



RESEARCH PAPER

Ethylene-sensitivity regulates proteolytic activity and cysteine protease gene expression in petunia corollas*

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Abstract

To investigate ethylene's role in petal senescence, a comparative analysis of age-related changes in total protein, protease activity, and the expression of nine cysteine protease genes in the corollas of ethylene-sensitive *Petunia × hybrida* cv. Mitchell Diploid (MD) and ethylene-insensitive (35S:*etr1-1*; line 44568) transgenic petunias was conducted. The later stages of corolla senescence in MD flowers were associated with decreased fresh weight, decreased total protein, and increased proteolytic activity. Corolla senescence was delayed by approximately 8 d in *etr-44568* transgenic petunias, and decreases in corolla fresh weight, protein content, and maximum proteolytic activity were similarly delayed. Protease inhibitor studies indicated that the majority of the protease activity in senescing petals was due to cysteine proteases. Nine cysteine proteases expressed in petals were subsequently identified. Northern blot analysis indicated that six of the nine cysteine proteases showed increased transcript abundance during petal senescence. One of these cysteine proteases, *PhCP10*, was detected only in senescing tissues. Expression of four of the senescence-associated cysteine proteases was delayed, but not prevented in *etr-44568* flowers. The other two senescence associated cysteine proteases had high levels of transcript accumulation in *etr-44568* corollas at 8 d after flower opening, when MD flowers were senescing. These patterns suggest that age-related factors, other than ethylene, were regulating the up-regulation of these genes during flower

ageing. The delay in visible symptoms and biochemical and molecular indicators of senescence in ethylene-insensitive flowers is consistent with the concept that ethylene modulates the timing of senescence pathways in petals.

Key words: Cysteine proteinases, *etr1-1*, flowers, KDEL-containing proteases, plants, petunias, *SAG12*, senescence.

Introduction

Senescence represents the last stage of flower development, ultimately culminating in the death of the petals. The senescence programme is regulated by co-ordinated changes in gene expression, and the later stages of senescence share many characteristics of programmed cell death (Rubinstein, 2000; Jones, 2004). Flower petals provide an excellent model system for studies of senescence because they have a finite lifespan and their death is under tight developmental control. While flowers serve an essential role in sexual reproduction, the maintenance of petals is costly in terms of respiratory energy and water loss (Stead, 1992). The programmed senescence of the petals (collectively called the corolla), once a flower is pollinated or no longer receptive to pollination, allows the plant to break down macromolecules and organelles and remobilize their constituents to developing tissues. In support of this recycling programme, a number of genes encoding hydrolytic enzymes have been identified in screens for genes

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Abbreviations: CP, cysteine protease; dao, days after opening; E-64, L-*trans*-epoxysuccinyl-leucylamide-(4-guanidino)-butane; EST, expression sequence tag; MD, Mitchell Diploid; PCD, programmed cell death; PMSF, phenylmethanesulphonyl fluoride; SA, senescence-associated; SAG, senescence-associated gene.

up-regulated during petal senescence (reviewed by Jones, 2004). Decreases in total nitrogen and phosphorus levels have also been detected in senescing petals (Nichols, 1976; Verlinden, 2003).

The degradation of proteins and the remobilization of amino acids to developing tissues is a prominent process during senescence (Solomon *et al.*, 1999). Endopeptidases, or proteases, which degrade proteins by hydrolysing internal peptide bonds, are subsequently one of the most well-characterized cell death proteins in plants (Beers *et al.*, 2000). These proteases are divided into the following subclasses based on their catalytic mechanisms: serine proteases (EC 3.4.21), cysteine proteases (EC 3.4.22), aspartic proteases (EC 3.4.23), metalloproteases (EC 3.4.24), and threonine proteases (EC 3.4.25). Experiments with class-specific inhibitors have attributed the protease activity associated with petal senescence primarily to the cysteine proteases (Stephenson and Rubinstein, 1998; Eason *et al.*, 2002; Wagstaff *et al.*, 2002). In support of their role in flower senescence, cysteine proteases up-regulated during petal senescence have been cloned from the petals of *Dianthus caryophyllus* (carnation) (Jones *et al.*, 1995), *Hemerocallis* spp. (daylily) (Valpuesta *et al.*, 1995; Guerrero *et al.*, 1998), *Alstroemeria peruviana* (Wagstaff *et al.*, 2002), *Sandersonia aurantiaca* (Eason *et al.*, 2002), *Narcissus pseudonarcissus* (daffodil) (Hunter *et al.*, 2002), and *Gladiolus grandiflora* (Arora and Singh, 2004).

Floral senescence in many species is regulated by the plant hormone ethylene (Woltering and van Doorn, 1988; Borochoy and Woodson, 1989). In these flowers, petal senescence is associated with an increase in endogenous ethylene production, and inhibitors of ethylene biosynthesis and action delay senescence (Lawton *et al.*, 1990; Woodson *et al.*, 1992; Jones and Woodson, 1997). The identification of mutants with impaired ethylene production or perception has allowed for a more precise analysis of ethylene function during senescence than could be obtained from inhibitor studies alone (Chang *et al.*, 1993). The *Arabidopsis thaliana* ethylene-insensitive mutant *etr1-1* exhibits a delayed leaf senescence phenotype, and this delay is accompanied by a corresponding delay in the expression of senescence-associated genes (SAGs) (Grbic and Bleeker, 1995). Although SAG induction was delayed in *etr1-1* plants, expression levels were similar to those detected in wild-type *Arabidopsis* leaves. This led to the conclusion that ethylene was affecting the timing of leaf senescence, but was not required for the execution of the senescence programme once it had begun. It has recently been reported that nuclear DNA fragmentation and the induction of a Co²⁺-dependent nuclease (PhNUC1) were delayed during corolla senescence in ethylene-insensitive, 35S:*etr1-1* transgenic petunias (Langston *et al.*, 2005). These studies suggest that ethylene is involved in modulating the timing of flower senescence, but it is still unclear

whether there are components of the senescence pathways in petals that are dependent on ethylene.

Petunia×*hybrida* has served as a model ethylene-sensitive plant for the molecular and biochemical analysis of flower senescence. Transformation of petunia with the *etr1-1* gene from *Arabidopsis* confers ethylene insensitivity or significantly reduced sensitivity and delays flower senescence (Wilkinson *et al.*, 1997; Gubrium *et al.*, 2000; Shibuya *et al.*, 2004). In this paper, 35S:*etr1-1* transgenic petunias have been used to study how ethylene regulates flower senescence. To compare the senescence programmes in ethylene-sensitive (MD) and ethylene-insensitive (*etr-44568*) flowers, a comparative analysis was conducted of age-related changes in total protein, protease activity, and the expression of nine cysteine protease genes in the petals of MD and *etr-44568* petunias. These characteristics were chosen for comparison because protein catabolism is a central component of the senescence programme in petals (Rubinstein, 2000; Jones, 2004). This is the first report of the differential regulation of individual cysteine protease genes by ethylene during flower development, and supports the idea that senescence-induced gene expression in petals occurs via ethylene-dependent and ethylene-independent signalling pathways.

Materials and methods

Plant materials

Petunia×*hybrida* cv. Mitchell Diploid (MD; wild type) and transgenic 35S:*etr1-1* (*etr-44568*, Wilkinson *et al.*, 1997; Shibuya *et al.*, 2004) plants were used in all experiments. All transgenic plants were homozygous for the transgene and were from the T₅ generation. Petunias were grown under greenhouse conditions, with temperatures set at 22/18 °C day/night and a 13 h photoperiod supplemented by metal halide and high pressure sodium lights. Seeds were treated with 100 µl l⁻¹ GA₃ for 24 h and sown in cell packs on top of soil-less mix (Promix BX, Premier Horticulture, Quebec, Canada). All plants were established in the greenhouse after seed germination, and were moved to 15 cm plastic pots after 4 weeks. Plants were fertilized at each irrigation with 150 mg l⁻¹ nitrogen from Peter's 15N-5P-15K (The Scotts Co., Marysville, OH).

Flowers were emasculated 1 d before flowers were fully open to prevent self-pollination and were left on the flower to senesce naturally. Corollas were collected from MD petunias at 0, 2, 4, 6, and 8 d after flower opening (dao) and from *etr-44568* petunias at 0, 2, 4, 6, 8, 10, 12, 14, and 16 dao. Styles (+stigmas) and ovaries were collected from MD flowers at 0 and 8 dao. MD flowers were pollinated by brushing pollen from freshly dehisced anthers onto the stigmas. Corollas, ovaries, and styles (+stigmas) were collected at 3 d after pollination when the flowers were wilted. Green, fully expanded leaves were collected from the top of the plant (green leaves) and naturally senescing leaves that were greater than 75% yellow were collected from the bottom (senescing leaves) of 24-week-old plants. Fully expanded green leaves were also collected 3 h after wounding with a wire brush (wounded leaves). Root and stem tissue was collected from 12-week-old plants and pollen was collected from flowers on the day of anther dehiscence. All tissues were frozen in liquid nitrogen and stored at -80 °C until used for protein extraction, protease activity, or RNA extraction. Fresh weights were measured

immediately before freezing. At least six corollas, styles, or ovaries were pooled for each time point. All experiments were conducted at least three times with independently collected and extracted tissues unless otherwise noted.

Total protein determination

Frozen tissue was powdered in liquid nitrogen and ground in 2 ml extraction buffer (100 mM NaPO₄, 2 mM EDTA, 20 mM DTT, 100 µM leupeptin, 5 µM pepstatin, 1 mM PMSF, 10 mM 1,10-phenanthroline, and 10 µM E-64) per corolla. Samples were centrifuged at 10 000 g at 4 °C for 15 min. The supernatant was used for protein quantification by the Bradford method (Bio-Rad Protein Assay Kit, Hercules, CA) using a BSA standard curve.

Protease activity

In vitro protease activity was determined using the synthetic substrate azocasein based on a method modified from Sarath *et al.* (1989). Corollas were powdered in liquid nitrogen and ground in ice-cold extraction buffer (2 ml per corolla) containing 100 mM NaPO₄ (pH 6.2) and 20 mM DTT. The samples were transferred to centrifuge tubes and centrifuged at 10 000 g at 4 °C for 15 min. The supernatant was stored at -80 °C until used in activity assays. Protease activity of total protein extracts was determined by adding 150 µl protein extract to 250 µl 100 mM NaPO₄ buffer (pH 6.2) containing 1% azocasein. Samples were incubated for 5 h at 37 °C. Optimum buffer composition, incubation duration and temperature, and assay pH for protease activity against azocasein were determined in preliminary experiments (data not shown). Reactions were terminated by adding 1.2 ml 10% TCA. Samples were mixed thoroughly and incubated at room temperature for 15 min. Samples were then clarified by centrifugation at 8000 g for 8 min and the supernatant was alkalized with 1.4 ml 1.0 M NaOH. Blanks were prepared by incubating the protein and azocasein separately and adding the substrate to the sample after the addition of TCA. Absorbance was measured at 490 nm using a Beckman DU640 Spectrophotometer (Beckman Coulter, Fullerton, CA). Sample protease activity was defined in arbitrary units where 1 unit was equivalent to a change of 0.01 absorbance units h⁻¹ at 490 nm.

Class-specific protease inhibitors were incorporated into the assay to determine the specificity of the protease activity detected in corolla

total protein extracts. Individual protease inhibitors were incubated with the total protein extract for 30 min at room temperature prior to the addition of the azocasein substrate. Protease inhibitors and final concentrations used included: E-64 (*L-trans*-epoxysuccinyl-leucylamide-(4-guanidino)-butane; 10 µM), leupeptin (100 µM), pepstatin (5 µM), 1,10-phenanthroline (10 mM), PMSF (phenylmethanesulphonyl fluoride; 1 mM) (Sigma, St Louis, MO). Blanks that included the specific protease inhibitors were prepared as described above.

Cysteine protease genes

Nine genes encoding putative cysteine proteases were identified from a search of the *Petunia* BlastQuest EST database developed at the University of Florida. These cysteine proteases were named *PhCP2* to *PhCP10* (*Petunia* × *hybrida* Cysteine Protease) and sequences have been submitted to GenBank (Accession nos AY662988–AY662996). Full-length sequencing of the selected cDNA clones was conducted by Amplicon Express (Pullman, WA). Sequence analysis was performed with Sequencher V4.1 (Ann Arbor, MI) and software available from NCBI (<http://www.ncbi.nlm.nih.gov/Genbank/>).

RNA extraction, RT-PCR, and gel blot analysis

Total RNA from *petunia* corollas, ovaries, styles, pollen, leaves, stems, and roots was isolated using Trizol (GibcoBRL, Rockville, MA). Two micrograms of total RNA was reverse transcribed at 37 °C for 2 h using Omniscript reverse transcriptase (Qiagen, Valencia, California). Two microlitres of cDNA was used as template for PCR amplification using Master Taq (Eppendorf, Hamburg, Germany) and specific primers to the nine cysteine protease genes (Table 1). Actin was amplified as a control. Actin primers were constructed to the tomato *TOM51* actin cDNA (GenBank accession no. U60481) and included F 5'-GTGTTGGACTCTGGTGATGG-3' and R 5'-TCAG-CAGTGGTGGTGAACAT-3'. PCR was conducted for 26 cycles of 94 °C for 2 min, 60 °C for 2 min, and 72 °C for 2 min using a Mastercycler gradient thermocycler (Eppendorf, Hamburg, Germany). PCR products were separated on 1.0% agarose gels and stained with ethidium bromide. Negative controls had no template DNA and positive controls included the cysteine protease or actin cDNAs in pBluescript (Stratagene, LaJolla, CA).

Table 1. Cysteine protease cDNAs from *Petunia* × *hybrida* and gene-specific primers used for RT-PCR and PCR labelling of probes

Gene name (GenBank accession number)	Size of cDNA clone (bp)	PCR primers
<i>PhCP2</i> (AY662988)	872	F 5'-GTCAAGACAGGCCAAAATCC-3' R 5'-CAAATGCACCAATGAAGTCG-3'
<i>PhCP3</i> (AY662989)	1041	F 5'-GCTCATGCTGGTCCTTCAGT-3' R 5'-GCATGAACTCCTCCACAT-3'
<i>PhCP4</i> (AY662990)	630	F 5'-GCTCCGACCCATACAGTATCA-3' R 5'-GCAAAGATTGACAAAGCA-3'
<i>PhCP5</i> (AY662991)	643	F 5'-GCAACAACCTTGTCTGACTGTGA-3' R 5'-ACAAACATTGCGGCCTCTAC-3'
<i>PhCP6</i> (AY662992)	830	F 5'-AAACCAAGGATGCAATGGAG-3' R 5'-CGATACCACACAACCCCTCT-3'
<i>PhCP7</i> (AY662993)	629	F 5'-AAGAACCAGGGTCAATGTGG-3' R 5'-TTGGCTGGTACATCACGGTA-3'
<i>PhCP8</i> (AY662994)	709	F 5'-AGGCATGGCTAGTACAACATGG-3' R 5'-GCAAATGCCCAACAACCTCC-3'
<i>PhCP9</i> (AY662995)	784	F 5'-GTAGCGAAAATGGCATGGAT-3' R 5'-TGTGCCTGCTAACATTGC-3'
<i>PhCP10</i> (AY662996)	765	F 5'-TCCGTACAAGGGAGAAGACG-3' R 5'-CGGATAAGAAGCTTCCGTTG-3'

Fifteen micrograms of total RNA was size separated through a denaturing 1.2% agarose gel containing formaldehyde, and subsequently, transferred overnight onto Hybond N membrane (Amersham Pharmacia Biotech, Piscataway, NJ) by capillary transfer in $10\times$ SSC. The RNA was cross-linked using a UV crosslinker at 50 mV cm^{-2} (Bio-Rad Laboratories, Hercules, CA). Radiolabelled, cDNA probes were generated by PCR using gene-specific primers (Table 1) and $\alpha^{32}\text{P}$ -CTP (PerkinElmer, Boston, MA). Prehybridization and hybridizations were performed as previously described (Jones *et al.*, 1995). Hybridization signals were visualized using a Storm 860 PhosphorImager and quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Transcript levels were normalized to rRNA levels to correct for any differences in RNA loading.

Western blot analysis

Frozen tissue was powdered in liquid nitrogen, ground in extraction buffer [100 mM TRIS-HCl, pH 7.6, 10 mM MgSO_4 , 10 mM DTT, complete mini protease inhibitor cocktail tablet (1 tablet per 10 ml; Roche Diagnostics Corporation, Indianapolis, IN)], and incubated on ice for 1 h with periodic vortexing. Samples were centrifuged at 20 000 g at 4 °C for 10 min. Protein in the supernatant was quantified by the Bradford method (Bio-Rad Protein Assay Kit, Hercules, CA) using a BSA standard curve. Equivalent amounts of protein (10 μg) were size-fractionated by SDS-PAGE on 10% acrylamide gels. After electroblotting on to Immun-Blot7 PVDF membrane (Bio-Rad, Hercules, CA), blots were blocked with 1% BSA in 50 mM TRIS-HCl, 150 mM NaCl, 0.3% Tween 20 (v/v), pH 8, overnight at room temperature. Blots were then incubated with primary antibody raised against the purified CysEP (diluted 1:1000 in blocking solutions, Schmid *et al.*, 1998) for 2 h, and subsequently washed with TRIS-buffered saline (TBS) containing 0.5% (v/v) Tween-20 for 5×5 min each. Immunoreactive proteins were visualized using the secondary antibody alkaline phosphatase-coupled sheep anti-rabbit IgG with nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (Roche Diagnostics Corporation, Indianapolis, IN) as a substrate.

Results

Corolla wilting and fresh weight declines are delayed by approximately 8 d in *etr-44568* transgenic petunias

MD petunias exhibited the first visible symptoms of senescence, wilting of the corolla, at approximately 8 d after flower opening (dao) (8.4 ± 0.26 d, $n=18$; Fig. 1A). Flower senescence in *etr-44568* flowers was significantly delayed, but more variability was observed in the number of days to corolla wilting. The average time to wilting was 15.6 ± 0.97 d, ($n=18$) with some flowers wilted on day 14 and some as late as day 18. Under these growing conditions the fresh weight of *etr-44568* flowers was slightly less than that of MD on the day of flower opening (0 dao) (Fig. 1B). At 2 dao the fresh weight of both MD and *etr-44568* flowers increased compared with 0 dao. Corolla fresh weights of both genotypes remained constant from 4–6 dao. By day 8, the fresh weight of MD corollas had decreased to approximately 84% of the corollas mean fresh weight at 0 d, and this decrease coincided with corolla wilting. The fresh weight of *etr-44568* corollas remained constant until 14–16 dao, which also coincided with wilting of the corolla.

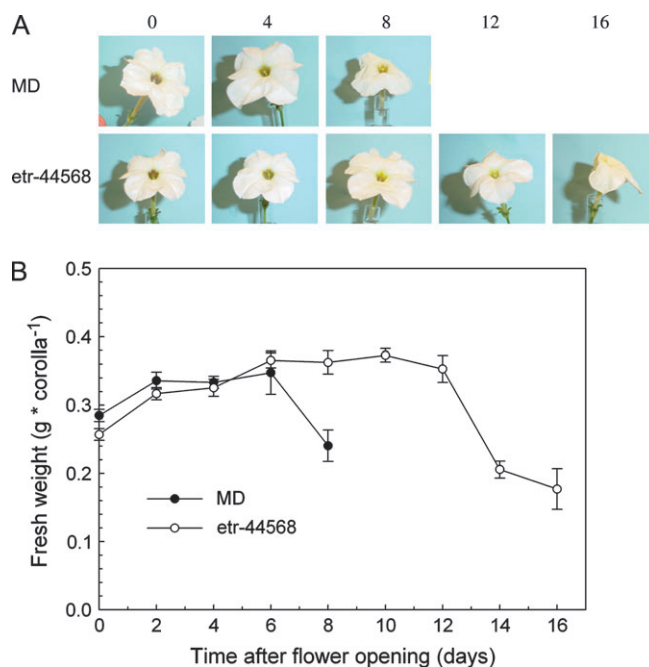


Fig. 1. Natural senescence of wild-type *Petunia x hybrida* cv. Mitchell Diploid (MD) and 35S:*etr-1* transgenic petunias (*etr-44568*). Visible symptoms of senescence (A) and fresh weight changes in corollas (B) during flower development. Data are the means of six replicates \pm SE. Experiments were conducted at least three times with similar results.

Comparative changes in total protein content and protease activity in the corollas of MD and *etr-44568* petunias

On the day of flower opening, *etr-44568* flowers had less total protein per corolla than MD flowers (Fig. 2A). The level of soluble proteins in MD corollas remained relatively constant from 0 to 4 dao and then began to decrease. The protein content of wilted corollas at 8 dao was only 46% of that measured from corollas at 0 d. Protein levels increased slightly in *etr-44568* corollas at 2 dao and remained relatively constant until 10 dao. By 16 dao, protein levels in *etr-44568* corollas had decreased to levels that were comparable with those measured from senescent MD corollas at 8 dao.

In vitro protease activity was determined against the synthetic protease substrate azocasein (Fig. 2B). Low levels of protease activity were detected in total protein extracts from MD and *etr-44568* corollas on the day of flower opening. Protease activity increased at 2 dao, and a greater than 2-fold increase in activity was subsequently measured from day 2 to day 4 in both MD and *etr-44568* corollas. Smaller increases in activity were measured between 4 and 6 dao in both genotypes. Protease activity was highest in MD corollas late in the senescence programme when petals were wilted (8 dao). Protease activity increased more gradually during the later part of *etr-44568* corolla senescence, and maximum protease activity was detected at 16 dao when petals were wilted. The maximum levels of

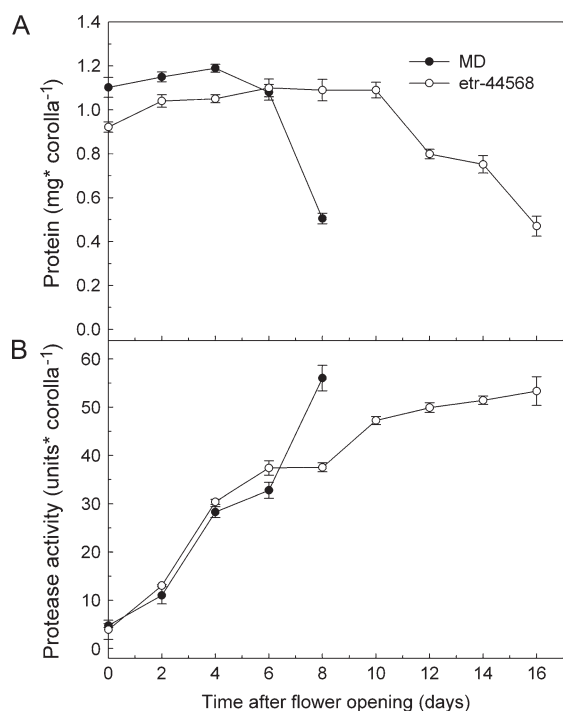


Fig. 2. Protein content (A) and protease activity (B) in wild-type *Petunia×hybrida* cv. Mitchell Diploid (MD) and 35S:*etr1-1* transgenic (etr-44568) petunia corollas during flower development. Corollas were collected from at least six flowers at various days after flower opening. Total protein was determined using the Bradford assay. Protease activity of corolla total protein extracts was determined against the synthetic protease substrate azocasein. Units of activity were measured in arbitrary units with one unit equivalent to a change of 0.01 absorbance units h^{-1} at 490 nm. Data are the means of three replicates \pm SE. Experiments were conducted at least twice with similar results.

protease activity measured in MD petals at 8 dao were similar to those measured in etr-44568 petals at 16 dao.

Class-specific protease inhibitors were incorporated into the *in vitro* protease activity assays to determine which classes of proteases were responsible for the increases in protease activity detected during the senescence of MD and etr-44568 corollas (Fig. 3). At 8 dao, when MD corollas had the highest activity, the greatest inhibition of protease activity was measured in total protein extracts incubated with E-64 and leupeptin. These samples had only 9% and 11%, respectively, of the activity detected in control extracts with no inhibitors. E-64 is an irreversible inhibitor of cysteine proteases, and leupeptin inhibits trypsin-like serine and most cysteine proteases. E-64 and leupeptin also inhibited protease activity in etr-44568 corolla extracts at 8 dao and 16 dao. *In vitro* protease activity in 16 d etr-44568 corollas was only 11% and 7% of control activity when treated with E-64 and leupeptin, respectively. The aspartic protease inhibitor, pepstatin, had no effect on protease activity in either MD or etr-44568 corollas. 1,10-phenanthroline, a metalloprotease inhibitor, resulted in a slight reduction in activity in both genotypes. PMSF, which inhibits serine

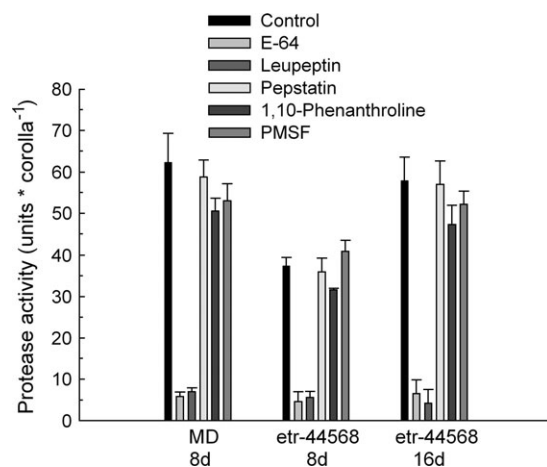


Fig. 3. Effect of class-specific inhibitors on protease activity in naturally senescing *Petunia×hybrida* cv. Mitchell Diploid (MD) and 35S:*etr1-1* transgenic (etr-44568) petunia corollas. Corollas were collected from MD flowers at 8 d after flower opening (dao) and from etr-44568 flowers at 8 dao and 16 dao. Total protein extracts were incubated with no inhibitor (control), E-64 (10 μM), leupeptin (100 μM), pepstatin (5 μM), 1,10-phenanthroline (10 mM), and PMSF (1 mM) for 30 min at 25 °C prior to the addition of azocasein substrate and incubation at 37 °C for 5 h. Units of activity were measured in arbitrary units with one unit equivalent to a change of 0.01 absorbance units h^{-1} at 490 nm. Data are the means of three replicates \pm SE. Experiments were conducted four times.

proteases and some cysteine proteases, had no significant effect on activity in either MD or etr-44568 corollas.

Differential regulation of cysteine protease genes during the senescence of MD and etr-44568 petunias

Inhibitor studies indicated that the majority of the protease activity during senescence was the result of proteases in the cysteine protease class. Therefore, putative cysteine protease genes were identified by searching ESTs generated by random sequencing of several petunia flower cDNA libraries. This search initially identified nine unique ESTs with homology to known cysteine proteases (CP). These clones were named *PhCP2–PhCP10* (*Petunia×hybrida* Cysteine Protease), and represent cDNAs ranging in size from 629 bp to 1041 bp. (Table 1). *PhCP1* was reserved for a previously identified petunia CP (PeTh3, GenBank no. U31094; Tournaire *et al.*, 1996). The PhCPs have the highest homology to members of the papain-like cysteine proteases (family C1A), and an NCBI conserved domain search identified putative Peptidase C1 domains in the predicted amino acid sequences of all nine PhCPs.

The predicted amino acid sequences of the petunia cysteine proteases were aligned with the most homologous plant proteases, including those identified from flowers, using ClustalW. The phylogenetic relationship of these proteases is shown in Fig. 4. *PhCP2* groups with *PhCP8* and *PhCP9* and other papain-type cysteine proteases that are up-regulated during senescence and by stresses including temperature, drought, and *Phytophthora infestans*

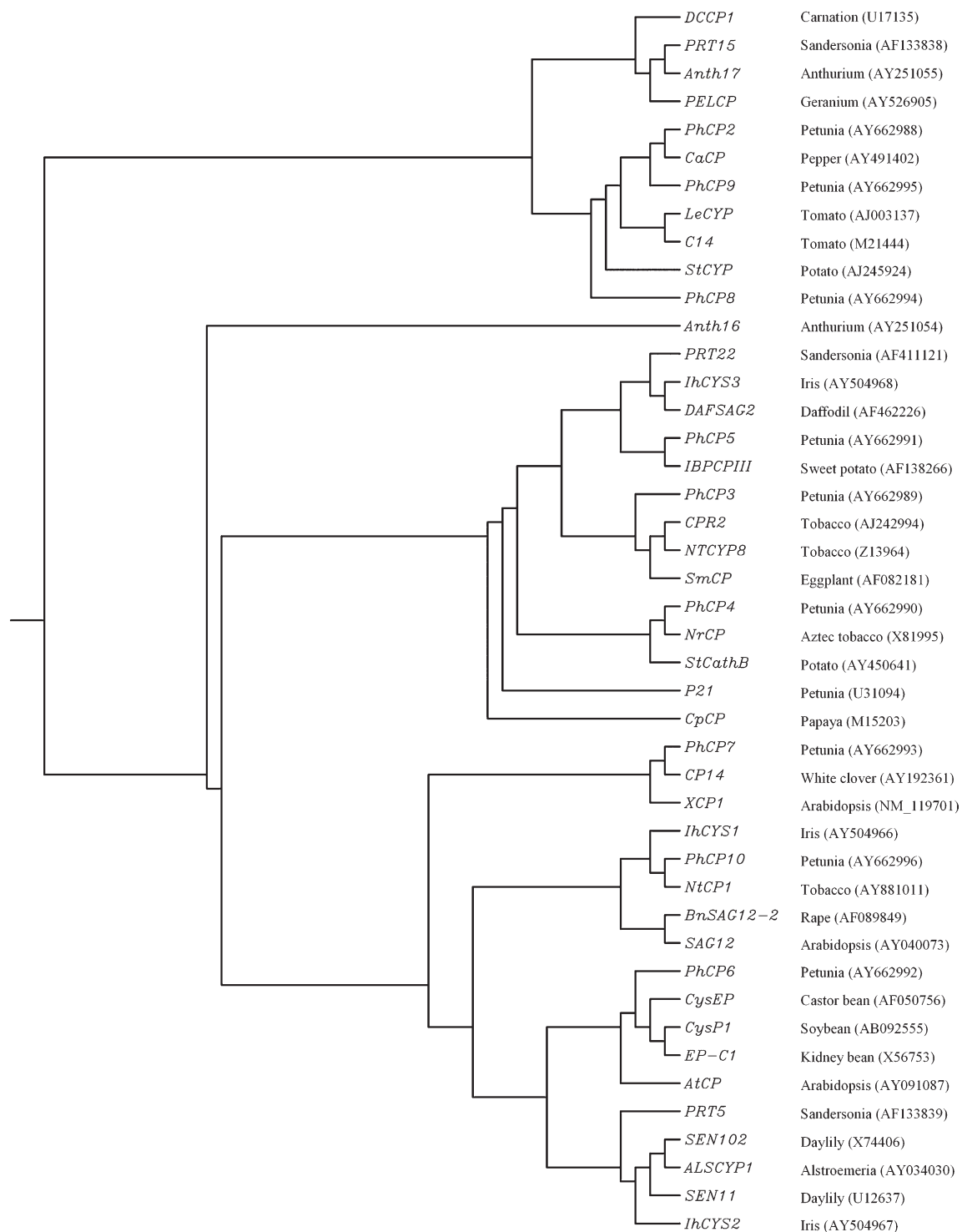


Fig. 4. Phylogenetic analysis of plant papain-type cysteine proteases with homology to the *Petunia*×*hybrida* cysteine proteases (*PhCP1*–*PhCP10*). Sequence alignment was obtained using ClustalW. GenBank accession numbers are in parenthesis.

infection (Drake *et al.*, 1996; Avrova *et al.*, 1999). *PhCP3* groups with a cluster of CPs up-regulated by drought, circadian rhythm, and wounding (Linthorst *et al.*, 1993). It is most homologous to a CP from *Solanum melongena* (egg plant) that is up-regulated during developmental events

associated with programmed cell death (Xu and Chye, 1999). *PhCP4* is most homologous to cathepsin B-like cysteine proteases from *Solanum tuberosum* (potato) (Avrova *et al.*, 2004) and *Nicotiana rustica* (Aztec tobacco) (Lidgett *et al.*, 1995). These genes are up-regulated

following *Phytophthora infestans* infection and wounding, respectively. *PhCP5* is most similar to CP isoform III from *Ipomea batatas* (sweet potato). It also groups with a number of CPs identified from the tepals of ethylene-insensitive flowers, including *PRT22* from *Sandersonia*, *IhCYS3* from *Iris×hollandica*, and *DAFSAF2* from daffodil (Eason *et al.*, 2002; Hunter *et al.*, 2002). The predicted polypeptide of *PhCP6* contains a C-terminal KDEL motif, and sequence alignments group it with other KDEL-containing cysteine proteases. This motif is thought to function as an endoplasmic reticulum retention signal for soluble proteins (Schmid *et al.*, 1999). *PhCP6* is most similar to Cys-EP, the *Ricinus communis* (castor bean) cysteine protease involved in programmed cell death of the endosperm. *PhCP7* has the highest homology to a CP from *Trifolium repens* (white clover) and a xylem endopeptidase from *Arabidopsis* involved in PCD during tracheary element differentiation (Zhao *et al.*, 2000; Asp *et al.*, 2004). *PhCP10* groups with a number of CP genes that have been reported to have senescence-specific patterns of transcript accumulation, including *SAG12* from *Arabidopsis*, and *SAG12* homologues from *Brassica napus* (rape) (*BnSAG12-2*) and *Nicotiana tabacum* (tobacco) (*NtCPI*) (Weaver *et al.*, 1998; Noh and Amasino, 1999).

RT-PCR experiments using gene-specific primers for all PhCPs were conducted to determine where in the plant the PhCP transcripts were detected and to determine if they were detectable in non-senescent (0) and senescing (3P and 8U) flower tissues (Fig. 5). Naturally senescing, unpollinated MD flowers were collected at 8 dao (8U), and pollinated MD flowers were collected at 3 d after pollination (3P) when they were senescent (corollas were wilted). All nine PhCPs were detected in flower corollas. Only *PhCP10* was detected in senescing but not in non-senescent corollas. *PhCP10* was also detected in senescing, but not in non-senescent, green leaves. Transcripts for *PhCP2*, *PhCP3*, *PhCP4*, *PhCP5*, *PhCP7*, and *PhCP9* were detected in flowers, leaves, stems, and roots. *PhCP6* transcripts were most abundant in flower tissues, and barely detectable in green leaves and stems. *PhCP8* was not detectable in pollen or roots. While *PhCP8* transcripts were detected in ovaries from flowers on the day of flower opening (0), they were not detected in ovaries from naturally senescing (8U) or pollinated flowers (3P).

Northern blot analysis was conducted to quantify changes in transcript abundance during corolla senescence, and to identify those cysteine proteases involved in petal senescence (Fig. 6). Some of the PhCPs had the highest transcript accumulation in senescing corollas, while some were down-regulated during flower development. In MD corollas *PhCP2*, *PhCP3*, *PhCP5*, *PhCP8*, *PhCP9*, and *PhCP10* showed patterns of up-regulation during senescence. These were referred to as senescence-associated cysteine proteases or SACPs. *PhCP8* and *PhCP10* had maximum transcript abundance at 6 dao, just before the

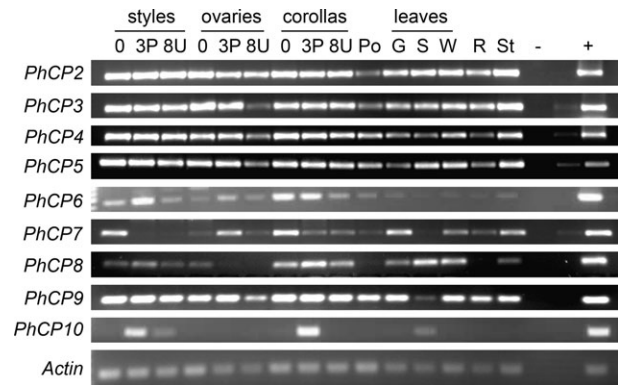


Fig. 5. RT-PCR analysis of the spatial and developmental regulation of cysteine protease transcript accumulation in *Petunia×hybrida* cv. Mitchell Diploid petunia floral and vegetative tissues. Styles, ovaries, and corollas were collected from non-senescent flowers on the day of flower opening (0), and from naturally senescing flowers at 8 d after flower opening (8U) and 3 d after pollination (3P). Pollen (Po) was collected from flowers on the day of anther dehiscence, approximately 2 d after the flowers opened. Green, fully expanded leaves were collected from the top of the plant (G) and naturally senescing leaves that were greater than 75% yellow were collected from the bottom (S) of 24-week-old plants. Fully expanded green leaves were also collected 3 h after wounding (W) with a wire brush. Root (R) and stem (St) tissue was collected from 12-week-old plants. Negative controls (–) had no template DNA and positive controls (+) included the cysteine protease or actin cDNAs in pBluescript. The gene specific primers used to amplify *PhCP2–PhCP10* are listed in Table 1 and actin was amplified as a normalization control. The experiment was performed twice with similar results.

fresh weight decline and visible symptoms of corolla wilting, while maximum transcript abundance of *PhCP2*, *PhCP3*, *PhCP5*, and *PhCP9* was detected at 8 dao, when corollas were wilted and protease activity was highest. Transcripts for all of the SACPs were detected in non-senescent corollas from 0 to 4 dao, except for *PhCP10*, which was specific to senescing corollas. *PhCP5*, which had relatively high constitutive levels of transcript at 0–6 dao, had the smallest up-regulation at the later stages of senescence when compared with the other SACP genes.

In *etr-44568* corollas, maximum transcript abundance of the SACPs, *PhCP2*, *PhCP8*, *PhCP9*, and *PhCP10* was delayed to 14–16 dao and corresponded with corolla wilting. By contrast, maximum transcript abundance of *PhCP3* was detected at 8 dao and had returned to lower, basal levels by 14–16 dao. Relative mRNA abundance of *PhCP5* was also similar in WT and *etr-44568* corollas at 8 dao, but mRNA levels continued to increase from 8–14 dao in the *etr-44568* corollas.

PhCP4, *PhCP6*, and *PhCP7* were down-regulated during the development of WT and *etr-44568* corollas. In *etr-44568*, *PhCP7* gradually decreased by 16 dao to levels detected in comparably wilted MD flowers at 8 dao. *PhCP4* and *PhCP6* transcript abundance decreased more rapidly than *PhCP7*, with large decreases detected at 2 dao and 4 dao in *etr-44568* and MD corollas, respectively.

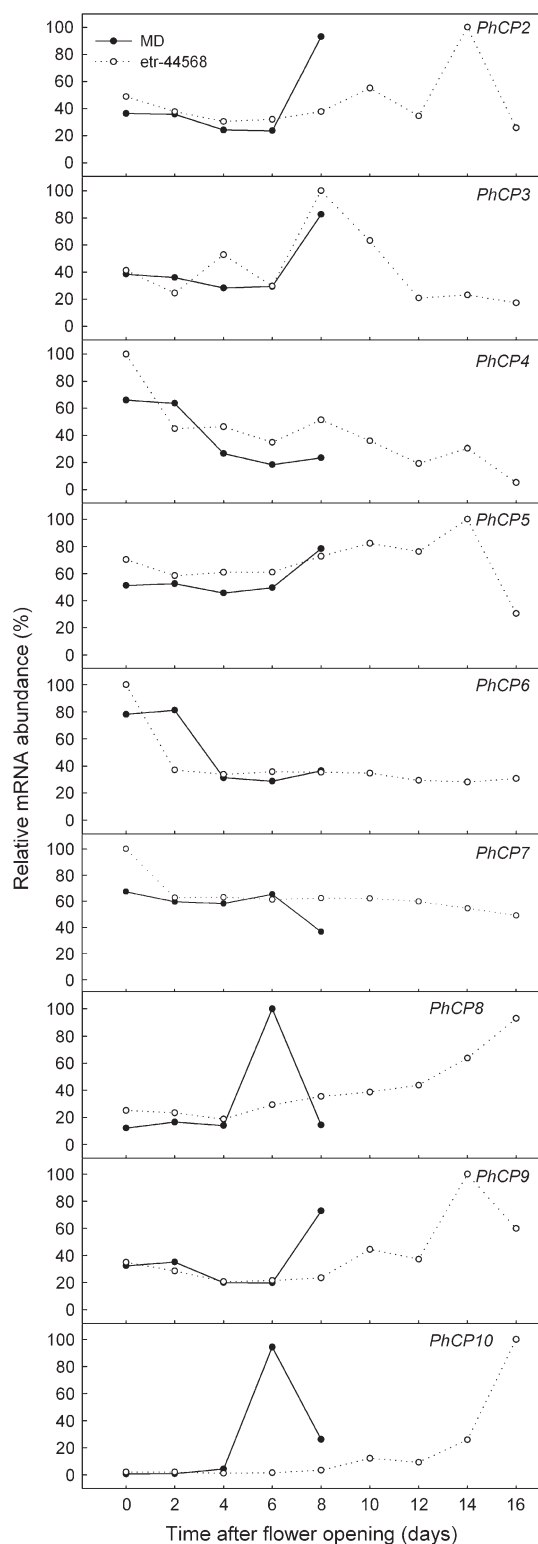


Fig. 6. RNA gel-blot analysis of temporal gene expression patterns for nine cysteine proteases during the natural senescence of wild type *Petunia* × *hybrida* cv. Mitchell Diploid (MD) and 35S:*etr1-1* transgenic (*etr-44568*) petunia corollas. PhosphorImager data are expressed for each individual blot in terms of percentage of the maximum signal detected on that blot. Transcript levels were normalized to rRNA levels to correct for any differences in RNA loading. The experiment was performed twice with similar results.

Western blot analysis of CysEP-like proteases

In contrast to the KDEL-containing CysEP-like proteases that have been identified in other flowers, *PhCP6* was found to be transcriptionally down-regulated during petunia petal senescence (Fig. 5; Guerrero *et al.*, 1998; Eason *et al.*, 2002). Western blot analysis of protein extracts from naturally senescing (0, 2, 4, 6, and 8 dao) petunia corollas confirmed that a CysEP-like protein decreased in abundance as MD corollas senesced. The antibody raised against CysEP (Schmid *et al.*, 1998) cross-reacted with one protein band from petunia corollas (*c.* 37.5 kDa; Fig. 7). The abundance of this 37.5 kDa protein declined from 0 dao to 8 dao.

Discussion

A common feature of programmed cell death during organ senescence is the degradation of proteins (Huffaker, 1990). Petal senescence has been associated with decreased protein levels, increased proteolytic activity, and the up-regulation of cysteine protease genes (reviewed in Jones, 2004). While one of the earliest reports of a cysteine protease involved in petal senescence was from ethylene-sensitive carnation flowers (Jones *et al.*, 1995), most studies of proteases and petal senescence have utilized species in which petal senescence is not regulated by ethylene (i.e. ethylene-insensitive flowers) (Guerrero *et al.*, 1998; Stephenson and Rubinstein, 1998; Eason *et al.*, 2002; Hunter *et al.*, 2002; Wagstaff *et al.*, 2002; Arora and Singh, 2004; Pak and van Doorn, 2005). Several senescence-associated cysteine protease genes have been reported to increase in abundance following ethylene treatment (Cervantes *et al.*, 1994; Alonso and Granell, 1995; Jones *et al.*, 1995; Weaver *et al.*, 1998; Cercos *et al.*, 1999; Chen *et al.*, 2002), but the role of ethylene in the initiation and execution of protein catabolism during petal senescence is not clear. A comparative study of age-related changes in total protein, protease activity, and cysteine protease gene expression in corollas from the ethylene-sensitive flowers of *Petunia* × *hybrida* cv. Mitchell Diploid (MD) and the ethylene-insensitive flowers of transgenic 35S:*etr1-1* petunias (*etr-44568*) has therefore been conducted.

Transformation of petunias with the mutated ethylene receptor gene (*etr1-1*) from *Arabidopsis* reduces ethylene sensitivity in flowers and delays senescence (Wilkinson *et al.*, 1997; Gubrium *et al.*, 2000; Shibuya *et al.*, 2004). Multiple independent transgenic lines have been generated with varying degrees of ethylene sensitivity. To determine the level of ethylene sensitivity of 35S:*etr1-1* lines at the molecular level, the induction of *PhEIL1*, a transcription factor involved in ethylene signal transduction, was determined following ethylene treatment of flowers (Shibuya *et al.*, 2004). In MD corollas, expression of *PhEIL1* was induced 370% by exogenous ethylene compared with

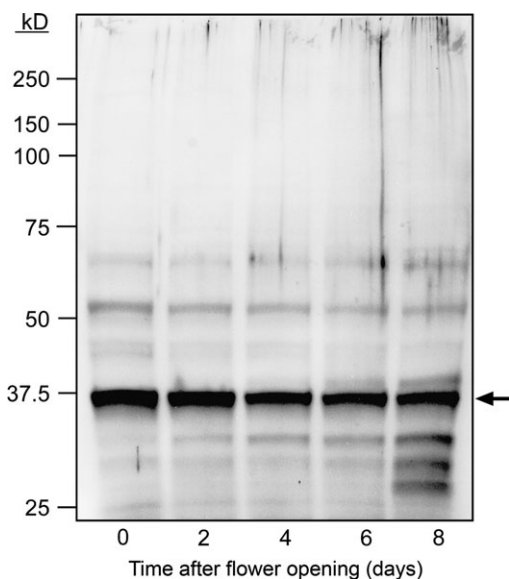


Fig. 7. Western-blot analysis of protein extracts from wild type *Petunia* × *hybrida* cv. Mitchell Diploid corollas from 0–8 d after flower opening. Blots were incubated with a primary antibody raised against the 35 kDa CysEP from castor bean ricinosomes (Schmid *et al.*, 1998). The experiment was performed twice with similar results.

untreated controls. *PhEIL1* expression was not induced in ethylene-treated etr-44568 flowers, indicating that these flowers are insensitive to ethylene or, at the very least, have greatly reduced ethylene sensitivity. By contrast, in etr-56, a transgenic line characterized as having reduced ethylene sensitivity, *PhEIL1* was induced 187% by ethylene.

Corolla senescence in both ethylene-sensitive MD and ethylene-insensitive etr-44568 petunias was accompanied by increased proteolytic activity and a decline in total protein levels. Quantitatively, the increase in proteolytic activity and the decrease in protein content were similar in etr-44568 and MD corollas, suggesting that the execution of protein catabolism during senescence is not dependent on ethylene signalling. On the other hand, the temporal regulation of protease activity and protein degradation differed between MD and etr-44568 flowers. Corolla senescence was delayed by approximately 8 d in etr-44569 flowers. Maximum protease activity and net protein losses were similarly delayed and corresponded with the wilting of the etr-44568 corollas. It was recently reported that DNA fragmentation and the induction of a senescence-specific nuclease (*PhNUC1*) were also delayed in 35S:*etr1-1* transgenic petunias (Langston *et al.*, 2005). Similarly, Burdon and Sexton (1993) reported that ethylene accelerates and coordinates petal abscission and senescence in raspberry flowers, as both processes will eventually occur even in the absence of ethylene. These studies support the role of ethylene as a modulator of senescence timing in petals.

While the senescence-related induction of proteolytic activity appeared to be merely delayed in etr-44568 corollas, it was not known whether the same proteases or

even the same class of proteases was responsible for the proteolytic activity measured in MD and etr-44568 corollas. It was found that the protease activity detected in protein extracts from both senescing MD and etr-44568 corollas was mainly due to cysteine proteases (~90%), and to a much lesser extent metalloproteases (<10%) (Fig. 3). In similar inhibitor studies with the ethylene-insensitive flowers, *Sandersonia* and *Iris*, approximately 50% of the protease activity measured in senescing tepals was attributed to cysteine proteases (Eason *et al.*, 2002; Pak and van Doorn, 2005). Treatment of *Sandersonia* and *Iris* flowers with the cysteine protease inhibitors, leupeptin and E-64, respectively, was also found to delay visible symptoms of senescence and decrease endogenous protease activity (Eason *et al.*, 2002; Pak and van Doorn, 2005). An increase in cysteine protease activity would appear to be a common feature of petal senescence in both ethylene-sensitive and ethylene-insensitive flowers.

In support of the role of cysteine proteases in petunia flower senescence, it was possible to identify nine cysteine protease genes that were expressed in corollas. Three of the nine cysteine protease genes (*PhCP4*, *PhCP6*, *PhCP7*) were down-regulated during flower ageing in both MD and etr-44568 (Fig. 6). Six cysteine protease genes (*PhCP2*, *PhCP3*, *PhCP5*, *PhCP8*, *PhCP9*, *PhCP10*) were up-regulated during the natural ageing of corollas, but the temporal expression patterns of some of these genes differed in MD and etr-44568 corollas.

The pattern of decreasing mRNA abundance of *PhCP4*, *PhCP6*, and *PhCP7* during flower ageing suggests that this group of cysteine proteases is most likely involved in the regulation of general protein turnover and cellular maintenance during the growth and development of the petals. Ethylene would not appear to be involved in co-ordinating the expression of these genes, as the patterns of down-regulation following flower opening were similar in MD and etr-44568 corollas. Of particular interest within this group of genes is *PhCP6*, which has high homology to CysEP from castor bean and other KDEL-containing cysteine proteases (Fig. 4). CysEP is localized within membrane-bound organelles called ricinosomes that are formed at the beginning of PCD (Schmid *et al.*, 1998). Acidification of the ricinosomes during the later stages of cell death causes activation and release of the CysEP following cleavage of the N-terminal pro-peptide and the C-terminal KDEL (Schmid *et al.*, 2001). Ricinosomes were originally identified in the endosperm of castor beans, but have also been detected in senescing daylily petals (Schmid *et al.*, 1999). In contrast to *PhCP6*, transcript abundance of the KDEL-containing cysteine proteases from *Sandersonia* (*PRT5*) and daylily (*SEN102* and *SEN11*) increased during tepal senescence (Guerrero *et al.*, 1998; Eason *et al.*, 2002). Western analysis using an antibody raised against the active form of CysEP (Schmid *et al.*, 1998) recognized a protein of approximately 37.5 kDa in petunias that also decreased

in abundance during corolla senescence. These experiments suggest that the CysEP-like proteases in petunia corollas are not likely to be involved in the large-scale proteolysis that accompanies the later stages of petal senescence, but that they may be involved in processes early in flower development that involve PCD.

The majority of the cysteine protease genes from petunia exhibited senescence-associated (SA) increases in transcript abundance (SACPs; *PhCP2*, *PhCP3*, *PhCP5*, *PhCP8*, *PhCP9*, and *PhCP10*) in MD corollas. All SACPs, except for *PhCP10*, were detected at basal levels in non-senescent tissues and increased in abundance during senescence. The expression of these SACPs suggests that their major role during senescence is catalysing the large-scale degradation of proteins that accompanies petal wilting, but that they may also have a role in protein turnover throughout flower development. The majority of the senescence-associated genes (SAGs) that have been identified from leaves and petals show a similar expression profile, and only a few SAGs have been detected only in senescing tissues (Buchanan-Wollaston, 1997; Jones, 2004).

PhCP10 was the only petunia SACP that had senescence-specific expression. Transcripts were not detectable in non-senescent floral or vegetative tissues, but were induced during the senescence of petals, styles, and leaves. *PhCP10* has high homology to *SAG12* from *Arabidopsis* and the *SAG12* homologues from tobacco and *B. napus* (Fig. 4). *SAG12* has no detectable expression in young leaves and is induced in older leaves during age-mediated senescence. Of the *Arabidopsis* senescence-associated genes, *SAG12* is suggested to be one of the best molecular markers of leaf senescence (Weaver *et al.*, 1998). Similarly, the *SAG12* homologue, *PhCP10*, would appear to be an excellent molecular marker for petal senescence.

It has been suggested by many researchers that senescence-associated proteases are functioning in the complete proteolysis that accompanies senescence and programmed cell death and allows for nutrient remobilization to sink tissues (Rubinstein, 2000; Jones, 2004). There is increasing evidence that proteases may have a regulatory role in plant growth and development by selectively cleaving and subsequently activating enzymes, including other proteases (Beers *et al.*, 2004). In this way, their function during senescence may be similar to the initiator caspases functioning in animal PCD pathways (Woltering *et al.*, 2002). *PhCP10* and *PhCP8* had maximum expression at 6 dao, before the visible symptoms of senescence, with decreasing abundance at 8 dao when the other SACPs had maximum transcript abundance. These patterns suggest that *PhCP10* and *PhCP8* may function earlier in the senescence programme, and they may be involved in initiating signalling pathways by cleaving other senescence-associated proteins. Three cysteine proteases that are up-regulated during tepal senescence have been reported in *Sandersonia* flowers (Eason *et al.*, 2002). One of these CPs, *PRT5*, was reported

to be specifically expressed during flower senescence, and was not detected in immature flowers or vegetative tissues. Like *PhCP10*, *PRT5* mRNAs began to accumulate earlier than the other senescence-associated CPs and before visible symptoms of senescence were apparent (Eason *et al.*, 2002).

A subgroup of the petunia SACPs (*PhCP2*, *PhCP8*, *PhCP9*, and *PhCP10*) also had senescence-associated increases in transcript abundance in *etr-44568* corollas at 14–16 dao when these flowers were wilting. Although expression was delayed, relative transcript abundance was similar to that measured in senescing MD corollas. Delays in leaf senescence in the *Arabidopsis etr1-1* mutant were also accompanied by corresponding delays in the expression of senescence-associated genes (Grbic and Bleecker, 1995). *PhCP2*, *PhCP8*, and *PhCP9* have high homology to the tomato cysteine protease, *C14 (SenU2)* (Fig. 4). This cysteine protease was originally detected in tomato fruits following low temperature stress (Schaffer and Fischer, 1988), but it is also involved in leaf senescence (Drake *et al.*, 1996). *C14 (SenU2)* transcripts were detected in young fully expanded leaves, increased in abundance during ageing, and reached maximum abundance at the later stages of leaf senescence (Drake *et al.*, 1996). In transgenic tomato plants deficient in ethylene biosynthesis, visible symptoms of leaf senescence and the enhanced accumulation of *SenU2 (C14)* mRNAs were delayed (Drake *et al.*, 1996). This is very similar to the expression profile of *PhCP2*, *PhCP8*, and *PhCP9* during corolla development in MD and *etr-44568* petunias, and it suggests that ethylene is similarly regulating the timing of proteolysis during the senescence of leaves and petals.

In contrast to the expression of *PhCP2*, *PhCP8*, *PhCP9*, and *PhCP10*, transcript accumulation of *PhCP3* and *PhCP5* was not delayed in *etr-44568* corollas. The temporal expression profile of *PhCP3* was similar in *etr-44568* and MD corollas. *PhCP3* reached maximum transcript abundance at 8 dao, and declined by 14–16 dao when the *etr-44568* corollas were wilting. Relative abundance of *PhCP5* transcripts was similar in MD and *etr-44568* corollas at 8 dao, but continued gradually to increase until 14 dao. These expression profiles suggest that *PhCP3* and *PhCP5* may encode the cysteine proteases responsible for the enhanced activity detected in *etr-44568* corollas at 8 dao. Phylogenetic analysis (Fig. 4) shows that *PhCP3* and *PhCP5* have high homology to CPs from the ethylene-insensitive flowers *Iris*, *Sandersonia*, and daffodil. Both *DAFSAG2* and *PRT22*, from daffodil and *Sandersonia*, respectively, were detected at low levels in young flowers and increased in abundance during the later stages of senescence (Eason *et al.*, 2002; Hunter *et al.*, 2002). *PhCP3* and *PhCP5* appear to be regulated during flower development independent of ethylene action, and may be regulated by age-related factors similar to those controlling senescence-associated expression of cysteine proteases in

ethylene-insensitive, non-climacteric flowers. Gene expression studies using *etr1-1 Arabidopsis* mutants led to similar conclusions regarding ethylene's regulation of abscission. While abscission could be accelerated by ethylene, it was not required for the normal abscission programme. These studies also identified both ethylene-dependent and ethylene-independent components of the abscission process in *Arabidopsis* (Patterson and Bleecker, 2004).

Conclusions

It has been shown that ethylene sensitivity regulates the timing of flower senescence in part by delaying the increased proteolytic activity and subsequent protein degradation that are an integral component of the senescence programme in petunia corollas. Expression profiles of the cysteine proteases during flower development and senescence identified both genes that were regulated by ethylene and those that were independent of ethylene. Expression of most of the senescence-associated cysteine protease genes was delayed but not prevented in *etr1-1* flowers, and these delays corresponded with corolla wilting. The delay in the progression of senescence in ethylene-insensitive flowers is consistent with the concept that ethylene acts as a modulator of senescence pathways.

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