove contaminating genomic DNA. Five micrograms of RNA was reverse transcribed into cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's instructions.

2.3. Primer design

All the primers used in this work are listed in Supplementary Table S1. For isolation of the *LlCYP* gene, degenerate primers CYPF and CYPR were designed from a comparison of conserved regions of senescence-associated cysteine proteases from monocotyledonous species in the GenBank database [7]. Primers for 18s rRNA (PUV1, PUV2) were also designed by comparison of ribosomal genes from available monocotyledonous species [25].

2.4. Cloning of LlCYP

A 340 bp fragment of a *L. longiflorum* KDEL protease gene was isolated from D4 outer tepal cDNA using degenerate primers CYPF and CYPR. The full-length cDNA was obtained using the BD SMART^{TM} RACE cDNA Amplification Kit (BD Biosciences Clontech, Palo Alto, CA) using gene specific primers GSPF and GSPR. The whole ORF was amplified from D4 cDNA using primers LlCYPclF and LlCYPclR containing the BamHI and NotI restriction sites respectively and inserted into the pET21b vector (Novagen, Darmstadt, Germany). Clones were sequenced and compared with database sequences using the BLAST programme (National Centre for Biotechnology Information, NCBI). The ORF sequence was deposited in Genbank under accession number HF968474. DNA sequences were analysed using Bioedit (v. 7.0.5.3 [26] and a phylogenetic tree was produced using MEGA4 [27]; SignalP and TargetP [28] were used to analyse the sequence for a signal sequence.

2.5. Real-time qPCR

Primers with optimal characteristics in relation to secondary structure, self-hybridisation, GC content (40–60%), Tm (55–70 °C) and amplicon length (90–130 bp) (LlCypF and LlCypR) were designed with Primer3 software [29]. qPCR was carried out in a 7300 real-time PCR system (Applied Biosystems) using 50 ng of cDNA and SYBR® green PCR master mix (Applied Biosystems). The thermal profile was: 95 °C × 2 min, followed by 40 cycles of 95 °C × 15 s, 64 °C × 1 min. Expression of the ribosomal 18S gene, used for internal normalisation, was analysed with PUV1 and PUV2 primers which amplify a 226 bp fragment. The thermal profile for 18S amplification was: 95 °C × 2 min, followed by 40 cycles of 95 °C × 15 s, 55 °C × 30 s, 72 °C × 30 s. The PCR products were further analysed by a dissociation curve programme (95 °C × 15 s, 60 °C × 1 min and 95 °C × 15 s) and all the reactions gave a single peak.

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