IDENTIFICATION AND CHARACTERIZATION OF SENESCENCE-ASSOCIATED PROTEINS IN PETUNIA COROLLAS

DISSERTATION

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By

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* * * * *

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ABSTRACT

Senescence is a degenerate process that leads to the death of plant cells, organs or whole plants. Senescence is not a passive process, but is an active developmental process regulated by plant age and other internal and external signals. The timing of natural senescence is not only controlled by developmental age, but also influenced by abiotic and biotic stimuli. The main purpose of leaf and petal senescence in plants is to remobilize and recycle nutrients from old and/or no longer necessary organs to developing parts of the plant, such as from senescing leaves to young leaves, flowers, fruits and seeds. The quality and subsequent value of both agricultural and horticultural crops is impacted by the senescence of vegetative and floral organs. Therefore, it is of practical importance to understand the molecular and biochemical mechanisms of senescence initiation, regulation and execution.

The goal of my project is to identify global protein changes that occur during petal senescence. To this end, I employed two-dimensional polyacrylamide gel electrophoresis (2-DE) based on proteomic approaches to identify protein changes during petunia corolla senescence. One hundred thirty three differentially expressed spots were selected to be sequenced by tandem mass spectrometry. The majority of up regulated
proteins were hydrolytic enzymes involved in macromolecular degradation and defense responses to abiotic and biotic stress. Protein sequencing data indicated that a number of proteins were post-translationally modified or processed during senescence. Our results not only support previous transcriptome studies, but also provide new insights into the post-translational regulation of senescence.

A senescence-specific nuclease (referred to as PhNUC2) from our 2-DE results was investigated. PhNUC2 transcripts were specifically induced in senescing flower tissues. We hypothesized that suppression of PhNUC2 activity would delay nucleic acid catabolism during senescence, and this would in turn delay petal senescence. Thus, virus-induced gene silencing (VIGS) was used to knock down PhNUC2 gene expression. PhNUC2 gene expression and PhNUC2 nuclease activity were significantly reduced in VIGS corollas during senescence. However, the flower longevity was not prolonged in VIGS experiments. The reasons are discussed.
Dedicated to my wife Ying, my son Enze and my parents
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CHAPTER 1

MOLECULAR EVENTS AND REGULATION OF PETAL SENESCENCE

OVERVIEW

What is senescence?

Senescence is a process that leads to the death of cells, organs or whole plants. Senescence is not a passive process, but is an active developmental process regulated by plant age and other internal and external signals (Buchanan-Wollaston, 1997; Hopkins et al., 2007; Lim et al., 2007). Although timing of natural senescence is controlled by developmental age, the senescence process is also influenced by other stimuli such as pollination, stresses and pathogen infections. The endogenous signals include age, developmental cues, reproductive development and phytohormones. The exogenous signals that influence senescence include various stresses such as light, temperature, drought, flooding, salt, ozone, nutrient deficiency, and pathogen and insect attacks. Hence, senescence is controlled by complex signaling networks. The regulation and execution of developmental senescence and stress-induced senescence share many
common pathways, but they also have distinct features (Buchanan-Wollaston et al., 2005; van der Graaff et al., 2006).

**Why does senescence happen?**

The main purpose of leaf and petal senescence in plants is to remobilize and recycle nutrients from dying parts to developing parts of the plant, such as from senescing leaves or petals to young leaves, flowers, fruits and seeds (Thomas et al., 2003). Flower longevity varies between species. Some flowers last for months, such as orchids, while some flowers last for a few hours, such as *Hibiscus trionum* (Stead, 1992). To avoid wasting energy to maintain flower attractiveness, flowers will die once they fulfill their role in reproduction. The death of petals facilitates nutrient remobilization and recycling, while reducing the risk of pathogen and insect attacks.

**PCD in plant senescence**

The hallmarks of plant PCD share some features of the process in animals, including condensation and shrinkage of the cytoplasm and nucleus, DNA laddering, caspase-like proteolytic activity, and cytochrome c release from mitochondria (Danon et al., 2000; Hoeberichts and Woltering, 2003). While the term senescence describes the degeneration process occurring at the tissue, organ or whole plant level, PCD is used when one discusses death at the cellular level. For convenience, the terms senescence and
PCD are used interchangeably. PCD occurs not only in leaf and flower senescence, but also in other plant developmental processes and defense and stress responses. Some examples include the degeneration of aleurone layer cells during seed germination, the formation of treachery elements, fruit ripening, the hypersensitive response (HR) in pathogen defense and other stress-induced cell death (Kuriyama and Fukuda, 2002). However, the PCD taking place during senescence is distinct from other types of PCD. It is a slower process, which allows cells to degrade macromolecules and remobilize the nutrients from dying cells to developing tissues or organs. Conversely, in the HR, cells quickly die to prevent the pathogen spreading from the affected sites following the pathogen infection (Lim et al., 2003).

**Ethylene affects on petal wilting and abscission**

Flower senescence symptoms vary from species to species. In most flowers, petals display clear symptoms before abscission, including discoloring and wilting. In other flowers, the petals abscise without exhibiting senescence symptoms (Stead, 1992; van Doorn, 2001). After testing hundreds of species, van Doorn (2001) classified petal senescence into four categories based on if petals wilted or abscised and if they responded to exogenous ethylene. These included ethylene-sensitive or ethylene-insensitive petal wilting, and ethylene-sensitive or ethylene- insensitive petal abscission. In dicotyledons, the petal wilting species were either ethylene-sensitive or ethylene-
insensitive, while the petal abscission species were all ethylene-sensitive. In most monocotyledons species, petals wilted before abscission, and were ethylene-insensitive. Only Alismataceae, Commelinaceae and a few species in Iridaceae such as Sisyrinchium angustifolium, S. californicum and S. laevigatum, had petals that wilted before abscission. These were in the ethylene-sensitive category. In Tulipa, all the tested cultivars were ethylene-insensitive. Some of these cultivars showed petal wilting, while others showed abscission without wilting or concomitant with wilting.

Markers of PCD, including DNA degradation and chromatin condensation, decreases in nuclear volume and DNA laddering were observed in the petal wilting species such as petunia and morning glory (Yamada et al., 2006; Yamada et al., 2007). However, in those species whose petal cells were still fully turgid before abscission, PCD hallmarks were also observed in the turgid cells of certain species (Yamada et al., 2007).

**Goals and objectives**

The goal of my research is to investigate global protein changes that occur during petal senescence. I used Petunia as my model system to study petal senescence, because petunia corollas have a defined life span, petals are ethylene sensitive, classic markers of program cell death (PCD) are observed, and these hallmarks coordinate with wilting symptoms before abscission.
Three objectives will be addressed in my dissertation:

1. Biochemical characterization of a senescence-specific nuclease PhNUC1.
2. Proteomic analysis of pollination induced petal senescence in petunia, and
3. Functional analysis of senescence-associated proteins that are identified from objective 2 using virus-induced gene silencing- a high throughput approach.

SENESCENCE TRANSCRIPTOME

**Senescence-related genes**

To identify and characterize the central components of the regulatory network and the individual steps and catabolic activities during senescence, large scale transcriptome studies have been performed on senescing *Arabidopsis* leaves during normal development and following exposure to various exogenous stimuli (Buchanan-Wollaston et al., 2003; Guo et al., 2004; Buchanan-Wollaston et al., 2005; van der Graaff et al., 2006). These studies showed that increased transcriptional activity is apparent before the onset of visible symptoms of senescence. As senescence proceeds, there are clear shifts of gene expression from genes encoding proteins involved in anabolism (such as photosynthetic genes) to those involved in catabolism (including fatty acid oxidation and nutrient mobilization). Those genes up regulated during senescence (defined as senescence-associated genes (SAGs) (Gan and Amasino, 1997), mainly encode transporters and the enzymes that function in the hydrolysis of macromolecules such as
proteins, carbohydrates, lipids and nucleic acids. In addition, numerous genes that function in defense and stress responses were identified, indicating that the senescing cells require these genes to maintain cell viability during nutrient degradation and remobilization. They may also serve to protect the leaves from pathogen and insect attack while senescence is progressing. It is apparent that senescence involves complex molecular signaling networks, and multiple regulatory proteins including kinases, phosphatases, ubiquitination-proteasome and transcription factors are coordinately involved in both developmental and stress-induced senescence.

Due to the lack of genome sequence availability, subtractive hybridization based cDNA microarray analyses have been used in petal senescence studies including those on *Alstroemeria*, *Iris*, carnation, four o’clock flower and morning glory (van Doorn et al., 2003; Breeze et al., 2004; Hoeberichts et al., 2007; Xu et al., 2007; Yamada et al., 2007). The majority of SAGs identified in these studies are classified into signaling, transcription factors, transporters, macromolecule degradation and defense and stress responses. A high proportion of these genes are shared with leaf senescence. Subtractive hybridization identified numerous kinases and transcription factors, but the roles of most these genes in flower senescence are still unclear. However, it has been suggested that these genes might be important for unraveling senescence initiation.

Temporally, senescence related genes including up and down regulated patterns can be classified into the following categories including initiation, regulation and
execution phases (Gan and Amasino, 1997; Nooden et al., 1997). Lim et al., (2003) further categorized these genes into: (1) genes that control aging and other endogenous signals during development; (2) genes that affect senescence in response to exogenous signals; (3) regulatory genes that up regulate down stream SAGs or down regulate biosynthetic or cellular maintenance activities; (4) genes that control degradation of regulatory genes; and (5) genes that function in senescence execution processes such as macromolecule degradation and nutrient remobilization.

Are SAGs also expressed in stress-induced senescence?

Large-scale transcriptome studies have shown that stress responses and leaf senescence share overlapping signaling pathways (Kolomiets et al., 2001; Chen et al., 2002; Buchanan-Wollaston et al., 2003; Buchanan-Wollaston et al., 2005; van der Graaff et al., 2006). Many, but not all of the SAGs that are induced during developmental senescence are also induced during stress-induced senescence (Buchanan-Wollaston et al., 2005). For example, in a microarray analysis of developmental senescence (NS), dark-induced senescence of attached leaves (DIS) and senescence of dark-incubated detached leaves (DES), 3513, 1833 and 2158 genes with significant changes in expression were identified, respectively (van der Graaff et al., 2006). There were 1232 overlapping genes among the three experiments, including 41% and 59% up and down regulated genes, respectively. Functional categories of SAGs from these three
experiments showed that in most categories, a similar proportion of genes were found in both DIS and DES (van der Graaff et al., 2006).

ENDOGENOUS SIGNALS OF SENESCENCE

Sugar

The endogenous signals of senescence include age, developmental cues and plant growth regulators. Once the differentiation of an organ is completed or the whole plant is developmentally mature, the senescence process is then initiated. It has been suggested that developmental and age-related processes influence the longevity and senescence of photosynthetic tissues (Hensel et al., 1993). Leaves accumulate sucrose and starch through photosynthesis. When the photosynthetic rate drops to a threshold level, leaf senescence is then initiated (Hensel et al., 1993; Bleecker and Patterson, 1997). Support for this hypothesis is provided by the fact that induction of the senescence-specific cysteine protease SAG12, is coordinated with the lowest expression of CHLOROPHYLL A/B BINDING PROTEIN (CAB) (Noh and Amasino, 1999). The up regulation of SAG12 was prevented by sucrose, glucose, and fructose treatments, indicating that the level of carbohydrate in the leaf may be linked to the initiation of senescence (Noh and Amasino, 1999). It has been reported that glucose is the regulator in various plant developmental processes including leaf senescence (Yanagisawa et al., 2003).
Sugar may influence initiation of flower senescence as well. Although petals usually do not photosynthesize, sugars are widely applied to prolong cut flower longevity (Pun and Ichimura, 2003). It has been demonstrated that supplements of sucrose and glucose antagonize the petal senescence process (Pun and Ichimura, 2003; Verlinden and Garcia, 2004; Hoeberichts et al., 2007). More recently, cDNA microarray analysis showed that silver thiosulphate (STS) and sugar treatments had similar effects on delaying the gene expression of the most SAGs in carnation petal senescence (Hoeberichts et al., 2007).

**Hormones**

Ethylene, a key plant hormone, not only induces flower wilting and abscission, but also regulates various plant growth and development processes (Yang and Hoffman, 1984; Fluhr and Mattoo, 1996). Flower species are classified into ethylene-sensitive and insensitive groups. For the ethylene-sensitive species, such as petunia, tobacco, carnation and orchids, developmental or environmental stimuli such as pollination and wounding trigger a burst of ethylene (Woltering and van Doorn, 1988; Stead et al., 1994). More recently, cDNA microarray analysis showed that accumulation of 90% of transcripts enriched from carnation senescing petals by subtractive hybridization was prevented by STS treatment (Hoeberichts et al., 2007). *Arabidopsis* mutants that are defective in ethylene signaling have been reported to display a delay of leaf senescence, indicating
that ethylene modulates or controls the timing of senescence (Grbic and Bleecker, 1995).

Functional studies of key genes in both the biosynthetic and signal transduction pathways have shown that ethylene is a major regulator of the senescence process in ethylene sensitive flower species, including Petunia (Wilkinson et al., 1997; Bovy et al., 1999; Chen et al., 2004; Shibuya et al., 2004; Kinouchi et al., 2006; Shibuya and Clark, 2006; Huang et al., 2007).

Other plant growth regulators have also been found to regulate leaf and flower senescence (Smart, 1994; van der Graaff et al., 2006). Cytokinins (CKs) regulate cell division and various metabolic and developmental processes, including senescence. In early senescing leaves, CK levels are reduced. It has been demonstrated that manipulating endogenous CK levels affected leaf and flower senescence. For example, transgenic *Arabidopsis* plants that overexpress the *ISOPENTENYLTRANSFERASE (IPT)* gene, which encodes an enzyme that catalyzes the rate-limiting step in cytokinin biosynthesis, have been shown to significantly delay leaf and flower senescence (Smart et al., 1991; Gan and Amasino, 1995; Chang et al., 2003). Jasmonic acid (JA) plays important roles in defense response and development processes including senescence (He et al., 2002; Turner et al., 2002). JA application has been reported to enhance senescence by inducing ethylene production (Porat et al., 1993; Porat et al., 1995). Salicylic acid (SA) is also involved in stress responses and senescence (Morris et al., 2000). Transcriptome comparisons between senescing *Arabidopsis* leaves from wild type plants and SA-
deficient *NahG* mutants found that expression of many SAGs is dependent on the SA-signaling pathway (Buchanan-Wollaston et al., 2005). A common set of SAGs are shared by both stress and hormone-mediated senescence, indicating that there is crosstalk among ethylene, SA and JA signaling pathways during senescence (Weaver et al., 1998; Turner et al., 2002; Li et al., 2004; Lin and Wu, 2004; Buchanan-Wollaston et al., 2005; Miao and Zentgraf, 2007), and that plant hormones in addition to ethylene are important.

**Phospholipids**

It has also been suggested that phospholipids (PLs) have a key role in petal senescence (Borochov et al., 1997; Thompson et al., 1998). Specifically, Phospholipase D (PLD) has been suggested to be a key enzyme mediating the first step in the degradation of phospholipids during the early stages of plant senescence (Thompson et al., 1998). Supporting this hypothesis, it has been shown that inhibition of PLD activity, by lysophosphatidylethanolamine, significantly delayed leaf (cabbage) and flower (snapdragon) senescence (Ryu et al., 1997).

There is also a correlation between ethylene levels and the phospholipid content of petals during the senescence process. Petunia is an ethylene sensitive species, and the pattern of ethylene production during petal senescence shows a steady, low level for the first few days after flower opening, followed by an increase that peaks when petals are wilted. The level of ethylene then shows a sharp decrease at the later stages of senescence.
The changes in ethylene accumulation are correlated to petunia petal wilting. The membrane lipid composition also changes during senescence. These changes include decreases in PL content, increases in diacylglycerol (DAG) and changes in membrane lipid microviscosity. However, these changes occur before peak ethylene production. Treatment with phorbol 12-myristate 13-acetate (PMA), an analog of DAG, increases ethylene production and accelerates the timing of the climacteric rise, concomitant with decreased flower longevity (Borochov et al., 1997). The effects of PMA were prohibited by pretreatment with STS, an ethylene perception inhibitor. These experiments suggest that some of the membrane degradation events precede ethylene production during the senescence process and may in fact help drive production. Since both DAG and PMA are known activators of protein kinases (Castagna et al., 1982), their enhancement of ethylene production and perception might be regulated through phosphorylation of components of the ethylene biosynthetic and/or signal transduction pathways (Spanu et al., 1994; Guo and Ecker, 2004).

KEY TRANSCRIPTION FACTORS IN THE SENESCENCE NETWORK

There are approximately 1500 predicted transcription factor genes in the Arabidopsis genome and 45% of these are plant specific (Riechmann et al., 2000). More than 100 transcription factors that positively or negatively regulate senescence were identified from previous expression studies of leaf senescence. Many transcription factor
families, such as WRKY, NAC, TLP, GRAS, bZIP and C2H2 are represented (Guo et al., 2004; Lin and Wu, 2004; Buchanan-Wollaston et al., 2005; van der Graaff et al., 2006).

The *Arabidopsis* WRKY family contains up to 100 members and all contain the WRKY domain, a 60-amino-acid domain with the conserved WRKYGQK motif at the N-terminal end, together with a specific zinc-finger motif (Eulgem et al., 2000). WRKY genes are expressed in many different plant tissues, in different developmental stages and in response to various stress stimuli; indicating that they participate in a wide variety of biological processes (Eulgem et al., 2000; Ulker and Somssich, 2004). Several WRKY genes were found to be induced during leaf senescence (Hinderhofer and Zentgraf, 2001; Robatzek and Somssich, 2001; Ulker et al., 2007) and are thought to be important in this process. For example, it has been suggested that WRKY53 plays a regulatory role in early leaf senescence (Hinderhofer and Zentgraf, 2001). RNAi and knock-out lines of *WRKY53* show delayed senescence phenotypes; conversely, overexpression causes accelerated senescence (Miao et al., 2004).

The function of other transcription factors has also been studied. For example, knock out of a NAC family transcription factor, AtNAP, causes delayed leaf senescence in *Arabidopsis* (Guo and Gan, 2006). Manipulation of *OsDOS*, encoding a CCCH-type transcription factor, shows that this gene negatively regulates leaf senescence in *Oryza sativa* (Kong et al., 2006). These studies suggest that transcription factors are key players in regulating senescence.
Expression studies have also identified a number of transcription factors induced
during petal senescence; some of which are the similar to factors already associated with
leaf senescence. For example, EIN3 has been identified as a key transcription factor in
the ethylene pathway in *Arabidopsis* and overexpression of *EIN3* confers a constitutive
ethylene response (Guo and Ecker, 2004). It has been found that ethylene prevents the
proteasome-mediated degradation of EIN3. The accumulated EIN3 represses downstream
ethylene response genes (Yanagisawa et al., 2003). Three EIN3-like proteins have been
identified in carnation petals (Dc-*EIL1-3*; Iordachescu and Verlinden, 2005). More
recently, one of these genes (Dc-*EIL*), was shown to accumulate during the early stages
of carnation petal senescence (Hoeberichts et al., 2007). The up regulation of the Dc-
*EIL3* gene was delayed or prevented by STS or sucrose treatment, respectively
(Hoeberichts et al., 2007). These results were consistent with previous studies of *EIN3* in
*Arabidopsis*, in which glucose was shown to antagonize ethylene responses by enhancing
the proteasome-mediated degradation of EIN3 (Yanagisawa et al., 2003). Therefore,
EIL3 might be a component of a master switch during senescence, which is regulated by
sugar as well as ethylene (Yanagisawa et al., 2003; Hoeberichts et al., 2007).
EXECUTION PHASE OF SENESCENCE

Lipid degradation

Membrane deterioration is one of the early events during senescence, and lipid degradation during senescence has been reviewed previously (Thompson et al., 1998). This process is coordinated with other catabolic processes of senescence such as degradation of nucleic acids, proteins, and carbohydrates (Buchanan-Wollaston et al., 2003). A number of genes encoding enzymes such as phospholipase D (PLD), phosphatidic acid phosphatase, lipoxygenase, and lipolytic acyl hydrolase (lipase) are induced during senescence, and these enzymes progressively function in lipid degradation. The released free fatty acids are used as substrates for peroxidation by lipoxygenases or are converted to sugar and used for energy during senescence (Smart, 1994; Thompson et al., 1998).

Evidence has shown that the maintenance of membrane integrity is important to prolong senescence. For example, a lipase encoding gene was shown to be induced following the ethylene burst at the initiation of carnation petal senescence (Hong et al., 2000). Transgenic Arabidopsis plants with suppressed lipase expression had delayed leaf senescence (Thompson et al., 2000). In addition, antisense transgenic Arabidopsis plants with reduced expression of an acyl hydrolase gene also showed a delayed senescence process (He and Gan, 2002). Taken together, these studies suggest that proteins involved in lipid degradation are not only catalyzing the catabolic processes of senescence, but
they might also regulate the senescence process.

**Protein degradation**

Proteolysis is important for plant growth, development and defense. Various proteases participate in protein degradation, N-mobilization, and protein maturation and localization (Schaller, 2004). The accumulation of transcripts for genes encoding proteases and proteasome components is consistent with protein catabolism being one of the major events in senescence (Jones, 2004; Pak and van Doorn, 2005). Senescence-associated cysteine proteases have been previously identified from many different flower species including carnation (Jones et al., 1995), *Alstroemeria* (Wagstaff et al., 2002; Breeze et al., 2004), *Sandersonia* (Eason et al., 2002) and petunia (Jones et al., 2005). Jones et al., (2005) have shown that six cysteine protease genes from petunia were up regulated with different temporal pattern during petunia flower senescence. Among these proteases, PhCP10 is an ortholog of *SAG12*, which was identified from Arabidopsis senescing leaves (Lohman et al., 1994; Weaver et al., 1998; Noh and Amasino, 1999). *SAG12* has been characterized as a senescence-specific gene that is regulated by developmental age and does not response to hormones or stress stimuli (Noh and Amasino, 1999). Another protease, PhCP5, is highly similar to cysteine proteases from ethylene insensitive flowers including *Iris* (van Doorn et al., 2003), *Sandersonia* (Eason et al., 2002), and daffodil (van Doorn et al., 2004). Gene expression profiles in wild type
and ethylene insensitive transgenic lines show that PhCP5 might be ethylene independent and regulated by aging (Jones et al., 2005). Recently, an aleurain-like cysteine protease BoCP5 was found to be induced during harvest-induced senescence in broccoli florets and leaf tissues. In the antisense transgenic plants, postharvest floret senescence (yellowing) is delayed, and florets contain significantly greater chlorophyll levels during postharvest storage than wild-type plants (Eason et al., 2005).

In plants, metacaspases, which are caspases-like cysteine proteases that specifically cleave proteins after aspartic acid residues, have been shown to be induced by stress or pathogen-induced PCD (Hoeberichts et al., 2003; He et al., 2008). In addition, metacaspase gene expression is induced during petunia corolla senescence (Jones, unpublished data). It has recently been postulated that metacaspases might function in the initiation and execution of PCD in planta, although evidence of functional characterization remains unclear (Uren et al., 2000; Woltering et al., 2002; Watanabe and Lam, 2005).

**Nucleic Acid Catabolism**

Nucleic acid catabolism during senescence involves the action of RNases, DNases, and nucleases (Sugiyama et al., 2000). The purpose of large-scale nucleic acid catabolism during senescence is to degrade DNA and RNA and remobilize their components including carbon, nitrogen and especially phosphorus, to sink tissues.
During this process, nuclear DNA becomes fragmented and the hydrolysis must be catalyzed by endonucleases capable of digesting both single-stranded and double-stranded DNA. DNA fragmentation during the senescence of leaves and petals has been detected in situ using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method or by visualizing the presence of the resulting 160 bp internucleosomal fragments (DNA ladders) on agarose gels (Orzaez and Granell, 1997; Yen and Yang, 1998; Panavas et al., 2000; Xu and Hanson, 2000; Wagstaff et al., 2003; Hoeberichts et al., 2005; Langston et al., 2005; Yamada et al., 2006). Correlating with the observed DNA fragmentation, the expression and the activity of senescence-associated nucleases were found to increase during both leaf and flower senescence.

**Cell wall degradation**

Transcript studies on leaf and petal senescence show that expression of a number of cell wall related genes is induced or suppressed during senescence. Up-regulated genes encode proteins functioning in cell wall loosening and degradation, while the down-regulated genes encode those proteins functioning in cell wall synthesis and expansion. For example, at the late stage of *Iris* tepal senescence, the cell walls were largely degraded and this corresponded to up regulation of beta-galactosidase and pectinesterase genes (van Doorn et al., 2003). The transcripts of beta-xylosidases genes are strongly induced during carnation petal senescence (Hoeberichts et al., 2007). Beta-xylosidases
have been implicated in senescence-related secondary cell wall metabolism (Goujon et al., 2003). In addition, the transcripts of expansin genes, whose gene products are involved in cell wall loosening during growth or disassembly, were induced in carnation and morning glory petal senescence (Cosgrove, 2000; Yamada et al., 2007).

PROTEOMIC APPROACHES AS A TOOL TO STUDY PETAL SENESCENCE

Although numerous SAGs that function in the network controlling petal senescence or the execution program have been identified (Rubinstein, 2000), only a few large-scale expression studies have been performed using cDNA microarrays (van Doorn et al., 2003; Breeze et al., 2004; Hoeberichts et al., 2007; Xu et al., 2007; Yamada et al., 2007). In addition, large-scale studies focused on changes at the protein level during senescence have been not reported. Although expression studies advance our understanding of senescence at the mRNA level, they remain largely elucidative on the protein level. It is known that transcript levels are not always correlated with the levels of protein in a cell. The production of proteins is affected by differing stability of mRNAs, and different efficiencies in translation. In addition, post-translational modification is often required for protein activation or deactivation or degradation. Therefore, concentrating on transcript levels will not reveal changes at this level. For example, PCD studies in an Arabidopsis cell suspension culture system showed that the genes encoding
some of the differentially expressed proteins detected in a 2-DE experiment were not
differentially expressed (Swidzinski et al., 2002; Swidzinski et al., 2004).

Recently, plant proteomics has been successfully used to study the protein
changes during various plant developmental processes such as leaf senescence, and
flower and fruit development (Wilson et al., 2002; Dafny-Yelin et al., 2005; Faurobert et
al., 2007), as well as drought, heat and salt-induced stress responses (Askari et al., 2006;
Horvath-Szanics et al., 2006; Huang et al., 2006; Hajheidari et al., 2007; Ito et al., 2007;
Veerasamy et al., 2007; Yang et al., 2007). These studies further indicate that proteomic
approaches can be successful.

VIRUS-INDUCED GENE SILENCING (VIGS)-A HIGH THROUGHPUT APPROACH
TO STUDY THE FUNCTION OF SAGS

Microarray experiments and 2-DE based proteomic approaches can identify a
large number of senescence-associated genes. Subsequent experiments must then be
carried out to demonstrate that these genes have important functions during the processes
of interest. It is therefore necessary for us to employ a high throughput approach, such as
VIGS, to investigate the function of identified SAGs. VIGS has many advantages
compared with other loss-of-function approaches (Baulcombe, 1999; Burch-Smith et al.,
2004). Conventional reverse genetic approaches require stable transformation, which is
time consuming and laborious. In addition, knock out mutations in a gene may not cause
any phenotype due to functional redundancy with other gene family members or gene
duplications. Alternatively, transgenic plants may not be obtained due to embryo lethality
if the target gene knocked out has an essential function early in development
(Baulcombe, 1999). VIGS is a desirable approach to circumvent the above problems. All
members of a multigene family can be silenced if the VIGS construct contains a sequence
that is complementary to a highly conserved sequence among the family members. Gene
silencing has been shown to tolerate between 10 to 20% mismatch among Ribulose-1,5-
Bisphosphate Carboxylase Oxygenase family members in antisense transgenic tobacco
plants (Dean et al., 1989; Quick et al., 1991; Hudson et al., 1992). More over, VIGS can
also be used to silence an entire gene family by including all gene members in tandem
within the construct (Chen et al., 2004; Chen et al., 2005). VIGS can be made gene
specific by choosing a region of the gene that does not have similarity to other family
members.

The mechanism of VIGS is based on post-transcriptional gene silencing (PTGS).
The PTGS phenomenon was initially observed as co-suppression of the *CHALCONE
SYNTHASE (CHS)* gene in CHS overexpression transgenic plants (Napoli et al., 1990).
Similar phenomena have also been found in fungi and animals, these are called quelling
and RNAi, respectively (Waterhouse et al., 2001). PTGS is triggered in the host cells
when an introduced sequence has sequence similarity to an endogenous gene. Double
stranded RNA species result, which cause degradation of endogenous genes and/or the transgenes (Burch-Smith et al., 2004).

Several viruses have been used for VIGS. It has been shown that Tobacco Rattle Virus (TRV) is a better silencing vehicle compared with other viruses (Brigneti et al., 2004). TRV has been widely used to study gene function in many species including tobacco, tomato, petunia, pepper, *Arabidopsis* and poppy (Liu et al., 2002; Brigneti et al., 2004; Chen et al., 2004; Chung et al., 2004; Chen et al., 2005; Fu et al., 2005; Hileman et al., 2005; Burch-Smith et al., 2006; Cai et al., 2006; Dong et al., 2007; Spitzer et al., 2007). Recently, TRV mediated VIGS has been used to study gene function during leaf senescence (Wang et al., 2005; Ahn et al., 2006; Lin et al., 2007), flower senescence (Chen et al., 2004; Chen et al., 2005; Xu et al., 2007) and fruit ripening (Liu et al., 2002; Fu et al., 2005; Xie et al., 2006). Therefore this system will be used for our studies.

SIGNIFICANCE OF RESEARCH

Senescence is responsible for the recycling of nutrients and energy reserves in plants. Macromolecules and organelles are degraded and nutrients are exported to sink organs such as ovaries, fruits and seeds during senescence. The initiation, regulation and execution of senescence are coordinated at the level of gene expression, protein synthesis and enzyme activities. Although expression studies advance our understanding of senescence at the mRNA level, they remain largely elucidative on the proteomic level.
The proteomic approach allows us to identify protein changes at the post-translational level during senescence. Furthermore, we will investigate the candidate genes using the VIGS technique, which is a high-throughput tool for gene functional analysis. Since age and stress-induced senescence during pre-harvest, harvest and post-harvest can have a detrimental affect on crop yield and quality, understanding how senescence is regulated in plants will enable us to delay senescence either by genetic improvement or by environmental control. This will enhance the quality and yield of agronomic and horticultural crops, which will benefit growers and consumers alike.
CHAPTER 2

INCREASES IN DNA FRAGMENTATION AND INDUCTION OF A SENESCENCE-SPECIFIC NUCLEASE ARE DELAYED DURING COROLLA SENESCENCE IN ETHYLENE-INSENSITIVE (ETR1-1) TRANSGENIC PETUNIAS

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 x 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 nonsenescing
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.

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 (Jones, 2004; Rubinstein, 2000). 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 labeling (TUNEL) method and by visualizing the presence of the resulting 160 bp
Internucleosomal fragments (DNA ladders) on agarose gels (Panavas et al., 2000; Serafini-Fracassini et al., 2002; Wagstaff et al., 2003; Xu and Hanson, 2000; Yen and Yang, 1998).

Nucleic acid catabolism during senescence involves the action of RNases, DNases, and nucleases (Sugiyama et al., 2000). The hydrolysis of genomic DNA must be catalyzed by endonucleases capable of digesting both single-stranded and double-stranded DNA. Plant endonucleases with activity against DNA have been classified as either Zn$^{2+}$-dependent or Ca$^{2+}$-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 Zn$^{2+}$-dependent and the Ca$^{2+}$-dependent classes. In addition, senescence-specific endonucleases that are dependent on Co$^{2+}$ have been identified from tomato and parsley leaves (Canetti et al., 2002; Lers et al., 2001). 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 (Canetti et al., 2002; Lers et al., 2001). 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 (ABA) 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 upregulated by treatment of daylily petals with ABA (Panavas et al., 1999), and nuclease transcripts from tomato and barley were also upregulated 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 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 senescence-associated genes (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. It therefore may not be energetically advantageous 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 program 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. Specifically, five nucleases with activity against single-stranded DNA (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 x hybrida* have identified seven nucleases using similar in-gel activity assays. In this paper we have chosen to focus on one nuclease, which shows activity only in petals showing visual symptoms of senescence. We have referred to this senescence-specific nuclease as PhNUC1, and its characterization is described as part of this report. This manuscript 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 programs of leaves and petals.

RESULTS

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

Wild type petunia flowers that were emasculated and left to naturally age on the plant exhibited visual symptoms of senescence (petal wilting) at 7 to 8 days after anthesis (daa) (Figure 2.1A). 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 to 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 our experimental conditions, this represented an 8-day delay in flower senescence compared to WT. While etr1-1 flowers always lasted longer than WT flowers, their longevity 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 (Figure 2.1B). Elevated ethylene production was detected from etr1-1 petals at 12 to 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 \textit{etr1-1} than WT corollas. 

Pollination accelerated the senescence of WT flowers, and corolla wilting was observed at 48 hours after pollination (hap) (Figure 2.2A). Maximum ethylene production coincided with corolla wilting (Figure 2.2B). Pollination did not accelerate the senescence of \textit{etr1-1} flowers, and corollas did not wilt until physically abscised from the flower by the growing ovary (data not shown). In light of this observation, it was determined that the best comparison between wild type and \textit{etr1-1} flowers would be obtained from unpollinated, naturally senescing flowers and pollinated \textit{etr1-1} flowers were not included in this study. A detailed analysis of the post-pollination response in \textit{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 aging and following pollination. At anthesis (0 hap and 0 daa), less than 5% of the nuclei in WT petals had fragmented DNA as determined by the TUNEL assay (Figure 2.1C and 2.2C). DNA fragmentation increased only slightly at 4 daa, but by 8 daa when corollas were completely wilted, DNA fragmentation was
observed in more than 90% of the nuclei. Increases in DNA fragmentation were delayed in unpollinated *etr1-1* flowers until 12 to 16 daa, but similar to 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 x 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 5 nucleases could be detected (data not shown). β-mercaptoethanol has previously been reported 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 x hybrida* nuclease), which has a molecular mass of about 43 kDa was identified in our preliminary studies, and we chose to focus on its regulation 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 (Figure 2.3 and 2.4). During the natural aging of unpollinated WT corollas, PhNUC1 activity was first detected at 8 daa (Figure 2.3). The induction of PhNUC1 was accelerated by pollination, and activity was detected at 48 to 72 hap when the corollas were wilted (Figure 2.4). Lower 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 µL 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 µg) 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. Additionally, DNA fragmentation and subsequent hydrolysis must involve the degradation of both double-stranded DNA (dsDNA) and single-stranded DNA (ssDNA). To determine substrate specificity of PhNUC1, single stranded (ssDNA), double stranded
DNA (dsDNA), or RNA were included in the SDS-PAGE activity gels (Figure 2.3 and 2.4). 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 to WT (Figure 2.3). 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 µL L⁻¹ ethylene, low levels of PhNUC1 activity were detected at 12 h and activity increased by 24 h (Figure 2.5). 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 to 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 nonsensceng (0 hap) and senescing (48 hap) WT
corolla extracts in Tris-HCl (pH 7.5) development buffers containing various cations as indicated in Fig 2.6. One millimolar EDTA 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 (Figure 2.6A). 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) (Figure 2.6B). Increasing the concentration of MnCl$_2$ in the development buffer resulted in only a slight increase in PhNUC1 activity, while increasing the concentration 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 (Figure 2.6B). 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 (Figure 2.6C).

**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 Figure 2.7. PhNUC1 activity was detected at a wide range of pH values, but maximum activity was detected at pH 7.5 -8.0.

**PhNUC1 is a glycoprotein**

Incubation of the carbohydrate-binding protein, Concanavalin A (ConA), with the total protein extracts from senescing petals (WT 48 hap) prior to electrophoresis resulted in an inhibition of PhNUC1 activity (Figure 2.8). This inhibition could be overcome by adding $\alpha$-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 evidenced by *in situ* labeling 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 (Figure 2.9). 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 programmed cell death.

A number of nucleases associated with programmed cell death 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 to 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 catalyzed 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 apoplastic-localized Zn\(^{2+}\)-dependent nucleases. PhNUC1 shares characteristics in common with both classes of nucleases, but in 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 (Canetti et al., 2002; Lers et al., 2001). 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 nonsenescing 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 (Canetti et al., 2002; Lers et al., 2001; this manuscript). The induction of Co\(^{2+}\)-requiring nucleases late in the senescence process indicates that large scale nucleic acid catabolism are components of both leaf and petal senescence, and provides evidence for some commonality among the senescence programs 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 (Canetti et al., 2002; Lers et al., 2001). Following exogenous treatment with ethylene, it is difficult to determine if
the observed affects 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 wild type (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 senescence-associated genes have been reported in flowers treated with chemical inhibitors of ethylene biosynthesis and action (Borochov et al., 1997; Burdon and Sexton, 1993; Jones et al., 1995; Lawton et al., 1990; Woodson et al., 1992). Delayed accumulation of senescence-associated genes 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 program once it had begun (Grbic and Bleecker, 1994). 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 programs. 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 program 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 wild type
petunia flowers suggests that nucleic acid catabolism in both of these senescence
programs 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 CaCl₂ and MgCl₂, 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 Co²⁺ (i.e. in
buffer containing only CaCl₂ or MgCl₂). 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 x 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 x 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, catalyzes 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.

MATERIALS AND METHODS

Plant materials

Petunia x hybrida ‘Mitchell’ 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 (Gubrium et al., 2000; Wilkinson et al., 1997). Experiments also utilized non-transformed wild type (WT)
Petunia x hybrida ‘Mitchell’. Seeds were treated with 100 mg L\(^{-1}\) GA\(_3\) 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 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 one day 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 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 wild type (WT) and \(etr1-1\) corollas was determined by sealing corollas in 22 mL vials (2 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, California). A total of 12 flowers were collected for each time point and sample collection and ethylene measurements were conducted twice. Graphed values represent the mean ethylene production (nL C$_2$H$_4$ g$^{-1}$FW h$^{-1}$) ± 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 µL 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-labeled 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 micrometer 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
µg mL⁻¹ proteinase K for 15 min and rinsed twice with 1X PBS. The tissue was then labeled with fluorescein for the detection of DNA fragmentation as per the manufacturer’s instructions (Apoptosis Detection System, Promega, Wisconsin). Co-staining with propidium iodide (Sigma, St. Louis, MO) was used to visualize all nuclei. Positive controls were treated with 2 µg mL⁻¹ DNase I (Sigma, St. Louis, MO) 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 hours after pollination (hap), unpollinated WT flowers at 0, 4, and 8 days after anthesis (daa) and etr1-1 flowers at 0, 4, 8, 12, and 16 daa. At least 3 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 to 400 nuclei, and the data presented are the average % of fragmented nuclei ± 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\textsubscript{2} and stored at –80° C until they were used for total protein extraction.

Corolla tops were powdered in liquid N\textsubscript{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 1,000 x g for 15 min at 4° C and the supernatant was stored in 1 mL aliquots at -80° C. Extractions included at least 4 corolla tops per time point and 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 µg mL\textsuperscript{-1} BSA. To identify DNase activity, gels contained either 15 µg mL\textsuperscript{-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 µg mL\textsuperscript{-1} petunia petal total RNA. Samples were equalized by loading 20 µL 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 µg 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 25º 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 37º 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 37º 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 µg mL⁻¹ ethidium bromide. Once conditions were optimized for PhNUC1 activity, all gels were incubated in development buffer that was supplemented with 100 µM CoCl₂.

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 µM CoCl₂, ZnCl₂, MnCl₂, CuCl₂, MgCl₂ or CaCl₂. Further investigation of divalent cation effects on PhNUC1 activity utilized various concentrations of CaCl₂, MnCl₂, CoCl₂, and ZnCl₂ in the presence or absence of the chelating agent EDTA (50 µM).
To determine if PhNUC1 is a glycoprotein, the ability to interact with the carbohydrate-binding protein, Concanavalin A, was evaluated as described by Thelen and Northcote (1989). A 10 µg sample of total protein in 50 mM Tris-HCl pH 7.5 was incubated with 12 µg Concanavalin A (Calbiochem, La Jolla, CA) for 10 min at 25 ºC. Competition assays with a lectin inhibitory sugar were conducted concurrently by adding α-methyl-D-mannoside (Calbiochem, La Jolla, CA) 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 ºC 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 MgCl₂, 0.1 mM EDTA, 1.0 mM DTT, 0.2 mM PMSF, 1.0 µg/mL pepstatin, 1.0 µg/mL leupeptin). Extracts were filtered through 1 layer of miracloth and centrifuged at 4,300 x g for 10 min at 4 ºC. 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 x g for 10 min at 4 ºC. The resulting supernatant represents the cytosolic fraction. Ten µg of protein from the nuclear and cytosolic fractions were run on ssDNA substrate gels using optimized conditions as determined in previous experiments.
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AUTHOR CONTRIBUTIONS

Brennick Langston and Michelle Jones planned experiments. Brennick Langston initiated and performed the ethylene measurements, TUNEL experiments and nuclease in-gel activity assays in his master studies. Figure 1, 2 and 5 are from his results. Shuangyi Bai performed nuclease activity in-gel assays including fig 3, 4, 6, 7, 8 and 9. Michelle Jones analyzed the results and wrote the manuscript. All authors discussed the results and commented on the manuscript.
Figure 2.1. Natural senescence of unpollinated wild type (WT) *Petunia x 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* labeling of fragmented DNA was determined by the TUNEL assay. Values represent the average ± SE.
A

Time after anthesis (days)

0 4 8 12 16 daa

WT

etr1-1

B

Ethylene production (nL g⁻¹ FW h⁻¹)

Time after anthesis (days)

WT

etr1-1

C

Fragmented Nuclei (%)

Time after anthesis (days)

WT

etr1-1
Figure 2.2. Pollination-induced senescence of wild type (WT) *Petunia x hybrida* ‘Mitchell’ flowers.

(A). Ethylene production (B) and nuclear DNA fragmentation (C) in corollas at various times after pollination. *In situ* labeling of fragmented DNA was determined by the TUNEL assay. Values represent the average ± SE.
Figure 2.3. 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 μL 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 μM CoCl₂.
Figure 2.4. 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 Figure 3. Total proteins were extracted from corollas at various times after pollination as indicated (hap; hours after pollination).
Figure 2.5. Induction of PhNUC1 by ethylene.

WT flowers at anthesis were treated with 2.0 µL 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 Figure 2.3.
Figure 2.6. 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 µM units. Each slice contained proteins from nonsenescing (0 hap) and senescing (48 hap) WT corollas (A) or just 48 hap corollas (B) and (C).
Figure 2.7. 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 Figure 2.3.
Figure 2.8. Interaction of PhNUC1 with the carbohydrate binding protein Concanavalin A.

Total protein samples were extracted from senescing WT corollas (48 hap). Total protein extract only (control), protein + Concanavalin A (ConA), protein + Concanavalin A + 0.2 M α-methyl-D-mannoside (ConA + 0.2M mm), and protein + Concanavalin A + 0.4 M α-methyl-D-mannoside (ConA + 0.4M mm) were resolved on SDS-PAGE containing ssDNA as described in Figure 3.
Figure 2.9. 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 µg) 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 µM CoCl₂.
ABSTRACT

Senescence is a highly regulated process, and is correlated with developmental and environmental cues. To globally analyze protein changes during pollination-induced petal senescence, we are using a proteomic approach to identify components of the senescence program in *Petunia x hybrida* cv Mitchell Diploid corollas. Total soluble proteins were extracted from petunia petals at 24, 48, and 72 hours after flower opening (i.e. unpollinated, nonsenescing flowers) and at 24, 48, and 72 h after pollination (i.e. senescing flowers). Two-dimensional gel electrophoresis was used to identify those proteins that were differentially expressed in nonsenescing (unpollinated) and senescing (pollinated) corollas. PDQuest image analysis (BioRad) software was used to identify those proteins up or down regulated by two fold in pollinated petals. Most of these were identified by comparing 72 h unpollinated with 72 h pollinated corollas. One hundred thirty-three differentially expressed protein spots were selected to be sequenced, and 73
of 133 were identified as single proteins. Liquid chromatography-tandem mass spectrometry (LC-tandem MS) was used to determine the identity of these proteins. Searching the NCBI nonredundant protein and petunia translated EST databases we have been able to assign a putative identification to greater than 90% of these proteins. The majority of the identified proteins are involved in defense and stress responses and many metabolic pathways including proteolysis, nucleic acid, cell wall and lipid catabolism. These results support previous senescence studies at the transcriptional level, and provide new insights into the post-translational regulation of senescence.

INTRODUCTION

Senescence, representing the last stage of plant development, leads to the death of a cell, an organ or a whole plant (Lim et al., 2003). This process is tightly regulated by signal transduction, gene expression, and metabolism in response to developmental and environmental cues (Buchanan-Wollaston, 1997). The purpose of senescence is not just the passive death of tissues, but it also allows the plant to mobilize and recycle nutrients for use by other developing parts of the plant including young leaves, flowers and fruits (Buchanan-Wollaston et al., 2003).

Cell death in senescing organs is genetically controlled, therefore this event is called programmed cell death (PCD) (Lam E, 2001). It has been found that PCD occurs throughout plant developmental processes including senescence and it is coordinated by
developmental cues and abiotic and biotic stresses (Gan and Amasino, 1997; Lam E, 2001; Kuriyama and Fukuda, 2002). Therefore, senescence at the cellular level could be referred to as a PCD event (Kuriyama and Fukuda, 2002).

Senescence is an integral process coordinated by developmental age and other internal and external signals. The timing of natural senescence is not only controlled by developmental age, but the senescence process is also influenced by other stimuli such as stresses, pests, and pathogen infection. The senescence of vegetative and floral tissues can have a detrimental impact on the quality and subsequent value of agricultural and horticultural crops. Therefore, it is important to understand the molecular and biochemical mechanisms of senescence initiation, regulation and execution.

Previous studies have reported that numerous genes are regulated during developmental senescence. These genes are referred as senescence-associated genes (SAGs) (Gan and Amasino 1997). SAGs encode proteins that function in the senescence process, including transcriptional regulators such as kinases and transcription factors. Other SAGs function as executors of senescence and include proteases, nuclease, lipid-, carbohydrate- and nitrogen-metabolizing enzymes. Finally, stress and defense responsive proteins are also differentially regulated during senescence (Buchanan-Wollaston et al., 2003; Guo et al., 2004; Guo and Gan, 2005). Recently, transcriptome analyses of leaf senescence have been conducted on a large-scale in Arabidopsis (Buchanan-Wollaston et al., 2003; Guo et al., 2004; Lin and Wu, 2004; Buchanan-Wollaston et al., 2005), Populus
Transcriptome analyses of flower senescence in *Iris*, *Alstroemeria*, *Mirabilis jalapa* (four o’clock flower) and morning glory have been performed using cDNA microarray analysis and other transcriptome approaches (van Doorn et al., 2003; Breeze et al., 2004; Xu et al., 2007; Yamada et al., 2007). These analyses reveal highly overlapping SAGs, and indicate that there are many commonalities in the molecular and biochemical regulation of developmental and stress-induced senescence.

Although transcriptome studies advance our understanding of senescence at the level of steady-state mRNA, relatively little data exists on the proteome in senescing tissues. Transcript levels are not always correlated with the levels of protein in a cell, because proteins are affected by differing stability of mRNAs and different efficiencies in translation. In addition, post-translational modification may be required for protein activation, deactivation or degradation. For example, PCD studies in an Arabidopsis cell suspension culture showed that some of the differentially expressed proteins in a 2-DE experiment did not exhibit the same expression patterns as those at the transcriptional level in a parallel microarray experiment (Swidzinski et al., 2002; Swidzinski et al., 2004). Recently, plant proteomics has been successfully used to study the protein changes in various plant developmental processes such as leaf senescence, flower and fruit development (Wilson et al., 2002; Dafny-Yelin et al., 2005; Faurobert et al., 2007), as well as drought, heat and salt-induced stress responses (Askari et al., 2006;
Horvath-Szanics et al., 2006; Huang et al., 2006; Hajheidari et al., 2007; Ito et al., 2007; Veerasamy et al., 2007; Yang et al., 2007). These studies further supported the importance of proteomic approaches in advancing our understanding of gene expression at the protein levels.

The objective of this experiment was to identify global protein changes that occur during flower senescence. To this end, we employed a proteomic approach, utilizing 2-DE, mass spectrometry and bioinformatic tools to identify and functionally classify the proteins that were differentially expressed in senescing and nonsenescing petals. In addition, to obtain samples with uniform senescence symptoms, we used pollinated corollas in our studies. Pollination serves as a trigger and accelerates petal senescence in many species (Stead, 1992). Pollination-induced petal senescence allows the plant to break down macromolecules and organelles from dying petals and remobilize nutrients to developing parts of the plant (Jones, 2004; Stead et al., 2006).

Proteins identified in our experiments were classified based on their biological processes. A few proteins that increase in abundance during senescence likely mediate regulatory processes. The majority of up regulated proteins are likely involved in defense responses to abiotic and biotic stress or are hydrolytic enzymes involved in macromolecular degradation. A number of protein isoforms were identified. Expression clustering analysis showed that most of these isoforms have similar expression patterns during senescence while others do not. Data from protein mass spectra and theoretical
and observed protein molecular masses indicated that some of these isoforms were post-translationally modified or processed during senescence. These results further support the idea that a combination of transcriptomic and proteomic approaches will be necessary to elucidate molecular and biochemical mechanisms of senescence. This work also supports the understanding that molecular and biochemical mechanisms of senescence are similar across the plant kingdom.

RESULTS

**Protein profiling during petunia corolla senescence**

To profile the differentially expressed proteins during pollination-induced corolla senescence, we chose 0, 24, 48 and 72-hour time points. No senescence symptoms were observed in unpollinated (U) corollas from 0 to 72 hours after flower opening or at 24 hours after pollination (P). Pollinated corollas were wilted at 48 and 72 hap, confirming that corolla senescence was accelerated after pollination (Fig.1A). About 600 distinct protein spots were detected in each gel. Molecular weights of these proteins ranged from 15 to 110 kDa (Fig.1B). Pixel intensity data were not normally distributed, though both natural log and log base 10 transformations were effective at creating a normal distribution of pixel intensity data. Analysis of variance detected highly significant differences between proteins (P<0.0001), pollination treatment (P < 0.0001), and timepoints (P<0.0001). The replication effect and all two-way interactions with replicate were
non-significant (0.760 < P > 1.00). The treatment by protein and timepoint by protein
interactions were both highly significant (P < 0.0001), indicating differential expression of
individual proteins due to pollination and time. Based on the mean squared error
associated with the ANOVA, we estimate that the experimental-wide cut-off for
differentially expressed spots was 2.1 fold at P < 0.05 and 3.6 fold at P < 0.001 (Kerr et al.,
2000). This analysis supplemented the identification of quantitative differences between
treatments by a t-test (P ≤ 0.05) using the PDQuest software. In addition, qualitative
differences (presence/absence) were also detected.

Comparison between unpollinated and pollinated samples at each time point were
used to identify select proteins for subsequent analysis. Up regulated proteins were
defined as those where the pixels associated with a spot increased in the pollinated
corollas compare to the unpollinated corollas, or proteins that were not detected in the
unpollinated corollas but were detected in the pollinated corollas. Down regulated
proteins were defined as those where pixel intensity decreased in unpollinated compared
to the pollinated corollas, or proteins that were detected in the unpollinated corollas but
not in the pollinated corollas. No significant protein changes were found between 24 U
and 24 P (Table 3.1). A comparison of protein profiles at 48 h (P versus U) identified 74
proteins that were up regulated and 41 proteins that were down regulated in pollinated
corollas, suggesting that about 15-20% of the total proteins were differentially regulated.
To characterize the protein changes at the late senescence stage, gels were compared at
72 h. At 72 h, 113 proteins were up regulated and 57 proteins were down regulated, suggesting that about 30% of the total proteins were differentially regulated.

We selected 133 differentially expressed protein spots, including 88 that were up regulated and 45 that were down regulated (Figure 3.2A and Figure 3.2B) for sequencing by tandem mass spectrometry. The CID spectra data were subjected to BLAST searches using blastp at the National Center for Biotechnology Information (NCBI: http://www.ncbi.nlm.nih.gov/BLAST/) and a translated Petunia x hybrida EST database (created at the Molecular and Cellular Imaging Center, The Ohio State University). Among the 133 spots, 47 up and 26 down regulated spots were identified as single proteins (Table 3.2 and Table 3.3). Relative abundance of each identified protein at 48 and 72 h is also summarized in Table 3.2 and Table 3.3. Forty-seven of the excised spots had more than one protein identified for each spot (Table 3.4 and Table 3.5). Thirteen (11 up and 2 down regulated) spots could not be identified from any database.

**Functional classification based on biological processes**

In order to begin functional analysis of the identified senescence-associated peptides, bioinformatic methods were used to predict the biological function of the identified proteins. Protein sequences were searched against NCBI and SGN Solanaceae databases using the TBLASN program to identify the most similar Arabidopsis genes. The locus identifiers of these Arabidopsis genes were then used to assign the biological
function of the identified proteins based on the TAIR gene ontology (GO) annotation database (http://www.arabidopsis.org/tools/bulk/go/index.jsp). Forty-seven up regulated proteins were classified into 10 groups including the function unclassified proteins and twenty-six down regulated proteins were classified into seven groups including unclassified proteins (Table 3.2 and Table 3.3). The majority of the up regulated proteins were classified as stress and defense response (9), carbohydrate metabolism (9), energy or electron transport (5), proteolysis (5) or lipid metabolism (4) (Figure 3.3A). The majority of down regulated proteins were classified as amino acid metabolism (9), carbohydrate metabolism (6), stress and defense responses (3), or cytoskeleton organization and biogenesis (3) (Figure 3.3B).

**Clustering analysis of protein expression pattern**

During the process of petal senescence, certain groups of protein will likely be co-regulated. To begin detecting such patterns, we applied hierarchical clustering to the 73 proteins from Table 3.2 and Table 3.3. The hierarchical clustering method, as described by Eisen *et al.* (Eisen et al., 1998), makes a dendrogram by calculating the pair wise average-linkage algorithm. The length of the branch on the tree represents the degree of similarity. We used the pair group method with arithmetic mean and correlation coefficient to define the similarity and the average-linkage to assemble the items.

Clustering revealed three groups, which included 24 U and 24 P, 48 U and 72 U,
and 48 P and 72 P, based on expression similarities (Figure 3.4) and corresponding to development stages from flower opening to senescence. Up and down regulated proteins were grouped into two main clusters. There were 8 and 6 subclusters of up regulated and down regulated proteins, respectively. The protein expression pattern of one representative protein from each subcluster is shown in Figure 3.5. In the up regulated clusters, four proteins from SB14-2 to SB14-20 were grouped in the first subcluster. Time course and pollination both affected protein expression, as expression levels in unpollinated and pollinated corollas were generally much higher compared to 0 h. Three proteins including SB14-24, SB51-14 and 36-23 were in the second subcluster. Protein abundance increased in both unpollinated and pollinated corollas at 48 and 72 h. The third subcluster included proteins SB36-19, SB36-14 and SB36-15. Compared to 0 h, expression of these proteins decreased in non-senescing corollas and slightly increased in 48 P and 72 P corollas. Nineteen proteins were included in the fourth subcluster including SB36-13 to SB44-7. This represented the largest subcluster, and included proteins with the highest expression levels at 48 P and 72 P. Most of these proteins were only detected in 48 P and 72 P corollas, and represented proteins involved in catabolic processes including carbohydrate, protein, nucleic acids and lipid degradation. Five proteins in the fifth subcluster, including SB14-51 to SB36-24, remained stable at non-senescing stages, and were highly expressed at 72 P. Nine proteins in the sixth subcluster, including SB36-10 to SB36-30, decreased in non-senescing stages from 24 U and reached the
lowest levels at 72 U, but they increased in abundance at 48 P and had maximum expression at 72 P. The seventh subcluster included only two proteins, SB14-9 and SB50-9, whose expression varied sharply during both time course and treatment. One protein, SB50-11, is included in the eighth subcluster, its expression level significantly increased only in 48 P corollas.

Among the down-regulated proteins, the first cluster included only SB49-24. Protein levels in this cluster were highest at 24 h, decreased at 48 h and then increased at 72 h in the unpollinated corollas. SB49-24 also decreased in 48 P corollas relative to 24 P corollas. Eight proteins were in the second cluster from SB44-15 to SB14-40, and their expression patterns showed that the amount of these proteins declined as the petals aged and after pollination. The decreases following pollination were steeper than those during the development of unpollinated corollas. Two proteins, SB44-5 and SB44-4, were in the third cluster in which the levels increased over time in unpollinated corollas and decreased in pollinated corollas. Fourteen proteins, SB44-23 to SB44-13, were in the fourth cluster, and their accumulation declined only upon pollination. Two proteins, SB44-21 and SB14-34, were in the fifth cluster, and their protein abundance increased in unpollinated corollas during aging and was lower in 72 P corollas when compared to 72 U. Only one protein, SB36-5, was in the sixth cluster. It accumulated from 24 to 48 h and decreased at 72 h in unpollinated corollas compared to 0 h. In the pollinated corollas it increased at 24 h and then decreased to the same level as 0 h at 72 h.
Protein isoforms were identified from multiple spots

Our proteome data showed that a high portion of differentially expressed proteins were identified as isoforms. A protein isoform is a protein with only small differences from other related proteins. Protein isoforms may be produced from distinct, but related genes, or result from alternative splicing or posttranslational modification. Three groups of isoforms were classified based on their expression patterns during senescence (Figure 3.6). The first group included seven isoforms represented by 15 up regulated spots (Figure 3.6A). These proteins included manganese superoxide dismutase (14-23 and 14-52), 1,4-benzoquinone reductase-like (14-22 and 36-17), beta-xylosidase 2 (44-7 and 14-13), vacuolar invertase (14-29, 36-21 and 36-22), lipoxygenase (SB14-6 and SB50-2), trypsin and kunitz-type protease inhibitor family protein (36-19 and 36-20), and an abscisic stress ripening-related protein (50-7 and 50-8). Each of these proteins was identified in two spots. Among these isoform proteins, 14-13 was identified as a C-terminally truncated form of beta-xylosidase 2, which was not detected in unpollinated corollas and increased in abundance from 48 P to 72 P. In addition, three spots from Table 3.4 were also identified as beta-xylosidase 2 including 14-53 which was identified as an N-terminally truncated form of beta-xylosidase 2. Lipoxygenase SB50-2 was up regulated at 48 P, while 14-6 was an N-terminally truncated form (14-6) that was only detected at 48 P and 72 P. The vacuolar invertases (14-29, 36-21 and 36-22) were identified as truncated forms that were accumulated or present 48 P and/or 72 P.
The second group included 3 down regulated isoforms represented by 11 distinct down regulated protein spots (Fig 6B). Four spots were identified as methionine synthase (14-31, 44-1, 44-2 and 44-3). Three of these four protein spots had similar MW with slightly different PIs. Four protein spots were identified as S-adenosyl-L-methionine synthetase (SAMS) (14-36, 14-37, 44-13 and 44-14). Three protein spots were identified as Caffeoyl-CoA-O methyltransferase (44-21, 44-22 and 44-23). The third group included Actin-depolymerizing factor 1 (50-10 and 49-21), Actin-depolymerizing factor 2 (51-14 and 49-22), S-adenosyl-L-methionine:benzoic acid/salicylic acid carboxyl methyltransferase (BSMT2) (50-11 and 14-40) and Photosystem II 23 kDa protein (14-25 and 49-20) (Figure 3.6C). Each of these proteins had two isoforms with opposite expression patterns. Two of the down regulated proteins, ADF1 (49-21) and ADF2 (49-22), were determined to be acetylated at their second amino acid based on the peptide molecular weight. However, we do not know if the two up regulated proteins, ADF1 (50-10) and ADF2 (51-14) were also acetylated, as that same peptide was not sequenced. Based on the peptide sequences, spots 50-11 and 14-40 were identified as BSMT2. Protein 50-11 appears to be a N-terminally truncated form with a different PI. Spot 14-25 was a truncated form of the full-length Photosystem II 23 kDa protein (49-20) and appeared at 48 P and 72 P, while spot 49-20 was down regulated at 48 P and 72 P.
DISCUSSION

2-DE based proteomic approaches identified senescence execution phase proteins

To characterize large-scale protein changes during corolla senescence, we used a 2-DE based proteomic approach to identify proteins that were differentially expressed during pollination-induced senescence. Protein expression profiles were mainly classified into up and down regulation. The number of up regulated proteins identified from 2-D gels was greater than the number of down regulated proteins. Most up regulated proteins were involved in catabolic processes, while the down regulated proteins were involved in anabolic processes. In addition, many of the up regulated proteins identified from our work were stress and defense response and hydrolytic enzymes. This is similar to the functional classes of senescence-associated genes identified from previous transcriptome studies of leaf senescence (Guo et al., 2004; Buchanan-Wollaston et al., 2005; van der Graaff et al., 2006; Gregersen and Holm, 2007) and flower senescence (van Doorn et al., 2003; Breeze et al., 2004; Yamada et al., 2007). As for the down regulated proteins, the majority of them are predicted to be involved in amino acid metabolism, stress and defense responses, carbohydrate metabolism, and cytoskeleton organization and biogenesis. Many of these processes are known to cease at the onset of senescence.

A number of spots were identified as protein isoforms. These isoforms are derived from gene family members, and chemical and truncated post-translational modification. Most of these isoforms have similar accumulation patterns, but a few have opposite
patterns. Isoforms in the up regulated cluster are involved in various biological processes, while the down regulated isoforms are mainly involved in methionine metabolism and S-adenosyl methionine biosynthesis.

**Stress Response and Defense**

It is well known that the expression and activities of antioxidant enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (A POD), and catalase (CAT) are up regulated during senescence or PCD (Swidzinski et al., 2004). In our 2-DE experiments, the accumulation of manganese superoxide dismutase (MnSOD) (14-23 and 14-52), iron superoxide dismutase (FeSOD) (36-16), and the nectarin 1 precursor (14-51) were seen to vary during this process. The nectarin 1 precursor was originally isolated from the protein mixtures that accumulate in the nectar of *Nicotiana* sp (Carter and Thornburg, 2000). Although no protein sequence similarity was found between the nectarin 1 precursor and MnSODs, it was determined to function as an MnSOD by colorimetric and in-gel activity assays (Carter and Thornburg, 2000). The function of all of these proteins is to detoxify the byproducts of respiration and ROS and protect cellular function to allow the senescence program to progress to completion. ROS play a role as secondary messengers in pathogen or other stress triggered PCD (Swidzinski et al., 2004). While plant cells employ the enzymes we identified to tightly regulate the ROS level in response to various stimuli, it is most plausible that they play a more general antioxidant
role during petal senescence.

Annexins are a multigene family of calcium-dependent, membrane-binding proteins that have peroxidase activity. The amount of annexin (36-23) protein increased in senescing corollas at 48 and 72 h after pollination. Coincident with our results, annexin gene expression was found to be enhanced during the senescence of Iris flowers (van Doorn et al., 2003). Although annexins have been well studied in animals, their role in salt stress and ABA-mediated stress responses in plants has only recently been revealed (Lee et al., 2004; Cantero et al., 2006). In Arabidopsis, Annat1, an annexin family member, is expressed in flowers, roots, leaves and stems, but is most abundant in stems (Clark et al., 2001). Annat1 expression is also induced in response to both oxidative stress and salicylic acid (Lee et al., 2004). Heterologous expression of Annat1 in E. coli rescues the ΔoxyR mutant from H₂O₂ stress, implying that this protein plays a role in oxidative stress responses (Gidrol et al., 1996). Annexin was shown to be present as a mixture of monomers and homodimers. Homodimerization is dependent on the presence of H₂O₂ or Ca²⁺, suggesting that annexins could function in oxidative stress as well as in calcium signaling pathways (Kopka et al., 1998; Clark et al., 2001; Gorecka et al., 2005). Although this protein has been argued to act as an antioxidant enzyme by eliminating H₂O₂ and protecting the cells from oxidative stress and apoptosis (Gorecka et al., 2005), further studies are required to elucidate the specific role of annexins in petal senescence.
Protein 36-23 is similar to tobacco NtPRp27, which was identified as a secreted protein in tobacco BY2 cells (Okushima et al., 2000). NtPRp27 is a pathogenesis-related (PR) protein, and NtPRp27 transcripts accumulate in response to various stresses including infection by TMV and wounding. It is also induced by the application of hormones including ethylene, ABA, JA and SA (Graham et al., 2002). NtPRp27 is mainly expressed in tobacco roots and slightly in flowers (Okushima et al., 2000). Given previous studies on NtPRp27, this protein is speculated to play a role in stress responses during petal senescence.

Auxin-response-like protein 50-4 is similar to Arabidopsis Dwarf in Light1 (DFL1). DFL1 belongs to an auxin-responsive GH3 gene family, functioning as an indole-3-acetic acid amido synthetase involved in auxin signal transduction (Nakazawa et al., 2001). The GH3 family is among the early or primary auxin responses and members of this gene family can negatively regulate auxin responses within minutes (Nakazawa et al., 2001)(Hagen and Guilfoyle, 2002). CcGH3, a GH3 gene in pepper, is highly expressed during the late stage of fruit ripening rather than the early stage, although auxin levels are usually higher at earlier stages. This indicates that auxin is not sufficient to induce CcGH3 expression in pepper fruits (Liu et al., 2005). Analysis showed that CcGH3 had both auxin and ethylene responsive elements within its promoter and that CcGH3 expression was mainly responsive to ethylene during fruit ripening (Liu et al., 2005). Furthermore, tomato fruit ripening in transgenic plants that overexpressed CcGH3
was faster than in controls following exogenous ethylene treatment (Liu et al., 2005).

These results also indicate that \textit{CcGH3} expression may be regulated by ethylene or that the signal crosstalk between auxin and ethylene during fruit development and ripening is involved in its regulation (Liu et al., 2005). This suggests that a CH3-like protein identified in our 2-DE experiments may be regulated by the ethylene during pollination-induced petal senescence.

\textbf{Carbohydrate metabolism and cell wall degradation}

Both carbohydrates and proteins are major components of plant cell walls. During senescence, carbohydrates need to be degraded and recycled from the senescing tissues to the growing portions of the plant. In our experiments a number of proteins were identified that may have a putative function in cell wall degradation. These results are consistent with the idea that once flowers are pollinated, carbohydrates are recycled from the loose cell wall of corollas to developing organs such as ovaries.

Beta-xylosidase 2 is an enzyme involved in cell wall polysaccharide disassembly or modification and may function in cell wall degradation during fruit ripening (Itai et al., 2003; Hayama et al., 2006). The up regulation of N- and C-terminally truncated forms (spot 14-53 and 14-13) of the beta-xylosidase 2 protein during senescence suggest that it may be targeted for degradation late in the senescence program or that post translational cleavage may be required to activate the enzyme during senescence. Interestingly,
another family member, beta-xylosidase 1, was also identified as an up regulated protein with a similar protein expression pattern as beta-xylosidase 2 during corolla senescence. Previous studies have shown that transcripts of beta-xylosidase 1 (LEXYL1) and beta-xylosidase 2 (LEXYL2) increased during tomato fruit ripening, but their gene expression patterns were different. LEXYL1 mRNA levels increased at the late fruit ripening stage, while LEXYL2 mRNA levels were higher in premature fruit and were much lower at the late ripening stage (Itai et al., 2003).

Sucrose metabolism is a key component of sink strength in flowers during PCD (van Doorn and Woltering, 2004). Invertase catalyses the hydrolysis of sucrose into glucose and fructose, and this enzyme is considered to play a key role in carbohydrate metabolism and the regulation of sucrose transport (HaouazineTakvorian et al., 1997). Induction of INVERTASE (INV) gene expression is coincident with the onset of PCD during flower development in four-o’clock flowers (Mirabilis jalapa L.) (van Doorn and Woltering, 2004). Recent research revealed that broccoli floret senescence was delayed in antisense acid invertase transgenic plants (Eason et al. 2007). In our experiments, three N-terminally truncated invertase proteins (14-29, 36-21 and 36-22) either were newly detected or were up regulated during corolla senescence, suggesting that these proteins might be potential candidates for manipulating flower senescence.

In contrast to the induction of proteins involved in cell wall degradation, many proteins that function in cell wall synthesis show reduced accumulation as senescence
proceeds. For example, three Caffeoyl-CoA O-methyltransferases (CCoAOMT) were found to be down regulated at senescence stages in our 2-DE experiments. CCoAOMT catalyzes the production of the lignin precursor, feruloyl-CoA, by utilizing SAM and caffeoyl-CoA (Zhong et al., 1998; Grimmig et al., 1999). Repression of CCoAOMT gene expression in transgenic tobacco and poplar plants dramatically reduces lignin content, supporting the role of CCoAOMTs in lignin biosynthesis (Zhong et al., 1998; Zhong et al., 2000). In addition, CCoAOMT is induced following pathogen infection to function in plant defense by enhancing cell wall strength (Matern et al., 1995; Grimmig et al., 1999; Maury et al., 1999). Recently, expression of this gene has been found at early stages of flower development in morning glory (Ipomoea nil), suggesting that this gene is involved in cell wall growth during petal expansion (Yamada et al., 2007). In our 2-DE experiments, three different CCoAOMTs were down regulated after pollination, concomitant with petal wilting. We speculate that high abundance of CCoAOMTs at the early stage of flower development might be correlated with cell expansion and once pollination occurs and senescence is initiated in the corollas, CCoAOMTs are down regulated.

**Cytoskeleton Organization and Biogenesis**

Actins are important cytoskeletal proteins encoded by diverse gene families. They make up a fundamental element of cellular cytoskeletons. In petunia, for example, a
superfamily of actins includes about 100 to 200 members that are divided into at least six highly divergent subfamilies (Mclean et al., 1990). In our 2-DE experiments, an actin protein, 14-30, was also found to be up regulated during corolla senescence, but the function of this protein during senescence is not clear.

Actin-depolymerizing factor (ADF) is one of the small actin-binding proteins that modulates actin cytoskeleton dynamics in eukaryotic cells (Mun et al., 2002; Thomas et al., 2006). In our 2-DE experiments, two PhADF1 (50-10 and 49-21) and two PhADF2 (50-14 and 49-22) proteins were identified. PhADF1 and PhADF2 encode polypeptides of 139 and 143 amino acids with a calculated molecular mass of 16.04 and 16.51 kDa, respectively. Previous studies have shown that the petunia PhADF1 and PhADF2 genes are highly expressed in petals, leaves and stems (Mun et al., 2000). Immunoblots showed that PhADF1 protein abundance significantly decreases in senescing petals (Mun et al., 2002). Interestingly, the two ADF1 proteins and the two ADF2 proteins showed opposite expression patterns in our experiments. Our data showed that the down regulated PhADF1 and PhADF2 isoforms were acetylated at the second amino acid of the N-terminus. We do not know if the two up regulated proteins, ADF1 (50-10) and ADF2 (51-14), were also acetylated, as that same peptide was not sequenced. Although there is no evidence to indicate that acetylation regulates ADF function, reversible phosphorylation of ADF proteins regulated by calmodulin-like domain protein kinase (CDPK) and phospholipase C affects the interaction between ADF and actins in maize
This indicates that post-translational modification plays an important role in the regulation of ADF function.

The actin cytoskeleton is a major target and regulator of signaling cascades responding to the internal and external stimuli in both animal and plant cells (Schmidt and Hall, 1998; Staiger, 2000). Overexpressing or supressing \textit{ADF1} in Arabidopsis transgenic plants affects F-actin organization, flowering, and cell and organ expansion, indicating that ADF proteins are key regulators for plant development (Dong et al., 2001). In addition, it has been reported that the dynamics of the actin cytoskeleton and the rate of actin polymerization or depolymerization is sufficient to induce PCD in yeast and animal cells. This PCD involves a caspase cascade that is activated following the cleavage of endogenous nuclease inhibitors (Posey and Bierer, 1999; Suria et al., 1999; Morley et al., 2003; Gourlay et al., 2004; Gourlay and Ayscough, 2005). Similarly, recent studies on PCD in self-incompatible \textit{Papaver rhoeas} pollen revealed that actin dynamics or polymer levels stimulate the activity of a caspase-3 like enzyme (Thomas et al., 2006). TUNEL assays further showed that both inhibition and stabilization of actin depolymerization could induce PCD (Thomas et al., 2006). Therefore, up and down regulations of PhADFs in our 2-DE experiments, highly suggests that these proteins may function as signals that trigger or modulate PCD during corolla senescence.
Nucleic Acid Catabolism

Nuclear condensation and nucleic acid degradation are well known hallmarks of PCD. 2-DE profiling identified an endonuclease (14-12) that was detected only at 48 P and 72 P. This endonuclease is most similar to an endonuclease (StEN1) from potato (Larsen, 2005). This endonuclease belongs to the nuclease I family, which can degrade single stranded DNA and RNA. Endonucleases have been identified as functioning during various plant growth and developmental processes including seed germination (Dominguez et al., 2004), xylem differentiation (Thelen and Northcote, 1989; Ito et al., 2002), stress response (Muramoto et al., 1999) and senescence (Perez-Amador et al., 2000; Xu and Hanson, 2000; Langston et al., 2005; Lers et al., 2001; Yamada et al., 2006; Yamada et al., 2006). The MW of this protein (43 kDa) is similar to that of a bifunctional nuclease, PhNUC1, which was previously shown to be up regulated during petunia petal senescence. In-gel activity assays have demonstrated that PhNUC1 activity required glycosylation (Langston et al., 2005). The amino acid sequence from other plant species indicate that type I nucleases have a molecular mass in the range of 31–39 kDa, and the larger MW of protein 14-12 may be explained by glycosylation or other post-translational modifications (Perez-Amador et al., 2000).
Methionine metabolism and S-adenosyl methionine biosynthesis

The synthesis of S-adenosyl-L-methionine (SAM or AdoMet), catalyzed by SAM synthetase from L-methionine utilizing ATP, is very important. SAM is not only an universal methyl-group donor for many biochemical pathways such as the methylation of nucleic acids, proteins, and lipids, but it is also a substrate for various plant biosynthesis pathways such as those for ethylene, polyamines, lignin and volatile fragrance and aroma compounds (Yang and Hoffman, 1984; Kende, 1993; Underwood et al., 2005; Roje, 2006). During senescence, hydrolytic enzymes involved in catabolic processes are up regulated, while enzymes involved in anabolic processes, such as methionine metabolism and cell wall biosynthesis are suppressed. Shen et al., (2005) showed that more than a 200-fold increase in free Methionine levels was observed in an Arabidopsis SAM synthetase 3 mutant (mto3) compared with the wild type. In our experiments, Methionine synthases and SAM synthetases, two important protein families involved in amino acid metabolism, as well as other processes, were suppressed during senescence. Because 80% of the methionine is consumed in SAM synthesis (Ravanel et al., 1998), the reduction of methionine synthases and SAM synthetases is correlated with the decreased demand on SAM biosynthesis and methionine usage during senescence.

In addition, the composition of the cell wall can be modified by differences in the levels of these enzymes. Evidence shows that plants respond to stresses by modifying cell wall components through changes of corresponding enzyme activities (Espartero et al.,
content is significantly decreased, supporting the hypothesis that lignin is a major metabolic sink for SAM (Shen et al., 2002). Studies have also showed that expression of SAM synthetase is induced in lignifying tissues of tomato plants responding to salt stress, indicating that lignification in vascular tissues is regulated by changes in SAM synthetase expression during stress responses (Sanchez-Aguayo et al., 2004). In our experiments, the down regulation of SAM synthetases, coordinated with the previously mentioned reduction of Caffeoyl-CoA O-methyltransferases, strongly suggesting that lignification levels decrease during petal senescence.

Floral scent is reduced upon pollination

The molecular and biochemical mechanisms underlying floral scent changes after pollination have been studied in many flower species such as petunia, snapdragon and rose (Lavid et al., 2002; Negre et al., 2003; Underwood et al., 2005; Spitzer et al., 2007). For example, among flower volatile organic compounds (VOCs), methyl benzoate is one of the major scent compounds emitted by these flowers (Dudareva et al., 2000; Kolosova et al., 2001; Verdonk et al., 2003). It is known that S-adenosyl-L-methionine (SAM) is the methyl donor for methyl benzoate synthesis and SAM synthesis is catalyzed by SAM synthetase (Kolosova et al., 2001; Negre et al., 2003). In our 2-DE experiments, protein abundance of multiple SAM synthetases was reduced at 48 h after pollination, suggesting
that SAM production was reduced significantly after pollination when flowers do not need to attract pollinators by emitting scent compounds.

SAM: benzoic acid/ salicylic acid carboxyl methyltransferases (BSMT) participate in the last step of methyl benzoate synthesis in snapdragon and petunia (Dudareva et al., 2000; Dudareva and Pichersky, 2000; Negre et al., 2003). In petunia, two SAM: benzoic acid/ salicylic acid carboxyl methyltransferase (PhBSMT1 and PhBSMT2) genes were identified, and their protein sequences are almost identical except for a three amino acid difference at the C-terminus (Negre et al., 2003). PhBSMT1 and PhBSMT2 gene expression and synthesis of methyl benzoate were inhibited by pollination-induced ethylene, indicating that pollination and pollination-induced ethylene not only plays an important role in regulating petal senescence but also has a role in regulating the emission of floral scent in petunia (Negre et al., 2003; Underwood et al., 2005). It has been reported that the emission of methyl benzoate was reduced up to 99% in transgenic PhBSMT RNAi flowers, and this decrease corresponded to suppressed PhBSMT mRNA levels (Underwood et al., 2005). These results demonstrate that PhBSMT1 and PhBSMT2 are responsible for synthesis of methyl benzoate in petunia. In our 2-DE experiments, the protein abundance of one PhBSMT2 (14-40) was reduced at 48 and 72 h after pollination, while another PhBSMT2 predicted as an N-terminally truncated form was induced at 48 and 72 h after pollination, supporting previous studies that the reduction of PhBMST protein is regulated at both the transcriptional and
post-translational levels after fertilization (Negre et al., 2003).

**Proteolysis mediated by proteases and the proteasome during corolla senescence**

Proteolysis is critical for plant growth, development, and defense and various proteases are responsible for selective protein degradation, protein maturation and localization by post-translational processing (reviewed in Schaller, 2004). Protein degradation always accompanies leaf and flower senescence (Jones 2004; Pak and van Doorn, 2005). One major category of proteins that we identified in our 2-DE experiments was proteolytic enzymes. Proteins involved in proteolysis including cysteine proteases, proteasome delta subunit, ubiquitin-conjugating enzyme family protein and serine carboxylase II-2 were identified as being up regulated in our 2-DE experiments, further supporting that protein catabolism is one of the major events during senescence.

Proteins involved in the ubiquitin-proteasome pathway have been suggested to be not only involved in protein degradation, but to participate in the regulation of signaling pathways including those regulating senescence and other plant development processes as well as stress and defense responses (Roberts et al., 2002; Vierstra, 2003). For example, *Nicotiana benthamiana* 26S proteasome subunit RPN9 was reported to function in virus defense by regulating vascular tissue formation, and this regulation has been illustrated by targeting a subset of regulatory proteins involved in both auxin transport and brassinosteroid signaling (Jin et al., 2006). In addition, a proteasome inhibitor
(Z-leu-leu-Nva-H) has been applied to slightly delay *Iris* tepal senescence, indicating that the proteasome may play a role in the induction of senescence in this plant (Pak and van Doorn, 2005). More recently, Vacca et al., (2007) showed that proteasome function is required for activation of programmed cell death in heat shocked tobacco Bright-Yellow 2 cells. Taken together, these studies imply that specific targeting of proteins for degradation could be an important mechanism for senescence regulation (Buchanan-Wollaston et al., 2005).

Another major protein category responsible for protein degradation comprises the cysteine proteases, which have been identified from many different flower species including carnation (Jones 1995), *Alstroemeria* (Wagstaff et al., 2002; Breeze et al., 2004), *Sandersonia* (Eason et al., 2002) and Petunia (Jones et al., 2005). In our experiments, we identified four different cysteine proteases (14-50, 14-45, 14-19 and 14-14). Although PhCP5 and PhCP10 were identified from the spots that have multiple proteins (see Table 4 and Table 5), they have been reported in previous petunia flower senescence studies (Jones et al., 2005). Jones et al., (2005) have shown that six out of nine cysteine protease genes from petunia, including *PhCP5* and *PhCP10*, were up regulated with different temporal patterns during petunia flower senescence. *PhCP10* is orthologous to *SAG12*, a senescence-specific gene which was identified from *Arabidopsis* leaves. The *PhCP5* expression profile in wild type and ethylene insensitive transgenic lines showed that *PhCP5* might be ethylene independent and regulated by
aging (Jones et al., 2005). Phylogenetic analysis showed that PhCP5 was highly homologous to cysteine proteases from ethylene insensitive flowers including iris (van Doorn et al., 2003), Sandersonia (Eason et al., 2002) and daffodil (van Doorn et al., 2004).

The cysteine protease P21 (14-50) was first identified from non-regenerating Petunia x hybrida cali that was subcultured on a low concentration of cytokinin media (Tournaire et al., 1996). P21 is very similar to SAG2 which is an Arabidopsis senescence-associated thiol protease (Hensel et al., 1993). Northern blot analysis showed that P21 is highly expressed in senescing leaves and mature flowers (Tournaire et al., 1996). According to previous studies on orthologous cysteine proteases including barley aleurain (Rogers et al., 1985), rice oryzain y (Watanabe et al., 1991) and human cathepsin H (Fuchs et al., 1988), it was postulated that the N-terminal signal peptide of P21 might be proteolytically cleavage during senescence to activate the protease (Tournaire et al., 1996). In our experiments, the molecular mass of P21 is about 30 kDa, smaller than the theoretical 39 kDa, which is concomitant with previous reports and could be indicative of signal peptide cleavage. Recently, an aleurain-like cysteine protease BoCP5 was increased during the harvest-induced senescence of broccoli florets and leaf tissues. In the antisense transgenic lines, postharvest floret senescence (yellowing) is delayed, and florets contain significantly greater chlorophyll levels during postharvest storage at 20 °C than wild-type plants (Eason et al., 2005). These results suggest that suppression of
certain cysteine proteases might delay flower senescence.

Serine carboxylase (SCP) is the largest class of proteases in plants. SCP as well as the chymotrypsin, subtilisin, share a common structural feature called the “catalytic triad”, which constitutes of a serine, an aspartic acid, and a histidine residue. In Arabidopsis, there are 51 serine carboxypeptidase homologs, and all of them have signal peptides at the N-terminus for endoplasmic reticulum targeting (Fraser et al., 2005). Serine carboxylase is highly express in the aleurone layer during wheat seed germination (Daldegan et al., 1994; Dominguez and Cejudo, 1998; Dominguez et al., 2002), as well as in the vascular tissues of germinated wheat seedlings in which cells were undergoing PCD (Dominguez et al., 2002). This indicates that this serine carboxylase not only functions in endosperm storage protein mobilization, but also participates in PCD during seed germination. Additional serine carboxylases are involved in wound stress (Walkersimmons and Ryan, 1980; Moura et al., 2001; Granat et al., 2003), and hormone signaling (Cercos et al., 2003; Li et al., 2001; Zhou and Li, 2005).

**Lipid degradation**

Membrane deterioration is one of the early events during senescence, and lipid degradation during senescence has been reviewed previously (Thompson et al., 1998). This process is coordinated with other catabolic processes of senescence such as the degradation of nucleic acids, proteins, and carbohydrates. In our experiments, a number
of enzymes functioning in this process, such as lipoxygenase and lipase were up
regulated during senescence. These findings support previous reports that membrane
breakdown is an active process during petal senescence. Moreover, evidence has been
shown that the maintenance of membrane integrity is important to prolong senescence.
For examples, a lipase gene was induced following ethylene treatment at the onset of
carnation petal senescence (Hong et al., 2000), and transgenic Arabidopsis with
suppressed lipase expression have delayed leaf senescence (Thompson et al., 2000). In
addition, antisense transgenic Arabidopsis plants with reduced expression of the acyl
hydrolase gene also showed a retarded senescence process (He and Gan, 2002). Taken
together, these studies suggest that proteins involved in lipid degradation are not only
important for the catabolic processes that execute senescence, but they might also
regulate the onset of senescence.

**Proteins of biological process unclassified**

*Putative acid phosphatase*

In our experiments, an N-terminally truncated putative acid phosphatase (36-27)
was identified from the up regulated protein spots, suggesting that the truncated acid
phosphatase might be either activated by post-translational processing or be degraded
during petal senescence. Acid phosphatase gene expression accumulates in response to Pi
starvation (Devaiah et al., 2007). Studies in leaf and petal senescence have shown that
phosphorus could be one of the targets for nutrient remobilization during senescence 
(Himelblau and Amasino, 2001; Chapin and Jones, 2007). In addition, protein 36-27 was 
also similar to tomato Acid Phosphatase-1 (APS-1) isolated from the cell cultures of a 
nematode resistant tomato cultivar (Williamson and Colwell, 1991). More evidence has 
shown that acid phosphatase gene expression responds to oxidative and salt stresses as 
well as the ABA and jasmonic acid treatments (del Pozo et al., 1999). These studies 
suggest that protein 36-27 might be involved in both phosphorus remobilization and 
stress responses. Further investigation is required to reveal its specific function during 
petal senescence.

*Similar to abscisic stress ripening protein*

Two protein spots (50-7 and 50-8) were identified as similar to abscisic stress 
ripening protein 2 (ASR2/DS2). The ASR (ABA, stress and ripening) gene family 
includes ASR1, ASR2, ASR3 and ASR4. ARS1, 3 and 4 are induced by dehydration, 
ABA and cold stresses, whereas ASR2 is ABA-independent and is specifically induced 
by dehydration (Doczi et al., 2005). Overexpression of tomato ASR1 or lily ASR1 in 
transgenic tobacco or Arabidopsis enhances tolerance to drought and salt stresses 
(Goldgur et al., 2007). ASR2 (DS2-type) genes were isolated in Solanum chacoense 
(ScDS2), Solanum tuberosum (StDS2), and Solanum lycopersicum (LeDS2) (Silhavy et 
al., 1995; Doczi et al., 2002; Doczi et al., 2005). Sequence analysis showed that DS2-type
proteins have a unique signature among the ARS family, which is a ten repeat hydrophilic GDDNK/TYGEKTSYG consensus sequence (Silhavy et al., 1995; Doczi et al., 2005). Expression analysis using promoter::GUS fusion lines showed that DS2 not only responds to dehydration but also is expressed in pollen, flowers and fruits, suggesting that it is regulated by both developmental cues and stress stimuli (Doczi et al., 2002; Doczi et al., 2005). The grape ARS1 ortholog and DS2 have also been hypothesized to be transcription factors (Silhavy et al., 1995; Cakir et al., 2003). Stress response proteins are induced during petal senescence, accompanied with petal dehydration and wilting. Therefore, these results suggest that the DS2-type proteins identified in our experiments might be a potential regulator involved in the ABA independent dehydration response during petal senescence.

While the majority of the identified proteins in our 2-DE experiment are involved in the processes of stress and defense responses, and macromolecule degradation, regulatory proteins, such as protein signal receptors and transcription factors, were not identified. This is not surprising because these regulators are usually encoded by low copy number genes and the proteins are transiently expressed at very low levels in the cell. It is also because we compared the total soluble proteins from whole corollas instead of membrane bound proteins in specific cell fractions. Therefore, special experimental design and techniques will be critical to characterize those proteins. For example, to identify the signal receptors, we might need to enrich the membrane proteins
from cells, as some of those proteins are located in the membranes. To identify transcription factors we might need to isolate nuclear proteins. Another factor that affected the detection of low abundant proteins is the sensitivity of the staining technique. The Gel-Code Blue staining based 2-DE approach is not sensitive enough to detect very small protein changes on the gels. To enhance sensitivity, silver staining, CYPRO Ruby staining or other specific staining methods including phosphorprotein staining might be helpful. However, just like other techniques, the 2-DE approach has its limits even though 2-DE techniques are improving. Therefore a combination of proteomic and genomic tools is essential to reveal the molecular and biochemical mechanisms of senescence.

CONCLUSION

Senescence is responsible for the recycling of nutrients and energy reserves in plants. Macromolecules and organelles are degraded and nutrients are exported to sink organs such as ovaries, fruits and seeds during senescence. The initiation, regulation and execution of senescence are coordinated by the regulation of gene expression, protein synthesis and enzyme activities. The proteins identified from our 2-DE studies not only support previous studies of gene changes at the transcriptional level during senescence, but also provide new insights into the post-translational regulation of senescence.
MATERIALS AND METHODS

Experimental design

*Petunia x hybrida* cv Mitchell Diploid petals (collectively, corollas) were collected from unpollinated and pollinated flowers at 0, 24, 48, and 72 h after flower opening. The experiment used a randomized complete block design with three replicates for each time point. Each replicate consisted of eight petals collected from at least three different plants and pooled prior to protein extraction. Each block contained pollinated and unpollinated corollas and three time points (24, 48 and 72 hours).

Prior to analysis, the distribution of protein intensities and residuals was inspected using the Univariate procedure of SAS. Transformation of the data was necessary in order to approximate a normal distribution. Both log_e [LN(value+1)] and log_{10} [Log(value+1)] transformations of the data approximated normal distributions, and the LN transformation was subsequently applied across all quantitative data prior to statistical analysis. Analysis of variance was conducted using a mixed model procedure in the SAS software and included main effects for protein, treatment (pollinated or unpollinated), time, and replicate. All factors were considered fixed. Sufficient degrees of freedom were available to test all two-way interactions which were included in the statistical model for analysis of variance.
**Protein extraction and 2-DE separation of proteins**

Unless otherwise indicated, all chemicals were purchased from Fisher Scientific (Fair Lawn, NJ, U.S.A.). Approximately 2.5 g corollas, including limbs and tubes, were ground in liquid nitrogen, then mixed with 8 ml of homogenization buffer (100 mM Hepes-KOH, pH 7.5, 5% glycerol, 15 mM EGTA [ethylene glycolbis (β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid], 5 mM EDTA, 0.5% polyvinylpyrrolidone, 3 mM dithiothreitol [DTT], 60 μl proteinase inhibitor cocktail (Sigma-Aldrich, St. Louis, U.S.A.). The homogenization was centrifuged at 10,000xg for 10 min at 4°C. After centrifugation, the supernatant was mixed with an equal volume of Tris-buffered phenol (pH 8) (EMD Chemicals Inc., Darmstadt, Germany) and was vortexed and incubated on ice for 5 min. The protein mixture was then centrifuged at 10,000xg for 5 min at 25°C. The phenol phase was back-extracted three times with back-extraction buffer (100 mM Tris, pH 8.4, 20 mM KCl, 10 mM EDTA, and 0.4% β-mercaptoethanol) and was precipitated with 5x vol of 100 mM ammonium acetate in methanol (vol/vol) at –20°C for 30 min. After washing the pellet with 80 % acetone, the protein pellet was suspended in 800 μl of rehydration buffer (8 M urea, 2 M Thiourea, 4 % 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS), 0.2% immobilized pH gradient (IPG) buffer, (pH 5 to 8; Bio-Rad Laboratories, Hercules, CA), 1% Trixon X-100, 0.5 % DTT). The suspended protein was sonicated for 2 min in a bath sonicator (Model PC5; L&R Manufacturing Co., Kearny, NJ), and was placed on a
shaking block at 120 rpm for 1.5 h at RT. The protein concentration was determined with the Amersham Biosciences 2-D quantification kit (Amersham Biosciences Corp., Piscataway, NJ).

Two hundred micrograms of total protein for each sample was rehydrated on 11-cm IPG strip (pH 5-8; Bio-Rad Laboratories, Hercules, CA) and then programmed isoelectric focusing (IEF) was conducted using the Protean IEF Cell (Bio-Rad Laboratories, Hercules, CA). Rehydration was running under passive conditions: 0 volts, 20 °C, 12-16 h, and IEF was running under the preset program: Start Voltage 0V, End Voltage 8000 V, Total Volt-Hours 35000V-hr at 20 °C. After IEF, IPG strips were equilibrated in equilibration buffer 1 (6 M Urea, 0.375 M Tris pH 8.8, 2 % SDS, 20 % glycerol, 2 % (w/v) DTT) and buffer 2 (6 M Urea ,0.375 M Tris pH 8.8, 2 % SDS, 20 % glycerol, 2.5 % (w/v) iodoacetamide), each for 10 min. The equilibrated strips were then subjected to SDS-PAGE (12.5 %) for second dimension separation. After running 2 h under constant 200 V, gels were stained with GelCode Blue (Pierce, Rockford, IL).

**Data analysis of 2-D gels**

Three replicate gels (3 biological replications of 8 corollas each) from each time point were analyzed. Gel images were analyzed with PDQuest v. 7.40 (Bio-Rad Laboratories, Hercules, CA, U.S.A.). For each time point, three replicate gel images for
pollinated and pollinated corollas were imported as a dataset. A synthetic Gaussian image from one of three replicate gel images was created as a reference for all subsequent quantification and protein analyses. Protein spots were visually inspected after automated detection and matching with the reference gel. To validate matching and spot intensities, manual editing and rematching among the images was needed. Due to the differences in protein loading and inaccurate intensities from saturated spots, the detected protein spots on each image were normalized with the reference gel based on the total protein pixel intensity.

Identification of proteins by mass spectrometry

Protein digestion and sequencing were conducted at the Cleveland Clinic Proteomics Laboratory. Protein spots of interest between pollinated and unpollinated corollas were excised from the SDS-PAGE gels as closely as possible to minimize excess polyacrylamide, divided into a number of smaller pieces and washed/destained in 50 % ethanol, 5 % acetic acid. The gel pieces were then dehydrated in acetonitrile and dried in a Speed-vac. In-gel proteolytic digestion using trypsin was accomplished by adding 5 μL 20 ng μL⁻¹ trypsin in 50 mM ammonium bicarbonate and incubating overnight at room temperature to achieve complete digestion. The peptides that were formed were extracted from the polyacrylamide in two aliquots of 30 μL 50 % acetonitrile with 5 % formic acid. These extracts were combined and evaporated to <30 μL for LC-MS analysis. The LC-MS
system was a Finnigan LCQ ion trap mass spectrometer system. The HPLC column was a self-packed 8 cm x 75 μm id Phenomenex Jupiter C18 reversed-phase capillary chromatography column. Two μL volumes of the extract were injected and the peptides eluted from the column by an acetonitrile/0.05 M acetic acid gradient at a flow rate of 0.2 μL/min were introduced into the source of the mass spectrometer on-line. The microelectrospray ion source is operated at 3.0 kV. The digest was analyzed using the data dependent multitask capability of the instrument acquiring full scan mass spectra to determine peptide molecular weights and product ion spectra to determine amino acid sequence in successive instrument scans. This mode of analysis produces approximately 2500 collisionally induced dissociation (CID) spectra.

The CID spectra data were searched against both the NCBI non-redundant protein database using the Mascot search program and the translated Petunia x hybrida EST database (created by MCIC, Ohio State University) using the Sequest search program. All matching spectra were verified by manual interpretation. If samples were identified by both the NCBI non-redundant protein database and translated Petunia EST database searches, the peptides identified in the Petunia search were compared to the NCBI identification. When this comparison indicated that the Petunia EST and NCBI entries were homologous, the NCBI database entry and the EST accession numbers were reported. For samples that were identified by the Petunia database search but not the NCBI search, the peptide sequences identified were used in a FASTA search in order to identify
homologous NCBI database entries. Samples that were not identified by either the NCBI or translated Petunia EST searches were then subjected to a search of a translated *Nicotiana benthamiana* and *Solanum tuberosum* EST database (created by Ohio State University) or they were identified by manual interpretation of the spectra.

**Protein expression pattern clustering**

Hierarchical clustering was conducted using Cluster software version 2.11 (http://rana.lbl.gov/EisenSoftware.htm). Protein expression patterns were clustered as described in the software user manual. Input data of protein expression were the log ratios from which percent volume of each protein spot at each time point were divided by percent volume of the same protein spot at 0 h. Correlation coefficient was chosen to define the similarity and the average-linkage to assemble the items.

**Functional classification based on biological processes**

In order to begin functional analysis of the identified senescence-associated peptides, bioinformatic methods were used to predict the functions of identified proteins. Protein sequences were searched against NCBI and SGN Solanaceae databases using the TBLASN program to identify the most similar Arabidopsis genes. Once most similar gene in Arabidopsis was identified, the locus identifier accession number of this gene was used to search in the TAIR gene ontology (GO) annotation database.
(http://www.arabidopsis.org/tools/bulk/go/index.jsp) to assign a biological function categorization.

ACKNOWLEDGMENTS

This work was supported by an OARDC Research Enhancement Competitive Grant, the Fred C. Gloeckner Foundation and the Ohio State University D.C. Kiplinger Endowment. We thank The Ohio State University Plant-Microbe Genomics Facility for use of PDQuest software, and David Mandich for excellent technical assistance. We thank Dr. Sophien Kamoun (OSU, Plant Pathology Dept) for sharing the Nicotiana benthamiana translated EST database and Ian Holford at the OARDC MCIC for bioinformatics assistance.
<table>
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<th>Time points and Treatments</th>
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<th>48 P vs 48 U</th>
<th>72 P vs 72 U</th>
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<tr>
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<td>Upregulated proteins $^x$</td>
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<td>Total %</td>
<td>No.</td>
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<td>0</td>
<td>74</td>
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<tr>
<td>Downregulated proteins $^y$</td>
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<td>0</td>
<td>41</td>
</tr>
<tr>
<td>Total</td>
<td>0</td>
<td>0</td>
<td>115</td>
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</table>

Table 3.1. Profiling of differentially expressed proteins during petunia corolla senescence.

$^x$ Proteins were defined as up regulated if they were not detected in unpollinated petals and were detected in pollinated petals or if they increased in abundance in pollinated petals.

$^y$ Proteins were defined as down regulated if they were detected in unpollinated but not in pollinated petals or if they decreased in abundance in pollinated petals.

$^z$ Significant differences among treatments within a timepoint were detected by the t-test (P$\leq$0.05).
Table 3.2. Functional classification of petunia proteins that were up regulated during pollination-induced corolla senescence.

Proteins were classified based on the biological processes according to homology to genes in the TAIR Gene Ontology database. Sample number indicates the spot sample for in-gel digestion in the sequencing reports. These sample numbers (without dash) were also used to indicate spots on the 2-D gels in Figure 3.2.

Matched Peptides indicate total number of peptides that matched to other proteins. The first and the second number in the parenthesis indicate the peptides with exact and homologous matches respectively.

Accession number indicates the sequence in the searched database identified from either NCBI non-redundant protein database or SGN petunia EST database (http://www.sgn.cornell.edu/index.pl).

Obs. pI/Mr, observed pI/Mr for each protein was calculated from the 2D-gels with Image PDQuest 7.4.0 software according to standard marker proteins.

Theor. pI/Mr, theoretical pI/Mr of the matched proteins.

Peptide coverage refers to the percentage of sequence coverage of the matched protein.

Mean % volume is the average of three replicated spot pixel intensities at 48 U, 48 P, 72 U and 72 P.

U is hours after flowers are fully opened without pollination (unpollinated).
P is hours after flowers are fully opened with pollination.

ud, spot volume was undetectable on the gel.
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<tr>
<th>Sample No.</th>
<th>Putative proteins and their biological process categories</th>
<th>Matched Peptides</th>
<th>Matched Species</th>
<th>Protein Accession No.</th>
<th>Observed Mr./PI</th>
<th>Theoretical Mr./PI</th>
<th>Peptide Coverage</th>
<th>Mean % Volume</th>
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<td>50-9</td>
<td>Plasma membrane polypeptide</td>
<td>16(4,12)</td>
<td><em>Nicotiana tabacum</em></td>
<td>2764992</td>
<td>24/5.1</td>
<td>22.9/5.0</td>
<td>62%</td>
<td>1068</td>
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<tr>
<td>14-23</td>
<td>Manganese superoxide dismutase</td>
<td>8 (4,4)</td>
<td><em>Gossypium hirsutum</em></td>
<td>3219353</td>
<td>26/6.9</td>
<td>22.1/8.5</td>
<td>53%</td>
<td>701</td>
</tr>
<tr>
<td>14-52</td>
<td>Manganese superoxide dismutase</td>
<td>7 (4,3)</td>
<td><em>Gossypium hirsutum</em></td>
<td>3219353</td>
<td>28/6.6</td>
<td>22.1/8.5</td>
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<td>563</td>
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<td>36-16</td>
<td>Iron superoxide dismutase</td>
<td>8 (6,2)</td>
<td><em>Solanum lycopersicum</em></td>
<td>33413303</td>
<td>26/6.0</td>
<td>27.9/6.6</td>
<td>34%</td>
<td>766</td>
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<td>14-51</td>
<td>Nectarin 1 precursor</td>
<td>6 (0,6)</td>
<td><em>Nicotiana tabacum</em></td>
<td>6090829</td>
<td>30/5.8</td>
<td>24.8/7.7</td>
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<td>36-12</td>
<td>Annexin</td>
<td>17 (11,6)</td>
<td><em>Capsicum annuum</em></td>
<td>1071660</td>
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<td>NtPRp27-like protein</td>
<td>11(11,0)</td>
<td><em>Petunia x hybrida</em></td>
<td>SGN-E520812</td>
<td>24/7.7</td>
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<td>50-4</td>
<td>Auxin-response-like protein</td>
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<td>68.9/5.5</td>
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<td>36-10</td>
<td>Mitochondrial formate dehydrogenase precursor</td>
<td>20 (17,3)</td>
<td><em>Solanum tuberosum</em></td>
<td>11991527</td>
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<td>42.0/6.6</td>
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<td><strong>Electron transport or energy pathways</strong></td>
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<td>36-5</td>
<td>Succinate dehydrogenase flavoprotein alpha subunit</td>
<td>15 (10,5)</td>
<td><em>Arabidopsis thaliana</em></td>
<td>8843734</td>
<td>49/6.3</td>
<td>69.7/5.9</td>
<td>31%</td>
<td>766</td>
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<tr>
<td>14-22</td>
<td>1,4-benzoquinone reductase-like protein</td>
<td>8 (4,4)</td>
<td><em>Arabidopsis thaliana</em></td>
<td>21539481</td>
<td>27/6.5</td>
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<td>36-17</td>
<td>1,4-benzoquinone reductase-like protein</td>
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<td>Photosystem II 23 kDa protein</td>
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#### Proteolysis

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<td>Cysteine proteinase P21</td>
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<td>Proteasome beta-subunit</td>
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#### Carbohydrate metabolism

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<td>Beta-xylosidase 2</td>
<td>Solanum lycopersicum</td>
<td>37359708</td>
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<td>2435</td>
<td>6521</td>
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<td>Vacuolar invertase (N or C-terminally truncated)</td>
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<tr>
<td>36-22</td>
<td>Vacuolar invertase (N-terminally truncated)</td>
<td>5</td>
<td>29893064</td>
<td>22/5.6</td>
<td>71.2/5.4 7%</td>
</tr>
<tr>
<td>36-21</td>
<td>Vacuolar invertase precursor (N-terminally truncated)</td>
<td>2</td>
<td>124701</td>
<td>24/6.9</td>
<td>70.1/6.1 16%</td>
</tr>
<tr>
<td>14-11</td>
<td>Putative beta-galactosidase (C-terminally truncated)</td>
<td>13</td>
<td>7939623</td>
<td>46/6.7</td>
<td>93.2/6.8 14%</td>
</tr>
<tr>
<td>50-3</td>
<td>Cytosolic phosphoglucomutase</td>
<td>21</td>
<td>8250624</td>
<td>59/6.7</td>
<td>63.5/6.0 44%</td>
</tr>
<tr>
<td>14-6</td>
<td>Lipoxygenase (N-terminally truncated)</td>
<td>21</td>
<td>899344</td>
<td>62/5.9</td>
<td>97.6/5.5 24%</td>
</tr>
<tr>
<td>50-2</td>
<td>Lipoxygenase</td>
<td>8</td>
<td>899344</td>
<td>65/5.7</td>
<td>97.6/5.5 12%</td>
</tr>
<tr>
<td>14-20</td>
<td>Putative GDSL-motif lipase</td>
<td>4</td>
<td>37789825</td>
<td>28/6.1</td>
<td>19.1/5.5 25%</td>
</tr>
<tr>
<td>36-14</td>
<td>Putative anther-specific proline-rich protein; carboxylic ester hydrolase/hydrolase</td>
<td>8</td>
<td>50910547</td>
<td>39/5.5</td>
<td>40.1/5.7 33%</td>
</tr>
<tr>
<td>14-9</td>
<td>UTP-glucose-1-phosphate uridyltransferase</td>
<td>21</td>
<td>21599</td>
<td>51/6.9</td>
<td>51.8/5.4 46%</td>
</tr>
<tr>
<td>14-12</td>
<td>Endonuclease</td>
<td>8</td>
<td>50657596</td>
<td>43/5.7</td>
<td>34.4/5.6 32%</td>
</tr>
<tr>
<td>36-33</td>
<td>Beta-ureidopropionase (PYD3)</td>
<td>3</td>
<td>30698009</td>
<td>48/6.4</td>
<td>45.5/5.9 13%</td>
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</tbody>
</table>

(continued)
Table 3.2 continued

<table>
<thead>
<tr>
<th>Cytoskeleton organization and biogenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>14-30 Actin 20 (17,3) <em>Nicotiana tabacum</em></td>
</tr>
<tr>
<td>50-10 Actin-depolymerizing factor 1 3 (2,1) <em>Petunia x hybrida</em></td>
</tr>
<tr>
<td>51-14 Actin-depolymerizing factor 2 7 (7,0) <em>Petunia x hybrida</em></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Amino acid metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylene tetrahydrofolate reductase 1(MTHFR1)</td>
</tr>
<tr>
<td>36-32 (C-terminally truncated) 4 (1,3) <em>Arabidopsis thaliana</em></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Unclassified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Putative acid phosphatase (N-terminally truncated) 4 (0,4) <em>Oryza sativa</em></td>
</tr>
<tr>
<td>Putative Kunitz-type proteinase inhibitor 3 (1,2) <em>Petunia x hybrida</em></td>
</tr>
<tr>
<td>Putative Kunitz-type proteinase inhibitor 5 (2,3) <em>Petunia x hybrida</em></td>
</tr>
<tr>
<td>Similar to abscisic stress ripening protein 2 (2,0) <em>Petunia x hybrida</em></td>
</tr>
<tr>
<td>Similar to abscisic stress ripening protein 1(1,0) <em>Petunia x hybrida</em></td>
</tr>
<tr>
<td>Putative aluminum-induced protein 6 (1,5) <em>Arabidopsis thaliana</em></td>
</tr>
<tr>
<td>Hypothetical protein 6 (5,1) <em>Petunia x hybrida</em></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>S-adenosyl-L-methionine:benzoic acid/salicylic acid carboxyl methyltransferase</th>
</tr>
</thead>
<tbody>
<tr>
<td>50-11 methyltransferase 21 (21,0) <em>Petunia x hybrida</em></td>
</tr>
</tbody>
</table>
Table 3.3. Functional classification of petunia proteins that were down regulated during pollination-induced corolla senescence.

Proteins were classified based on the biological processes according to homology to genes in the TAIR Gene Ontology database.

Sample number indicates the spot sample for in-gel digestion in the sequencing reports. These sample numbers (without dash) were also used to indicate spots on the 2-D gels in Figure 3.2.

Matched Peptides indicate total number of peptides that matched to other proteins. The first and the second number in the parenthesis indicate the peptides with exact and homologous matches respectively.

Accession number indicates the sequence in the searched database identified from either NCBI non-redundant protein database or SGN petunia EST database (http://www.sgn.cornell.edu/index.pl).

Obs. pI/Mr, observed pI/Mr for each protein was calculated from the 2D-gels with Image PDQuest 7.4.0 software according to standard marker proteins.

Theor. pI/Mr, theoretical pI/Mr of the matched proteins.

Peptide coverage refers to the percentage of sequence coverage of the matched protein.

Mean % volume is the average of three replicated spot pixel intensities at 48 U, 48 P, 72 U and 72 P.

U is hours after flowers are fully opened without pollination (unpollinated).

P is hours after flowers are fully opened with pollination.

ud, spot volume was undetectable on the gel.
<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Putative proteins and their biological process categories</th>
<th>Matched Peptides</th>
<th>Matched Species</th>
<th>Protein Accession No.</th>
<th>Obser. Mr./PI</th>
<th>Theor. Mr./PI</th>
<th>Peptide Coverage Mean % Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>44-12</td>
<td>3-phosphoshikimate 1-carboxyvinyltransferase</td>
<td>19 (19,0)</td>
<td>Petunia x hybrida</td>
<td>114176</td>
<td>49/6.5</td>
<td>55.5/8.0</td>
<td>39%</td>
</tr>
<tr>
<td>51-11</td>
<td>Putative pyridoxine biosynthesis</td>
<td>11 (11,0)</td>
<td>Nicotiana tabacum</td>
<td>46399271</td>
<td>36/6.6</td>
<td>33.1/5.9</td>
<td>32%</td>
</tr>
<tr>
<td>49-24</td>
<td>Copper-zinc superoxide dismutase</td>
<td>5 (3,2)</td>
<td>Solanum lycopersicum</td>
<td>13445918</td>
<td>18/6.0</td>
<td>14.8/5.3</td>
<td>45%</td>
</tr>
<tr>
<td></td>
<td>Amino acid metabolism</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14-31</td>
<td>Methionine synthase</td>
<td>22 (16,6)</td>
<td>Solanum tuberosum</td>
<td>8439545</td>
<td>80/6.6</td>
<td>84.7/5.9</td>
<td>32%</td>
</tr>
<tr>
<td>44-1</td>
<td>Methionine synthase</td>
<td>2 (2, 0)</td>
<td>Solanum tuberosum</td>
<td>8439545</td>
<td>73/5.7</td>
<td>84.7/5.9</td>
<td>5%</td>
</tr>
<tr>
<td>44-2</td>
<td>Methionine synthase</td>
<td>43 (24, 19)</td>
<td>Solanum tuberosum</td>
<td>8439545</td>
<td>81/6.7</td>
<td>84.7/5.9</td>
<td>63%</td>
</tr>
<tr>
<td>44-3</td>
<td>Methionine synthase</td>
<td>24 (17,7)</td>
<td>Solanum tuberosum</td>
<td>8439545</td>
<td>80/6.6</td>
<td>84.7/5.9</td>
<td>30%</td>
</tr>
<tr>
<td>14-37</td>
<td>S-adenosyl-L-methionine synthetase</td>
<td>19 (19,0)</td>
<td>Petunia x hybrida</td>
<td>559506</td>
<td>45/6.2</td>
<td>43.8/5.5</td>
<td>73%</td>
</tr>
<tr>
<td>14-36</td>
<td>S-adenosyl-L-methionine synthetase</td>
<td>21 (14,7)</td>
<td>Petunia x hybrida</td>
<td>5726594</td>
<td>45/6.0</td>
<td>43.7/5.4</td>
<td>71%</td>
</tr>
<tr>
<td>44-13</td>
<td>S-adenosyl-L-methionine synthetase</td>
<td>16 (8,8)</td>
<td>Nicotiana tabacum</td>
<td>33340517</td>
<td>47/6.5</td>
<td>43.1/5.8</td>
<td>55%</td>
</tr>
<tr>
<td>44-14</td>
<td>S-adenosyl-L-methionine synthetase</td>
<td>12 (10,2)</td>
<td>Nicotiana tabacum</td>
<td>33340517</td>
<td>48/6.6</td>
<td>43.1/5.8</td>
<td>37%</td>
</tr>
<tr>
<td>49-18</td>
<td>Hydroxymethyltransferase</td>
<td>28 (12,16)</td>
<td>Arabidopsis thaliana</td>
<td>2244749</td>
<td>55/7.9</td>
<td>51.7/6.8</td>
<td>65%</td>
</tr>
</tbody>
</table>

(continued)
Table 3.3 continued

<table>
<thead>
<tr>
<th>Cytoskeleton organization and biogenesis</th>
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<tbody>
<tr>
<td>14-34 Alpha tubulin</td>
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<tr>
<td>49-21 Actin-depolymerizing factor 1</td>
</tr>
<tr>
<td>49-22 Actin-depolymerizing factor 2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Carbohydrate metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xyloglucan endotransglycosylase-hydrolase</td>
</tr>
<tr>
<td>51-13 XTH7</td>
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</table>

<table>
<thead>
<tr>
<th>Ribulose bisphosphate carboxylase</th>
</tr>
</thead>
<tbody>
<tr>
<td>44-15 large chain precursor</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ribulose 1,5-bisphosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>44-26 carboxylase small chain precursor</td>
</tr>
<tr>
<td>44-21 Caffeoyl-CoA -O methyltransferase</td>
</tr>
<tr>
<td>44-22 Caffeoyl-CoA O-methyltransferase</td>
</tr>
<tr>
<td>44-23 Caffeoyl-CoA O-methyltransferase</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nitrogen metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutamine synthetase shoot</td>
</tr>
<tr>
<td>44-18 isozyme</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Electron transport or energy pathways</th>
</tr>
</thead>
<tbody>
<tr>
<td>44-4 Transketolase 1</td>
</tr>
<tr>
<td>49-20 Photosystem II 23 kDa protein</td>
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</tbody>
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(continued)
Table 3.3 continued

<table>
<thead>
<tr>
<th></th>
<th>Unclassified</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>44-5</strong></td>
<td>Similar to Acyl-activating enzyme 11; Similar to AMP-dependent</td>
</tr>
<tr>
<td></td>
<td>synthetase and ligase family protein2 (2,0)</td>
</tr>
<tr>
<td></td>
<td>S-adenosyl-L-methionine:benzoic acid/salicylic acid carboxyl</td>
</tr>
<tr>
<td></td>
<td>methyltransferase 17 (17,0)</td>
</tr>
<tr>
<td></td>
<td>51-12</td>
</tr>
</tbody>
</table>
Table 3.4. Up regulated spots with multiple protein identities.

1 Proteins were classified based on the biological processes according to homology to genes in the TAIR Gene Ontology database.
2 Sample number indicates the spot sample for in-gel digestion in the sequencing reports. These sample numbers (without dash) were also used to indicate spots on the 2-D gels in Figure 3.2. As more than one proteins were identified from each single spot, the intensity of each protein at each analyzed stage could not be determined.
3 Matched Peptides indicate total number of peptides that matched to other proteins. The first and the second number in the parenthesis indicate the peptides with exact and homologous matches respectively.
4 Accession number indicates the sequence in the searched database identified from either NCBI non-redundant protein database or SGN petunia EST database (http://www.sgn.cornell.edu/index.pl).
5 Obs. pI/Mr, observed pI/Mr for each protein was calculated from the 2D-gels with Image PDQuest 7.4.0 software according to standard marker proteins.
6 Theor. pI/Mr, theoretical pI/Mr of the matched proteins.
7 Peptide coverage refers to the percentage of sequence coverage of the matched protein
<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Putative proteins and their biological process categories</th>
<th>Matched Peptides</th>
<th>Matched Species</th>
<th>Protein Accession</th>
<th>Obser. Mr./PI</th>
<th>Theor. Mr./PI</th>
<th>Peptide Coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>14-18</td>
<td>Response to stress and defense response</td>
<td>10 (2,8)</td>
<td><em>Nicotiana tabacum</em></td>
<td>2764992</td>
<td>31/5.5</td>
<td>22.9/5.0</td>
<td>50%</td>
</tr>
<tr>
<td>14-15</td>
<td>Peroxidase</td>
<td>6 (4,2)</td>
<td><em>Nicotiana tabacum</em></td>
<td>5381253</td>
<td>37/5.7</td>
<td>34.4/9.4</td>
<td>22%</td>
</tr>
<tr>
<td>14-49</td>
<td>Cytosolic ascorbate peroxidase</td>
<td>10 (5,5)</td>
<td><em>Dimocarpus longan</em></td>
<td>57339046</td>
<td>31/6.1</td>
<td>23.1/4.7</td>
<td>58%</td>
</tr>
<tr>
<td>36-18</td>
<td>Putative universal stress protein</td>
<td>4 (1,3)</td>
<td><em>Cicer arietinum</em></td>
<td>45720184</td>
<td>25/5.9</td>
<td>19.7/5.7</td>
<td>27%</td>
</tr>
<tr>
<td>50-5</td>
<td>Fumarase</td>
<td>9 (6,3)</td>
<td><em>Solanum tuberosum</em></td>
<td>1488652</td>
<td>50/7.3</td>
<td>53.3/6.5</td>
<td>19%</td>
</tr>
<tr>
<td></td>
<td><strong>Electron transport or energy pathways</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14-17</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>6 (5,1)</td>
<td><em>Nicotiana tabacum</em></td>
<td>4539543</td>
<td>33/6.4</td>
<td>36.7/7.7</td>
<td>24%</td>
</tr>
<tr>
<td>14-3</td>
<td>Succinate dehydrogenase flavoprotein alpha subunit</td>
<td>11 (8,3)</td>
<td><em>Arabidopsis thaliana</em></td>
<td>21700795</td>
<td>68/6.3</td>
<td>69.7/5.9</td>
<td>27%</td>
</tr>
<tr>
<td>14-41</td>
<td>Phosphoglycerate kinase</td>
<td>7 (5,2)</td>
<td><em>Nicotiana tabacum</em></td>
<td>1161602</td>
<td>44/6.3</td>
<td>42.3/5.7</td>
<td>23%</td>
</tr>
<tr>
<td>36-31</td>
<td>Early nodulin-like protein 2 precursor</td>
<td>6 (4,2)</td>
<td><em>Petunia x hybrida</em></td>
<td>SGN-E528550</td>
<td>16/5.6</td>
<td>29.9/9.4</td>
<td>27%</td>
</tr>
<tr>
<td>36-9</td>
<td>Plastidic aldolase NPALDP1</td>
<td>9 (9,0)</td>
<td><em>Nicotiana tabacum</em></td>
<td>4827251</td>
<td>40/6.2</td>
<td>42.6/6.9</td>
<td>34%</td>
</tr>
<tr>
<td></td>
<td><strong>Proteolysis</strong></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14-14</td>
<td>Cysteine proteinase</td>
<td>3 (1,2)</td>
<td><em>Solanum lycopersicum</em></td>
<td>20334377</td>
<td>42/5.7</td>
<td>38.5/5.0</td>
<td>11%</td>
</tr>
<tr>
<td>14-19</td>
<td>Cysteine proteinase (CP10)</td>
<td>7 (7,0)</td>
<td><em>Petunia x hybrida</em></td>
<td>52546926</td>
<td>31/5.9</td>
<td>16.8/6.1</td>
<td>42%</td>
</tr>
<tr>
<td>14-45</td>
<td>Cysteine proteinase (CP5)</td>
<td>4 (0,4)</td>
<td><em>Petunia x hybrida</em></td>
<td>52546916</td>
<td>36/6.3</td>
<td>21.2/5.1</td>
<td>20%</td>
</tr>
</tbody>
</table>

(continued)
<table>
<thead>
<tr>
<th>Entry</th>
<th>Description</th>
<th>Accession</th>
<th>Mw/PI</th>
<th>Molecular weight</th>
<th>PI</th>
<th>% Mw</th>
<th>% PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>14-19</td>
<td>P21</td>
<td>5 (4,1)</td>
<td>Petunia x hybrida</td>
<td>945081</td>
<td>31/5.9</td>
<td>39.3/5.8</td>
<td>18%</td>
</tr>
<tr>
<td>14-15</td>
<td>20S proteasome alpha 6 subunit</td>
<td>9 (7,2)</td>
<td>Nicotiana benthamiana</td>
<td>22947842</td>
<td>37/5.7</td>
<td>29.9/5.1</td>
<td>36%</td>
</tr>
<tr>
<td>14-47</td>
<td>Putative alpha 7 proteasome subunit</td>
<td>8 (6,2)</td>
<td>Nicotiana tabacum</td>
<td>14594925</td>
<td>31/6.4</td>
<td>27.5/6.1</td>
<td>38%</td>
</tr>
<tr>
<td>36-4</td>
<td>Serine carboxypeptidase</td>
<td>3 (0,3)</td>
<td>Arabidopsis thaliana</td>
<td>15228281</td>
<td>68/6.3</td>
<td>57.3/5.2</td>
<td>9%</td>
</tr>
<tr>
<td>14-28</td>
<td>Leukotriene-A4 hydrolase</td>
<td>9 (3,6)</td>
<td>Arabidopsis thaliana</td>
<td>22136844</td>
<td>66/5.6</td>
<td>69.3/5.1</td>
<td>20%</td>
</tr>
<tr>
<td>36-18</td>
<td>Glucose acyltransferase /serine carboxypeptidase</td>
<td>3 (0,3)</td>
<td>Solanum berthaultii</td>
<td>4101705</td>
<td>25/5.10</td>
<td>52.1/5.2</td>
<td>6%</td>
</tr>
<tr>
<td></td>
<td>ATP-dependent clp protease</td>
<td></td>
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<tr>
<td></td>
<td>ATP-binding subunit clpA homolog</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50-1</td>
<td>CD4B</td>
<td>23 (21,2)</td>
<td>Solanum lycopersicum</td>
<td>399213</td>
<td>67/5.6</td>
<td>102.2/5.9</td>
<td>32%</td>
</tr>
<tr>
<td></td>
<td><strong>Carbohydrate metabolism</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14-5</td>
<td>Beta-xylosidase 2</td>
<td>13 (10,3)</td>
<td>Solanum lycopersicum</td>
<td>37359708</td>
<td>65/5.9</td>
<td>68.9/8.0</td>
<td>27%</td>
</tr>
<tr>
<td>14-7</td>
<td>Beta-xylosidase 2</td>
<td>13 (9,4)</td>
<td>Solanum lycopersicum</td>
<td>37359708</td>
<td>60/6.1</td>
<td>68.9/8.0</td>
<td>23%</td>
</tr>
<tr>
<td></td>
<td>Beta-xylosidase 2 (N-terminally truncated)</td>
<td>4 (2,2)</td>
<td>Solanum lycopersicum</td>
<td>37359708</td>
<td>27/5.9</td>
<td>68.9/8.0</td>
<td>9%</td>
</tr>
<tr>
<td>14-17</td>
<td>Xyloglucan endo-transglycosylase-like protein</td>
<td>4 (1,3)</td>
<td>Arabidopsis thaliana</td>
<td>2827712</td>
<td>33/6.4</td>
<td>31.1/6.4</td>
<td>19%</td>
</tr>
<tr>
<td>36-1</td>
<td>Polygalacturonase-like protein-like</td>
<td>7 (1,4)</td>
<td>Solanum tuberosum</td>
<td>81074755</td>
<td>66/5.8</td>
<td>51.9/5.7</td>
<td>21%</td>
</tr>
<tr>
<td>36-8</td>
<td>Alpha-galactosidase</td>
<td>7 (4,2)</td>
<td>Petunia x hybrida</td>
<td>34765755</td>
<td>43/6.0</td>
<td>31.3/4.9</td>
<td>26%</td>
</tr>
<tr>
<td>36-9</td>
<td>Fructokinase</td>
<td>13 (5,8)</td>
<td>Arabidopsis thaliana</td>
<td>7434221</td>
<td>40/6.2</td>
<td>24.7/6.9</td>
<td>54%</td>
</tr>
<tr>
<td></td>
<td><strong>Lipid metabolism</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50-1</td>
<td>Lipoxygenase</td>
<td>22 (9,13)</td>
<td>Nicotiana tabacum</td>
<td>899344</td>
<td>67/5.6</td>
<td>97.6/5.5</td>
<td>26%</td>
</tr>
<tr>
<td>36-2</td>
<td>Lipoxygenase</td>
<td>3 (0,3)</td>
<td>Nicotiana tabacum</td>
<td>899344</td>
<td>62/5.8</td>
<td>97.6/5.5</td>
<td>5%</td>
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</table>

(continued)
<table>
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<tr>
<th>Table 3.4 continued</th>
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<tr>
<td><strong>Enoyl-ACP reductase</strong></td>
</tr>
<tr>
<td>Putative GDSL-motif lipase/hydrolase protein</td>
</tr>
<tr>
<td><strong>Isopentenyl diphosphate isomerase 2</strong></td>
</tr>
<tr>
<td><strong>Nucleotide/nucleoside/nucleobase metabolism</strong></td>
</tr>
<tr>
<td>Adenosine kinase isoforms 1S</td>
</tr>
<tr>
<td><strong>Nitrogen metabolism</strong></td>
</tr>
<tr>
<td>Putative protein</td>
</tr>
<tr>
<td>Glutamine synthetase GS1</td>
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<tr>
<td><strong>Amino acid metabolism</strong></td>
</tr>
<tr>
<td>Glutamine synthetase synthase</td>
</tr>
<tr>
<td><strong>Other metabolisms</strong></td>
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<td>Globulin-like protein</td>
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<tr>
<td>Isovaleryl-CoA dehydrogenase</td>
</tr>
<tr>
<td>Vacuolar H+-APTase A1 subunit isoform</td>
</tr>
<tr>
<td>1-aminocyclopropane-1-carboxylate oxidase 1</td>
</tr>
<tr>
<td><strong>Unclassified</strong></td>
</tr>
<tr>
<td>Purple acid phosphatase</td>
</tr>
<tr>
<td>Similar to putative PrMC3</td>
</tr>
<tr>
<td>Dehydration stress-induced protein</td>
</tr>
</tbody>
</table>
Table 3.5. Down regulated spots with multiple protein identities.

Proteins were classified based on the biological processes according to homology to genes in the TAIR Gene Ontology database.

Sample number indicates the spot sample for in-gel digestion in the sequencing reports. These sample numbers (without dash) were also used to indicate spots on the 2-D gels in Figure 3.2. As more than one proteins were identified from each single spot, the intensity of each protein at each analyzed stage could not be determined.

Matched Peptides indicate total number of peptides that matched to other proteins. The first and the second number in the parenthesis indicate the peptides with exact and homologous matches respectively.

Accession number indicates the sequence in the searched database identified from either NCBI non-redundant protein database or SGN petunia EST database (http://www.sgn.cornell.edu/index.pl).

Obs. pI/Mr, observed pI/Mr for each protein was calculated from the 2D-gels with Image PDQuest 7.4.0 software according to standard marker proteins.

Theor. pI/Mr, theoretical pI/Mr of the matched proteins.

Peptide coverage refers to the percentage of sequence coverage of the matched protein.
### Putative proteins and their biological process categories

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Putative proteins and their biological process categories</th>
<th>Matched Peptides</th>
<th>Matched Species</th>
<th>Protein Accession</th>
<th>Obser. Mr./PI</th>
<th>Theor. Mr./PI</th>
<th>Peptide Coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>14-32</td>
<td>ATP synthase beta subunit</td>
<td>10 (0,10)</td>
<td><em>Nicotiana tabacum</em></td>
<td>19685</td>
<td>55/5.5</td>
<td>59.9/6.0</td>
<td>26%</td>
</tr>
<tr>
<td>14-39</td>
<td>Putative mitochondrial NAD-dependent malate dehydrogenase</td>
<td>6 (5,1)</td>
<td><em>Solanum tuberosum</em></td>
<td>21388550</td>
<td>41/6.0</td>
<td>36.2/8.5</td>
<td>25%</td>
</tr>
<tr>
<td>44-19</td>
<td>Similar to alcohol dehydrogenase</td>
<td>2 (2,0)</td>
<td><em>Petunia x hybrida</em></td>
<td>SGN-E526120</td>
<td>47/6.9</td>
<td>31.5/9.6</td>
<td>9%</td>
</tr>
<tr>
<td>44-19</td>
<td>Similar to alcohol dehydrogenase class III</td>
<td>2 (2,0)</td>
<td><em>Petunia x hybrida</em></td>
<td>SGN-E520836</td>
<td>47/6.9</td>
<td>21.6/10.3</td>
<td>23%</td>
</tr>
<tr>
<td>51-9</td>
<td>Alcohol dehydrogenase</td>
<td>14 (0, 14)</td>
<td><em>Arabidopsis thaliana</em></td>
<td>21592656</td>
<td>47/6.9</td>
<td>42.8/8.3</td>
<td>38%</td>
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<tr>
<td>44-27</td>
<td>Enolase</td>
<td>4 (4,0)</td>
<td><em>Solanum lycopersicum</em></td>
<td>1161573</td>
<td>49/6.3</td>
<td>35.1/6.3</td>
<td>21%</td>
</tr>
<tr>
<td>44-29</td>
<td>Mitogen-activated protein kinase 4</td>
<td>10 (10, 0)</td>
<td><em>Nicotiana tabacum</em></td>
<td>1076640</td>
<td>47/6.2</td>
<td>45.1/5.6</td>
<td>39%</td>
</tr>
<tr>
<td>44-30</td>
<td>Phenylalanine ammonia-lyase</td>
<td>12 (9,3)</td>
<td><em>Nicotiana tabacum</em></td>
<td>129594</td>
<td>76/6.7</td>
<td>77.8/6.3</td>
<td>21%</td>
</tr>
<tr>
<td>49-19</td>
<td>Phenylcoumaran benzylic ether reductase homolog F1 (Isoflavone reductase homolog P3)</td>
<td>8 (2,6)</td>
<td><em>Forsythia intermedia</em></td>
<td>7578895</td>
<td>36/6.0</td>
<td>34.0/5.9</td>
<td>30%</td>
</tr>
<tr>
<td>44-16</td>
<td>Ascorbate free radical reductase</td>
<td>4 (3,1)</td>
<td><em>Solanum lycopersicum</em></td>
<td>832876</td>
<td>45/5.8</td>
<td>47.0/5.8</td>
<td>13%</td>
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<tr>
<td>44-30</td>
<td>Methionine synthase</td>
<td>3 (2,1)</td>
<td><em>Solanum tuberosum</em></td>
<td>8439545</td>
<td>76/6.7</td>
<td>84.7/5.9</td>
<td>6%</td>
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<tr>
<td>14-35</td>
<td>S-adenosyl-L-methionine synthetase</td>
<td>17 (17,0)</td>
<td><em>Petunia x hybrida</em></td>
<td>5726594</td>
<td>45/5.8</td>
<td>43.7/5.4</td>
<td>63%</td>
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(continued)
Table 3.5 continued

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<th>M</th>
<th>V</th>
<th>M/V</th>
</tr>
</thead>
<tbody>
<tr>
<td>44-11</td>
<td>S-adenosyl-L-methionine synthase</td>
<td>15 (8.7)</td>
<td><em>Medicago sativa</em></td>
<td>48093937</td>
<td>47/5.9</td>
</tr>
<tr>
<td>44-28</td>
<td>S-adenosyl-L-methionine synthetase</td>
<td>13 (10.3)</td>
<td><em>Solanum lycopersicum</em></td>
<td>429106</td>
<td>49/6.2</td>
</tr>
<tr>
<td>44-29</td>
<td>S-adenosyl-L-methionine synthetase</td>
<td>11 (11.0)</td>
<td><em>Petunia x hybrida</em></td>
<td>559506</td>
<td>47/6.2</td>
</tr>
<tr>
<td>44-10</td>
<td>S-adenosyl-L-homocysteine hydrolase</td>
<td>29 (26.3)</td>
<td><em>Nicotiana tabacum</em></td>
<td>441217</td>
<td>52/6.1</td>
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<tr>
<td>44-10</td>
<td>Similar to Tyrosine/dopa decarboxylase</td>
<td>6 (6.0)</td>
<td><em>Petunia x hybrida</em></td>
<td>SGN-E528060</td>
<td>52/6.1</td>
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**Protein metabolism**

<table>
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<th>M</th>
<th>V</th>
<th>M/V</th>
</tr>
</thead>
<tbody>
<tr>
<td>14-32</td>
<td>Chaperonin, putative</td>
<td>4 (0.4)</td>
<td><em>Arabidopsis thaliana</em></td>
<td>30696748</td>
<td>55/5.5</td>
</tr>
<tr>
<td>44-11</td>
<td>Eukaryotic translation initiation factor</td>
<td>10 (8.2)</td>
<td><em>Nicotiana tabacum</em></td>
<td>19697</td>
<td>47/5.9</td>
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**Cytoskeleton organization and biogenesis**

<table>
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<th>V</th>
<th>M/V</th>
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<tr>
<td>14-33</td>
<td>Alpha tubulin</td>
<td>7 (0.7)</td>
<td><em>Physcomitrella patens</em></td>
<td>25396545</td>
<td>49/5.5</td>
</tr>
<tr>
<td>44-17</td>
<td>Actin</td>
<td>15 (14.1)</td>
<td><em>Gossypium hirsutum</em></td>
<td>32186890</td>
<td>44/5.8</td>
</tr>
<tr>
<td>14-35</td>
<td>Actin</td>
<td>2 (0.2)</td>
<td><em>Solanum tuberosum</em></td>
<td>21536</td>
<td>45/5.8</td>
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</table>

**Carbohydrate metabolism**

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<th>V</th>
<th>M/V</th>
</tr>
</thead>
<tbody>
<tr>
<td>44-16</td>
<td>Ribulose bisphosphate carboxylase/oxygenase activase</td>
<td>14 (9.5)</td>
<td><em>Solanum lycopersicum</em></td>
<td>10720247</td>
<td>45/5.8</td>
</tr>
<tr>
<td>14-38</td>
<td>RuBisCo activase</td>
<td>14 (8.6)</td>
<td><em>Nicotiana tabacum</em></td>
<td>445628</td>
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**Nitrogen metabolism**

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<th>M</th>
<th>V</th>
<th>M/V</th>
</tr>
</thead>
<tbody>
<tr>
<td>44-17</td>
<td>Glutamine synthetase shoot isozyme</td>
<td>7 (4.3)</td>
<td><em>Oryza sativa</em></td>
<td>50912511</td>
<td>44/5.8</td>
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</tbody>
</table>

**Other metabolisms**

<table>
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<th>V</th>
<th>M/V</th>
</tr>
</thead>
<tbody>
<tr>
<td>44-28</td>
<td>Putative LytB protein</td>
<td>6 (2.4)</td>
<td><em>Oryza sativa</em></td>
<td>50540738</td>
<td>49/6.2</td>
</tr>
<tr>
<td>14-38</td>
<td>Flavanone 3-beta-hydroxylase</td>
<td>13 (13.0)</td>
<td><em>Petunia x hybrida</em></td>
<td>2465434</td>
<td>43/5.7</td>
</tr>
</tbody>
</table>

(continued)
Table 3.5 continued

| 118 | 3-phosphoshikimate                        | 10 (10,0) | *Petunia x hybrida* | 114176 | 49/6.3 | 55.5/8.0 | 24% |
| 51-9 | Glycine-rich RNA-binding protein           | 9 (8,1)   | *Nicotiana tabacum* | 2119043 | 19/5.6 | 16.6/5.3 | 52% |
| 14-32 | ATPase alpha subunit                      | 3 (0,3)   | *Atropa belladonna* | 20068316 | 55/5.5 | 55.5/5.3 | 7%  |
| 14-39 | Unclassified                              |           |                    |        |        |          |     |
| 51-9 | S-adenosyl-L-methionine:benzoic acid/salicylic acid carboxyl methyltransferase | 18 (18,0) | *Petunia x hybrida* | 28629495 | 41/6.0 | 40.7/5.7 | 55% |
| 49-23 | Similar to nucleic acid binding protein    | 4 (4,0)   | *Petunia x hybrida* | SGN-E536589 | 19/5.6 | 18.0/5.3 | 17% |
Figure 3.1. Protein changes during petunia corolla development and senescence.

(A) Pollination accelerates petunia flower senescence. *Petunia x hybrida* ‘Mitchell Dipolid’ (MD) unpollinated (U) flower at 0, 24, 48 and 72 h and pollinated (P) flower at 24, 48 and 72 h. (B) 2-D gel profiling of protein changes corresponding to corolla senescence. Representative Gel-code blue stained 2-D gels of petunia corolla proteome variation during pollination-induced senescence. First dimension was performed using 200 g total soluble proteins on linear gradient IPG strips with pH 5-8. In the second dimension, 12% SDS-PAGE gels were used and proteins were visualized using Gel-code blue staining. About 600 spots were detected on each gel. Three biological replicates at each time point were conducted.
Figure 3.2. Representative 2-DE comparisons of unpollinated and pollinated gels at 72h.

(A) All the down regulated protein spots labeled with arrows are shown on the 72U gels. (B) All the up-regulated protein spots labeled with arrows are shown on 72P gels. The numbers labeled on the each spot are the sample numbers (without dash in the middle) in Table 3.2, 3.3, 3.4 and 3.5. The assigned identities of up and down regulated proteins and their expression data are also shown in the Table 3.2, 3.3, 3.4 and 3.5.
Figure 3.3. Biological processes classification of up (A) and down (B) regulated proteins.

Forty-seven up and twenty-six down regulated proteins were assigned to putative biological process categories according to the TAIR GO annotation database (http://www.arabidopsis.org/tools/bulk/go/index.jsp).
Figure 3.4. Hierarchical cluster analysis of the identified spots with the single protein identities.

Protein expression levels are presented as the Log ratio relative to the 0 h unpollinated reference. Color ranges from green to red represent protein expression from the highest down-regulation to the highest up-regulation, respectively. Black color indicates no change compared to 0 h. Two main clusters were formed, representing up and down regulated proteins during senescence. The similarities of protein expression patterns represented by the distance of tree branches are shown on the left side. The spot sample numbers with putative protein identification are indicated on the right side of the heat map.
Figure 3.5. Representative proteins with expression patterns of 14 subclusters.

Protein expression patterns of each of 14 subclusters from the hierarchical tree are shown by one representative protein. Spots that were up or down regulated are indicated with arrows on the 2-D gels. The expression patterns with quantitative data are shown on the right side of the 2-D gels. The solid and dot lines indicate the protein expression changes in pollinated and unpollinated corollas from 0 to 72 h, respectively.
1: 14-50 cysteine protease

2: 51-14 Actin depolymerizing factor 2

3: 36-19 Trypsin and kunitz-type protease inhibitor

4: 14-12 Endonuclease

5: 14-30 Actin

6: 36-20 Trypsin and kunitz-type protease inhibitor

7: 50-9 Plasma membrane polypeptide

(continued)
Figure 3.5 continued

8: SB50-11 S-adenosyl-L-methionine:benzoic acid/salicylic acid carboxyl methyltransferase

9: 49-24 Copper-zinc superoxide dismutase

10: SB44:2 Methionine synthase

11: SB44:4 Transketolase 1

12: 44-18 Glutamine synthetase shoot isozyme

13: 44-21 Caffeoyl-CoA -O methyltransferase

14: 36-5 Succinate dehydrogenase flavoprotein alpha subunit
Figure 3.6. Protein isoforms and their expression patterns during corolla development and pollination-induced senescence.

(A) and (B) Up and down regulated proteins isoforms showing similar expression patterns. (C) Protein isoforms with opposite expression patterns. Spots that were up or down regulated are indicated with arrows on the 2-D gels. The expression patterns with quantitative data are shown on the right side of the 2-D gels. The solid and dot lines indicate the protein expression changes in pollinated and unpollinated corollas from 0 to 72 h, respectively.
A

14-13 Beta-xylosidase 2 (C-terminally truncated)

14-23 Manganese superoxide dismutase

14-52 Manganese superoxide dismutase

14-22 1,4-benzoquinone reductase-like

36-17 1,4-benzoquinone reductase-like

(continued)
Figure 3.6 continued

A

14-6 Lipoxigenase (N-terminally truncated)

50-2 Lipoxigenase

36-19 Trypsin and Kunitz-type protease inhibitor

36-20 Trypsin and Kunitz-type protease inhibitor

50-7 Similar to abscisic stress ripening protein

50-8 Similar to abscisic stress ripening protein

(continued)
Figure 3.6 continued

A

14-29 Vacuolar invertase (N or C-terminally truncated)

36-21 Vacuolar invertase (N-terminally truncated)

36-22 Vacuolar invertase (N-terminally truncated)

(continued)
Figure 3.6 continued

B

14-31 Methionine synthase

44-1 Methionine synthase

44-2 Methionine synthase

44-3 Methionine synthase

44-21 Caffeoyl-CoA -O methyltransferase

44-22 Caffeoyl-CoA -O methyltransferase

44-23 Caffeoyl-CoA -O methyltransferase

(continued)
Figure 3.6 continued

B

14-36 S-adenosyl-L-methionine synthetase

14-37 Methionine adenosyltransferase (SAMT)

44-13 S-adenosyl-L-methionine synthetase

44-14 S-adenosyl-L-methionine synthetase

(continued)
Figure 3.6 continued

C

50-10 Actin-depolymerizing factor 1

49-21 Actin-depolymerizing factor 1

51-14 Actin-depolymerizing factor 2

49-22 Actin-depolymerizing factor 2

14-40 S-adenosyl-L-methionine:benzoic acid/salicylic acid carboxyl methyltransferase

50-11 S-adenosyl-L-methionine benzoic acid/salicylic acid carboxyl methyltransferase

(continued)
Figure 3.6 continued

C

14-25 Photosystem II 23 kDa protein

49-20 Photosystem II 23 kDa protein
CHAPTER 4

FUNCTIONAL CHARACTERIZATION OF A SENESCENCE-SPECIFIC NUCLEASE PHNUC2

ABSTRACT

Senescence is a highly programmed process, allowing plants to degrade macromolecules and remobilize nutrients to developing tissues or organs. Nucleic acid catabolism, as one of these programmed events, is accompanied by induction of nuclease activities and nuclear DNA fragmentation. In chapter 2, a senescence-specific nuclease, PhNUC1, was characterized as a bifunctional nuclease that degrades both DNA and RNA. Recently, a senescence-specific endonuclease (referred to hereafter as PhNUC2) was identified from Petunia x hybrida corollas undergoing pollination-induced senescence using a 2-DE based proteomic approach (chapter 3). Although the molecular weight of PhNUC1 on the gels is the same as that of PhNUC2 on the 2-D gels, we do not know if these two proteins are the same, due to lack of sequence information. We therefore isolated the PhNUC2 full-length cDNA based on peptide sequencing data obtained from our 2-DE experiments. The analysis of the deduced amino acid sequence of PhNUC2 showed that PhNUC2 is highly similar to the senescence-associated bifunctional nuclease BFN1 from Arabidopsis. Characterization of PhNUC2 expression
showed that PhNUC2 transcripts specifically increased in senescing floral tissues. Suppression of PhNUC2 gene expression using virus-induced gene silencing (VIGS) dramatically reduced the nuclease activity that corresponds to PhNUC1, indicating that PhNUC1 and PhNUC2 may be the same protein or very close family members. Silencing PhNUC2 did not change flower longevity; possible reasons for this will be discussed below.

INTRODUCTION

Senescence is a process that leads to the death of a cell, an organ or a whole plant. The purpose of senescence is to remobilize the nutrients from dying tissues to developing tissues, as well as to survive under abiotic and biotic stresses (Greenberg, 1996; Pontier et al., 1998; Lam et al., 1999; Pontier et al., 1999; Buchanan-Wollaston et al., 2003; Buchanan-Wollaston et al., 2007). Senescence is tightly controlled by both endogenous and exogenous stimuli. At the plant level, senescence represents the final stage of development. The physiological, morphological and biochemical changes during senescence are concomitant with the activation of a number of genes and enzyme activities that function as regulators and executioners in the senescence network. Thus, at the cellular level, senescence is also called programmed cell death (PCD).

The term senescence-associated genes (SAGs), refers to genes that are up-regulated during senescence. SAGs have been reported in many recent studies and reviews (Buchanan-Wollaston et al., 2003; van Doorn et al., 2003; Breeze et al., 2004; Guo et al., 2004; Jones, 2004; Buchanan-Wollaston et al., 2005; Stead et al., 2006; Hopkins et al., 2007; Lim et al., 2007; Yamada et al., 2007). These SAGs are mainly
classified into signal transduction, transcriptional regulation, and biological processes involved in stress and defense responses and the hydrolysis of macromolecules including nucleic acids, proteins, carbohydrates and lipids.

As one of the execution events in leaf and flower senescence, nucleic acid catabolism, involving RNases, DNases and nucleases, results in the recycling of nucleotides and phosphorus from senescing tissues to developing tissues (Bleecker, 1998; Thomas et al., 2003). Endonucleases catalyze the hydrolysis of RNA and genomic DNA. Induction of gene expression or activities of a number of endonuclease were identified during leaf senescence (Blank and McKeon, 1989; Perez-Amador et al., 2000; Lers et al., 2001; Canetti et al., 2002) and flower senescence (Panavas et al., 1999; Xu and Hanson, 2000; Langston et al., 2005). Induction of endonucleases were also identified from various PCD processes during plant growth and development, including seed germination (Aoyagi et al., 1998; Fath et al., 1999) and differentiation of tracheary elements (TEs) (Mittler and Lam, 1995; Fukuda, 2000; Ito and Fukuda, 2002). In addition, endonucleases were induced in stress and defense responses such as salt stress (Muramoto et al., 1999) and the hypersensitive response (HR) to plant microbial pathogens (Mittler and Lam, 1995; Ryerson and Heath, 1996; Mittler and Lam, 1997; Matousek et al., 2007). Based on differential requirements for divalent cations for nuclease activity, at least three groups, including Zn$^{2+}$, Ca$^{2+}$ and Co$^{2+}$-dependent endonucleases, have been identified (Sugiyama et al., 2000; Lers et al., 2001; Canetti et al., 2002; Langston et al., 2005).

To date, only a few cDNA or protein sequences of PCD or senescence-induced nucleases, including Arabidopsis *BFNI*, barley *BENI*, dayliy SA6, and Zinnia *ZEN1*, *ZEN2* (NucZe1) and *ZEN3* (NucZe2), have been reported (Thelen and Northcote, 1989;
Aoyagi et al., 1998; Panavas et al., 1999; Perez-Amador et al., 2000). These nucleases belong to the nuclease I family and are glycoproteins that degrade RNA and single stranded DNA endonucleolytically. The molecular masses of these enzymes range from 31 to 42 kD. Their enzyme activities are optimal at acidic pH, they require Zn$^{2+}$ for activation and stability, and they are inhibited by EDTA (Aoyagi et al., 1998; Perez-Amador et al., 2000). More recently, a senescence-specific endonuclease, PhNUC2, was identified during petal senescence using 2-DE based proteomic approaches (chapter 3). Interestingly, previous in-gel activity assays have shown that PhNUC1, a glycoprotein that degrades both RNA and DNA, was also specifically induced during petal senescence in petunia (Langston et al., 2005). Although the molecular weight of PhNUC1 calculated from the in-gel activity assay is the same as that of PhNUC2, we do not know if these two nucleases are the same protein due to lack of sequence information for PhNUC1.

Functional studies of endonucleases have only been reported in a few plant species. In Arabidopsis plants overexpressing BFN1, no phenotypic changes during plant growth and development were observed compared with the wild type plants (Perez-Amador et al., 2000). After introducing an antisense construct of the nuclease, ZEN1, into Zinnia elegans suspension cells, nuclear DNA degradation was suppressed during TE differentiation (Ito and Fukuda, 2002). In addition to these functional studies, suppression of LX ribonuclease in transgenic tomato plants was found to delay leaf senescence and abscission (Lers et al., 2006). However, so far no functional studied of the nucleases induced during flower senescence have been reported. Therefore, isolation and characterization of petunia genes encoding endonucleases, especially those induced
during petal senescence, will advance our understanding of the role of nucleases in senescence.

In this chapter, we isolated the PhNUC2 gene based on information obtained from peptide sequences from our 2-DE experiments and the conserved regions of other senescence-induced nucleases in the GenBank database. Preliminary results showed that the regulation of PhNUC2 at the transcriptional level correlated with the patterns of protein accumulation in our 2-DE experiments. We also identified another nuclease, PhNUC3, using the petunia EST database in the SOL genomics network. PhNUC3 was predicted to be another bifunctional nuclease based on the deduced amino acid sequence, but transcript levels were not upregulated during petal senescence.

We hypothesized that knocking down PhNUC2 gene expression would reduce nuclease activity and delay corolla senescence. Since Tobacco Rattle Virus (TRV) mediated virus-induced gene silencing has been used to study gene function during leaf senescence (Wang et al., 2005; Ahn et al., 2006; Lin et al., 2007), flower senescence (Chen et al., 2004; Chen et al., 2005) and fruit ripening (Liu et al., 2002; Fu et al., 2005; Xie et al., 2006), we employed this approach to test our hypothesis. The mechanism of VIGS is based on post-transcriptional gene silencing (PTGS). The PTGS phenomenon was initially observed as co-suppression of the chalcone synthase (CHS) gene in CHS overexpressing transgenic plants (Napoli et al., 1990). Similar phenomena have been found in fungi and animals, which are called quelling and RNAi, respectively (Waterhouse et al., 2001). PTGS is triggered in the host cells when an introduced sequence shares homology to the endogenous gene, resulting in the degradation of the endogenous gene or transgene (Waterhouse et al., 2001). In our VIGS experiment,
PhNUC2 transcript levels were decreased and nuclease activity corresponding to that of PhNUC1 was also less in senescing VIGS corollas. However, the flower senescence process was neither delayed nor accelerated in our VIGS plants. The reasons are discussed below.

RESULTS

Full-length cDNA isolation of PhNUC2 and PhNUC3

Previous 2-DE experiments (chapter 3) identified PhNUC2 as a senescence-associated protein. PhNUC2 spots were not detected in nonsenescing corollas, including 0 to 72 h unpollinated and up to 24 h after pollination (hap), but PhNUC2 proteins were highly abundant in senescing corollas at both 48 and 72 hap (Figure 4.1). To isolate the cDNA that encoded the petunia PhNUC2, a 697 bp cDNA fragment was amplified using RT-PCR. Primers were constructed according to the information obtained from the PhNUC2 peptide sequencing data and alignment with other senescence-associated nuclease protein sequences (Figure 4.2). We then conducted 5’ and 3’ RACE to obtain a 1254 bp PhNUC2 full-length cDNA. The full-length PhNUC2 cDNA encodes 301 amino acids, with a predicted molecular weight of 34.1kDa and a PI of 5.2 (Figure 4.2). Three putative N-glycosylation sites are located at amino acids 119, 137 and 211, based on the presence of the consensus sequence Asn-Xaa-Ser/Thr (Figure 4.2). The first 25 amino acids of PhNUC2 are predicted to encode a signal peptide, which potentially targets PhNUC2 to the secretory pathway. The mature peptide of PhNUC2 is predicted to be 31.4kDa after signal peptide cleavage.
A blast search using the PhNUC2 sequence against the petunia EST database (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/Blast/index.cgi) identified another endonuclease fragment (PhNUC3; Accession No. CV299186). The coding region of the PhNUC3 fragment share 62% identity with the corresponding region of the PhNUC2 gene, which is found at the 5’ end. We conducted 3’ RACE to extend the sequence to the end of PhNUC3. We then obtained a 1354 bp full-length PhNUC3 cDNA that encodes 293 amino acids with a predicted molecular weight of 33.8 kDa and PI of 6.2. Five N-glycosylation sites are predicted at amino acids 92, 116, 134, 208 and 222, respectively (data not shown). The first 25 amino acids of PhNUC3 are also predicted to encode a signal peptide that targets PhNUC3 to the secretory pathway. The mature peptide of PhNUC3 is 31.0 kDa after the signal peptide is cleaved.

**Comparison of PhNUC2 and PhNUC3 with other related nucleases**

Multiple alignments of the deduced amino acid sequences of PhNUC2, PhNUC3 and other endonucleases were performed by ClustalW. PhNUC2 and PhNUC3 share many common features with the bifunctional nucleases that were characterized previously, including the residues that bind zinc atoms and form disulfide bonds, the glycosylation sites, and the RNase and DNase catalytic sites (Aoyagi et al., 1998; Perez-Amador et al., 2000) (Figure 4.3A).

According to the phylogenetic analysis, PhNUC2, PhNUC3 and homologous nucleases are grouped into two subfamilies (Figure 4.3B). PhNUC2 falls into a group that includes potato StEN1, tomato TBN1, Arabidopsis BFN1, daylily Sa6 and zinnia ZEN1. PhNUC3 (CV299186) falls into the other group that includes tomato SGN-322719, zinnia
ZEN2 and ZEN3. PhNUC2 shares 84.8% and 84.4% amino acid similarity with StEN1 and TBN1, respectively. Petunia PhNUC2 and Arabidopsis BFN1 share 65.9% similarity at the amino acid level, which is much higher than that of the 48.6% amino acid similarity shared between BFN1 and PhNUC3.

**Transcripts of PhNUC2, not PhNUC3, were induced during senescence**

Semi-quantitative RT-PCR showed that the expression pattern of PhNUC2 at the transcript level was correlated with the protein level during pollination-induced corolla senescence (Figure 4.4A). To further characterize PhNUC2 expression during pollination-induced senescence, we performed real-time PCR. While almost no transcripts of PhNUC2 were detected in non-senescing corollas, PhNUC2 transcripts were highly abundant in senescing corollas at 48 and 72 h after pollination (hap) (Figure 4.4B). To investigate the spatial expression of PhNUC2, non-senescing and senescing flower parts were collected. Real-time PCR analysis showed that PhNUC2 transcript abundance was the highest in senescing styles at 72 hap, while very low levels of PhNUC2 transcripts were detected in other non-senescing tissues (Figure 4.5).

To test if PhNUC3 is also a senescence-associated gene, we conducted semi-quantitative RT-PCR to compare the gene expression patterns between PhNUC2 and PhNUC3 during senescence. PhNUC2 transcripts were induced during natural senescence and pollination-induced senescence (Figure 4.6). PhNUC3 transcripts were relatively low at all time points. Compared with PhNUC2 expression patterns, PhNUC3 expression was highest at 0 h and slightly decreased during senescence (Figure 4.6).
PhNUC2 expression was enhanced by exogenous ethylene treatment and was not inhibited in ethylene insensitive flowers

To determine if PhNUC2 expression was enhanced by ethylene, detached petunia flowers were sealed in a 24 L chamber and treated with 2 µL L⁻¹ ethylene for 12 h or 36 h. Controls flowers were sealed in another 24 L chamber without ethylene for 36 h. Real-time PCR analysis showed that PhNUC2 transcripts were not detected in control flowers, while PhNUC2 transcripts were induced after both 12 h and 36 h exogenous ethylene treatment. The PhNUC2 transcript level at 12 h after ethylene treatment was comparable to that of 48 h pollinated corollas. After 36 h of ethylene treatment, PhNUC2 expression was 5 times higher than at 12 h of treatment (Figure 4.7A). To determine if PhNUC2 expression was inhibited in ethylene-insensitive corollas, we also compared PhNUC2 expression in MD and transgenic petunia (35S: etr1-1) corollas by real-time PCR. PhNUC2 transcripts accumulated to similar levels at the late senescence stages in MD corollas at 8 days and etr1-1 corollas at 18 days (Figure 4.7B).

PhNUC2 expression levels were reduced in TRV2-CHS-NUC2 VIGS corollas

To characterize the function of PhNUC2 during corolla senescence, we utilized virus-induced gene silencing (VIGS) to knock down PhNUC2 expression. The chalcone synthase (CHS) gene was used as a reporter system to give a visual indicator of silencing (Chen et al., 2004). The fragment of PhNUC2 was choosen from the region corresponding to bases 304-606, including both DNase and RNase active sites. In the TRV-CHS-NUC2 construct, the inserts from the fragment of the CHS gene and PhNUC2 gene were tandemly ligated. Seedlings at different ages were inoculated with an
Agrobacteria mixture containing TRV1 and TRV2 (or TRV2 derivatives). The inoculated leaves showed symptoms of necrosis within a few days after inoculation and then recovered from virus infection within 2 weeks. Although both young and older seedlings showed the silencing symptoms, the seedlings with 4 to 6 true leaves showed the best silencing symptoms compared to inoculating older plants (data not shown). It took VIGS plants about one month to produce CHS gene silenced flowers if they were inoculated at the young seedling stage with 4 to 6 true leaves. In addition, the CHS gene silenced flowers ranged from almost completely white to purple flowers with white sectors, indicating that the effect of VIGS was variable (Figure 4.8).

The PhNUC2 transcript level in the VIGS experiments was determined by real-time PCR. A region of the PhNUC2 gene not included in the TRV-CHS-PhNUC2 construct was chosen for PCR amplification. Compared with buffer (mock), TRV2 and TRV2-CHS controls, real-time PCR analysis indicated that PhNUC2 gene expression was significantly suppressed in naturally senescing corollas collected from TRV2-CHS-PhNUC2 infiltrated plants (data not shown). In the senescing corollas at 48 hap, the relative expression of PhNUC2 in TRV2-CHS-NUC2 was 1.74, which was 37% and 65% lower than that of the buffer and TRV2-CHS treatments, respectively (Figure 4.9A). Expression levels of PhNUC2 in TRV2 and TRV2-CHS controls were much higher than that of mock infiltrated plants (Figure 4.9A). To determine if PhNUC3 transcripts were reduced in TRV2-CHS-NUC2 senescing corollas, we checked the PhNUC3 expression using real-time PCR. No significant reduction in PhNUC3 was detected among all the treatments, indicating that the VIGS was specifically knocking down PhNUC2 gene expression in TRV2-CHS-NUC2 plants (Figure 4.9B).
Nuclease activity was reduced in TRV2-CHS-NUC2 VIGS corollas

To determine if knocking down PhNUC2 gene expression in TRV2-CHS-NUC2 corollas also reduced nuclease activities, we performed nuclease in-gel activity assays. The 43 kDa nuclease activity previously identified as PhNUC1 by in-gel activity assays (Langston et al., 2005) was significantly reduced in TRV2-CHS-NUC2 senescing corollas compared with senescing corollas from mock, TRV2, or TRV2-CHS controls. The suppressed activity of this 43 kDa nuclease was observed when single stranded DNA, double stranded DNA or RNA were used as substrates (Figure 4.10).

Senescence was not delayed in TRV2-CHS-NUC2 silenced corollas

To determine if suppression of PhNUC2 gene expression and activity delayed flower senescence, we recorded flower longevities. Forty-eight flowers from Buffer (mock), TRV2, TRV2-CHS and TRV-CHS-NUC2 were evaluated in growth chambers. An analysis of variance (ANOVA) was performed using the General Linear Model procedure of SAS (PC version 9.1, the SAS Institute, Cary, NC) to evaluate the statistical model:

\[ Y_{hijk} = \mu + \text{Treat}_h + \text{Exp}_i + \text{Treat}_h*\text{Exp}_i + \text{Treat}_h*\text{Plant}_j(\text{Exp})_i + \varepsilon_{hijk} \]

Differences between treatments were statistically significant based on ANOVA (P < 0.001). No other effects in the model were significant. Treatment explained 28.5% of the total variance with uncontrolled error accounting for the remainder of the variance (data not shown). The lifespan of flowers from plants treated with buffer only averaged 9.9 days (Table 4.1). Flowers from plants treated with the TRV2 vector control lasted an average of 9.4 days, a difference that was statistically different from the buffer only
control (P < 0.01). VIGS constructs containing either CHS or PhNUC2 were not statistically different from each other, with average days to wilt of 8.8 and 8.5, respectively. However, these two treatments were significantly different from buffer treated controls and from the TRV2 construct (P < 0.01) (Table 1).

DISCUSSION

Senescence allows the plants to remobilize the nutrients from senescing leaves or petals to developing tissues. This process is tightly controlled by a number of genes that function in signal transduction, gene regulation, macromolecular degradation and transportation. Among senescence-associated genes, endonucleases that function in nucleic acid catabolism have been found to be induced during leaf and flower senescence, correlating with increases in DNA fragmentation and nuclease activity (Blank and McKeon, 1989; Perez-Amador et al., 2000; Lers et al., 2001; Canetti et al., 2002; Yamada et al., 2006a; Yamada et al., 2006b). Endonucleases are also involved in other plant PCD processes. Based on their divalent cation requirement, endonucleases have been classified into Zn$^{2+}$-dependent and Ca$^{2+}$-dependent groups (Sugiyama et al., 2000). Zn$^{2+}$-dependent nucleases were found to be involved in various processes of plant PCD such as seed germination, TE differentiation and leaf senescence (Blank and McKeon, 1989; Aoyagi et al., 1998; Wood et al., 1998; Perez-Amador et al., 2000; Ito and Fukuda, 2002). Ca$^{2+}$-dependent nucleases were induced during the hypersensitive response (Mittler and Lam, 1995). Recently a third group has been found including the senescence-specific nucleases tomato LeNUC1 and LeNUC2 (Lers et al., 2001), parsley PcNUC1 and PcNUC2 (Canetti et al., 2002) and petunia PhNUC1 (Langston et al., 2002).
The activity of these nucleases is optimal at neutral pH (slightly basic) and is enhanced by Co\(^{2+}\). Ca\(^{2+}\)-dependent nucleases have been suggested to function during the initial fragmentation of nuclear DNA. The majority of the nuclear DNA is then degraded by vacuolar and apoplastic Zn\(^{2+}\)-dependent nucleases after the rupture of the membranes (Sugiyama et al., 2000; Ito and Fukuda, 2002). Co\(^{2+}\)-dependent nucleases may be similar to the Ca\(^{2+}\) type and also function at the initial stage of PCD (Langston et al., 2005).

Biochemical characteristics of PCD induced endonucleases share many common features with the Nuclease I family (Sugiyama et al., 2000). The properties of Zn\(^{2+}\)-dependent nucleases are also equivalent to plant Nuclease I enzymes (Sugiyama et al., 2000). Nuclease I enzymes are defined as heat stable glycoproteins that have both DNase and RNase sites that function in the degradation of DNA and RNA (Iwamatsu et al., 1991; Maekawa et al., 1991; Perez-Amador et al., 2000). Zn\(^{2+}\) is required for their stability and activation (Volbeda et al., 1991). Their activities are optimal at acidic pH and inhibited by EDTA. The molecular sizes of Nuclease I enzymes range from 33-44 kDa (Sugiyama et al., 2000).

The characteristics of Nuclease I enzymes are conserved across the plant, fungi and bacteria (Perez-Amador et al., 2000; Sugiyama et al., 2000). Sequence analyses showed that PhNUC2 shares many common features of the Nuclease I family of endonucleases. For example, PhNUC2 has three N-glycosylation sites that are also found in other nucleases. The molecular weight of PhNUC2 is about 43kDa on our 2-D gels, which is greater than the predicted molecular weight of 34 kDa based on PhNUC2 cDNA. This suggests that the increase in the actual molecular weigh of PhNUC2 is due to the glycosylation of PhNUC2 protein.
Previous studies have identified PhNUC1, which is a senescence-specific nuclease induced during petal senescence in petunia (Langston et al., 2005). Biochemical characterization showed that PhNUC1 is a bifunctional nuclease that can degrade both RNA and DNA (Langston et al., 2005). PhNUC1 has been characterized as a glycoprotein and glycosylation is required for the nuclease activity (Langston et al., 2005). Neither PhNUC1 protein nor \textit{PhNUC1} cDNA sequence information is available, but in-gel activity assays show that the molecular weight of PhNUC1 is about 43 kDa, which is close to the size of PhNUC2 on our 2-D gels.

PhNUC2 was predicted to have a signal peptide that results in the secretion of the protein to the extracellular space. These signal peptides are also conserved among the nuclease I proteins that have been reported to date (Perez-Amador et al., 2000). In barley, BEN1 was secreted from the aleurone layer to the endosperm to degrade nuclear DNA during endosperm degeneration (Aoyagi et al., 1998). In some examples, it is not clear that the nuclease I protein is being secreted to the extracellular space. In transgenic tobacco, ZEN1 did not seem to be secreted, but was transported to the vacuole during tracheary element differentiation, suggesting that hydrolytic enzymes degrade intracellular component such as genomic DNA after the collapse of the tonoplast (Aoyagi et al., 1998). In petunia corolla senescence, in-gel activity assays showed that PhNUC1 accumulated in the nuclear fractions and activity was optimal at neutral pH (slightly basic), suggesting that activation of PhNUC1 may require transport of PhNUC1 to the nucleus during corolla senescence (Langston et al., 2005). Therefore, it is unclear whether the signal peptide leads PhNUC2 to be secreted into the extracellular space or to be targeted to a specific organelle.
PhNUC2 expression was enhanced by ethylene treatment, suggesting that PhNUC2 is regulated by ethylene. The nuclease activities of LeNUC1, PcNUC1, PcNUC2 and PhNUC1 have been shown to be induced by exogenous ethylene treatment (Lers et al., 2001; Canetti et al., 2002; Langston et al., 2005). In transgenic ethylene insensitive petunias (35S::etr1-1 lines), the induction of PhNUC1 nuclease activity was not inhibited but was delayed, and activity corresponded with the postponed senescence symptoms of the etr1-1 corollas (Langston et al., 2005). Similarly, the induction of PhNUC2 gene expression was shown to accumulate to similar levels in unpollinated senescing etr1-1 corollas at 18 days compared to unpollinated senescing MD corollas at 8 days. These results further indicate that ethylene is modulating the timing of plant PCD.

Since in-gel activity assays showed that the molecular weight of PhNUC1 is about 43 kDa, which is the same as the molecular weight of PhNUC2 on the 2D gels, we speculated that PhNUC1 and PhNUC2 were the same protein. This hypothesis was further supported by the VIGS experiments. In the PhNUC2 gene silenced corollas, PhNUC2 gene expression was significantly suppressed during senescence. Nuclease in-gel activity assays showed that PhNUC1 activity was significantly reduced in PhNUC2 silenced corollas during senescence.

Nucleases were also found to be induced in the hypersensitive response (HR) to plant microbial pathogens (Mittler and Lam, 1995; Ryerson and Heath, 1996; Mittler and Lam, 1997; Matousek et al., 2007). Activation of a Ca\(^{2+}\) dependent nuclease was identified from TMV infected tobacco plants. This nuclease was specifically induced in pathogen induced PCD due to the HR, because its activity was not detected during tobacco leaf senescence (Mittler and Lam, 1995). In our VIGS experiments, PhNUC2
transcripts in TRV2 and TRV2-CHS senescing corollas were much higher than the
transcript levels in the buffer (mock) controls (Figure 4.9), suggesting that PhNUC2
might play a role in defense responses following TRV inoculation and systemic spread.
Sequence alignments showed that PhNUC2 is also highly similar to the tomato nuclease
TBN1 (84.8% identical at the amino acid level). Gene expression and nuclease activity of
TBN1 was found to be induced in tomato vein tissues of symptomatic leaves following
spindle tuber viroid (PSTVd) infection (Matousek et al., 2007). New vascular tissues
were established in the symptomless plants that contained a higher ratio of xylem to
phloem in the vein tissues than that of diseased plants. This suggests that TBN1 might
function in TE differentiation during viroid defense (Matousek et al., 2007). According to
the results of our VIGS experiment, PhNUC2 is likely to respond to both HR and
senescence-induced PCD.

The functional investigation of a senescence-specific nuclease has been reported
in Arabidopsis leaves (Perez-Amador et al., 2000). Overexpression of Arabidopsis BFN1
in transgenic plants did not shown any morphological effects on plant growth and
development. Similarly, flower longevity was not delayed in our VIGS experiments,
possibly because PhNUC2 is expressed at the later stage of senescence, functioning as an
executor rather than a regulator of senescence. Although flower longevity was not
delayed in our VIGS experiments, nuclear degradation might be altered during PCD in
PhNUC2 silenced corollas. Studies showed that the introduction of antisense ZEN1
retarded nuclear degradation during TE differentiation in Zinnia elegans cells (Ito and
Fukuda, 2002).
Since our 2-DE-based proteomic studies identified a number of senescence-associated genes, it was necessary for us to employ a high throughput technique to investigate the function of these senescence-associated genes. To establish a rapid and efficient way to investigate senescence-associated genes in our lab, we utilized VIGS. TRV mediated VIGS has been used to study gene function during leaf senescence (Wang et al., 2005; Ahn et al., 2006; Lin et al., 2007), flower senescence (Chen et al., 2004; Chen et al., 2005) and fruit ripening (Liu et al., 2002; Fu et al., 2005; Xie et al., 2006).

VIGS has many advantages compared with other loss-of-function approaches (Baulcombe, 1999; Burch-Smith et al., 2004). VIGS can be used to silence a multigene family if the region chosen for silencing from the target gene is highly conserved in the family. Moreover, VIGS can also be used to silence an entire gene family by including all gene members in the construct (Chen et al., 2004; Chen et al., 2005). Conversely, to avoid silencing a highly homologous gene family, a specific region from the target gene should be chosen for making the virus construct. Unlike the RNAi approach, VIGS does not require stable transformation which is time consuming and laborious. More importantly, VIGS avoids embryo lethality that can result from completely knocking out a target gene.

It has been shown that TRV is a better silencing system compared with other virus systems (Brigneti et al., 2004). TRV has been widely used to study gene function in tobacco, tomato, petunia, pepper, Arabidopsis and poppy (Liu et al., 2002; Brigneti et al., 2004; Chen et al., 2004; Chung et al., 2004; Chen et al., 2005; Fu et al., 2005; Hileman et al., 2005; Burch-Smith et al., 2006; Cai et al., 2006; Dong et al., 2007; Spitzer et al., 2007). In our experiments, the TRV2 based system did have minor viral symptoms and
spread systemically very well after plants recovered from the inoculation.

VIGS also has its limitations. First, TRV2 itself might affect the flower longevity, as the longevity of TRV2 control flowers were half a day shorter than that of buffer controls according to the statistic analysis (Table 4.1). Second, VIGS could not produce a uniform silencing phenotype among plants, and even in the same plant. We employed the CHS gene as an indicator of silencing, as previous studies have shown that there was a reliable correlation between silencing the CHS reporter gene and the tandemly ligated target gene (Chen et al., 2004; Chen et al., 2005). To minimize the difference among VIGS samples, we therefore collected the silenced flowers indicated by almost white corollas. However, the flower longevity of TRV2-CHS controls was 1.1 day and 0.6 day shorter than that of buffer and TRV2 controls, respectively, indicating suppression of CHS might also affect flower longevity (Table 4.1). It has been reported that CHS transcripts were accumulated in pathogen defense responses (Ryder et al., 1984; Dhawale et al., 1989). Therefore, the TRV2-CHS should be the proper controls when one investigates a gene function using the TRV2-CHS tandemly ligated system. Third, it is possible that a senescence phenotype was not apparent due to the remaining function of the target gene. For example, in our VIGS experiments, PhNUC2 gene expression was not totally knocked out. If VIGS does not completely knock out gene expression, the remaining expression of the target gene can still function if the suppression does not reach the threshold level required to inhibit the response.
MATERIAL AND METHODS

Plant material

*Petunia x hybrida* cv. Mitchell Diploid (MD, wild type) transformed with 35S::etr1-1 (etr1-1 line 44568) were originally obtained from Dr. David Clark, University of Florida. The Arabidopsis *etr1-1* gene encodes a mutated receptor that confers dominant ethylene insensitivity. The *etr1-1* transgenic plants are insensitive to ethylene and have a delayed flower senescence phenotype (Chang et al., 1993; Wilkinson et al., 1997). MD and *etr1-1* line 44568 seeds were treated with 100 mg L-1 GA3 for 24 h and sown in cell-packs on the top of soil-less mix (Promix BX, premier Horticulture, Quebec, Canada). These plants were grown in the greenhouse with temperatures set at 24/16 °C (day/night) and a 13 h photoperiod supplemented by metal halide and high pressure sodium lights. *Petunia x hybrida* cv. Fantasy Blue seeds were generously provided by Goldsmith Seeds (Gilroy, CA, USA). Plants were grown in growth chambers at 22°C under 16 h light/8 h dark cycles. These plants were inoculated at the 4-6 true leaf stage and then used in VIGS analysis experiments.

Flowers were emasculated 1 d before flowers were fully open to prevent self-pollination. To study pollination-induced senescence, 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. Zero h after pollination (hap) and 0 d represent unpollinated flowers on the day of flower opening.
RNA isolation and cDNA synthesis

Total RNA was isolated from petunia corollas using TRIzol Reagent (Invitrogen, Carlsbad, CA). Two micrograms of total RNA was treated with RNase-free DNase I (Promega, Madison, WI, USA) to remove any contaminating genomic DNA. RNA was then reverse transcribed at 37 °C for 1 h using Omniscript reverse transcriptase kit (Qiagen, Valencia, CA). Synthesized first-strand cDNAs were used as the templates for RT-PCR or real-time PCR.

Isolation and sequence analysis of petunia nucleases PhNUC2 and PhNUC3

All primers were designed with Integrated DNA Technologies (IDT) Primer Quest and synthesized by IDT (Coralville, IA). Sequencing was performed at the Molecular and Cellular Imaging Center (The Ohio State University/OARDC, Wooster, OH). A partial petunia PhNUC2 sequence was amplified by RT-PCR using the forward primer 5’-TGGAGCAAGARGGNCA-3’ and the reverse primer 5’-TTTCNCCRGCTTCRACACCTT-3’. The primers for this fragment were designed according to the alignment of the peptide sequencing data from our previous 2-DE experiments and other senesence-associated nucleases. The remaining 5’ and 3’ cDNA sequences were isolated by rapid amplification of cDNA ends (RACE) according to the user manual from the SMART RACE cDNA amplification kit (Qiagen, Valencia, CA). The primers for amplification of the full-length PhNUC2 cDNA are listed as follows:

3’ RACE 5’- TGGCGACTTATCGGCCCTCTGCGTGTGG -3’
5’ RACE 5’-ACTTGGTCCCGGCCACACGCAGAGGC -3’
A partial EST sequence of PhNUC3 (Genbank accession CV299186) was identified from the petunia EST database (http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/Blast/index.cgi) by tblastn using the PhNUC2 full length cDNA sequence. The forward primer and reverse primer for RT-PCR amplification of PhNUC3 were 5’-TCTGACAGAAGATGCTTTGGCTGC -3’ and 5’-AGTGGTAGTGGAACCGAACCTGAT -3’, respectively. The PCR conditions were: 1 cycle of 94 °C for 3 min; 27 cycles of 94 °C for 30 s, 61 °C for 30 s and 72 °C for 30 s; 1 cycle of 72 °C for 7 min. The remaining 3’cDNA of PhNUC3 was isolated from MD corolla cDNA using the SMART RACE kit. The PhNUC3 3’ RACE primer sequence was 5’-GCTCATGGGCAGATCAGGTTCGGTTCCA-3’.

Sequence analysis

The PhNUC2 deduced amino acid sequence was generated by ORF finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). N-glycosylation sites of PhNUC2 were predicted by NetNGlyc analysis (http://www.cbs.dtu.dk/services/NetNGlyc-1.0). The PhNUC2 signal peptide was predicted by TargetP 1.1 (http://www.cbs.dtu.dk/services/TargetP).

The nucleotide sequences of PhNUC2, PhNUC3, and homologous nucleases from other plant species were obtained from the Genbank (http://www.ncbi.nlm.nih.gov/) and tomato and potato EST database in the SOL genomics network (http://www.sgn.cornell.edu) using the BLASTN program. The deduced amino acid sequences of PhNUC2 and PhNUC3 were compared with those of homologous nucleases including StEN1 from potato (accession no. AY676603), TBN1 from tomato (accession
no. AM238701), BFN1 from Arabidopsis (accession no. U90204), ZEN1, ZEN2 and ZEN3 from zinnia (accession nos. AB003131, U90265 and U90266, respectively), SA6 from daylily (accession no. AF082031), potato (EST no. SGN-U272930) and tomato (EST no. SGN-U322719). Sequence alignment of selected nucleases were generated by CLUSTALW (http://www.ebi.ac.uk/Tools/clustalw/) (Thompson et al., 1994). Aligned sequences were formatted for viewing using BOXSHADE 3.21 (www.ch.embnet.org/software/BOX form.html). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura et al., 2007). The phylogenetic tree was generated using the Neighbor-Joining method (Saitou and Nei, 1987). Bootstrap values were obtained by 1000 bootstrap replicates (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 217 positions in the final dataset.

Characterization of PhNUC2 and PhNUC3 expression during senescence in MD

RT-PCR was used to detect PhNUC2 expression during senescence in a pilot experiment under the following conditions: 1 cycle of 94 °C for 3 min; 27 cycles of 94°C for 30s, 61°C for 30s and 72 °C for 30 s; 1 cycle of 72 °C for 7 min. The forward and reverse primers were 5’-ATGATGACTTGTCGAATCGCCCAGGA-3’ and 5’-AGAGGGCCGATAAGCTGAAATCGCCCATTAACA-3’, respectively.
PhNUC2 expression in MD corollas during senescence was determined using real-time PCR. A 155 bp PCR fragment of the PhNUC2 gene including partial 3’ UTR region corresponding to bases 952-1106 was amplified by real-time PCR. The forward and reverse primers were 5’-CCACTTGCAGCAACTTGATCAACCA-3’ and 5’-TCGACGACGACGAACGATACTTGT-3’, respectively. To characterize PhNUC3 expression in MD corollas during senescence using real-time PCR, a 110 bp PCR fragment corresponding to bases 207-316 of the PhNUC3 gene was amplified. The forward and reverse primers were

5’-TCTGACAGAAGATGCTTTGGCTGC-3’ and
5’-AGTGGTAGTGGGAACCGAACCTGAT-3’, respectively.

One microliter cDNA was added in a 20 μL reaction volume of iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). Real-time PCR was conducted for 40 cycles of 94 °C for 10s, 61 °C for 30s, 72 °C for 30s using the iCycler Thermal Cycler (Bio-Rad, Hercules, CA). The signals were collected by the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Relative PhNUC2 or PhNUC3 expression was normalized to PhACTIN expression for each cDNA sample. Three replicates of each cDNA sample were run in each experiment. The PhACTIN forward and reverse primers were

5’-AGCCAACAGAGAGAAGATGACCCA-3’ and
5’-ACACCATCACCAGAGTCCAACACA-3’.
PhNUC2 expression in response to ethylene treatment

Two sets of six deattached petunia MD flowers were sealed in a 24 L chamber and treated with 2 µL L$^{-1}$ ethylene for 12 h or 36 h. Control flowers were sealed in another 24 L chamber without ethylene treatment for 36 h. Real-time PCR analysis of PhNUC2 was performed as described above. Experiments were repeated twice.

Plasmid construction of VIGS

The TRV1 and TRV2 vectors for VIGS were generously provided by Dr. Dinesh-Kumar, Yale University, USA. The strategy of TRV tandem constructs was as described in Chen et al., (2004). Briefly, to generate the TRV2-CHS construct, a 194 bp fragment of the CHS gene corresponding to bases 654–847 of petunia CHS (GenBank accession number X14599) was amplified by RT-PCR from petunia ‘V26’ corolla cDNA using primers 5’-cggaattcACCATTGGGCATTTCATG-3’ with an EcoRI restriction site and 5’-cgctctagaAGCCTTTCTCATTTCA-3’ with a XbaI restriction site. The amplified fragment was cloned into the TRV2 empty vector in the sense orientation to generate the TRV2-CHS construct.

To generate the TRV2-CHS-NUC2 construct, a 303 bp fragment of the PhNUC2 gene corresponding to bases 304-606 of the PhNUC2 gene was amplified by RT-PCR from petunia corolla cDNA using primers.

F: 5’-catctccatggTCAAGCCCTCTCCACTTCAT-3’ with a NcoI restriction site and R: 5’-catgtggtaccGTGCAAGTTTGACTTGCC-3’ with a KpnI restriction site. PCR products were cloned into TRV2-CHS to generate the TRV2-CHS-NUC2 construct.
**Agrobacterium-mediated infiltration**

The constructs, TRV1 (TRV RNA1 construct) and TRV2 (TRV RNA2 construct) or its derivatives (TRV2-CHS and TRV2-CHS-NUC2) were transformed into *Agrobacterium* strain GV3101 by heat shock. The infection procedures of VIGS were as described in Liu et al. (2002) and Chen et al., (2004). The Agrobacteria were cultured overnight at 28 °C in LB medium with appropriate antibiotics (Gentamicin 25 μg μL⁻¹, Kanamycin 50 μg μL⁻¹, Rifampicin 10 μg μL⁻¹). A 250 μl culture at a 1:100 ratio was inoculated into 25 ml LB-MESA media (10 mM MES pH 5.7, 20 μM acetosyringone) with antibiotics (Gentamicin 25 μg μL⁻¹, Kanamycin 50 μg μL⁻¹, Rifampicin 10 μg μL⁻¹). The Agrobacterium cells were then harvested and resuspended in inoculation buffer (10 mM MgCl₂, 20 mM MES pH 5.7, 100 μM acetosyringone) to an O.D. of 1.0 and left overnight at room temperature.

The bacteria containing TRV1 and the bacteria containing TRV2 or its derivatives were then mixed together in a 1:1 ratio. For each treatment, eight *Petunia x hybrida* cv. Fantasy Blue seedlings at 1 month old were used for infiltration. The leaves of petunia plants were infiltrated with the mixed Agrobacterial culture using a 1 ml disposable syringe without a needle.

**Characterization of PhNUC2 and PhNUC3 expression during senescence in VIGS plants**

PhNUC2 and PhNUC3 expression levels in Buffer, TRV2-CHS controls and TRV2-CHS-NUC2 silenced corollas during senescence were conducted using real-time PCR. Two corollas were collected from each of three plants in a treatment. Six corollas
of each treatment were pooled together before RNA was extracted. Totally, two sets of corolla tissues were collected.

The primers used were the same as those previously described for real-time PCR analysis of PhNUC2. This pair primers has been designed to avoid amplification of the TRV2-CHS-NUC2 construct. The same samples in PhNUC2 VIGS experiments were also used for real-time PCR of PhNUC3. The primers of PhNUC2 and PhNUC3 and the real-time PCR procedures are as described previously.

**In-gel nuclease activity assay**

Three unpollinated corollas at 9 days after opening (one corolla from each of three plants) in each VIGS treatment (Buffer, TRV2, TRV2-CHS and TRV2-CHS-NUC2) were collected. Total proteins were extracted from each corolla and used for in-gel nuclease activity assays using single stranded DNA, double stranded DNA, and RNA as substrates. The methods of in-gel nuclease activity assays were as described in Langston et al 2005 (chapter 2). The experiments were repeated twice.

**Flower longevity evaluation**

Flowers were tagged 24 h before they were fully open. The next day, when flowers were fully open is referred to as day 0. Flower longevity was determined as the number of days from flower opening until the corolla showed more than half collapse (wilted). The experiments were repeated twice and a total of 48 flowers (6 flowers from each of 4 plants per treatment, 2 replicates) from each of Buffer, TRV2, TRV2-CHS and TRV2-CHS-NUC2 were tagged and longevity was recorded. An analysis of variance
(ANOVA) was performed using the General Linear Model procedure of SAS (PC version 9.1, the SAS Institute, Cary, NC) to evaluate the statistical model:

\[ Y_{hijk} = \mu + \text{Treat}_h + \text{Exp}_i + \text{Treat}_h \times \text{Exp}_i + \text{Treat}_h \times \text{Plant}_j \times (\text{Exp})_l + \epsilon_{hijk} \]

Where \( \mu \) is the mean across all experiments and treatments, \( \text{Treat}_h \) is the effect of treatment (Buffer, Viral construct), \( \text{Exp}_i \) was one of the two experimental replicates, \( \text{Treat}_h \times \text{Plant}_j \times (\text{Exp})_l \) is the effect of individual plants within experiments, and \( \epsilon_{hijk} \) is the uncontrolled error. All effects in the model were considered fixed. The variance associated with each variable was estimated using restricted maximum likelihood (REML) in the Varcomp procedure. Mean separations were analyzed based on the least significant difference at the 0.01 level.

ACKNOWLEDGEMENTS

We thank Dr Dinesh-Kumar, Yale University, for providing the TRV constructs. We thank Dr Sophien Kamoun, The Sainsbury Laboratory, UK, for advice on the VIGS experiments and Dr. David Francis for assistance with statistical analysis. We thank Goldsmith Seeds for generously donating Petunia seeds.
Figure 4. 1. PhNUC2 protein expression in unpollinated and pollinated corollas in *Petunia x hybrida* cv. Mitchell Diploid.

PhNUC2 protein spots were detected on 2-D gels and are indicated by arrows. U, unpollinated corollas collected at 0, 24, 48 and 72 h after flower opening. P, pollinated corollas collected at 24, 48 and 72 h after pollination.

(B) PhNUC2 protein expression patterns in unpollinated and pollinated corollas. Relative PhNUC2 expression was the average quantity data of PhNUC2 protein spot intensities obtained from PDQuest v. 7.40 (Bio-Rad Laboratories, Hercules, CA). The black bar represents the average value of three replicates. The error bar indicates SD.
Figure 4.2. The sequences of PhNUC2 nucleotide and the predicted protein sequence.

The PhNUC2 cDNA encodes a protein of 301 amino acids. The solid lines indicate the PhNUC2 peptides that were sequenced from 2-D gels. The dashes show the regions of the primers that were used to amplify the PhNUC2 697 bp fragment. Three predicted glycosylation sites are located at 119, 137 and 211 (gray boxes). The arrow indicates the cleavage site of the signal peptide.
Figure 4.3. Comparison of deduced amino acid sequences of PhNUC2 and PhNUC3 homologs.

(A) Deduced amino acid sequences of PhNUC2, PhNUC3 and similar nuclease I enzymes were aligned using CLUSTALW. Dark and gray boxes indicate identical or similar residues. Dashes, gaps introduced to produce the alignment; asterisks (*), residues involved in the binding of zinc atoms; plus signs (+), residues involved in forming disulfide bonds; number symbols (#), structurally important glycosylation sites. Active sites for RNase and DNase activities in nucleases (His residues at positions 85 and 157, respectively, in PhNUC2) are also indicated under the alignments (Sugiyama et al., 2000). The region between red letters was used for making the TRV2-CHS-NUC2 VIGS construct.

(B) Phylogenetic analysis of PhNUC2, PhNUC3 and other similar nucleases. Phylogenetic analyses were conducted in MEGA4. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances are in the units of the number of amino acid substitutions per site.
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Figure 4.4. Relative PhNUC2 gene expression in unpollinated non-senescing and pollinated senescing corollas in *Petunia x hybrida* cv. Mitchell Diploid.  

(A) Semi-quantitative RT-PCR of PhNUC2 gene expression in unpollinated and pollinated corollas from 0 to 72 h. *ACTIN* was the internal control. PCR was performed for 24 cycles. (B) Real-time PCR of PhNUC2 gene expression in unpollinated and pollinated corollas from 0 to 72 h. U, unpollinated. P, pollinated. Relative PhNUC2 expression was normalized to *ACTIN* expression for each cDNA sample. The black bar represents the average value of three replicates. The error bar indicates SD.
Figure 4.5. Real-time PCR of PhNUC2 gene expression in flower parts in *Petunia x hybrida* cv. Mitchell Diploid.

Styles and ovaries were collected from non-senescing flowers on the day of flower opening (0), and from naturally senescing flowers at 8 d after flower opening (8d) and 72 h after pollination (72 P). Pollen was collected from flowers on the day of anther dehiscence, approximately 2 d after the flowers opened. The black bar represents the average value of three replicates. The error bar indicates SD.
Figure 4.6. Gene expression patterns of PhNUC2 and PhNUC3 during natural and pollination-induced senescence in *Petunia x hybrida* cv. Mitchell Diploid.

Gene expression analysis for PhNUC2 and PhNUC3 was conducted by semi-quantitative RT-PCR. *ACTIN* was used as an internal control. PCR was performed for 27 cycles. Unpollinated, corollas were collected from unpollinated flowers at 0, 24, 48, 72 h and 192 h. Pollinated, corollas were collected from pollinated flowers at 24, 48 and 72 h. Unpollinated flowers at 192 h and pollinated flowers at 72 h were senescent and corollas were wilted.
Figure 4.7. Response of PhNUC2 gene expression to ethylene. (A) Real-time PCR analysis of PhNUC2 gene expression that were regulated by ethylene treatment. Detached flowers were sealed in three 24 liter chambers. Control, detached flowers were set in a sealed chamber of air for 36 hours as a control; Treatments, detached flowers were set in the sealed chamber and treated with 2 µL L⁻¹ ethylene for 12 or 36 hours, respectively. (B) Real-time PCR analysis of PhNUC2 gene expression in MD and etr1-1 lines. Non-senescing corollas were collected from MD and etr1-1 line at 0 day; senescing corollas were collected from MD at 8 days and etr1-1 line at 18 days. The black bar represents the average value of three replicates. The error bar indicates SD.
Figure 4.8. TRV2-CHS-NUC2 VIGS and control flowers.

(A) Flowers represented from left to right are Buffer (mock), TRV2-CHS (positive control) and TRV2-CHS-NUC2 infiltration. Pictures were taken at 5 weeks after infiltration.

(B) Flowers represented from left to right indicate VIGS silencing effects were variable. The white color reflecting CHS silencing as a reporter for target gene silencing ranged from almost white to white with purple sectors.
Figure 4.9. Relative PhNUC2 and PhNUC3 expression in senescing corollas of VIGS plants.

(A) Real-time PCR analysis of VIGS effects on PhNUC2 gene expression levels among senescing corollas at 48 h after pollination. Flowers were collected from Buffer, TRV2, TRV2-CHS and TRV2-CHS-NUC2 infiltrated plants. RNA was isolated from 6 pooled corollas in each treatment. (B) Real-time PCR analysis of VIGS effects on PhNUC3 gene expression levels among senescing corollas at 48 h after pollination. The same RNA samples used for PhNUC2 real-time PCR were used to determine PhNUC3 gene expression. The black bar represents the average value of three replicates. The error bar indicates SD. All experiments were repeated twice.
Figure 4.10. PhNUC1 activity during corolla senescence in VIGS plants.

(A) Senescing and non-senescing flowers in different treatments in VIGS experiments. a, Buffer (mock) infiltration; b, TRV2 infiltration; c, TRV2-CHS infiltration; d, TRV2-CHS-NUC2 infiltration. For each treatment, the first flower is a non-senescing flower and the last three are senescing flowers.

(B) Nuclease in-gel activity assay with single strand DNA, double strand DNA and RNA as substrates, respectively. C (control), proteins extracted from MD 48 hap corollas; Y, non-senescing corollas; S, senescing corollas. PhNUC1 activity in each assay is indicated by the arrow.
<table>
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<th>Treatment</th>
<th>Flower longevity (days) (N=48)</th>
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<td>Buffer</td>
<td>$9.9 \pm 1.0 \text{ A}$</td>
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<td>TRV2</td>
<td>$9.4 \pm 0.9 \text{ B}$</td>
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<td>TRV2-CHS-NUC2</td>
<td>$8.5 \pm 0.9 \text{ C}$</td>
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Table 4.1. Flower longevity comparisons among Buffer (mock), TRV2, TRV2-CHS and TRV2-CHS-NUC2 infiltrated plants.

Flower longevity of each flower was determined as the number of days from flower opening until the corolla showed more than half collapsed (wilted). Flower longevity reported represents the mean of 48 flowers for each treatment (2 replicates, 6 flowers from each of 4 plants for each replicate). Values represent means±SD. Mean separations were analyzed based on the least significant difference (LSD) at 0.01 level. Means with different letters are significantly different.
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