



RESEARCH PAPER

Increases in DNA fragmentation and induction of a senescence-specific nuclease are delayed during corolla senescence in ethylene-insensitive (*etr1-1*) transgenic petunias

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Abstract

The programmed senescence of flower petals has been shown to involve the fragmentation of nuclear DNA. Nuclear DNA fragmentation, as determined by the TUNEL assay, was detected in *Petunia* × *hybrida* corollas during both pollination-induced and age-related senescence. DNA fragmentation was detected late in the lifespan of the flower when corollas were wilting and producing ethylene. The induction of a 43 kDa nuclease (PhNUC1) correlated with increased DNA fragmentation. PhNUC1 is a glycoprotein with activity against DNA and RNA and a pH optimum of 7.5. EDTA was found to inhibit PhNUC1 activity, but the addition of Co^{2+} restored activity in the presence of the chelating agent. When total protein extracts from senescing petals were fractionated by differential centrifugation, PhNUC1 activity was detected in the nuclear but not the cytoplasmic fraction. Activity of PhNUC1 was induced in non-senescing corollas by treatment with ethylene. Delayed increases in PhNUC1 activity observed in ethylene-insensitive flowers (35S:*etr1-1*) suggest that ethylene modulates the timing of PhNUC1 induction, but that it is not an absolute requirement for its activation.

Key words: Cobalt, ethylene, flowers, petal senescence, pollination.

Introduction

Senescence in plants has been defined as a form of programmed cell death (PCD) (Rubinstein, 2000). Flowers provide a good system for studying PCD in plants because they have a short, well-defined life span and morphological changes in the petals can easily be associated with biochemical changes. During flower senescence, developmental cues, hormonal signals, and environmental stimuli result in the up-regulation of genes encoding hydrolytic enzymes involved in the breakdown and relocation of cellular constituents (Rubinstein, 2000; Jones, 2004). This programmed degradation allows the plant to recycle nutrients from transient tissues like petals and styles to sink tissues like fruits and seeds.

The purpose of large-scale nucleic acid catabolism during senescence is to degrade DNA and RNA and to remobilize their constituents, primarily phosphorus, to sink tissues (Thomas *et al.*, 2003). During this hydrolysis process, nuclear DNA becomes fragmented. DNA fragmentation during the senescence of leaves and petals has been detected *in situ* using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) method and by visualizing the presence of the resulting 160 bp internucleosomal fragments (DNA ladders) on agarose gels (Yen and Yang, 1998; Panavas *et al.*, 2000; Xu and Hanson, 2000; Serafini-Fracassini *et al.*, 2002; Wagstaff *et al.*, 2003).

Nucleic acid catabolism during senescence involves the action of RNases, DNases, and nucleases (Sugiyama *et al.*,

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Abbreviations: ConA, Concanavalin A; daa, days after anthesis; dsDNA, double-stranded DNA; hap, hours after pollination; PhNUC1 nuclease, *Petunia* × *hybrida* nuclease; PCD, programmed cell death; SAG, senescence-associated gene; ssDNA, single-stranded DNA; WT, wild type.

2000). The hydrolysis of genomic DNA must be catalysed by endonucleases capable of digesting both single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA). Plant endonucleases with activity against DNA have been classified as either Zn²⁺-dependent or Ca²⁺-dependent based on their divalent cation requirements (Sugiyama *et al.*, 2000). Nuclease activity has been shown to increase during the senescence of barley (Wood *et al.*, 1998), wheat (Blank and McKeon, 1989), rice (Hosseini and Mulligan, 2002), parsley (Canetti *et al.*, 2002), *Arabidopsis* (Perez-Amador *et al.*, 2000), and tomato (Lers *et al.*, 2001) leaves. Senescence-associated endonucleases include members of both the Zn²⁺-dependent and the Ca²⁺-dependent classes. In addition, senescence-specific endonucleases that are dependent on Co²⁺ have been identified from tomato and parsley leaves (Lers *et al.*, 2001; Canetti *et al.*, 2002). Nucleases that were induced during petal senescence concomitant with increases in DNA fragmentation have also been reported in daylily and *Petunia inflata* corollas (Panavas *et al.*, 2000; Xu and Hanson, 2000).

Increasing evidence suggests that the activation of endonucleases and subsequent digestion of nuclear DNA may be modulated by hormones. The activity of senescence-specific nucleases from tomato and parsley was enhanced by treatment with ethylene (Lers *et al.*, 2001; Canetti *et al.*, 2002). DNA fragmentation was also accelerated in pea carpels that had been treated with ethylene, while inhibitors of ethylene action delayed this degradation (Orzaez and Granell, 1997). In ethylene-insensitive flowers like daylily, abscisic acid applications caused premature senescence and correspondingly earlier increases in DNA fragmentation and nuclease activity (Panavas *et al.*, 1998, 2000). Accumulation of mRNA from a putative S1-type nuclease (DSA6) was up-regulated by treatment of daylily petals with ABA (Panavas *et al.*, 1999), and nuclease transcripts from tomato and barley were also up-regulated by ABA treatment of leaves (Lers *et al.*, 1998; Muramoto *et al.*, 1999).

While senescence-promoting ethylene treatments have been shown to result in accelerated up-regulation of senescence-associated genes (SAGs) and activities, these changes may be a primary response to ethylene itself or a primary response to senescence induced by these treatments (Weaver *et al.*, 1998). In ethylene-insensitive *Arabidopsis* plants (*etr1-1* mutants), delayed leaf senescence coincided with delayed induction of SAGs (Grbic and Bleecker, 1995). These studies indicated that ethylene was not required for senescence to occur, but was merely regulating the timing of SAG expression and the onset of the senescence process in leaves.

In petunia, as observed in many ethylene-sensitive flowers, pollination accelerates ethylene production and corolla wilting. The hydrolysis of macromolecules during flower senescence allows for remobilization of nutrients from petals to the developing ovary and seeds following compatible pollination. Therefore, it may not be energetically advanta-

geous for flowers to remobilize constituents of the petals to developing leaves or other sink tissues, when an unpollinated flower senesces. The first objective of this research was to determine if nucleic acid catabolism is a component of the senescence programme in petals from pollinated and unpollinated flowers. The second objective was to investigate the role of ethylene in regulating senescence-associated nuclease activity and DNA fragmentation.

In 2000, Xu and Hanson reported that the advanced stages of petal senescence in *Petunia inflata* were associated with DNA laddering and increased nuclease activity (Xu and Hanson, 2000). Specifically, five nucleases with activity against ssDNA were detected in total protein extracts from *P. inflata* petals using in-gel activity assays, and activities of all five nucleases increased during the senescence of pollinated flowers. Preliminary experiments in *Petunia*×*hybrida* have identified seven nucleases using similar in-gel activity assays. The characterization of a single senescence-specific nuclease, referred to as PhNUC1, is described in this report. This paper presents the first investigation of ethylene's regulation of DNA fragmentation and nuclease activity using ethylene-insensitive transgenic plants (35S:*etr1-1*) with delayed senescence. It is also the first report of a senescence-specific nuclease that is enhanced by cobalt during the senescence of flower petals, and provides evidence for commonality among the senescence programmes of leaves and petals.

Materials and methods

Plant materials

Petunia×*hybrida* 'Mitchell' plants transformed with 35S:*etr1-1* (line Z00-35-10) were obtained from Dr David Clark (University of Florida). These plants are insensitive to ethylene and have a delayed flower senescence phenotype (Wilkinson *et al.*, 1997; Gubrium *et al.*, 2000). Experiments also utilized non-transformed wild-type (WT) *Petunia*×*hybrida* 'Mitchell'. Seeds were treated with 100 mg 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 glasshouse after seed germination, and were moved to 10 cm pots after 4 weeks. Plants were fertilized once a week with N at 300 mg l⁻¹ from 15N-5P-15K Cal Mag (Peters soluble fertilizer, The Scotts Co., Marysville, OH). Tap water was used for all other irrigations.

Flowers were emasculated 1 d before flower opening (anthesis) to prevent self-pollination. Flowers were pollinated at anthesis by brushing pollen from freshly dehisced anthers onto the stigma. Alternatively, flowers were emasculated and left unpollinated to senesce naturally. Corollas were collected from WT flowers at various times after pollination for the determination of ethylene production, DNA fragmentation, or nuclease activity (see below). Zero hours after pollination (hap) represents unpollinated flowers at anthesis. Corollas were also collected from unpollinated WT and *etr1-1* flowers at various times after anthesis.

Measurement of ethylene production and ethylene treatment

Ethylene production by WT and *etr1-1* corollas was determined by sealing corollas in 22 ml vials (two corollas per vial) with septa in the lids. After a 30 min incubation period, a 1 ml sample of the headspace in the vials was removed for analysis using a Varian CP-3800 gas

chromatograph equipped with an FID and HaysepR packed column (Varian, Walnut Creek, CA). A total of 12 flowers were collected at each time point, and sample collection and ethylene measurements were conducted twice. Graphed values represent the mean ethylene production ($\text{nl C}_2\text{H}_4 \text{ g}^{-1} \text{ FW h}^{-1}$) \pm standard error (SE).

To treat flowers with ethylene, WT flowers were harvested from the plant at anthesis and placed in test tubes of distilled water. Six flowers were placed in a 24 l chamber into which ethylene was injected to yield a final concentration of $2.0 \mu\text{l l}^{-1}$. Flowers were treated for 0, 12, 24, or 36 h. Control flowers were placed in a chamber of air for 36 h.

DNA fragmentation (TUNEL assay)

The fragmentation of genomic DNA leads to an increase in the number of DNA molecules with 3'-hydroxyl termini. The TUNEL assay, which incorporates fluorescein-labelled dUMP at 3'-hydroxyl termini using terminal deoxynucleotidyl transferase, can then be used to detect DNA fragmentation *in situ*.

Fresh tissue pieces, collected from the distal margins of the corolla, were fixed in FAA (85% ethanol, 5% glacial acetic acid, and 10% formaldehyde) at room temperature for approximately 12 h. This tissue was dehydrated through a series of ethanol/xylene washes and embedded in paraffin. Ten-micrometre sections were mounted on poly-L-lysine coated slides (Sigma, St Louis, MO), deparaffinized with xylene, and rehydrated through a graded series of ethanol washes. Corolla sections were digested with $20 \mu\text{g ml}^{-1}$ proteinase K for 15 min and rinsed twice with $1 \times$ PBS. The tissue was then labelled with fluorescein for the detection of DNA fragmentation as per the manufacturer's instructions (Apoptosis Detection System, Promega, WI). Co-staining with propidium iodide (Sigma, St Louis, MO) was used to visualize all nuclei. Positive controls were treated with $2 \mu\text{g ml}^{-1}$ DNase I (Sigma) for 10 min at room temperature. Samples that were treated with a reaction mix without terminal deoxynucleotidyl transferase were used as negative controls. Corollas were collected from WT flowers at 0, 24, 48, and 72 hap, unpollinated WT flowers at 0, 4, and 8 daa and *etr1-1* flowers at 0, 4, 8, 12, and 16 daa. At least three different corollas for each time point were observed, and the experiment was conducted twice. Slides were examined with a Leica TCS Confocal Microscope (Leica Microsystems, Wetzlar, Germany). The total number of nuclei staining with propidium iodide and the nuclei staining with fluorescein were counted, and the percentage of nuclei with fragmented DNA was calculated. This represented approximately 350–400 nuclei, and the data presented are the average percentage of fragmented nuclei \pm SE.

Nuclease activity assays

WT corollas were collected at various times after pollination and unpollinated WT and *etr1-1* corollas were collected at various times after anthesis. Only the upper part of the corollas, which showed the first visual symptoms of senescence, was used in these assays. Corolla tops were frozen in liquid N_2 and stored at -80°C until they were used for total protein extraction.

Corolla tops were powdered in liquid N_2 in a mortar and pestle and then transferred to a 30 ml centrifuge tube. Tissue was extracted by vortexing in 0.5 ml of homogenization buffer [50 mM TRIS-HCl (pH 7.6), 2 mM DTT] per corolla top. Samples were centrifuged at 1000 g for 15 min at 4°C and the supernatant was stored in 1 ml aliquots at -80°C . Extractions included at least four corolla tops per time point. Sample collection, extraction, and activity gels were replicated at least three times.

Nuclease gel activity assays were performed as described by Blank and McKeon (1991) with some modifications. SDS-PAGE was performed using a 15% (w/v) resolving gel that contained $100 \mu\text{g ml}^{-1}$ BSA. To identify DNase activity, gels contained either $15 \mu\text{g ml}^{-1}$ double-stranded salmon sperm DNA (Stratagene, La Jolla, CA) or DNA that had been made single stranded by boiling for 3 min. To

identify RNase activity, gels contained $40 \mu\text{g ml}^{-1}$ petunia petal total RNA. Samples were equalized by loading $20 \mu\text{l}$ of total protein extract (equal volume per corolla) to correct for the large decreases in total protein that accompany corolla senescence. Gels equalized based on total protein ($10 \mu\text{g}$ of total protein) were used for comparison. Total protein was determined using the Bradford method (Bio-Rad Protein Assay Kit, Hercules, CA). Running buffer and sample loading buffer were prepared as recommended by the manufacturer (Criterion Gel Application Guide, Bio-Rad, Hercules, CA). Gels were run at 120 V for 2 h at 25°C . After electrophoresis, nucleases were renatured by incubating gels in renaturation buffer [0.1 M TRIS-HCl (pH 7.4), 1% Triton X-100] at 37°C with gentle shaking for 1 h. Gels were rinsed twice in 0.1 M TRIS-HCl (pH 7.4) and incubated in development buffer [50 mM TRIS-HCl (pH 7.5), 20 mM NaCl] overnight at 37°C . To visualize bands of nuclease activity, gels were stained for 1 h at room temperature in 50 mM TRIS (pH 7.0) containing $0.5 \mu\text{g ml}^{-1}$ ethidium bromide. Once conditions were optimized for PhNUC1 activity, all gels were incubated in development buffer that was supplemented with $100 \mu\text{M}$ CoCl_2 .

Biochemical characterization of PhNUC1 activity was conducted using total protein from WT corollas at 48 hap using ssDNA substrate activity gels. Multiple samples were loaded and the gels were sliced after electrophoresis so that different development conditions could be investigated. To investigate pH effects on PhNUC1 activity, individual gel slices were incubated in TRIS-HCl buffers at various pH values. Final pH values were confirmed following incubation. All subsequent experiments utilized the pH determined to be optimal for PhNUC1 activity. To determine divalent cation requirements for PhNUC1 activity, the development buffer included either $100 \mu\text{M}$ CoCl_2 , ZnCl_2 , MnCl_2 , CuCl_2 , MgCl_2 , or CaCl_2 . Further investigation of divalent cation effects on PhNUC1 activity utilized various concentrations of CaCl_2 , MnCl_2 , CoCl_2 , and ZnCl_2 in the presence or absence of the chelating agent EDTA ($50 \mu\text{M}$).

To determine if PhNUC1 is a glycoprotein, the ability to interact with the carbohydrate-binding protein, Concanavalin A (ConA), was evaluated as described by Thelen and Northcote (1989). A $10 \mu\text{g}$ sample of total protein in 50 mM TRIS-HCl (pH 7.5) was incubated with $12 \mu\text{g}$ ConA (Calbiochem, La Jolla, CA) for 10 min at 25°C . Competition assays with a lectin inhibitory sugar were conducted concurrently by adding α -methyl-D-mannoside (Calbiochem) to two independent samples to a final concentration of 0.2 M and 0.4 M. Prior to electrophoresis, all samples were incubated at 37°C for 15 min.

The subcellular localization of PhNUC1 activity was determined following cellular fractionation. Two grams of corolla tops from WT flowers at 48 hap were ground in ice-cold extraction buffer [50 mM TRIS-HCl (pH 7.5), 0.3 M sucrose, 15 mM KCl, 5.0 mM MgCl_2 , 0.1 mM EDTA, 1.0 mM DTT, 0.2 mM PMSF, $1.0 \mu\text{g ml}^{-1}$ pepstatin, $1.0 \mu\text{g ml}^{-1}$ leupeptin). Extracts were filtered through one layer of miracloth and centrifuged at 4300 g for 10 min at 4°C . The pellet, which contained mainly nuclei was resuspended in protein isolation buffer [10 mM TRIS-HCl (pH 7.5), 1.0 mM DTT, 0.4 M NaCl (pH 7.4)]. This fraction is referred to as the nuclear fraction. The supernatant, now devoid of nuclei, was then centrifuged at 10 000 g for 10 min at 4°C . The resulting supernatant represents the cytosolic fraction. Ten micrograms of protein from the nuclear and cytosolic fractions were run on ssDNA substrate gels using optimized conditions, as determined in previous experiments.

Results

Ethylene production and flower senescence in ethylene-sensitive and -insensitive petunias

Wild-type petunia flowers that were emasculated and left to age naturally on the plant exhibited visual symptoms of

senescence (petal wilting) at 7–8 daa (Fig. 1A). In comparison, the senescence of ethylene-insensitive, *etr1-1* flowers was delayed and they did not begin to show visual symptoms of senescence until 12–16 daa. At 12 daa, *etr1-1* flowers began to show slight inrolling around the corolla margins, and by 16 daa the corollas were completely wilted. Under the present experimental conditions, this represented an 8 d delay in flower senescence compared with WT. While *etr1-1* flowers always lasted longer than WT flowers, their longe-

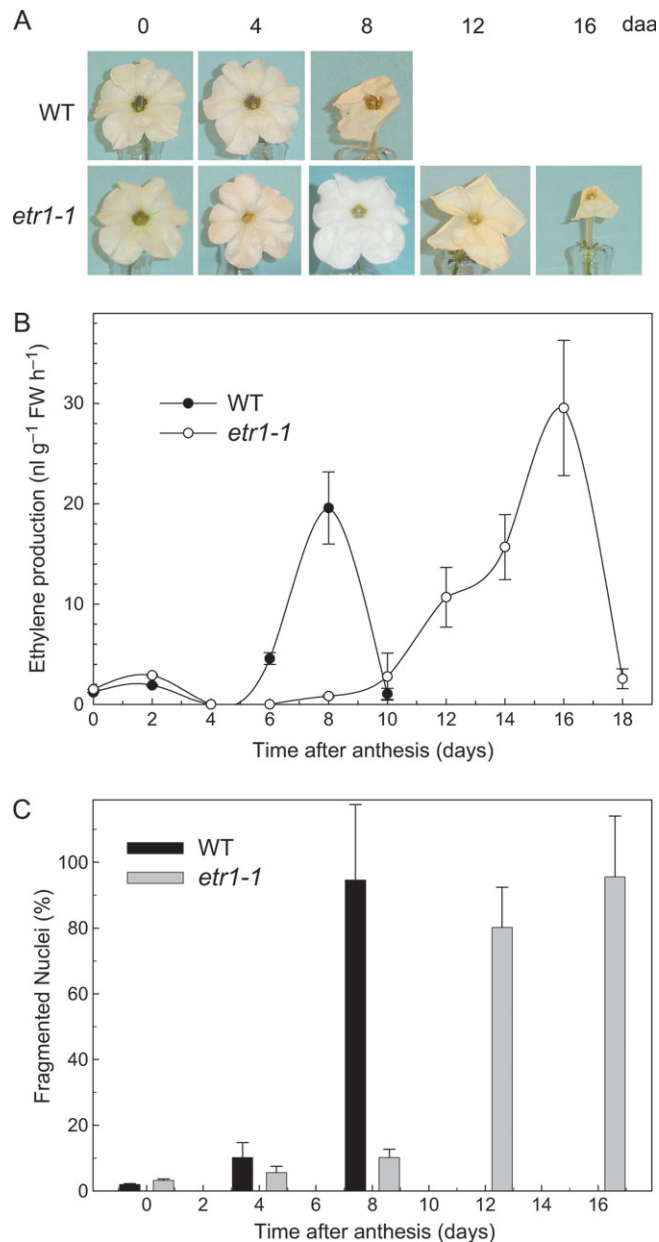


Fig. 1. Natural senescence of unpollinated wild-type (WT) *Petunia* × *hybrida* ‘Mitchell’ flowers and *P. hybrida* transformed with 35S:*etr1-1* (A). Ethylene production (B) and nuclear DNA fragmentation (C) in corollas at various times after anthesis. *In situ* labelling of fragmented DNA was determined by the TUNEL assay. Values represent the average ±SE.

vity was dependent on growing conditions, especially temperatures. A temperature effect on *etr1-1* flower longevity has previously been reported by Gubrium *et al.* (2000).

The senescence of WT flowers was accompanied by a peak of ethylene production that corresponded with petal wilting (Fig. 1B). Elevated ethylene production was detected from *etr1-1* petals at 12–16 daa. These time points also corresponded with the visual symptoms of petal senescence. The maximum levels of ethylene produced during flower senescence were slightly higher in *etr1-1* than WT corollas.

Pollination accelerated the senescence of WT flowers, and corolla wilting was observed at 48 hap (Fig. 2A). Maximum ethylene production coincided with corolla wilting (Fig. 2B). Pollination did not accelerate the senescence of *etr1-1* flowers, and corollas did not wilt until physically

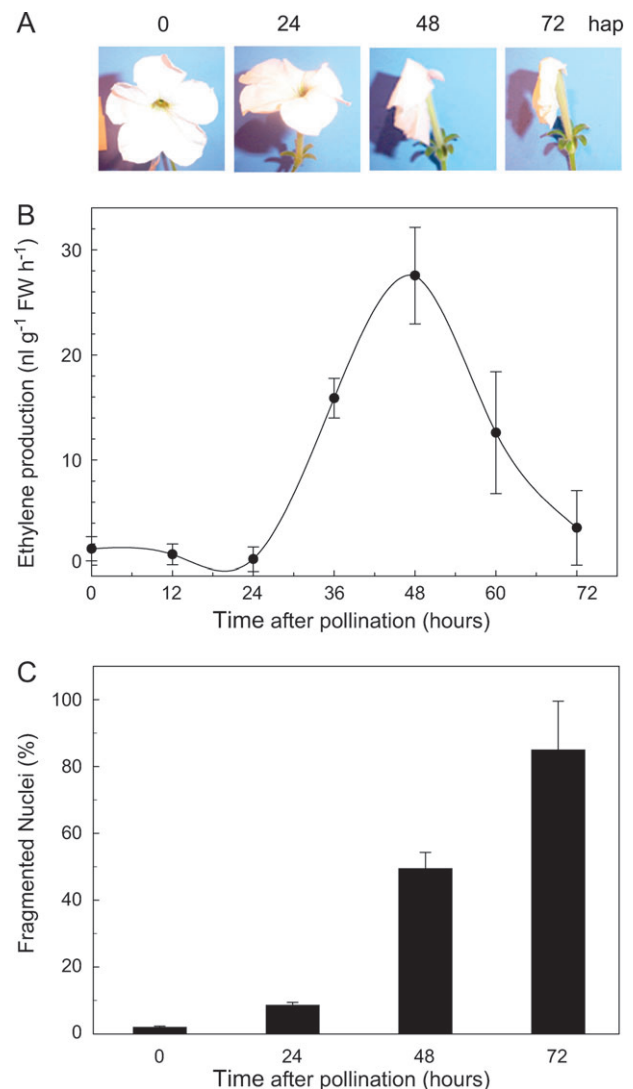


Fig. 2. Pollination-induced senescence of wild-type (WT) *Petunia* × *hybrida* ‘Mitchell’ flowers (A). Ethylene production (B) and nuclear DNA fragmentation (C) in corollas at various times after pollination. *In situ* labelling of fragmented DNA was determined by the TUNEL assay. Values represent the average ±SE.

abscised from the flower by the growing ovary (data not shown). In light of this observation, it was determined that the best comparison between WT and *etr1-1* flowers would be obtained from unpollinated, naturally senescing flowers and pollinated *etr1-1* flowers were not included in this study. A detailed analysis of the post-pollination response in *etr1-1* flowers is underway.

DNA fragmentation is associated with petal wilting in ethylene-sensitive and -insensitive petunias

Increased DNA fragmentation was associated with the advanced stages of corolla senescence during natural ageing and following pollination. At anthesis (0 hap and 0 daa), <5% of the nuclei in WT petals had fragmented DNA as determined by the TUNEL assay (Figs 1C, 2C). DNA fragmentation increased only slightly at 4 daa, but by 8 daa when corollas were completely wilted, DNA fragmentation was observed in >90% of the nuclei. Increases in DNA fragmentation were delayed in unpollinated *etr1-1* flowers until 12–16 daa, but as in WT flowers, maximum DNA fragmentation was associated with corolla wilting and ethylene production. Following pollination, DNA fragmentation in WT corollas increased slightly at 24 hap, and was nearly 50% by 48 hap. At 72 hap, when corollas were completely wilted, 85% of the nuclei had fragmented DNA.

Senescence-specific PhNUC1 has activity against both DNA and RNA

Preliminary experiments in *Petunia*×*hybrida* identified bands on in-gel activity assays corresponding to seven nucleases (data not shown). Five of these bands were similar in size to those previously reported in *Petunia inflata* (Xu and Hanson, 2000). When β -mercaptoethanol was included in the extraction buffer, only five nucleases could be detected (data not shown). β -Mercaptoethanol has been reported previously to inhibit nuclease activities (Lers *et al.*, 2001). While multiple nucleases were reported to increase their activities during the progression of senescence in *P. inflata*, one nuclease was shown to have senescence-specific activity (D1; Xu and Hanson, 2000). A similar senescence-specific nuclease (PhNUC1, *Petunia*×*hybrida* nuclease), which has a molecular mass of about 43 kDa was identified in preliminary studies, and it was decided to focus on the regulation of this nuclease and investigate its role in nucleic acid catabolism during petal senescence. The conditions used for in-gel activity assays examining temporal changes in nuclease activity were those determined to be optimal for PhNUC1 activity.

PhNUC1 activity was only detected in corollas at advanced stages of senescence when visual symptoms of corolla wilting were apparent (Figs 3, 4). During the natural ageing of unpollinated WT corollas, PhNUC1 activity was first detected at 8 daa (Fig. 3). The induction of PhNUC1 was accelerated by pollination, and activity was detected at 48–72 hap when the corollas were wilted (Fig. 4). Lower

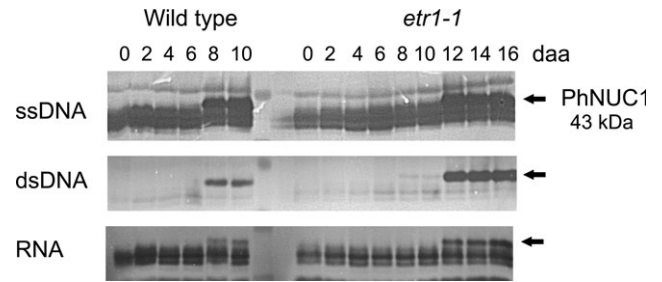


Fig. 3. Changes in PhNUC1 activity during the senescence of unpollinated WT and *etr1-1* corollas. Substrate specificity of PhNUC1 was determined using SDS-PAGE nuclease activity gels containing ssDNA, dsDNA, or RNA. Total proteins were extracted from corollas at various times after anthesis as indicated (daa, days after anthesis). Sample loading was equalized per corolla by loading 20 μ l of extract per lane. Proteins were resolved on 15% SDS-PAGE. Following renaturation and washing as described in the Materials and methods, the resolving gel was developed in 50 mM TRIS-HCl buffer (pH 7.5) containing 100 μ M CoCl_2 .

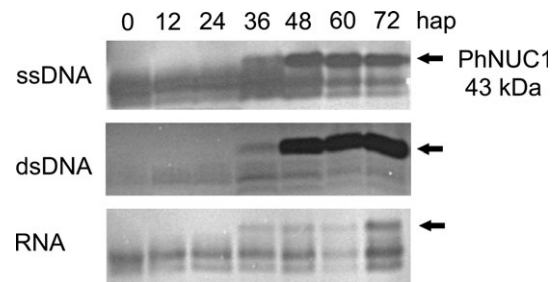


Fig. 4. Changes in PhNUC1 activity during the senescence of pollinated WT corollas. Substrate specificity of PhNUC1 was determined using SDS-PAGE nuclease activity gels containing ssDNA, dsDNA, or RNA as described in Fig. 3. Total proteins were extracted from corollas at various times after pollination as indicated (hap, hours after pollination).

levels of activity could also be detected at 36 hap when some flowers were showing early symptoms of wilting at the corolla margins. PhNUC1 activity increased slightly from 48 to 72 hap. This increase was most apparent on RNA substrate gels. To examine the temporal changes in PhNUC1 activity during senescence, corolla samples for SDS-PAGE were equalized by loading 20 μ l of total protein extract (equal volume per corolla) to correct for the large decrease in total protein that accompanies corolla senescence. Activity gels loaded on the basis of total protein (10 μ g) were also run for comparison, and the pattern of PhNUC1 activity was similar (data not shown).

Catabolism of nucleic acids during senescence must involve the combined action of RNases and DNases or the activity of endonucleases that degrade both DNA and RNA. In addition, DNA fragmentation and subsequent hydrolysis must involve the degradation of both dsDNA and ssDNA. To determine substrate specificity of PhNUC1, ssDNA, dsDNA, or RNA was included in the SDS-PAGE activity gels (Figs 3, 4). PhNUC1 had activity against dsDNA, ssDNA, and RNA, but substrate preference could not be

determined from these experiments. Patterns of PhNUC1 induction were similar in pollinated and unpollinated flowers independent of the substrate.

The timing of PhNUC1 induction is modulated by ethylene

In unpollinated *etr1-1* flowers, induction of PhNUC1 activity was delayed compared with WT (Fig. 3). An activity band at 43 kDa was not detected until 12 daa. This corresponded with the detection of fragmented DNA in *etr1-1* corollas and with visual symptoms of senescence. When WT flowers were treated with $2.0 \mu\text{l l}^{-1}$ ethylene, low levels of PhNUC1 activity were detected at 12 h and activity increased by 24 h (Fig. 5). PhNUC1 activity at 36 h was similar to that detected at 24 h. While these flowers were not wilted when removed from the ethylene at 36 h, this treatment was sufficient to accelerate senescence and flowers wilted within the next 12–24 h. PhNUC1 was not detected in untreated flowers (0 h ethylene) or when control flowers were treated with air for 36 h.

PhNUC1 activity is enhanced by cobalt and inhibited by zinc

Divalent cation requirements of PhNUC1 were investigated by incubating ssDNA activity gel slices that included both non-senescing (0 hap) and senescing (48 hap) WT corolla extracts in TRIS-HCl (pH 7.5) development buffers containing various cations as indicated in Fig. 6. EDTA (1 mM) was included in the sample-loading buffer to chelate any cations present in the protein extract. Following multiple washes in 50 mM TRIS-HCl (pH 7.5) and incubation in development buffer [50 mM TRIS-HCl (pH 7.5), 20 mM NaCl] containing no supplemental cations, the induction of PhNUC1 could be detected in 48 hap samples (Fig. 6A). Low levels of PhNUC1 activity were also detected when 100 μM CaCl_2 , MgCl_2 , MnCl_2 , or ZnCl_2 were included. Only when CoCl_2 (100 μM) was added to the development buffer was PhNUC1 activity enhanced over levels detected when no supplemental cations were added. PhNUC1 activity was enhanced by incubating activity-gel slices in increasing concentrations of CoCl_2 (0 μM , 20 μM , 100 μM , and 200 μM) (Fig. 6B). Increasing the concentration of MnCl_2 in the development buffer resulted in only a slight increase in PhNUC1 activity, while increasing the concen-

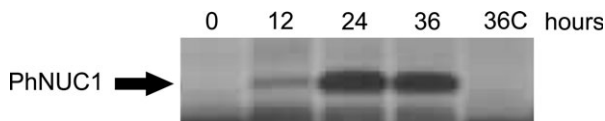


Fig. 5. Induction of PhNUC1 by ethylene. WT flowers at anthesis were treated with $2.0 \mu\text{l l}^{-1}$ ethylene for 0, 12, 24, or 36 h. Control flowers were treated with air for 36 h (36C). Total protein samples from WT corollas were extracted and resolved on SDS-PAGE containing ssDNA as described in Fig. 3.

tration of CaCl_2 did not increase activity. While PhNUC1 was detectable at low levels in ssDNA substrate gels not incubated in development buffer containing CoCl_2 , activity against dsDNA was not detectable in the absence of Co^{2+} (data not shown). PhNUC1 exhibited sensitivity to EDTA and was not detectable when 50 μM EDTA was added to the development buffer (Fig. 6B). Activity was restored by increasing concentrations of CoCl_2 (100 or 200 μM) and, to a lesser degree, MnCl_2 (100 or 200 μM) but not by CaCl_2 . The enhanced activity detected in the presence of CoCl_2 was inhibited by the addition of increasing concentrations of ZnCl_2 to the development buffer (Fig. 6C).

PhNUC1 has optimal activity around pH 7.5

The pH optimum of PhNUC1 was determined by incubating ssDNA activity gel slices that included senescing (48 hap) WT corolla extracts in TRIS-HCl buffers of varying pH values as indicated in Fig. 7. PhNUC1 activity was detected at a wide range of pH values, but maximum activity was detected at pH 7.5–8.0.

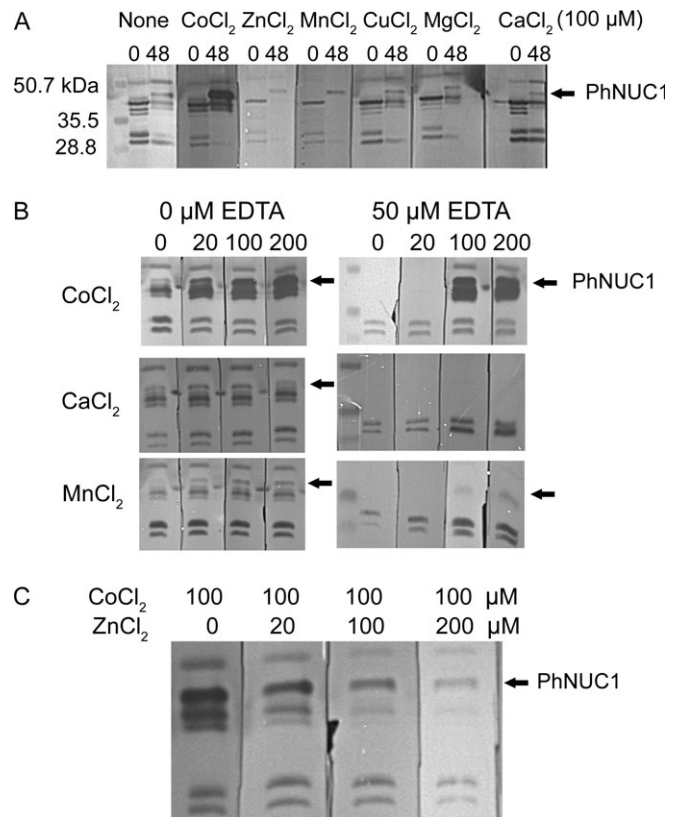


Fig. 6. Effect of bivalent cations on PhNUC1 activity. Total protein samples from WT corollas were extracted and resolved on SDS-PAGE containing ssDNA. Following renaturation and washing as described in the Materials and methods, the gel was sliced and individual strips were incubated in 50 mM TRIS-HCl (pH 7.5) development buffer containing various ion combinations as indicated. All concentrations are in micromolar units. Each slice contained proteins from non-senescing (0 hap) and senescing (48 hap) WT corollas (A) or just 48 hap corollas (B) and (C).

PhNUC1 is a glycoprotein

Incubation of the carbohydrate-binding protein, ConA, with the total protein extracts from senescing petals (WT 48 hap) prior to electrophoresis resulted in an inhibition of PhNUC1 activity (Fig. 8). This inhibition could be overcome by adding α -methyl-D-mannoside (0.2 or 0.4 M final concentration), a lectin inhibitory sugar that competes with glycoproteins for the binding of ConA.

PhNUC1 activity is detected in the nuclear fraction of corolla protein extracts

During senescence, the integrity of the nucleus is maintained until very late in the senescence process and DNA degradation takes place in the nucleus as shown by *in situ* labelling of fragmented DNA within the petunia corolla nuclei. Following differential centrifugation, nuclear and cytosolic extracts from senescing petunia corollas (WT 48 hap) were run on ssDNA-activity gels (Fig. 9). PhNUC1 activity was not detected in the cytosolic fraction but was detected in the nuclear-enriched fraction.

Discussion

The programmed senescence of petunia flowers was associated with nucleic acid catabolism as evidenced by increased DNA fragmentation and induction of nuclease activity within senescing petals. Activity of the senescence-specific nuclease, PhNUC1, was only detected at the later stages of senescence when petals were visually wilted. Increased DNA fragmentation within the nuclei of petunia

petals was also detected late in the senescence process and corresponded with the induction of PhNUC1 activity. The neutral pH optimum and activity within the nuclear protein fraction of senescing petals indicate that PhNUC1 may be localized within the nucleus. Activity against both DNA and RNA suggests that PhNUC1 may not be specific to DNA degradation, but may play a more general role in nucleic acid catabolism during senescence. Senescence is an active process that requires transcription, therefore the activation of nucleases and subsequent degradation of nuclear DNA would be expected to occur during the later, irreversible stages of PCD.

A number of nucleases associated with PCD in plants have been identified (Sugiyama *et al.*, 2000). These endonucleases have been classified as either Zn^{2+} -dependent or Ca^{2+} -dependent, based on divalent cation requirements, pH optima, and substrate specificity (Sugiyama *et al.*, 2000). Zn^{2+} -dependent endonucleases are sensitive to EDTA and their activity is dependent on Zn^{2+} . Their pH optimum is in the acidic range and they prefer RNA and ssDNA to dsDNA. Zn^{2+} -dependent endonucleases tend to be monomeric glycoproteins in the 33–44 kDa range, and most plant nucleases characterized to date belong to this class. Ca^{2+} -dependent nucleases, as the name suggests, are dependent on Ca^{2+} and in some cases may be inhibited by Zn^{2+} . Their pH optimum tends to be in the neutral region and their substrate preference is ssDNA over RNA (Sugiyama *et al.*, 2000).

Both classes of endonucleases have been identified in senescing leaves (Blank and McKeon, 1989; Wood *et al.*, 1998), and it has been proposed that the different endonucleases play different roles in the catabolism of nucleic acids during senescence (Sugiyama *et al.*, 2000). The initial fragmentation of nuclear DNA is thought to be catalysed by Ca^{2+} -dependent nucleases, which have maximal activity at neutral pH. Following the rupture of membranes and acidification of the cell, further degradation of nucleic acids would then be carried out by vacuolar- or apoplasmic-localized Zn^{2+} -dependent nucleases. PhNUC1 shares characteristics in common with both classes of nucleases, but in

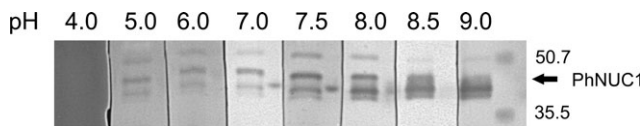


Fig. 7. Effect of pH on PhNUC1 activity. Protein samples from 48 hap WT corollas were extracted and resolved on SDS-PAGE containing ssDNA as described in Fig. 3.

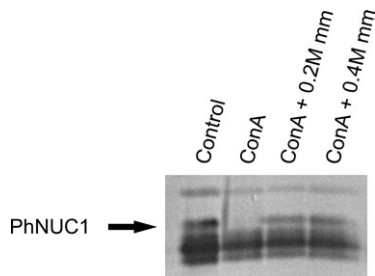


Fig. 8. Interaction of PhNUC1 with the carbohydrate-binding protein Concanavalin A (ConA). Total protein samples were extracted from senescing WT corollas (48 hap). Total protein extract only (control), protein+ConA (ConA), protein+ConA+0.2 M α -methyl-D-mannoside (ConA+0.2 M mm), and protein+ConA+0.4 M α -methyl-D-mannoside (ConA+0.4 M mm) were resolved on SDS-PAGE containing ssDNA as described in Fig. 3.

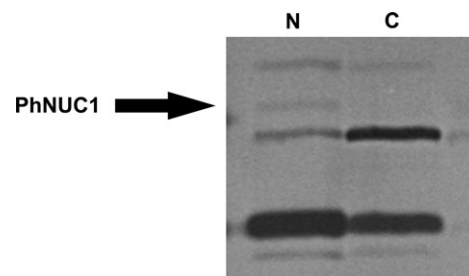


Fig. 9. Subcellular localization of PhNUC1. Nuclear (N) and cytosolic (C) cellular fractions were obtained from senescing WT corollas (48 hap) following differential centrifugation as outlined in the Materials and methods. Protein (10 μ g) was resolved on SDS-PAGE containing ssDNA. Following renaturation and washing, the resolving gel was developed in 50 mM TRIS-HCl buffer (pH 7.5) containing 100 μ M $CoCl_2$.

light of its potential nuclear localization, neutral pH optimum, and inhibition by zinc, it shows more similarity to the Ca^{2+} -dependent nucleases characterized to date.

Despite its similarities to the Ca^{2+} -dependent nucleases, PhNUC1 activity is dependent on Co^{2+} rather than Ca^{2+} . Other Co^{2+} -dependent nucleases have recently been reported in parsley and tomato (Lers *et al.*, 2001; Canetti *et al.*, 2002). LeNUC1 from tomato and PcNUC1 and PcNUC2 from parsley are similar in size to PhNUC1 with estimated sizes of 41 kDa, 43 kDa, and 40 kDa, respectively. Similar to PhNUC1, their activity is senescence-specific and is not detected in non-senescent tissues. PcNUC1 and PcNUC2 are associated with dark-induced and natural leaf senescence (Canetti *et al.*, 2002). LeNUC1 activity was also detected in naturally senescing leaves, but was not detected in ripening fruit (Lers *et al.*, 2001). Activity of these senescence-specific nucleases was not investigated in petals. All of the Co^{2+} -dependent nucleases, including PhNUC1, are glycoproteins that can degrade both RNA and DNA, are inhibited by Zn^{2+} , and have pH optima in the neutral to slightly basic range (Lers *et al.*, 2001; Canetti *et al.*, 2002; this paper). The induction of Co^{2+} -requiring nucleases late in the senescence process indicates that large-scale nucleic acid catabolism is a component of both leaf and petal senescence, and provides evidence for some commonality among the senescence programmes of different tissues.

Ethylene treatment of petunia flowers at anthesis resulted in premature senescence and induction of PhNUC1 activity. Similar ethylene induction was detected for the senescence-specific nucleases identified in tomato and parsley (Lers *et al.*, 2001; Canetti *et al.*, 2002). Following exogenous treatment with ethylene, it is difficult to determine if the observed effects are the direct result of the ethylene signal or a secondary response to ethylene (i.e. a primary response to senescence). To investigate ethylene's role in nucleic acid catabolism during petunia corolla senescence, DNA fragmentation and PhNUC1 activity were compared between WT (ethylene-sensitive) and ethylene-insensitive, *etr1-1* petunias. Senescence was delayed in *etr1-1* flowers, and this delay in corolla wilting coincided with delayed ethylene production. Nucleic acid catabolism during senescence was independent of ethylene perception, and only the timing of PhNUC1 induction and subsequent DNA fragmentation were affected in *etr1-1* flowers. Similar delays in petal senescence, ethylene production, and the induction of SAGs have been reported in flowers treated with chemical inhibitors of ethylene biosynthesis and action (Lawton *et al.*, 1990; Woodson *et al.*, 1992; Burdon and Sexton, 1993; Jones *et al.*, 1995; Borochoy *et al.*, 1997). Delayed accumulation of SAGs in the leaves of the ethylene-insensitive *Arabidopsis* mutant *etr1-1* led to the conclusion that ethylene regulated the timing of senescence, but was not required for execution of the senescence programme once it had begun (Grbic and Bleeker, 1995). The

accelerated induction of PhNUC1 by ethylene and its delayed induction in *etr1-1* petunia flowers supports the role of ethylene as a modulator of senescence timing in petals.

Pollination induces corolla senescence in many ethylene-sensitive flowers (Stead, 1992; van Doorn, 1997). This is often described as an acceleration of the natural senescence process in unpollinated flowers, although few studies have directly compared the senescence programmes. The accelerated degradation of macromolecules within the corolla following compatible pollination allows for the removal of a metabolically costly tissue that has served its function in pollinator attraction, while facilitating the recycling of nutrients from the senescing petals to the developing ovary. When a flower is not pollinated, and its ovary is also senescing, it is reasonable to assume that the programme of petal senescence may not be identical to that of a pollinated flower. In this instance, it may not be as efficient for the plant to recycle petal nutrients to sink tissues outside of an individual flower. Despite these assumptions, the increases in DNA fragmentation and the induction of PhNUC1 during pollination-induced and natural senescence of WT petunia flowers suggests that nucleic acid catabolism in both of these senescence programmes is similar.

A nuclease referred to as D1, which is similar in size to PhNUC1, was previously identified from *Petunia inflata* corollas following pollination (Xu and Hanson, 2000). The induction of D1 was senescence-specific and corresponded with petal wilting as reported in the present study. When activity gels were developed in TRIS-HCl buffer containing CaCl_2 and MgCl_2 , D1 had activity against ssDNA but did not have any detectable activity against dsDNA. While PhNUC1 had activity against ssDNA and dsDNA, only activity against ssDNA could be detected in the absence of Co^{2+} (i.e. in buffer containing only CaCl_2 or MgCl_2). These limited comparisons suggest that PhNUC1 and D1 represent the same nuclease, and provide evidence supporting a common mechanism of nucleic acid catabolism during pollination-induced senescence of the self-compatible *Petunia* × *hybrida* and the self-incompatible *P. inflata*.

Nuclear DNA fragmentation and the induction of a nuclease with activity against ssDNA, dsDNA, and RNA correlated with endogenous ethylene production and corolla wilting in *Petunia* × *hybrida*. The Co^{2+} requirement of the senescence-specific nuclease, PhNUC1, suggests that a third group of endonucleases, in addition to the Ca^{2+} - and Zn^{2+} -dependent nucleases, catalyses nucleic acid degradation in plants. The pH optimum of 7.5, localization to nuclear enriched protein fractions, and timing of PhNUC1 induction relative to the occurrence of DNA fragmentation support a role for PhNUC1 in nuclear DNA catabolism during petal senescence. The delayed induction of PhNUC1 activity and DNA fragmentation in *etr1-1* petunias indicates that ethylene modulates the timing of nucleic acid catabolism during petal senescence.

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References

- Blank A, McKeon TA.** 1989. Single-strand-preferring nuclease activity in wheat leaves is increased in senescence and is negatively photoregulated. *Proceedings of the National Academy of Sciences, USA* **86**, 3169–3173.
- Blank A, McKeon TA.** 1991. Three RNases in senescent and non-senescent wheat leaves: characterization by activity staining in sodium dodecyl sulfate polyacrylamide gels. *Plant Physiology* **97**, 1402–1408.
- Borochov A, Spiegelstein H, Philosoph-Hadas S.** 1997. Ethylene and flower petal senescence: interrelationship with membrane lipid catabolism. *Physiologia Plantarum* **100**, 606–612.
- Burdon JN, Sexton R.** 1993. Ethylene co-ordinates petal abscission in red raspberry (*Rubus idaeus* L.) flowers. *Annals of Botany* **72**, 289–294.
- Canetti L, Lomaniec E, Elkind Y, Lers A.** 2002. Nuclease activities associated with dark-induced and natural leaf senescence in parsley. *Plant Science* **163**, 873–880.
- Grbic V, Bleecker AB.** 1995. Ethylene regulates the timing of leaf senescence in *Arabidopsis*. *The Plant Journal* **8**, 595–602.
- Gubrium EK, Clevenger DJ, Clark DG, Barrett JE, Nell TA.** 2000. Reproduction and horticultural performance of transgenic ethylene-insensitive petunias. *Journal of the American Society for Horticultural Science* **125**, 277–281.
- Hosseini R, Mulligan BJ.** 2002. Application of rice (*Oryza sativa* L.) suspension culture in studying senescence *in vitro*. I. Single strand preferring nuclease activity. *Electronic Journal of Biotechnology* **5**, 42–54.
- Jones ML.** 2004. Changes in gene expression during senescence. In: Nooden L, ed. *Plant cell death processes*. San Diego, CA: Elsevier Science, 51–71.
- Jones ML, Larsen PB, Woodson WR.** 1995. Ethylene-regulated expression of a carnation cysteine proteinase during flower petal senescence. *Plant Molecular Biology* **28**, 505–512.
- Lers A, Khalchitski A, Lomaniec E, Burd S, Green PJ.** 1998. Senescence-induced RNases in tomato. *Plant Molecular Biology* **36**, 439–449.
- Lers A, Lomaniec E, Burd S, Khalchitski A.** 2001. The characterization of LeNUC1, a nuclease associated with leaf senescence in tomato. *Physiologia Plantarum* **112**, 176–182.
- Lawton KA, Raghobama KG, Goldsbrough PB, Woodson WR.** 1990. Regulation of senescence-related gene expression in carnation flower petals by ethylene. *Plant Physiology* **93**, 1370–1375.
- Muramoto Y, Watanabe A, Nakamura T, Takabe T.** 1999. Enhanced expression of a nuclease gene in leaves of barley plants under salt stress. *Gene* **234**, 315–321.
- Orzaez D, Granell A.** 1997. DNA fragmentation is regulated by ethylene during carpel senescence in *Pisum sativum*. *The Plant Journal* **11**, 137–144.
- Panavas T, LeVangie R, Mistler J, Reid PD, Rubinstein B.** 2000. Activities of nucleases in senescing daylily petals. *Plant Physiology and Biochemistry* **38**, 837–843.
- Panavas T, Pikula A, Reid PD, Rubinstein B, Walker EL.** 1999. Identification of senescence-associated genes from daylily petals. *Plant Molecular Biology* **40**, 237–248.
- Panavas T, Walker EL, Rubinstein B.** 1998. Possible involvement of abscisic acid in senescence of daylily petals. *Journal of Experimental Botany* **49**, 1987–1997.
- Perez-Amador MA, Abler ML, De Rocher EJ, Thompson DM, van Hoof A, LeBrasseur ND, Lers A, Green PJ.** 2000. Identification of BFN1, a bifunctional nuclease induced during leaf and stem senescence in *Arabidopsis*. *Plant Physiology* **122**, 169–179.
- Rubinstein B.** 2000. Regulation of cell death in flower petals. *Plant Molecular Biology* **44**, 303–318.
- Serafini-Fracassini D, Del Duca S, Monti F, Poli F, Sacchetti G, Bregoli AM, Biondi S, Della Mea M.** 2002. Transglutaminase activity during senescence and programmed cell death in the corolla of tobacco (*Nicotiana tabacum*) flowers. *Cell Death and Differentiation* **9**, 309–321.
- Stead AD.** 1992. Pollination-induced flower senescence: a review. *Plant Growth Regulation* **11**, 13–20.
- Sugiyama M, Ito J, Aoyagi S, Fukuda H.** 2000. Endonucleases. *Plant Molecular Biology* **44**, 387–397.
- Thelen MP, Northcote DH.** 1989. Identification and purification of a nuclease from *Zinnia elegans* L.: a potential molecular marker for xylogenesis. *Planta* **179**, 181–195.
- Thomas H, Ougham HJ, Wagstaff C, Stead AD.** 2003. Defining senescence and death. *Journal of Experimental Botany* **54**, 1127–1132.
- van Doorn WG.** 1997. Effects of pollination on floral attraction and longevity. *Journal of Experimental Botany* **48**, 1615–1622.
- Wagstaff C, Malcolm P, Rafiq A, Leverentz M, Griffiths G, Thomas B, Stead A, Rogers H.** 2003. Programmed cell death (PCD) processes begin extremely early in *Alstroemeria* petal senescence. *New Phytologist* **160**, 49–59.
- Weaver LM, Gan S, Quirino B, Amasino RM.** 1998. A comparison of the expression patterns of several senescence-associated genes in response to stress and hormone treatment. *Plant Molecular Biology* **37**, 455–469.
- Wilkinson JQ, Lanahan MB, Clark DG, Bleecker AB, Chang C, Meyerowitz EM, Klee HJ.** 1997. A dominant mutant receptor from *Arabidopsis* confers ethylene insensitivity in heterologous plants. *Nature Biotechnology* **15**, 444–447.
- Wood M, Power JB, Davey MR, Lowe KC, Mulligan BJ.** 1998. Factors affecting single strand-preferring nuclease activity during leaf aging and dark-induced senescence in barley (*Hordeum vulgare* L.). *Plant Science* **131**, 149–159.
- Woodson WR, Park KY, Drory A, Larsen PB, Wang H.** 1992. Expression of ethylene biosynthetic-pathway transcripts in senescing carnation flowers. *Plant Physiology* **99**, 526–532.
- Xu Y, Hanson MR.** 2000. Programmed cell death during pollination-induced petal senescence in petunia. *Plant Physiology* **122**, 1323–1333.
- Yen C-H, Yang C-H.** 1998. Evidence for programmed cell death during leaf senescence in plants. *Plant and Cell Physiology* **39**, 922–927.