Cysteine protease gene expression and proteolytic activity during senescence of *Alstroemeria* petals

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Abstract

The functional life of the flower is terminated by senescence and/or abscission. Multiple processes contribute to produce the visible signs of petal wilting and inrolling that typify senescence, but one of the most important is that of protein degradation and remobilization. This is mediated in many species through protein ubiquitination and the action of specific protease enzymes. This paper reports the changes in protein and protease activity during development and senescence of *Alstroemeria* flowers, a Liliaceous species that shows very little sensitivity to ethylene during senescence and which shows perianth abscission 8–10 d after flower opening. Partial cDNAs of ubiquitin (*ALSUQ1*) and a putative cysteine protease (*ALSCYP1*) were cloned from *Alstroemeria* using degenerate PCR primers and the expression pattern of these genes was determined semi-quantitatively by RT-PCR. While the levels of *ALSUQ1* only fluctuated slightly during floral development and senescence, there was a dramatic increase in the expression of *ALSCYP1* indicating that this gene may encode an important enzyme for the proteolytic process in this species. Three papain class cysteine protease enzymes showing different patterns of activity during flower development were identified on zymograms, one of which showed a similar expression pattern to the cysteine protease cDNA.

Key words: *Alstroemeria*, flower, protease, protein, senescence.

Introduction

Senescence is the final event in the life of many plant tissues and is a highly regulated process that involves structural, biochemical and molecular changes that in many cases bear the hallmarks of programmed cell death (Buchanan-Wollaston and Morris, 2000; Rubinstein, 2000; Noh and Amasino, 1999; Makrides and Goldthwaite, 1981). The two senescent organs best studied are leaves and flowers. An advantage of flower petals, compared to leaves, as a system for studying organ senescence is that the process is irreversible and has tight developmental control. Within a given species it is possible to predict exactly when a bud will open and how rapidly the petals will senesce. In addition, a number of morphological and physiological changes are evident that allow the process to be readily documented.

Floral senescence in many species is regulated by ethylene biosynthesis (Woltering and van Doorn, 1988). In these species a burst of endogenously produced ethylene initiates senescence and co-ordinates the expression of genes required for the process (Jones et al., 1995). However, other species are insensitive to exogenous ethylene and floral senescence operates independently of ethylene production in these species. It remains unclear in these species how the senescence process is initiated and, importantly, how the senescence process is regulated. Inhibitors of protein synthesis, such as cycloheximide, can delay the senescence of both flowers which are regulated by ethylene and those which are ethylene insensitive (Wulster et al., 1982; Lay-Yee et al., 1992). Thus de novo protein synthesis is a requirement for floral senescence and therefore senescence must be an active process in both types of flowers.

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An alternative way of distinguishing patterns of floral senescence is based upon whether the perianth loses fresh weight and turgidity, thus wilting, or if the perianth abscises with little or no loss of fresh weight or other cellular constituents (Stead and van Doorn, 1994). In species that show wilting of the perianth, an important function of senescence may be to allow remobilization of key metabolites from the senescing organ back into the plant. These metabolites are then redirected to the development of other organs such as the developing ovary (Nichols and Ho, 1975; Feller and Keist, 1986) or tuber formation (Fischer et al., 1998). Protein degradation forms an important part of this remobilization process; therefore the activity of proteolytic enzymes is an essential element of senescence. Genes encoding putative proteolytic enzymes are routinely identified in screens for senescence-related genes from leaves (Huffaker, 1990; teolytic enzymes are routinely identified in screens for element of senescence. Genes encoding putative pro-

forms an important part of this remobilization process; therefore the activity of proteolytic enzymes is an essential element of senescence. Genes encoding putative proteolytic enzymes are routinely identified in screens for senescence-related genes from leaves (Huffaker, 1990; Buchanan-Wollaston, 1997; Gan and Amasino, 1997) and flowers (Jones et al., 1995). These proteolytic enzymes have been divided into several different groups depending upon the specific site at which they cleave target proteins and those most commonly identified are the cysteine proteases. Cysteine proteases are comprised of a number of different classes, but the majority of senescence-associated cysteine proteases behave as typical papain-family members (Granell et al., 1998). The expression patterns of putative cysteine proteases associated with leaf organ senescence vary considerably. Some, such as LSC790 from Brassica napus (Buchanan-Wollaston, 1997), are up-regulated relatively late in senescence, whereas others, such as SAG12 from Arabidopsis leaves, are up-regulated from the first visible signs of senescence (Lohman et al., 1994). In contrast, SEN102, another cysteine protease, is actually down-regulated during leaf senescence in Hemerocallis (Guerrero et al., 1998).

The same gene, however, is up-regulated during floral senescence of the same species (Valpuesta et al., 1995). Thus, patterns of gene expression may vary both spatially between organs and temporally within the same organ and, in one case at least, is inducible by treatment with ethylene (Jones et al., 1995). These cysteine proteases may therefore have a variety of functions reflecting the fact that remobilization is a continual process during development as well as during senescence.

Part of the regulation of senescence is brought about by targeting specific proteins for degradation, which may be mediated via the ubiquitination pathway. Ubiquitin is a highly conserved 76 amino acid protein that is covalently attached to a variety of cellular proteins to target them for proteolysis by the 26S proteasome (Wilkinson, 1995). There is evidence for up-regulation of ubiquitin expression in some floral systems (Courtney et al., 1994). The level of protein ubiquitination may offer some control over rates of proteolysis, but it is a general targeting mechanism for protein degradation and proteolysis rates will depend upon the activity of the specific proteolytic enzymes. Up-regulation of genes encoding proteolytic enzymes is, therefore, a common feature of senescent tissues.

Alstroemeria flowers are not usually regarded as being ethylene-sensitive (Woltering and van Doorn, 1998) and commercial treatments with ethylene inhibitors or ant-agonists are only recommended to prevent leaf yellowing (Dai and Paull, 1991), their effect on floral vase life being minimal. Petals from this species were therefore used in the present study as a system in which to investigate events associated with protein degradation during ethylene-independent floral senescence. This species is widely grown as a cut flower, particularly in the cool climates typical of Northern Europe, and has a vase life of approximately 10 d. During this time the coloured buds open and the whole developmental process has been classified into seven distinct stages (Wagstaff et al., 2001) encompassing petal senescence and ending with perianth abscission.

In this paper the cloning of a papain-class cysteine protease from Alstroemeria is reported, that is up-regulated during the senescence process. The expression of this gene is associated with concomitant changes in cysteine protease activity of the petals.

Materials and methods

Plant material

Seven stages of Alstroemeria peruviana var. Samora petal development and senescence were used (Wagstaff et al., 2001). Flowers were removed from the plant (at stage 1) 2 d before flower opening (outer sepals fully pigmented) and transported back to the laboratory dry. Individual petals were then removed from each inflorescence and placed into dH2O. The flowers were fully open at stage 2 (day 0) when the sepals were reflexed. At stage 3 (day +2) the top three anthers had anthesed and 2 d later at stage 4 (day +4) the bottom three anthers had done likewise. Stage 5 (day +6) was defined by the separation of the stigmatic lobes and by stage 6 (day +8) the petals were showing signs of discoloration and inrolling. Abscission of the perianth occurred at stage 7 (day +10). Petals from each stage were used for protein and RNA extraction.

Protein extraction

Protein was extracted from each stage of development by grinding 400 mg tissue in liquid nitrogen (number of petals/sepals in each sample was also recorded) in 1.5 ml buffer containing 50 mM NaPO4, 2 mM EDTA, 5 mM PCMP5, 1 mM PMSF, 5 mM iodoacetamide, and 20 μM leupeptin. The contents of the mortar were transferred to an Eppendorf tube and placed on ice for 10 min. 500 mM DTT was added to bring the final concentration to 10 mM and the samples were then incubated on ice for a further 10 min prior to microcentrifugation (4 °C, 12000 g for 10 min). The supernatant was split into 200 μl aliquots and 1.2 ml ice-cold acetone was added to each aliquot. Samples were incubated on ice for 10 min followed by
centrifugation as before. The supernatant was discarded and the pellet resuspended in 200 μl 1% SDS and stored at −20 °C. Total protein concentration was determined by a dye binding assay in which absorbance was measured at 550 nm following addition of 200 μl Bicinchoninic acid (Sigma) and 40 μl CuSO₄·5H₂O to 5 μl protein solution (Smith et al., 1985). Total protein was calculated both g⁻¹ FW and per organ.

**Total protease activity**

Total protease activity of the petals was determined (Nieri et al., 1998) using azocasein as a synthetic substrate with additional modifications. A crude extract was prepared by weighing and then grinding individual petals from all seven stages in 500 μl ice-cold 50 mM TRIS–HCl pH 7.4. The samples were transferred to microcentrifuge tubes and centrifuged for 3 min at 10000 g, 4 °C. The supernatant was retained and stored at −80 °C until required. Protease activity of the crude extract was determined by adding 100 μl from each extract to 200 μl 50 mM Na-acetate pH 5.0 containing 0.5% (w/v) azocasein. The samples were incubated at 37 °C for 24 h and the reactions were then terminated by the addition of 50 μl 50% TCA and incubation on ice for 1 h. A duplicate reaction for each sample was prepared with the TCA added at the start to act as a blank. The samples were then centrifuged as before and 100 μl aliquots were placed on a microtitre plate and then alkalinized with 15 μl 10 M NaOH per well prior to reading at 492 nm. Sample activity was defined in arbitrary units with 1 unit equivalent to 10 μM NaOH per well prior to reading at 492 nm. Zymograms were prepared using 11 μl zymogram loading buffer (50 mM TRIS–HCl pH 6.8, 10% (v/v) glycerol, 1% (w/v) SDS, and 0.01% (w/v) bromophenol blue). The proteins were run under denaturing conditions at 180 V for 30 min. The gels were then rehydrated for 1 h in 2.5% Triton X-100 and developed overnight (15–18 h) at 37 °C in 50 mM sodium citrate, 5 mM DTT, 5 mM CaCl₂, and 1 mM ZnCl₂, pH 5.0. Gels were stained in 0.05% Coomassie R-250 and destained in 10% acetic acid, 40% methanol solution. Areas of protease activity were revealed as cleared bands on a blue background.

Protease inhibitors were used to determine the specificity of the bands visualized by zymography. Proteases were inhibited by including either 2 μM leupeptin in dH₂O (inhibits serine and cysteine proteases) or 100 μM l-trans-epoxysuccinyl-leucylamido (4-guanidino) butane (E-64, Sigma) in 50% ethanol (inhibits cysteine proteases) in the overnight development buffer.

**RNA extraction**

Two batches of 1 g petals were extracted from each stage (Wilkins and Smart, 1996) including the amendments suggested recently (Hunter and Reid, 2001). Total RNA (2.5 μg) from each stage was DNase treated (Promega) and then used to make cDNA using Superscript II MMLV reverse transcriptase (Gibco). Controls lacking reverse transcriptase were included in the cDNA synthesis to verify the complete removal of genomic DNA in the DNase treatment step.

**Cloning of cysteine protease and ubiquitin partial cDNAs from Alstroemeria**

Degenerate primers to cysteine protease genes (CYPF: GACATGASCWRSSAGGARTT and CYPR: AAYTSGAAY-GCATARTCCCAT) were designed from a comparison of conserved regions of senescence-associated cysteine proteases from monocotyledonous species in the database. Degenerate primers for ubiquitin (UQF: GCNAAGATCCAGGACAA and UQR: ATSARVCGTGCTGTTG) were also designed by comparison of ubiquitin genes from available monocotyledonous species. Partial cDNAs were amplified from Alstroemeria cDNA using 0.625 units of Quiagen Taq polymerase, Quiagen buffer, 125 ng of cDNA from petals of flowers at stages 1, 3 and 5, 1.5 mM MgCl₂, 0.2 mM dNTPs, and 1 μg of each PCR primer. Reactions were cycled in a Hybrid OMNE machine for 35 cycles of (94 °C 1 min, 60 °C 1 min, 72 °C 1 min). PCR products were cloned into pGEMEasy T (Promega) and sequenced using an ABI377 automated sequencer.

**Semi-quantitative RT-PCR**

Specific Alstroemeria PCR primers were designed from the partial cysteine protease and ubiquitin-like cDNA clones (cysteine protease: CYPAF: AGGAGTTCTAGAACCATT and CYPAR: TCGAATGCTAGTCTGAT; ubiquitin: UQAF: GCAAGATCCAGGACAA and UQAR: ATCAGACGCTGCTGTTG). An initial PCR reaction was conducted using the Alstroemeria specific primers on 100 ng of the cloned partial cDNA to estimate the optimal cycle number for exponential amplification. PCR conditions were as described above except that 54 °C and 50 °C, respectively, were used for the cysteine protease and ubiquitin primers. Tubes were removed from the thermocycler at 16, 18, 20, 22, 24, 26, 28, and 30 cycles and the products were analysed on a 1.5% agarose gel. 22 cycles produced a barely visible band and was subsequently used for semi-quantitative RT-PCR in which 125 ng of cDNA from the seven defined stages of Alstroemeria petals was used as a template under the conditions described above.

Spectrophotometry and ethidium bromide-stained gels were used to equalize RNA for the RT-PCR. PCR products were separated on 1.5% agarose gels and Southern blotted onto nylon membrane to enhance the signal for quantitation. Pre-hybridization and hybridization were performed at 60 °C in 5× Denharts, 6× SSC, 0.1% SDS, 5% PEG, 0.1% tetrasodium pyrophosphate, and 100 μg ml⁻¹ denatured herring sperm DNA. Random primed probes were prepared as described previously (Feinberg and Vogelstein, 1983). The probe used was the respective PCR product. Washes were in 2× SSC, 0.1× SDS at 60 °C. Blots were exposed to phosphorimaging film (Kodak) and images were analysed in a Bio-Rad phosphorimager using a Quantity One (Bio-Rad) image analysis package.

**Results**

**Total protein concentration and protease activity**

Changes in protein levels and protease activity were investigated over the duration of the flower vase life. When protein levels were calculated g⁻¹ FW the level of protein within the petal tissue fell over the first two stages...
of development and then remained level for the remainder of senescence, until perianth abscission (Fig. 1). A slight rise in protein levels over the last stage is a reflection of the extensive loss of fresh weight occurring at this time. Total protease activity in the petals increased dramatically at stage 5 (day $q_6$) (Fig. 1), and continued to rise until abscission occurred.

**Characterization of the protease activity by zymography**

Zymography revealed protease activity in three distinct bands visible in the lower part of the gel ranging from 12 to 26 kDa (Fig. 2A). No protease activity was visible prior to stage 3 (day $q_2$). Band I (26 kDa) of protease activity showed down-regulation from stage 3 (day $q_2$) onwards, activity of band II (16 kDa) fluctuated during flower development and band III (12 kDa) was up-regulated over the same period. Zymograms were conducted with specific inhibitors to identify the classes of proteases represented by these bands of activity. Leupeptin inhibits both serine and cysteine proteases (Fig. 2B), whereas E64 inhibits cysteine proteases specifically (Fig. 2C). Both treatments eliminated all three bands of protease activity leading to the conclusion that all represented cysteine proteases. The optimal pH for zymography was found to be pH 5.0–5.5 (data not shown) indicating that the activities may be localized in a relatively acidic region of the cell, such as the vacuole.

**Isolation and characterization of a partial Alstroemeria cysteine protease cDNA**

Using degenerate PCR primers designed from an alignment of senescence associated monocotyledonous cysteine proteases a 338 bp partial cDNA (ALSCYP1) was obtained from Alstroemeria petals from stages 4–6. (The nucleotide sequence reported here will appear in the EMBL, Genbank and DDBJ Nucleotide Sequence Databases under Accession number AY034030.) Analysis of the sequence suggests that this gene is a papain-like cysteine protease. The predicted amino acid sequence of ALSCYP1 begins approximately 390 bp from the N terminus of other plant cysteine proteases and contains several of the elements characteristic of the structure of papain (Light et al., 1964; Mitchel et al., 1970). The three highly conserved amino acids (Cys, His and Asn) that make up the catalytic triad characteristic of the active site of cysteine proteases (Storer and Menard, 1994) are present. In addition, the sequence obtained contains four conserved Gly residues that are involved in maintaining an active enzyme conformation (Kamphuis et al., 1985), two conserved Cys residues that form disulphide bridges (Koehler and Ho, 1990) and a Gln active site residue (Kambouris et al., 1992). The partial cDNA does not, however, extend to cover the region that would indicate localization to the ER (by means of a KDEL ER...
retention signal such as that observed in the TPE4A cysteine protease from senescent ovaries of pea (Cercos et al., 1999)). Alignment of the putative protein sequence deduced from the partial cDNA with cysteine proteases identified in floral tissue shows 53–65% homology at the amino acid level with the same regions of SEN102 (Hemerocallis), SEN11 (Hemerocallis), DCCP1 (Dianthus), and cysteine proteases from Phalaenopsis and Sandersonia (Fig. 3).

The 278 bp ubiquitin partial cDNA (ALSUQ1) obtained from Alstroemeria (Wagstaff et al., 2001; Accession number AY034031) spans the repeat sequence that is conserved among eukaryotic organisms. The repeat is 228 bp in length and the primers used in subsequent semi-quantitative experiments were such that total ubiquitin expression was accurately reflected.

Expression programme of ALSCYP1 and ALSUQ1 during petal development and senescence. Semi-quantitative RT-PCR was used to investigate the expression programme of ALSCYP1 in petals. A continuous increase in ALSCYP1 mRNA levels during flower vase life was found (Fig. 4A) with no expression in the -RT controls, indicating the absence of any contaminating genomic DNA. Quantitation of the RT-PCR signal revealed a 30-fold increase in signal from stage 1 (day −2) to stage 7 (day +10) indicating strong up-regulation of this gene during petal senescence (Fig. 4B). This is in contrast to the levels of ubiquitin expression determined using the same method from the identical batch of cDNA. Ubiquitin expression remained more or less constant throughout vase life (Fig. 5A) with the maximal variation in signal less than 1.5 times that of the minimum signal recorded during quantification (Fig. 5B). The appearance of multiple bands on the autoradiograph is presumably due to amplification of multiples of the repeat sequence that is characteristic of the ubiquitin gene.

Discussion

The fall in protein levels g⁻¹ FW found in Alstroemeria petals showed a similar pattern to that observed in many

![Fig. 3. Alignment of ALSCYP1 cysteine protease cloned from Alstroemeria with other floral papain-class cysteine proteases namely SEN102 (Hemerocallis), SEN11 (Hemerocallis), DCCP1 (Dianthus), and cysteine proteases from Phalaenopsis and Sandersonia (Accession numbers P43156, U12637, U17135, U34747, and AAD28477, respectively). Alignment of the same region of the cDNA as ALSCYP1 was conducted using Clustal method analysis in Megalign (DNASTAR Inc.) Amino acids matching the consensus are shaded. (♦) Cys residue forming a disulphide bridge; (□) Gly residues maintaining an active enzyme conformation; (+) Gln active site residue; (▲) catalytic Cys, His and Asn triad of the active site.](image-url)
Examples of floral and leaf senescence (Stephenson and Rubinstein, 1998; Jones et al., 1995; Smart, 1994; Celikel and van Doorn, 1995). Protease activity rose sharply from stage 5 although protein levels remained fairly constant at this time and the actual rate of protease activity was very small compared to the calculated protein pool available. This suggests that the substrates for the protease activity measured may not contribute significantly to the total protein pool, or that protein synthesis is occurring during this time to replenish those proteins that have been degraded.

Previous studies with cysteine protease have indicated that multiple cysteine proteases are present during daylily flower senescence (Stephenson and Rubinstein, 1998). In the present study zymography indicated the presence of several cysteine proteases that showed both up- and down-regulation during petal development in *Alstroemeria*. The down-regulated protease (band I) is presumably involved in regulation of protein turnover and is part of the general cell maintenance machinery. The up-regulated protease band (III), identified by zymography, shows a similar pattern to those identified in *Hemerocallis* petals (Stephenson and Rubinstein, 1998; Guerrero et al., 1998) that show up-regulation during development, but is rather larger in size. However, since the petal extracts were run under native conditions without boiling prior to loading it is possible that the proteins may have a different migration pattern to the denatured protein markers. The pattern of increase shown by band III was the same as the acidic endopeptidases, of which cysteine proteases are a part, detected by the azocasein assay, in that both show a clear increase from stage 5.

The *Alstroemeria* cysteine proteases observed by zymography were not detectable in the bud or at the time of flower opening, despite the observation that total protease activity increases prior to these stages and loss of total protein is also occurring. This earlier activity could be due to the limits of the sensitivity of zymography or due to proteases that cannot digest the gelatin substrate. The cysteine protease partial cDNA also shows up-regulation throughout development, with a pattern of expression that mimics that of one of the cysteine proteases identified by zymography. Clearly, further experiments would be required to establish whether the cysteine protease activity is encoded by this gene and whether it has an important role in *Alstroemeria* petal senescence. The transcript is clearly present from the earliest stage of *Alstroemeria* petals examined, before cysteine protease activity is detectable by zymography. This expression pattern is in contrast to that seen in *Hemerocallis* petals where thiol protease activity is detected by zymography at the same stage as the expression of *SEN11* and *SEN102* when visualized by Northern blotting (Guerrero et al., 1998).

The expression pattern of *ALSCYP1* shows a continuous increase in transcript levels from the opening bud to the abscising flower, a period of approximately 11 d. *Hemerocallis* *SEN11* and *SEN102* transcripts show relatively low levels of expression in petals as the bud opens, but a marked increase during the later stages of petal collapse (Guerrero et al., 1998; Valpuesta et al., 1995). This difference in the early expression of the cysteine protease genes in these two lilies may be due to the different sensitivity of the detection methods used, or may be a reflection of the different physiological strategies of the two species (rapid wilting compared to a slower, perianth abscising, system).

Protein turnover is in part mediated through targeted proteolysis, involving the ubiquitination of proteins prior to degradation and subsequent cleavage by specific protease enzymes. Expression of the putative *Alstroemeria* ubiquitin cDNA, *ALSUQ1* however, showed very little change throughout flower development and senescence. An increase in protein ubiquitination during flower senescence has been reported in other species such as *Hemerocallis* (Courtney et al., 1994) and it may play a regulatory role in this case. However, overall up-regulation of ubiquitin expression in *Hemerocallis* was less obvious than the more striking changes in the patterns of ubiquitinated proteins revealed by Western blotting.
(Courtney et al., 1994). The same ubiquitin antibody as was used to probe ubiquitinated proteins in Hemerocallis was used in Western blots of Alstroemeria petals. However, very little change in ubiquitinated proteins was detected (data not shown). This difference in ubiquitination between Hemerocallis and Alstroemeria might again reflect (subtly) different levels of control relating to different remobilization strategies.

The gradual increase in expression of the cysteine protease gene in Alstroemeria is paralleled by a steady loss in total protein levels (1.4-fold) and loss in fresh mass throughout vase life (Wagstaff et al., 2001). This suggests that the petals are starting to senesce from stage 1 (day –2), i.e. almost from the time that the flowers open. Hemerocallis flowers show a much more dramatic (6-fold) protein loss from the closed bud to the fully senescent flower (Lay-Yee et al., 1992). This is coupled with a rise in fresh mass that correlates with flower opening prior to a rapid decline as is seen in Alstroemeria. Similarly, large protein losses are seen in other wilting flowers, e.g. Ipomoea (Matile and Winkenbach, 1971) in which protein levels decline 2.8-fold per corolla, and unpollinated Petunia corollas which decline 8-fold during senescence (Lucas, 1989). By contrast, protein levels in Digitalis flowers (Stead and Moore, 1979) decline just 1.5-fold per corolla prior to abscission. These data demonstrate that, as with Alstroemeria, the extent of protein loss is least in species showing petal abscission rather than petal wilting.

Alstroemeria would therefore appear to be a complex flower that shows little protein loss and little change in fresh weight, although some of the classical signs of senescence (petal inrolling and colour change) are evident. Proteolytic activity in this species is clearly evident from the data shown, and cysteine protease gene expression thus appears to be a common feature of leaf and flower senescence (Buchanan-Wollaston, 1997; Guerrero et al., 1998; Valpuesta et al., 1995) that is universal across wilting and abscissing species. Although there is clearly a difference in overall protein loss between wilting and abscissing species there are no data available at present to compare the levels of protease activity between species with different senescence strategies. The prediction, if patterns of protein loss follow patterns of protease activity, would clearly be that wilting species would show a more marked increase in proteolytic activity than abscissing species during senescence. Since proteolytic activity frequently precedes loss in fresh weight it might be hypothesized that wilting is driven by increased protease activity during senescence. This will be exacerbated by the increased membrane lipid degradation occurring during senescence (MK Leverentz et al., unpublished results) which may result in exposure of proteins to the action of the expressed proteases.

The study of proteolytic enzymes provides an important clue to the regulation of floral senescence in systems where ethylene appears not to be an important co-ordinating factor. This may reveal common elements of all senescence pathways that are used as the default in systems not reliant upon ethylene as the senescence regulator.

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