IMPROVING THE POSTPRODUCTION QUALITY OF FLORICULTURE CROPS

DISSERTATION

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By

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ABSTRACT

Senescence is the last stage in the life cycle of plants and eventually leads to organ or plant death. The senescence process is regulated by plant hormones including ethylene and abscisic acid. During senescence, the cell components are broken down and resources are redistributed. Proteases play a crucial role during nutrient degradation. Senescence can be accelerated during environmental stresses and the quality of crops during periods of stress can be reduced. Floriculture crops are often exposed to many environmental stresses during shipping and retailing, and these stresses often result in damaged crops and profit losses. Understanding the senescence process will allow us to develop floriculture crops with higher postproduction quality and to improve the quality of crops under various environmental conditions.

The long-term goal of this research is to improve the postproduction quality of floriculture crops by developing methods to prevent or reduce damage from senescence. To accomplish these goals, a putative senescence-specific protease from *Petunia ×hybrida* (*PhCP10*) was characterized and its functional role during petal senescence was investigated. Secondly, the effects of exogenous applications of ABA on drought stress tolerance were evaluated in several important floriculture crops. The *Petunia ×hybrida* cysteine protease, *PhCP10,* is up-regulated relatively early during petal senescence. Expression patterns and high sequence homology to the senescence-specific

cysteine protease from *Arabidopsis* (*SAG12*), suggested that *PhCP10* might be involved in senescence. Transcript levels of *PhCP10* increased during senescence regardless of the cause (aging, pollination or abiotic stresses). Drought-stressed and nutrient deprived leaves had increased *PhCP10* expression only when the leaves were senescing. Cloning and sequencing of the *PhCP10* promoter also revealed a senescence-specific region homologous to that of the *SAG12* promoter. A putative enhancer element was also identified through expression of GFP driven by the *PhCP10* promoter.

To further understand how to delay stress-induced senescence ABA applications were investigated as a way to enhance drought tolerance and extend the shelf life of important floriculture crops. ABA enhanced tolerance to drought stress by closing stomata to reduce water loss. Exogenous ABA applications delayed wilting and allowed plants to survive during short periods of drought stress. However, leaf chlorosis in some bedding plants was observed following ABA application. To prevent the development of leaf chlorosis additional plant hormones known to delay senescence, including cytokinin (benzyladenine or BA), gibberellic acid (GA_{4+7}) or the ethylene perception inhibitor, 1-methylcyclopropene (1-MCP), were applied prior to ABA treatments. Although the individual application of these hormones had no effect on the development of s-ABA-induced leaf chlorosis, application of a mixture of benzyladenine and gibberellic acid (BA + GA_{4+7}) prevented leaf chlorosis. The application of ABA alone or a combination of ABA and BA + GA_{4+7} will allow floriculture crops to tolerate temporary drought stress without significant loss of postproduction quality.

DEDICATION

This document is dedicated to my husband and family.

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CHAPTER 1:

IMPROVING THE POSTPRODUCTION QUALITY OF FLORICULTURE CROPS

Importance of the floriculture industry in the United States

Floriculture crop production is a $4.2 billion dollar industry in the United States, with bedding and garden plants accounting for approximately 44% of the total wholesale value (USDA, 2009). While overall sales of floriculture crops may have declined in recent years, bedding and garden plant sales have increased (USDA, 2009). A large number of ancillary industries (suppliers, distributors and individuals associated with marketing) also benefit from floriculture crop sales and their sales are not reflected in these estimates. Therefore, the true value of floriculture industry in the United States is unknown.

Retailing and postproduction handling of floriculture crops

In the last several decades, there have been several changes in the retailing of floriculture crops that have necessitated a stronger emphasis on postproduction quality. One of these changes is the retail location. Fewer floriculture crops are purchased from traditional garden centers and local florists, where plants were cared for by horticultural professionals, and more customers are purchasing floriculture crops from big-box retailers (mass merchandisers and club centers) and general retailers (department stores

and supermarkets) (Yue and Behe, 2008). Those retailers often obtain their plants from distant producers. Prolonged shipping increases exposure to less desirable environmental conditions, which can result in decreased crop quality (Starman et al., 2007).

Another major change to the retailing of floriculture crops is the utilization of new distribution systems called pay-by-scan (PBS) or vendor managed inventory (VMI) systems. PBS/VMI is a distribution system in which the supplier (vendor) provides products to the retailers, but the retailer never takes responsibility for the products until it is sold to the consumer (Naab, 2006). This allows the retailer to avoid taking possession of the products and the retailers do not have to record possible markdowns on their accounts which protects them from devaluation of their assets. PBS/VMI also allows the supplier to obtain electronic invoices faster (possibly receiving quicker payment) and to adjust the quantity and variety of merchandise to reflect consumers' preferences (Naab, 2006). In this system, the suppliers are completely responsible for their products and have to determine the most effective way to retail the product before quality decreases. For some growers, this is a new retailing challenge, placing a new responsibility on them to maintain the postproduction quality of their crops (van Iersel et al., 2009). This change in retailing requires additional staffing and training in order to maintain the postproduction quality.

These changes in retailing can result in a lack of care leading to decreased crop quality in the retail setting. This lack of care means floriculture crops may often endure environmental stresses such as water deprivation (Armitage, 1993; Starman et al., 2007). Water deprivation or drought stress is a major cause of postproduction loss and plants are

constantly under drought stress from infrequent or inconsistent watering in the retail environment (Barrett and Campbell, 2006; Waterland et al., 2010a; Waterland et al., 2010b). When plants are exposed to environmental stresses, senescence can be accelerated and plants quickly become unmarketable (Barrett and Campbell, 2006: van Iersel et al., 2009).

Senescence

Senescence is the final stage of development for plants and their organs. Natural or developmental senescence is induced by endogenous factors such as aging and hormone levels, but senescence can also result from exposure to various environmental stresses (Munné-Bosch and Alegre, 2004). Although stresses can severely impact plant quality, most floriculture research has focused on traditional breeding to developing new crops and improving current varieties (Chandler and Tanaka, 2007). Unfortunately, very little information is currently available on the physiological evaluation of senescence as induced by environmental stresses.

Senescence associated with environmental conditions

Exogenous factors such as environmental stresses can accelerate senescence in plants. These environmental stresses include drought, wounding, waterlogging, high and low irradiation, extreme temperatures, ozone, salinity, and nutrient deprivation (Munné-Bosch and Alegre, 2004). Various senescence associated genes (SAGs) showing increased transcript abundance under various stresses have been indentified in leaves

(Weaver et al., 1998). These genes, involved in responding to environmental stresses, are also associated with the regulation of senescence (Lim et al., 2007). Of the 43 transcription factors that were reported to be involved in senescence, 28 of those genes were also shown to be induced by stress treatments. While this suggests an overlap in senescence and stress response mechanisms, the role of specific SAGs in stress related responses is not well understood (Chen et al., 2002; Lim et al, 2007, Woo et al, 2004). Among genes that are upregulated during senescence, we are particularly interested in cysteine proteases. Cysteine proteases are involved in protein degradation and remobilization of nutrients. Transcripts of cysteine proteases accumulate under environmental stresses such as low temperature, salinity, drought and nutrient deprivation (Forsthoefel et al., 1998; Kingston-Smith et al., 2005; Koizumi et al., 1993; Schaffer and Fischer, 1988).

Plant hormones involved in plant senescence

In addition to differential gene expression of SAGs, changes in endogenous hormone levels also occur during senescence. Plant hormones are naturally produced substances that influence plant growth and development at low concentrations. Abscisic acid (ABA), ethylene, cytokinins, and gibberellins will be focused on in this research. These plant hormones play important roles in promoting or inhibiting plant senescence (Gan, 2004; Smart, 1994).

Ethylene. Ethylene is a gaseous plant hormone involved in ripening, stress responses, and senescence in many plants (Bleecker and Kende, 2000). Ripening of climacteric fruit and senescing of ethylene sensitive flowers are associated with a burst of respiration and an increase in ethylene synthesis (Gan, 2004, Kadar, 1992; Serek et al., 2006). Inhibition of ethylene synthesis and/or perception results in delayed ripening and/or senescence (Gan, 2004: Kadar, 1992). In postproduction/postharvest environments, exposure to ethylene results in premature leaf chlorosis and senescence and accelerated abscission and senescence of petals and/or inflorescences (Blankenship and Dole, 2003; Bleecker and Kende, 2000; Serek and Reid, 2000). Flower and leaf senescence and abscission decreases postproduction quality by decreasing the aesthetic beauty (Serek and Reid, 2000).

In senescence research, the manipulation of ethylene biosynthesis or perception has received a great deal of attention. Transgenic plants with altered ethylene perception or plants treated with chemicals that reduce ethylene biosynthesis or perception show delayed senescence (Jones et al., 2005; Serek et al., 2006; Wilkinson et al., 1997). Characterization of cysteine proteases during petal senescence revealed that expression of these proteases was delayed in the ethylene receptor mutant, *etr1-1*, and this delayed expression coincided with a delay in corolla senescence (Jones et al., 2005).

Besides molecular manipulation of genes to inhibit perception of ethylene, inhibitors that block ethylene synthesis or perception have also been extensively studied (Blankenship and Dole, 2003; Serek et al., 2006). Treatment of floriculture crops with ethylene perception inhibitors delayed senescence in a variety of crops including oriental

lily (*Lilium* sp.), carnation (*Dianthus caryophyllus*), snapdragon (*Antirrhinum majus*), and petunia (*Petunia ×hybrida*) (Blankenship and Dole, 2003; Celikel et al., 2002; Serek et al., 2006). Two commonly studied inhibitors of ethylene perception are silver thiosulfate (STS) and 1-methylcyclopropene (1-MCP). STS is generally applied as a pretreatment to cut flowers (Macnish et al., 2010; Serek et al., 2006). Because of the mobility and persistence of STS, it is very effective at delaying flower senescence after only one application. However the application and disposal of STS poses an environmental risk because it contains the heavy metal silver (Macnish et al., 2010). Another ethylene perception inhibitor used in floriculture crops is 1-MCP. Commercial 1-MCP is released as a gas when mixed with water or buffer. Treatments with 1-MCP are also very effective at delaying senescence, but multiple applications may be required. 1-MCP is not readily transported within the plant. The effectiveness of 1-MCP may also be limited because plants regain ethylene sensitivity as new receptors are synthesized (Serek et al., 2006).

Although applications of ethylene perception inhibitors or genetic modification of ethylene receptors can be used to delay senescence, senescence is never completely prevented. This suggests that there are additional factors regulating flower senescence. Daylilies (*Hemerocallis* spp.), for example, do not experience an increase in ethylene production prior to flower senescence (van Doorn and Stead, 1994). For daylily flowers, ABA applications accelerate cell death in petals and induce other biochemical changes associated with natural senescence (Panavas et al., 1998). Treatment of various flowers with exogenous ethylene sources such as ethephon (2-chloroethylphosphonic acid), is

accompanied by increased ABA levels in a variety of plants including cleavers (*Galium aparine*) and rose (*Rose ×hybrida*) (Abeles et al. 1992; Hansen and Grossmann, 2000). Senescence is clearly regulated via the interaction of multiple plant hormones, with ethylene having a central role.

Abscisic acid. Abscisic acid (ABA) is a plant hormone that has been associated with leaf abscission, bud dormancy, seed dormancy, suppression of elongation and stomatal closure (Taiz and Zeiger, 2002). The plant hormone ABA is most noted for the primary role in controlling seed and bud dormancy as well as for controlling water loss by regulating stomatal opening. ABA is also associated with other processes such as abscission, fruit ripening and senescence but it is unclear if ABA is playing a primary role in these processes (Noodén, 1988). ABA is believed to be a strong factor promoting leaf senescence (Gan, 2004). Whether ABA interacts with ethylene during leaf senescence is still unclear (van Doorn and Woltering, 2008). It has been documented that exogenous applications of ABA resulted in increases in ethylene production that could hasten senescence (Abeles et al. 1992). ABA is recognized as playing a role in senescence but its contribution to senescence may be mediated by responding to plant hormones such as ethylene. Applications of exogenous ABA leads to increased leaf chlorosis in *Viola ×wittrockiana* (pansy) and *Viola cornuta* (viola), which does not appear to be associated with increased ethylene production or sensitivity (Waterland et al., 2010b).

Cytokinins. Cytokinins are associated primarily with cell division, but are also known to be involved in leaf senescence, nutrient mobilization, apical dominance, apical meristem formation, floral development, seed germination and breaking of bud dormancy (Taiz and Zeiger, 2002). During senescence, cytokinin levels decrease and expression of genes involved in cytokinin breakdown increase (Gan, 2004; Lim et al., 2007; van Doorn and Woltering, 2008). Cytokinins reduce leaf yellowing and senescence by delaying chlorophyll degradation (Gan and Amasino, 1995; Gan and Amasino, 1997). Manipulation of endogenous cytokinin levels affects leaf and flower senescence. Transgenic petunia and *Nicotiana tabacum* (tobacco) plants over-producing cytokinins (P_{SAG12}:*IPT,* isopentenyl transferase) showed delayed leaf senescence and reduced symptoms of leaf chlorosis following drought stress (Chang et al., 2003; Clark et al., 2004; Gan and Amasino, 1995; Lim et al., 2007). Increased cytokinin levels also delayed ethylene production and enhanced tolerance to ethylene treatment (Chang et al., 2003). Application of cytokinins such as BA (benzyladenine), delay the development of leaf yellowing in floriculture crops including lily (*Lilium* sp.), tulip (*Tulipa gesneriana*), geranium (*Pelargonium ×hortorum*), gerbera daisy (*Gerbera jamesonii*), goldenrod (*Solidago canadensis*), miniature rose (*Rosa ×hybrida*) and zinnia (*Zinnia elegans*) (Franco and Han, 1997; Kim and Miller, 2009; Padhye and Runkle, 2008; Philosoph-Hadas et al., 1996; Pinto et al., 2005; Tjosvold et al., 1994). Although cytokinins are known to delay senescence, the exact mechanism by which cytokinins interact with other plant hormones to suppress senescence is unknown.

Gibberellins. Gibberellins are also associated with a variety of responses including cell elongation, seed germination, floral initiation and development and fruit set (Taiz and Zeiger, 2002). Gibberellins are thought to inhibit degradation of chlorophyll, proteins and nucleic acids during leaf senescence (Gan, 2004). The application of gibberellic acid, specifically GA_{4+7}, also effectively enhances postproduction quality by reducing the occurrence of leaf chlorosis and senescence. Basal leaf yellowing is reduced or prevented by GA_{4+7} application in Easter and Oriental (*Lilium* sp.) lilies (Han, 1997; Ranwala and Miller, 2000; Ranwala et al., 2000; Ranwala et al., 2003). In some species, including Easter lilies (*Lilium longiflorum*), leaf senescence is not associated with ethylene production and it is prevented by the application of cytokinins (BA) and/ or gibberellins (GA_{4+7}) (Franco and Han, 1997). Application of GA_3 delayed increases in ethylene production and delayed wilting in carnations (Saks et al., 1992; van Doorn and Woltering, 2008). Exogenous application of GA_3 delayed chlorophyll degradation caused by chilling stress in the herbaceous perennial Himalayan Paris (*Paris polyphylla*) (Li et al., 2010). The relationship between gibberellins and other plant hormones associated with senescence is also unclear.

Gene expression of cysteine proteases during senescence

Microarray, subtractive hybridization, and proteomic analyses have been used in a variety of flowering crops to identify genes associated with senescence (Bai et al., 2010; Breeze et al., 2004; Buchanan-Wollaston et al., 2003; Thompson et al., 2004). Many of these SAGs are involved in degradation, remobilization and responses to hormones and

stresses (reviewed in Jones, 2004; Bai et al., 2010; Breeze et al., 2004). The exact function of each of these genes is not completely understood and the specific mechanism of gene interaction with each other is also unknown.

Among SAGs, cysteine proteases are upregulated during senescence. Cysteine proteases have been associated with senescence in a variety of plants including *Arabidopsis*, tomato, maize, citrus, daylily, carnation and petunia (reviewed in Jones, 2004). Cysteine proteases are thought to be associated with breakdown, remobilization and recycling of proteins in the dying cells of the corolla so that they can be used in other parts of the plant. Increased cysteine protease activity has also been measured following exposure to environmental stresses and pathogen infection (Beers et al., 2000).

Rationale and significance of research

Postproduction/postharvest losses in floriculture crops can range from 5 to 20%, depending on the retail environment (Armitage, 1993; Healy, 2009). A large portion of these losses are associated with accelerated senescence. During shipping and retailing floriculture crops are likely to encounter various stresses, such as drought, that can hasten senescence. Drought and other stresses result in accelerated senescence and a decrease in aesthetic beauty. Understanding the senescence process will allow us to develop/maintain floriculture crops with increased postproduction quality under stressful conditions during shipping and retailing.

Goals and objectives

The long-term goal of this research is to improve the postproduction quality of floriculture crops. To reach this goal, it would be useful to gain an understanding of the mechanisms of senescence and develop methods to prevent or reduce damage from accelerated senescence-induced by exposure to environmental stresses (i.e. drought stress). By delaying senescence, we can improve/maintain the postproduction quality of floriculture crops. In this research, two different approaches were taken. First, cysteine proteases were characterized during the senescence process. Secondly, the effectiveness of exogenous applications of ABA in delaying drought stress-induced senescence on several species of bedding plants and chrysanthemums, was evaluated.

The four specific objectives to accomplish these research goals are:

1. to characterize a putative senescence-specific cysteine protease in petunia.

2. to determine if applications of abscisic acid enhance drought stress tolerance in bedding plants.

3. to determine if exogenous benzyladenine and gibberellic acid application prevents abscisic acid-induced leaf chlorosis in pansy and viola.

4. to determine if abscisic acid applications delay wilting in drought-stressed chrysanthemums without the development of phytotoxcity.

CHAPTER 2:

CHARACTERIZATION OF A SENESCENCE-SPECIFIC CYSTEINE PROTEASE, *PHCP10*, IN PETUNIA

Abstract.

Plant senescence can lead to large economic losses during the postproduction handling of horticulture crops. Although senescence is a natural process, it can be hastened as a result of exposure to biotic and abiotic stresses. Senescence associated genes or SAGs are transiently upregulated during senescence. Among SAGs, a group of proteases called cysteine proteases have been identified as important to the senescence process, but the function of these cysteine proteases in senescence is unclear. *SAG12*, a cysteine protease from *Arabidopsis*, is consistently upregulated only in senescing tissues. Analysis of the promoter region of the *SAG12* gene has led to the identification of a putative regulatory region that drives senescence-specific expression. The goal of this project was to investigate the function of the *Petunia* ×*hybrida* Cysteine Protease 10 (*PhCP10*), a putative cysteine protease from petunia, in age-related and stress-induced plant senescence. Expression analysis and high sequence homology of *PhCP10* to *SAG12* strongly suggested that *PhCP10* might be involved in senescence. A functional characterization of *PhCP10* during age-related, pollination-induced and abiotic stress-

induced senescence also indicated that regulation of *PhCP10* was senescence dependent. However, down regulation of *PhCP10* utilizing RNAi technology resulted in no difference in flower longevity. Cloning and sequencing of the *PhCP10* promoter revealed a putative senescence-specific region, homologous to that of the *SAG12* promoter. A putative enhancer element was also identified through promoter deletion analysis. Further investigation of the *PhCP10* promoter to identify the senescence-specific element, interacting factors, and identification of the target protein(s)/residue(s) of PhCP10 is desired to understand the functional role and regulation of *PhCP10* during flower senescence.

Senescence is the final stage in the life cycle of plants. For horticulture crops, accelerated senescence caused by drought or other stresses can lead to severe profit losses. Delaying senescence is important for floriculture crops as the customer appeal and marketability of these products depends heavily on the beauty of the floral display. The first step to understanding senescence and developing crops with prolonged flower longevity is to identify genes associated with floral senescence. To identify those genes, microarray and subtractive hybridization methods have been used in a variety of flowering crops (Breeze et al., 2004; Buchanan-Wollaston et al., 2003; and Thompson et al., 2004). Some of the genes identified by these methods are upregulated in senescing tissues and are called senescence associated genes (SAGs) (Lohman et al., 1994). SAGs include proteases, nucleases, metallothioneins, stress-related genes and genes associated

with ethylene production and perception (Breeze et al., 2004; reviewed in Jones, 2004). The functional role of most of these genes in the senescence process is still unclear.

Many SAGs are suspected to be involved in the regulation of the senescence process. SAGs can be divided into two groups based on their putative function. The first group of SAGs is involved in nutrient remobilization during age-related (or natural) senescence, while SAGs in the second group are associated with protection and stress responses (Noh and Amasino, 1999a).

SAGs including proteases, nucleases, and transporters play a central role in nutrient remobilization during senescence. During nutrient remobilization cell components are broken down and reallocated to other parts of the plant. A large portion of the nutrient remobilization process involves degradation of proteins into amino acids by proteases (Solomon et al., 1999). Proteases are therefore one of the most well characterized proteins associated with senescence in plants (Beers et al., 2000).

Proteases in both plants and animals can be divided into the following five different classes based on their catalytic mechanism: serine proteases, cysteine proteases, aspartic proteases, metalloproteases, and threonine proteases. Among the five classes, cysteine proteases play a crucial role in morphological changes during plant cell death (Kusaka et al., 2004). Cysteine proteases show an increase in expression during flower senescence in a wide variety of crops including Alstroemeria (*Alstroemeria peruviana*), broccoli (*Brassica oleracea*), carnation (*Dianthus caryophyllus*), daylily (*Hemerocallis* spp.), daffodil (*Narcissus pseudonarcissus*), gladiolus (*Gladiolus grandiflora*), iris (*Iris* ×*hollandica*), Chinese-lantern lily (*Sandersonia aurantiaca*), and petunia (*Petunia*

×*hybrida*) (Arora and Singh, 2004; Coupe at el, 2003; Eason et al., 2002; Guerrero et al., 1998; Hunter et al., 2002; Jones et al., 1995; Jones et al., 2005; Pak and van Doorn, 2005; Stephenson and Rubinstein, 1998; Valpuesta et al., 1995; Wagstaff et al., 2002). Reduction or inhibition of cysteine protease expression appears to effectively delay senescence. Down-regulation of cysteine protease, *BoCP5*, in broccoli resulted in a delay of senescence associated floret yellowing (Eason et al., 2005). Protease inhibitors also delay flower senescence in Iris (*Iris* ×*hollandica* cv. Blue Magic), and Chinese-lantern lily (*Sandersonia aurantiaca*) (Eason et al., 2002; Pak and van Doorn, 2005).

In addition to their involvement in senescence, SAGs also show increased gene expression under various abiotic stresses, including wounding, darkness, dehydration and ethylene exposure (Weaver et al., 1998). Increases in cysteine protease activity have also been associated with wounding, high salinity, pathogen infection, seed germination, senescence and nitrogen deficiency (Beers et al., 2000; Kingston-Smith et al., 2005). Transcripts of cysteine proteases accumulate under other environmental stresses such as low temperature, high salinity, and drought (Forsthoefel et al., 1998; Koizumi et al., 1993; Schaffer and Fischer, 1988). There appears to be a functional overlap between SAGs and stress response genes (Weaver et al., 1998), but the role of specific SAGs such as cysteine proteases in stress related responses is not clear.

Ethylene is a plant hormone that regulates the timing of fruit ripening and flower senescence (Bleecker and Kende, 2000). Ethylene-insensitive *etr1-1* petunias have provided valuable information about the functional role of ethylene during senescence (Wilkinson et al., 1997). *Etr1-1* petunias display a delayed flower senescence phenotype

(Wilkinson et al., 1997; Langston et al., 2005; Jones et al., 2005). The delayed senescence of *etr1-1* flowers is accompanied by an increase in protease activity and an increase in cysteine protease transcript abundance (Jones et al., 2005). *SAG12*, a senescence-specific cysteine protease from *Arabidopsis*, has also been reported to be upregulated by ethylene only after senescence is induced (Weaver et al., 1998).

Senescence associated cysteine proteases have also been indentified from petunia. A putative cysteine protease, *PhCP10*, has been cloned (Jones et al., 2005). Expression of *PhCP10* increases in senescing corollas and appears to be senescence-specific (Jones et al., 2005). The sequence of *PhCP10* showed high homology to that of *SAG12* from *Arabidopsis*. *SAG12* is a senescence-specific cysteine protease in leaves but its involvement in flower senescence is not known (Grbic, 2002; Lohman et al., 1994; Noh and Amasino, 1999a; Noh and Amasino, 1999b; Weaver et al., 1998).

Due to the sequence similarity of *PhCP10* and *SAG12* it was hypothesized that the *PhCP10* promoter could function in a similar manner to the *SAG12* promoter. The *SAG12* promoter drives expression only in senescing tissues. This senescence specificity is due to a highly conserved senescence-specific region within the promoter that is believed to regulate senescence inducibility (Noh and Amasino, 1999b). A highly conserved senescence-specific region similar to that of *SAG12* has also been identified in the promoters of *Brassica napus SAG12* homologs (*BnSAG12-1* and *BnSAG 12-2*; Noh and Amasino, 1999b). The identification of these regions in various plant species suggests that the function of the *SAG12* promoter could be conserved across species,

including petunia. The *SAG12* promoter also contains an enhancer region that doubles the senescence-specific expression in *Arabidopsis* (Noh and Amasino, 1999b).

The goal of this research was to characterize *PhCP10* during age-related and stress-induced senescence. The specific objectives of this research were 1) to determine whether *PhCP10* expression is senescence-specific, 2) to determine if *PhCP10* is a primary ethylene response gene and 3) to isolate and characterize the *PhCP10* promoter.

Materials and Methods

Source and handling of plant material. *Petunia* ×*hybrida* 'Mitchell Diploid' ('MD') were grown under normal greenhouse conditions in Wooster, OH, unless otherwise stated. Greenhouse temperature settings were 23.9 to 21.1ºC during the day and 18.3 to 15.6ºC at night. 'MD' seeds were treated with 100 $mg \cdot L^{-1}$ GA_3 overnight prior to being sown in cell-packs containing soilless media (Pro-Mix-BX-Mycorise-Pro®, Premier Horticulture, Quebec, Canada). After seedlings had 3 to 4 true leaves, plantlets were transplanted to 15-cm pots (unless otherwise stated). Plants were maintained using natural irradiance with supplemental lighting provided by high-pressure sodium and metal halide lamps (GLX/GLS e-systems GROW lights, PARSource, Petaluma, CA) when external photosynthetic photon flux (*PPF*) was less than 800 $\mu mol \cdot m^{-2} \cdot s^{-1}$ as measured by a weather station located outside of the greenhouses. Supplemental lighting was provided from 0500 to 1900 HR daily. Plants were irrigated daily with 150 mg $N \cdot L^{-1}$ 20N-3P-19K (Jack's Professional™ Water-Soluble Petunia FeED; J.R. Peters, Inc., Allentown, PA). Four weeks after transplanting, plants received an application of

Soluble Trace Element Mix (STEM; Scotts-Sierra Horticulture Products Co., Marysville, OH). All flowers were emasculated 1 d before anthesis (flower opening). Flowers were pollinated at anthesis with newly dehisced pollen from other flowers on the same plant.

Temporal and spatial expression of PhCP10 in flowers and other plant tissues - All tissues for Real-Time PCR analysis were immediately frozen in liquid N_2 after collection and stored at -80ºC until RNA was extracted. Stems and roots were collected from 7 week old plants. Anthers were collected prior to dehiscence and pollen was collected from newly dehisced anthers. Nonsenescing corollas were collected at 0 h after pollination (0 hap or at anthesis) and senescing corollas were collected at 48 hap. Corollas with or without pollination were collected for time course experiments. Pollinated corollas were collected every 12 h from anthesis until 72 hap and unpollinated corollas were collected at 0, 2, 4, 6, 7, 8, 9, and 10 d after anthesis (daa). Pollinated and unpollinated corollas were visually wilted at 48 hap and Day 7, respectively.

PhCP10 expression in ethylene treated corollas - For ethylene treatment experiments, unpollinated flowers at anthesis were detached and placed in deionized water. Test tubes containing one flower each were placed in a 24-L chamber and flowers were treated with 2 $\mu L \cdot L^{-1}$ C_2H_4 for 0, 0.5, 1, 2, 4, 12, 24 or 36 h as previously described in Chang et al., 2003. Control flowers were treated with air (0 $\mu L \cdot L^{-1}$ C_2H_4) for 36 h. Corollas were collected immediately after ethylene or air treatment. To further determine the effect of ethylene on regulation of *PhCP10* expression, unpollinated flowers were pretreated with the protein synthesis inhibitor, cycloheximide, prior to a 4 h ethylene treatment. Detached flowers at anthesis were placed in test tubes containing cycloheximide (50 mM)

or no cycloheximide (0 mM) for 4 h. After the 4 h, cycloheximide pretreatment flowers were place in test tubes filled with deionized water. These flowers were then divided into two groups that were treated with ethylene (2 $\mu L \cdot L^{-1}$ for 4 h) or air (0 $\mu L \cdot L^{-1}$ for 4 h). All flowers were treated in 24-L sealed chambers. Corollas were collected 4 h after ethylene or air treatment.

PhCP10 expression in drought-stressed leaves - To determine if *PhCP10* expression was induced by drought stress, seven week old petunia 'MD' seedlings were transplanted into 11-cm pots (as stated above) and grown in a growth chamber. Growth chambers (Conviron, model BDW 80; Controlled Environments Ltd., Winnipeg, Manitoba, Canada) were maintained at 20 ºC/20 ºC day/night temperatures with 16 h of lighting at an average photosynthetic photon flux of ≈ 218.6 $\mu moles \cdot m^{-2} \cdot s^{-1}$ from high pressure sodium lights. Prior to drought stress experiments, plants were watered until leaching occurred (container capacity). All drought-stressed plants were allowed to dry-down until they showed visible symptoms of leaf wilting. The plants remained wilted for twenty-four hours and were then rewatered. The cycle was repeated three times until visual and measureable leaf chlorosis (i.e. senescence) had occurred. Control plants were watered daily. Leaf chlorosis was measured with a handheld chlorophyll content meter (SPAD-502; Konica Minolta Sensing, Inc., Osaka, Japan). Senescing leaves were collected from a similar drought stress experiment in which the leaves were allowed to senesce until they were very chlorotic. RNA was extracted from three basal leaf halves divided down the midvein. Gene expression of *PhASR (Petunia ×hybrida* ABA, stress, and ripening) a marker gene for drought stress was also measured.

PhCP10 expression in nutrient deprived leaves –Nutrient deprivation analysis of *PhCP10* expression was performed on seven week old petunia 'MD' seedlings that were transplanted to 11-cm pots. Each 11-cm pot contained three seedlings in coarse perlite (Thermo-O-Rock East, Inc., New Eagle, PA), and 1/2 tsp of Soil Moist Granules (JRM Chemical, Inc., Cleveland, OH) and a felt wick. Pots were placed in a plastic storage container that was modified to hold nutrient solution and allowed to wick nutrient solution up from the bottom of the pot. Plants were also irrigated overhead daily with the appropriate nutrient solution and grown under normal greenhouse conditions as stated above from 9 July to 14 Aug., 2007. Seedlings received a modified nutrient solution one week after transplanting (Machlis and Torrey, 1956). Control plants received complete nutrient solution. The components for the nutrient solutions were formulated according to Machlis and Torrey (1956). Complete nutrient solution included 5.3 mM $Ca(NO_3)_2$, 5.3 mM KNO_3, 2.1 mM $MgSO_4$, 1.1 mM KH_2PO_4, 0.7 mM Fe DTPA, 0.5 mM H_3BO_3, 90 µM $MnCl_2 \cdot 2H_2O$, 8.1 µM $ZnCl_2$, 2.9 µM $CuCl_2 \cdot 2H_2O$, and 1.4 µM $NaMoO_4 \cdot 2H_2O$. All plants in the nutrient deprivation experiments received the complete nutrient solution except each major nutrient component as listed below. For nitrogen deprivation experiment 5.3 mM $CaCl_2$ and 5.3 mM KCl were used instead of $Ca(NO_3)_2$ and KNO_3; for phosphorus deprivation experiment 1.1 mM KCl was used instead of KH_2PO_4 and for potassium deprivation 5.3 mM $NaNO_3$ and 1.1 mM NaH_2PO_4 instead of KNO_3 and KH_2PO_4. Leaves were collected three weeks after application of nutrient deprived solutions. Plants were arranged in a randomized block design with 2 pots per treatment.

Expression analysis – RT-PCR (qRT-PCR) was performed with an iQ5 Real-Time PCR Detection System (BioRad, Hercules, CA) using iQ SYBR Green Mastermix (BioRad, Hercules, CA) to determine relative abundance of transcripts. RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). RNA was treated with RQ1 RNAse free DNase (Promega, Madison, WI). cDNA was synthesized with Omniscript Reverse Transcriptase Kit (Qiagen, Valencia, CA) and 2 µg of RNA was treated with 2 units of DNAse. qRT-PCR experiments were conducted as stated in Chapin and Jones (2009) except for the annealing temperature was 60 ºC instead of 65 ºC. Primers used to determine *PhCP10* (GenBank Accession no AY662996) expression were AATTCGTGGGGCAGTAATTG as a forward primer (F) and GAGGGGAAAGCAATGACAAG as a reverse primer (R) and were normalized to *PhACTIN* primers (F primer; AGCCAACAGAGAGAAGATGACCCA and R primer; ACACCATCACCAGAGTCCAACACA). Melt curves were generated to validate amplification of a single PCR product for each primer set. All gene expression in this study was normalized to *PhACTIN*.

Generation of transgenic PhCP10 RNAi petunias. To determine the function role of *PhCP10* RNAi transgenic plants were generated. An RNAi construct of *PhCP10* with 455 base pairs of the 3´ end of the *PhCP10* coding region was made in pGSA1276 (http://www.chromdb.org/rnai/pGSA1276.html). The pGSA1276 vector was designed to allow easy insertion of the sense and antisense fragments flanking a GUS stuffer. The sense and antisense fragments were ligated at *Asc*I and *Swa*I sites, and *Bam*HI and *Spe*I sites, respectively (Fig. 2.1). The RNAi construct was driven by the (Cauliflower Mosaic

Virus 35S) CaMV35S promoter. This construct was transformed into petunia 'MD' leaf tissue using *Agrobacterium tumeficiens* LBA4404, according to the modified procedure by Jorgensen et al., (1996). Positive transgenic plants were identified by using a primer set that spanned an intron of the *PhCP10* gene, to identify transgene and not endogenous gene.

Evaluation of flower longevity was performed on pollinated and unpollinated flowers of *PhCP10* RNAi transgenic plants at the T_0 and T_1 generations. All flowers were emasculated at one d before anthesis and pollination was performed using pollen from other flowers on the same plant at anthesis. Three lines with lowest *PhCP10* expression in the T_0 generation were selected for further evaluations in the T_1 generation.

PhCP10 expression in RNAi transgenic petunia. The level of *PhCP10* expression in *PhCP10* RNAi lines were measured by semi-qRT-PCR for T_0 plants and by qRT-PCR for T_1 plants using senescing corollas after pollination. In preliminary experiments 18 RNAi plants at T_0 generation, four negative plants, and three 'MD' plants were evaluated. Preliminary PCRs were also performed to optimize primer annealing temperature and cycle numbers for semi-qRT-PCR. Primers for semi-qRT-PCR and qRT-PCR were designed to produce 326 bp and 130 bp fragments, respectively and both reverse primers' sequences were chosen from the 3' UTR.

Cloning of the PhCP10 promoter. Previously the last exon of the *PhCP10* coding region was cloned and Thermal Asymmetric InterLaced-PCR (TAIL-PCR) was performed to obtain the remaining coding region and a 1.601 kb region of the promoter (Fig. 2.1). TAIL-PCR was designed to amplify the target region using combinations of specific

primers and arbitrary degenerated (AD) primers (Liu and Whittier, 1995). Primers were designed as stated in Liu and Whittier (1995) (Table 2.1). Three rounds of TAIL-PCR were conducted to obtain the *PhCP10* promoter. The first TAIL experiment used specific primers (TAIL primers) 1 to 3 and AD primers 1 to 4 (Table 2.1). The second TAIL experiment used TAIL primers 4 to 6 and AD primers 1 to 4 (Table 2.1). The third TAIL experiment used TAIL primers 6 to 8 (Table 2.1) and AD primers 1 to 10 (Table 2.1). The cloned putative promoter region was sequenced and compared to the sequences of *SAG12* promoter. *PhCP10* F and *PhCP10* R primers were used as controls for each TAIL-PCR experiment (Table 2.1).

Generation of PhCP10 promoter deletion constructs. A putative senescence-specific and basal regions of *PhCP10* promoter were predicted by localized BLASTs with *Arabidopsis SAG12* promoter sequences (Fig. 2.2). To validate the predicted enhancer, senescence specific and basal regions of the promoter, promoter deletion analysis was performed. For the promoter deletion constructs, three different fragments containing various promoter regions of *PhCP10* were amplified by PCR using *PfuUltra* II Fusion HS DNA Polymerase (Stratagene, La Jolla, CA) and ligated into pGEM-11Zf (+) at *Sal*I and *Not*I sites (Figs. 2.3 and 2.4A). pPhCP10-1, pPhCP10-2 and pPhCP10-3 contained only the basal promoter region (263 bp), the putative senescence-specific and basal regions (562 bp) and the entire cloned promoter (1.601 kb), respectively (Fig. 2.3). All promoter constructs were driving Green Fluorescent Protein (GFP) expression.

The GFP coding region was amplified using pMCS-sgfp-nos as a template (Chiera et al., 2007) with primers containing *Sph*I extension at the 5' end and *Xba*I and

*Hin*dIII extension at the 3'end (Fig. 2.4A). A PCR fragment containing GFP was ligated into pGEM-11Zf (+) vector at *Sph*I and *Hin*dIII sites and then the various *PhCP10* promoter fragments were ligated to create three promoter deletion constructs. *PhCP10* promoter–GFP cassettes were transferred into pJL10 (Fig. 2.4B) for all transient expression analyses. CaMV35S promoter driving GFP expression in pJL10 was used as a positive control and CaMV35S promoter was cloned from CaMV35S-GFP (Chiera et al., 2007). These expression cassettes were also ligated into pCAMBIA2200 for stable transformation in 'MD' leaf tissue as stated above (Fig. 2.4C). Positive transgenic plants were identified by PCR analyses with primers designed to amplify GFP sequence and a portion of promoter contained in each construct.

Transient expression of GFP driven by PhCP10 promoter deletions in lima beans.

Transient GFP expression of the three promoter deletion constructs (Fig. 2.3) and control CaMV35s-GFP construct was measured in lima bean cotyledons (Chiera et al., 2007; Dhillon et al., 2009). Lima bean cotyledons were excised and bombarded with tungsten particles coated with each DNA construct using the Particle Inflow Gun (PIG) (Finer et al., 1992). Images of lima beans expressing GFP were taken every h for 75 h as stated in Chiera et al., (2007). Images were registered and aligned utilizing ImageJ software (Rasband 1997-2007). An image area (400 x 300 pixel) was selected for quantifying GFP expression (Chiera et al., 2007). Images were separated into red, green and blue channels to remove background fluorescence. Total expression was determined as area multiplied by the mean gray scale value of red and green channels (Chiera et al., 2007).

Transient expression of GFP driven by PhCP10 promoter deletions in petunia flowers. To perform transient expression analysis in petunia flowers, *Agrobacterium tumefaciens* GV3101 was transformed by heat shock with the same constructs used in transient expression experiments in lima beans. Agroinfiltrations were preformed in the same manner as stated in Lindbo (2007) except infiltration was performed on the adaxial side of petunia flowers. Flowers were detached from the plants and infiltrated one d before anthesis. All flowers were pollinated at anthesis. Flowers were photographed hourly for 96 hap by an automated image collection system (Buenrostro-Nava et al., 2006; Chiera et al., 2007).

GFP expression in stable transgenic petunias. Generation of stably transformed promoter deletion constructs was accomplished by excising the promoter deletion constructs out of expression cassettes mentioned earlier and ligating them into pCAMBIA2200. Positive transgenic plants were screened by PCR using primers to amplify the corresponding promoters and the 5' end of GFP. Southern blot analysis was performed on the two lines of pPhCP10-3 expressing GFP as stated in Buenrostro-Nava et al., (2006) and Sambrook et al. (1989). DNA was extracted using methods stated in Murray and Thompson (1980) with modification as in Fulton et al., (1995) (Hernandez-Garcia et al., 2009). Ten and 20 micrograms of DNA from two transgenic plants containing the pPhCP10-3 promoter constructs were digested with *Xba*I. Ten picograms of pPhCP10-3 plasmid DNA was used as a positive control. NEB 2-Log ladder (New England BioLabs Inc., Ipswich, MA) was used as a molecular weight marker. For the probe 40 nanograms of full length GFP PCR product were labeled with P^{32}-dCTP using

Prime-It II Random Primer Labeling Kit (Stratagene, La Jolla, CA). Unincorporated nucleotides were removed with Micro Bio-Spin P-30 Tris Chromatography Columns (BioRad, Hercules, CA). The membrane was hybridized overnight at 60 ºC and washed at 60 ºC. Probed membranes were placed on a phosphor screen for 24 h and imaged with the Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA) (Buenrostro-Nava et al., 2006). Minimum expected size of fragment is ≈ 2.7 kb from right broader (RB) to *Xba*I site at the 3'end of GFP.

Statistical analysis. Flower longevity values were analyzed using Proc GLM (generalized linear model) with LSD means separation (least significant difference test) ($P \leq 0.05$). Values were analyzed by SAS Version 9.1.3 (Statistical Analysis System, SAS Institute, Inc., Cary, NC).

Results

Temporal and spatial expression of PhCP10 in flowers and other plant tissues. Very low levels of *PhCP10* expression were observed in all tissues tested, while the relative expression increased (approximately 378 times) in pollinated senescing corollas compared to nonsenescing corollas (Fig. 2.5). All senescing petunia corollas showed increased *PhCP10* expression (Figs. 2.6 and 2.7). Regardless of whether corolla senescence was induced by aging (unpollinated) or by pollination. The increase in *PhCP10* transcript abundance also coincided with the first visible symptom of flower wilting (Figs. 2.6 and 2.7). As senescence progressed, *PhCP10* expression continued to increase until the corollas were nearly desiccated in unpollinated flowers (Fig. 2.6).

Pollinated corollas, however, showed a large increase in *PhCP10* expression between 36 and 48 hap when the first visible sign of senescence was apparent, followed by a decrease in expression (Fig. 2.7).

PhCP10 expression in ethylene treated corollas - Treatment of corollas with ethylene also resulted in increased *PhCP10* expression (Fig. 2.8). Expression started to increase 12 h after ethylene treatment and peaked at 24 h, while treatment with air did not increase expression of *PhCP10* (Fig. 2.8). Treatment of corollas with cycloheximide prior to ethylene treatment resulted in no increase in *PhCP10* expression (Fig. 2.9). Only ethylene treated corollas without cycloheximide pretreatment had an increase in *PhCP10* expression.

PhCP10 expression in drought-stressed and nutrient deprived petunia leaves – In petunia leaves, expression of *PhCP10* was not increased during the course of drought stress and subsequent wilting unless visible leaf chlorosis developed (decrease in chlorophyll content by 82.1%) (Fig. 2.10A). Expression of a drought stress marker gene from petunia, *PhASR*, increased during the first visible signs of drought stress or two d without water (Fig. 2.10B) and decreased when plants were rewatered. *PhASR* expression was decreased in senescing leaves contrary to *PhCP10* expression (Fig. 2.10B).

PhCP10 expression in leaves deprived of nitrogen, phosphorus or potassium also increased only when leaves were visibly chlorotic (i.e. senescing) (Fig. 2.11). The highest *PhCP10* expression was observed from petunia leaves deprived of nitrogen. Nitrogen deprivation resulted in the most chlorotic leaves followed by phosphorus deprivation (Fig. 2.11). Phosphorus deprived leaves had interveinal chlorosis, while

potassium deprived leaves had no visible signs of chlorosis and *PhCP10* expression was also marginal in those leaves (Fig. 2.11).

PhCP10 expression in RNAi transgenic petunia. Eighteen positive *PhCP10* RNAi plants at the T_0 generation were identified by PCR screening. Three lines with the greatest reduction in *PhCP10* expression were selected for further evaluation (Fig. 2.12). RNAi lines 75, 68 and 81 showed a 96.8, 92.8 and 67.1% reduction in *PhCP10* transcript abundance, respectively, compared to that of 'MD' (Fig. 2.12). Pollinated flowers of RNAi lines 75, 68 and 81 had 3.3, 3.6, 3.2 d flower longevity compared to 3.2 d for 'MD' controls (Statistical Analysis System, SAS Institute, Inc., Cary, NC) (Table 2.2). Flowers of the same *PhCP10* RNAi lines that were not pollinated had 11.5, 11.6 and11 d of longevity compared to 11.4 d for 'MD' flowers ($P \leq 0.05$) (Statistical Analysis System, SAS Institute, Inc., Cary, NC) (Table 2.2). Flowers of transgenic petunias with reduced *PhCP10* expression (*PhCP10* RNAi) showed no altered flower longevity (Table 2.2).

Isolation of the PhCP10 gene. The PCR fragments containing the entire coding region and a 1.6 kb promoter of *PhCP10* were obtained by TAIL-PCR. Amino acid sequence of PhCP10 (GenBank accession # AAU81596) was aligned with *Arabidopsis* SAG12 (GenBank accession # AAC49135) and its homologs BnSAG12.1 (GenBank accession # AAD53011) and BnSAG12.2, (GenBank accession # AAD53012) in *Brassica napus* (rape) and NtCP1 (GenBank accession # AAW78661) in *Nicotiana tabacum* (tobacco) (Fig. 2.13). Analysis of sequence comparison showed the conserved amino acid residues for cysteine protease activity (arrows) and conserved cysteine residues involved in disulfide bridges (boxes) (Fig. 2.13). The highest amino acid sequence similarity (85%)

was observed between PhCP10 and the "senescence-specific" protease CP1 from tobacco (NtCP1). Sequences of SAG12 from *Arabidopsis* and BnSAG12.1 and BnSAG12.2 from rape showed 54, 56, and 52% similarity to that of *PhCP10*, respectively (Fig. 2.13).

PhCP10 promoter isolation and characterization. To further investigate the senescence specificity of *PhCP10*, the promoter was cloned and sequenced. The *PhCP10* promoter sequence (pPhCP10) was compared to *Arabidopsis SAG12* to identify putative enhancer, senescence-specific and basal regions of the promoter. The *Arabidopsis SAG12* and *PhCP10* promoter sequences showed 59.7% and 58.3% identity in putative senescence-specific region and putative basal promoter region, respectively (Fig. 2.2). All three pPhCP10 promoter constructs transiently expressed GFP with lower expression levels than the control (CaMV35s::GFP) in lima bean cotyledons (Fig. 2.14). However, there were differences in GFP expression driven by different promoter deletion constructs in lima bean cotyledons. The highest total expression of GFP was observed with the full length promoter construct (pPhCP10-3::GFP), while the deletion constructs pPhCP10-1 and pPhCP10-2 drove somewhat lower levels of GFP expression (Fig. 2.14). The upper graph in Fig 2.14 is comparing all the promoter deletion constructs to the Cauliflower Mosaic Virus 35S promoter (CaMV35S). CaMV35S is removed in the lower graph to better visualize difference among the PhCP10 promoter deletion constructs. Transient expression of the three promoter deletion constructs in agroinfiltrated petunia corollas showed constitutive expression of GFP, especially around the veins. No difference in intensity of the GFP signal was measured over the course of flower senescence (Fig. 2.15).

Stable transgenic petunias harboring pPhCP10-1::GFP (13 plants), pPhCP10-2::GFP (6 plants), pPhCP10-3::GFP (5 plants) constructs along with control plants containing pCaMV35S::GFP (7 plants) were successfully generated. GFP could not be detected in transgenic petunias containing either the pPhCP10-1::GFP or pPhCP10-2::GFP constructs. Two petunias containing pPhCP10-3::GFP (plant # 46 and 106) showed a high level of GFP expression, which became more confined around veins of corollas as senescence progressed. GFP expression of plant # 46 (pPhCP10-3::GFP) is shown in Fig. 2.16. Southern blot analysis revealed that two plants # 46 and 106 contained pPhCP10-3::GFP constructs, but they appeared to be the result of the same integration event (Fig. 2.17).

Discussion

Cysteine proteases are upregulated during senescence in various plant species and appear to be involved in senescence. However, their functional roles during petal senescence are unknown. In petunia, nine putative cysteine proteases have been identified (Jones et al., 2005). Among those proteases, expression of *PhCP10* occurs relatively early during flower senescence (Jones et al., 2005). In this research, the increase in *PhCP10* expression coincided with the onset of wilting in both unpollinated and pollinated corollas, suggesting a possible involvement of *PhCP10* in petal senescence (Figs 2.5, 2.6 and 2.7).

When nutrient deprivation and drought stress induced leaf senescence (i.e. visible leaf chlorosis), transcripts of *PhCP10* increased. This indicated that *PhCP10* expression

was induced by exposure to stresses that caused senescence. Expression of *NtCP1*, a senescence-specific cysteine protease from tobacco, was also detected only in senescing leaves and was not induced in mature green leaves exposed to drought or heat stress (Beyene et al., 2006). Similar findings were also observed with *SAG12* expression in dehydrated leaves. *Arabidopsis* leaves that were severely wilted but not chlorotic showed no increase in *SAG12* expression (Weaver et al., 1998). Taking these findings into consideration, *PhCP10* appeared to be a senescence-specific protease of petunia.

To investigate whether *PhCP10* is a primary ethylene response gene, *PhCP10* expression was evaluated following ethylene. Transcript levels of *PhCP10* in petunia corollas exposed to ethylene did not increase as rapidly as genes that were primarily regulated by ethylene (Chapin and Jones, 2009; Weaver et al., 1998). If *PhCP10* was primarily regulated by ethylene, increases in expression would have been visible within hours of ethylene treatment. This finding was further investigated with a cycloheximide pretreatment prior to ethylene exposure. If *PhCP10* were a primary ethylene response gene, a rapid increase of transcript abundance would be expected in response to ethylene treatment without *de novo* protein synthesis (i.e. in the presence of cycloheximide). Otherwise, *de novo* protein synthesis would have been required and cycloheximide pretreatment of the flowers would have blocked increased *PhCP10* expression. No increase in *PhCP10* expression was observed in petunia corollas treated with cycloheximide prior to ethylene exposure, suggesting that *PhCP10* was not primarily regulated by ethylene. Although *SAG12* expression in ethylene treated flowers has not yet been examined, *SAG12:IPT* transgenic petunias showed an increase in *IPT* expression

as a result of ethylene exposure (Chang et al., 2003). Whether this upregulation of *IPT* expression was a result of senescence induced by ethylene treatment or the promoter responding to the ethylene treatment itself was unclear (Chang et al., 2003).

The flower longevity of *PhCP10* RNAi lines with near knockout levels of *PhCP10* expression was not altered, suggesting that *PhCP10* might not play a role in the induction of senescence, but possibly act as an executioner. Because many proteases are active during senescence, the lack of phenotype could be due to gene or functional redundancy.

In order to further investigate senescence-specificity of *PhCP10* gene, the promoter of *PhCP10* was cloned and promoter deletion analysis was performed. In stably transformed plants, GFP expression was observed from the cells containing a 1.6 kb promoter driving GFP (pPhCP10-3), while no GFP expression was detected from the cells containing the putative senescence-specific and basal regions (pPhCP10-2) or putative basal region (pPhCP10-1) of the promoter. However, in a transient GFP expression study utilizing detached flowers, there was no difference in GFP expression over the course of flower senescence among all three promoter constructs. These experiments indicated that the basal region of the promoter was sufficient to drive GFP expression in the detached flowers. The observation of GFP expression patterns in stably transgenic plants and in transient analyses suggested that the putative "enhancer" region of the *PhCP10* promoter was required for visible expression of GFP in a whole plant and that "factor(s)" regulating expression of *PhCP10* might come from other parts of the plant. Transient expression of GFP driven by the same set of promoter constructs was

performed in lima bean cotyledons and increased expression of GFP was also observed with the full length promoter construct (pPhCP10-3::GFP). This result supported the presence of a possible enhancer element in the promoter. This 1.6 kb region of the *PhCP10* promoter corresponded to the *SAG12* promoter which included an enhancer element at -1345 to -1181 that greatly increased the expression of *SAG12* (Noh and Amasino, 1999a). Expression studies in stable transgenic plants needs to be repeated due to an insufficient number of transgenic plants containing the pPhCP10-3::GFP construct. Southern blot analysis was performed on only two transgenic plants (pPhCP190-3 #46 and #106). It is unknown if the other promoter constructs were incorporated properly. Weak or no GFP expression observed in stable transgenic plants containing pPhCP10-1::GFP or pPhCP10-2::GFP could be due to very low activity from promoter truncation. Additional promoter deletion constructs containing a full length promoter without a putative senescence specific region could verify its senescence specificity.

It was also noticed that GFP expression was more visible in the veins as the corollas began to senesce. Leaf senescence in general is known to be associated with remobilization of nutrient from senescing leaves to developing sink organs and tissues (Noodén, 1988; Smart, 1994). Flowers appeared to remobilize nitrogen and phosphorus during senescence (Chapin and Jones, 2009; Verlinden et al., 2003). It has also been postulated that cysteine proteases are involved in nutrient remobilization (Chen et al., 2002; Jones et al., 2005). GFP expression in the vein area could suggest the involvement of *PhCP10* in nutrient remobilization. Further investigation into whether *PhCP10* plays a role in nutrient remobilization is desired.

Due to the fact that *PhCP10* responded only to senescence, *PhCP10* could be used as a marker gene for leaf and flower senescence. The promoter of *PhCP10* could also be utilized as a tool for senescence related studies. Although the functional role of *PhCP10* during flower senescence is still unknown, *PhCP10* is indeed a senescence-specific protease. Identifying transcription factors that regulate the expression of *PhCP10* and specific substrates of the protease will help us to better understand the role of *PhCP10* in senescence.

Table 2.1. Primers used for TAIL-PCR of *PhCP10* promoter region.

AD1	TGW GNA GWA NCA SAG A
AD2	AGW GNA GWA NCA WAG G
AD3	CAW CGI CNG AIA SGA A
AD4	TCS TIC GNA CIT WGG A
AD5	NGT CGA SWG ANA WGA A
AD6	TGW GNA GSA NCA SAG A
AD7	AGW GNA NCA GWA WAG G
AD8	STT GNT AST NCT NTG C
AD9	NTC GAS TWT SGW GTT
AD10	WGT GNA GWA NCA NAG A
Tail 1	CAG ACA CCG TCT TCT CCC TTG
Tail 2	CCA CGG ACA CTG GCT GGT TA
Tail 3	ACC ATA TCC CAC CGC TGT AA
Tail 4	CAG AAA ATG CCC AGC AGC AC
Tail 5	GAG GCA AGC TGC TGT GGA AA
Tail 6	GGA AAA GCC CTT TTG ACT GAC ATG
Tail 7	GCG TTT ATG GAG CAG TTT GTT GC
Tail 8	TAA AAT GTG TCT TGG GCT TTG AGG
PhCP10 F	TCC GTA CAA GGG AGA AGA CG
PhCP10 R	CGG ATA AGA AGC TTC CGT TG

W = A or T, S = G or C, N = A, T, C or G, and I = Inosine

Table 2.2. Days until flowers of *Petunia ×hybrida* (petunia) *PhCP10* RNAi lines or 'Mitchell Diploid' ('MD') were visibly wilted.[z]

	Flower longevity (d)	
	Pollinated corollas	Unpollinated corollas
PhCP10 RNAi line 68	3.6a	11.6a
PhCP10 RNAi line 75	3.3a	11.5a
PhCP10 RNAi line 81	3.2a	11.0a
'MD'	3.2a	11.4a

[z]Values are means of three flowers from each of plant. Flower longevity was determined from five T_1 plants of RNAi event 68 (n=15), ten T_1 plants from event 75 (n=30), three T_1 plants from event 81 (n=9) and 4 'MD' plants (n=12). Values in columns followed by different letters are statistically different at $P \leq 0.05$ (least significant difference test). Values are the average number of days ± SD from anthesis until flowers were visibly senescing (i.e. wilted).

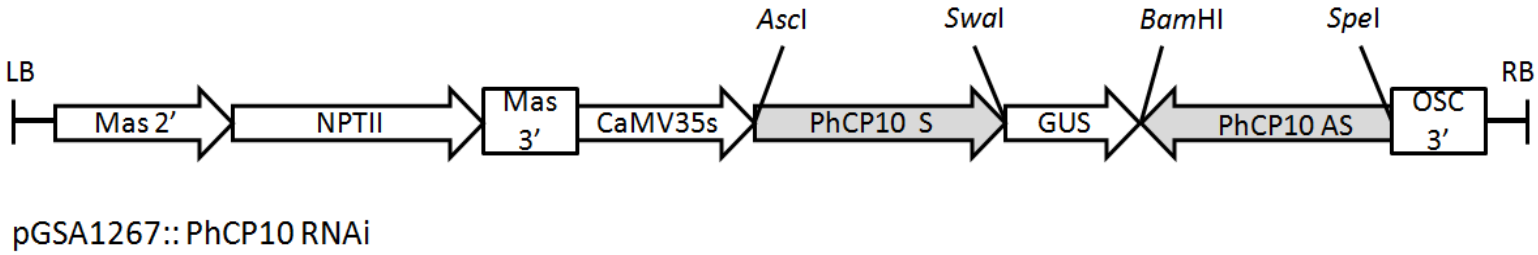

Fig. 2.1. Diagram of RNAi construct. *Petunia* ×*hybrida* 'Mitchell Diploid' (petunia) plants were used to generate RNAi transgenic plants. RNAi constructs were made using 455 base pairs of the 3′ end of the *PhCP10* coding region (gray arrows) in pGSA1276. The sense and antisense fragments were ligated at *Asc*I and *Swa*I sites, and *Bam*HI and *Spe*I sites, respectively. The RNAi construct was driven by the (Cauliflower Mosaic Virus 35S) CaMV35S promoter.

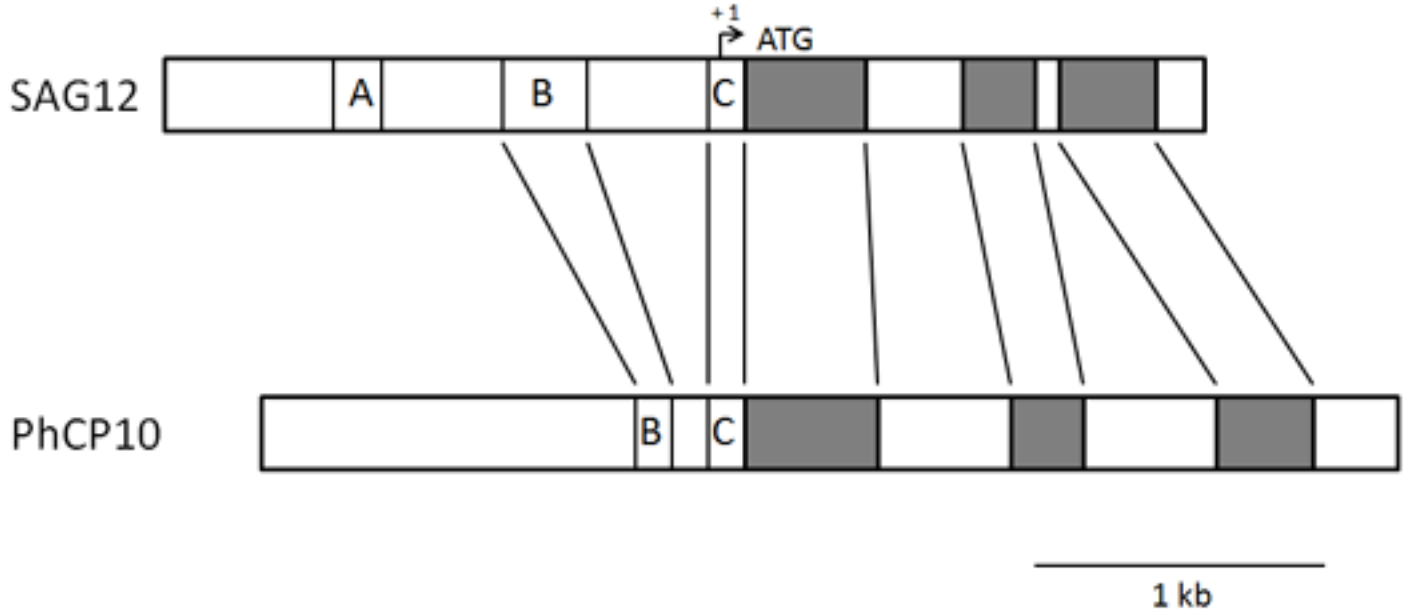

Fig. 2.2. Organization of genomic DNA of *SAG12* and *PhCP10*. Transcription start site of *SAG12* is indicated by +1. Transcription start site of *PhCP10* was not determined. Exons are indicated by gray boxes. Region A is an enhancer element of *SAG12*. Region B is the senescence-specific region (SS) of *SAG12* and putative SS region of *PhCP10*. Region C indicates the basal promoter region (BP) of *SAG12* and putative BP of *PhCP10*. The black bar is equal to 1 kb.

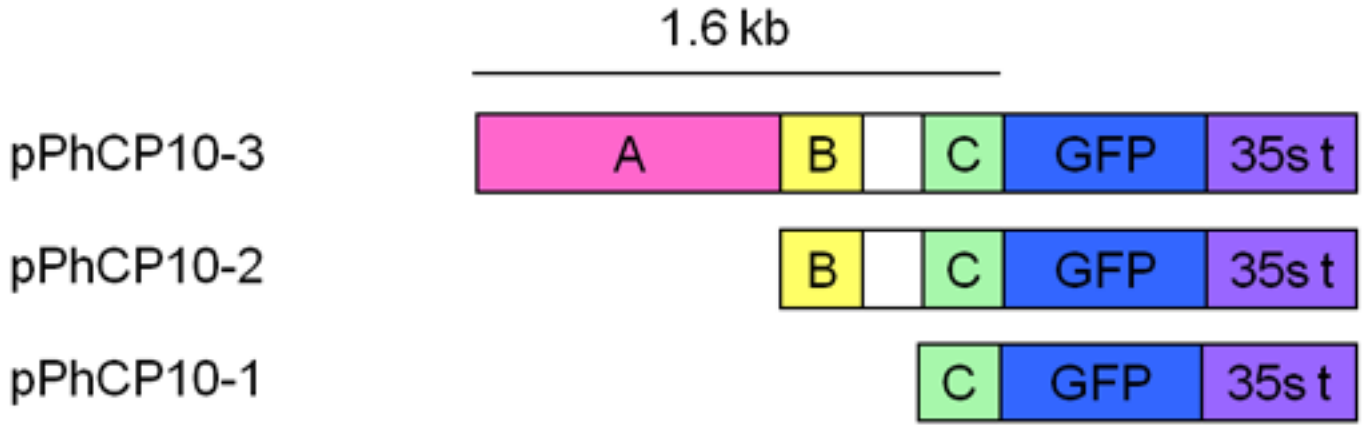

Fig. 2.3. Promoter deletion constructs of *PhCP10*. A, B and C indicate a putative enhancer, senescence-specific and basal regions of *PhCP10* promoter, respectively. The length of A, B and C are 1039, 139 and 263 bp, respectively. Region A may include a putative enhancer element.

Fig. 2.4. Diagrams of *PhCP10* promoter deletion constructs for transient and stable transformation. Different regions of the *PhCP10* promoter were amplified by PCR and ligated into pGEM-11 at *Sal*I and *Not*I sites (A). GFP was prepared by PCR using primers containing *Sph*I extension at the 5' end and *Xba*I and *Hin*dIII extension at the 3'end. A PCR fragment containing GFP was ligated into pGEM-11 vector at *Sph*I and *Hin*dIII sites and then the various *PhCP10* promoter fragments were ligated to create three promoter deletion cassettes (A). *PhCP10* promoter–GFP cassettes were transferred to pJL10 vector (B) for all transient expression analyses. CaMV35S (Cauliflower Mosaic Virus 35S) promoter driving GFP expression in pJL10 vector was used as a positive control. For generation of stably transformed *Petunia* ×*hybrida* 'Mitchell Diploid' (petunia) expression cassettes were ligated into pCAMBIA2200 using the *Sal*I and *Bam*HI (C). pPhCP10 promoter constructs and CaMV35s control construct contained the *NPTII* gene for screening (C).

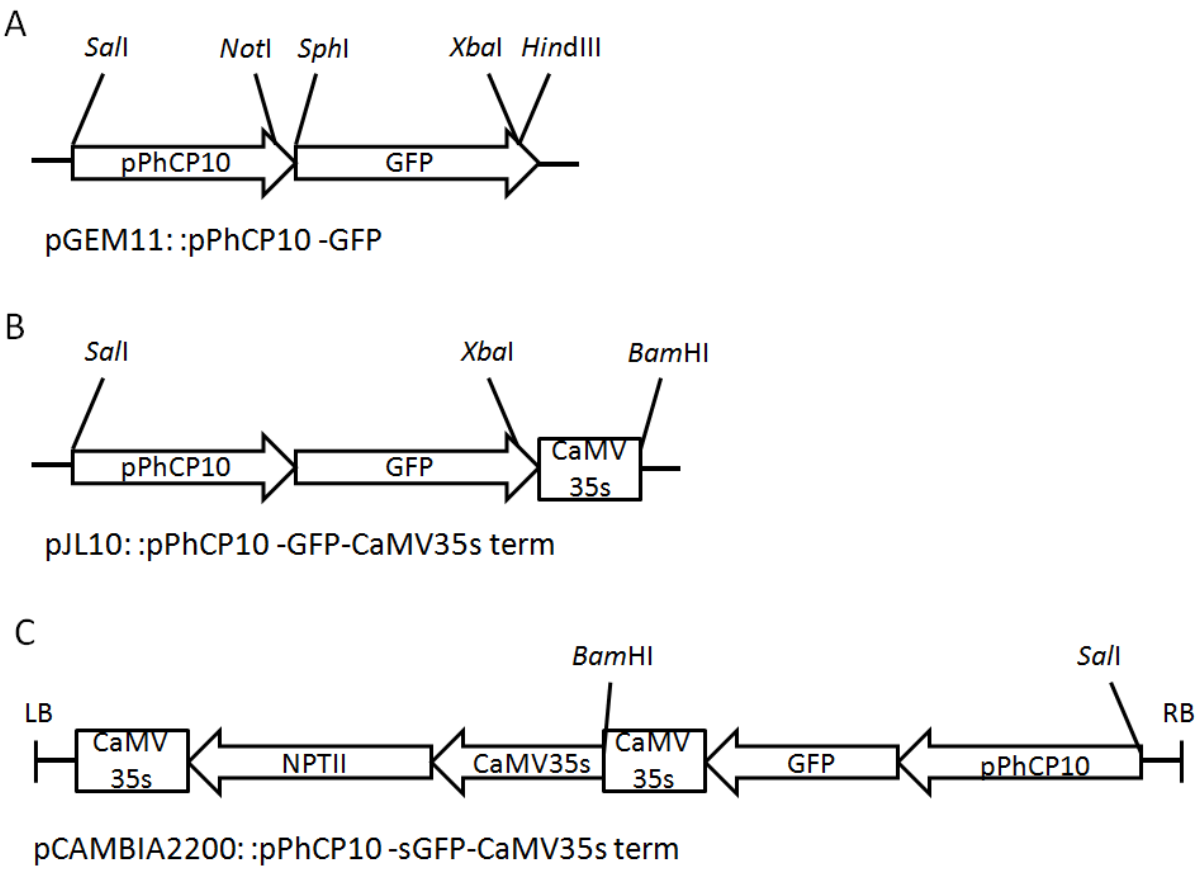
A
SalI
NotI
SphI
XbaI
HindIII
pPhCP10
GFP
pGEM11: :pPhCP10 -GFP
B
SalI
XbaI
BamHI
pPhCP10
GFP
CaMV 35s
pJL10: :pPhCP10 -GFP-CaMV35s term
C
BamHI
SalI
LB
RB
CaMV 35s
NPTII
CaMV35s
CaMV 35s
GFP
pPhCP10
pCAMBIA2200: :pPhCP10 -sGFP-CaMV35s term

Fig. 2.4.

Fig. 2.5. Relative expression of *PhCP10* in various tissue types of *Petunia* ×*hybrida* ‘Mitchell Diploid’ (petunia). Stems and roots were collected from 7 week old plants. Anthers were collected at the nondeshiscent stage and pollen was collected from newly dehisced anthers. Non senescing corollas were collected at 0 hours after pollination (hap) (or at anthesis) and senescing corollas were collected at 48 hap

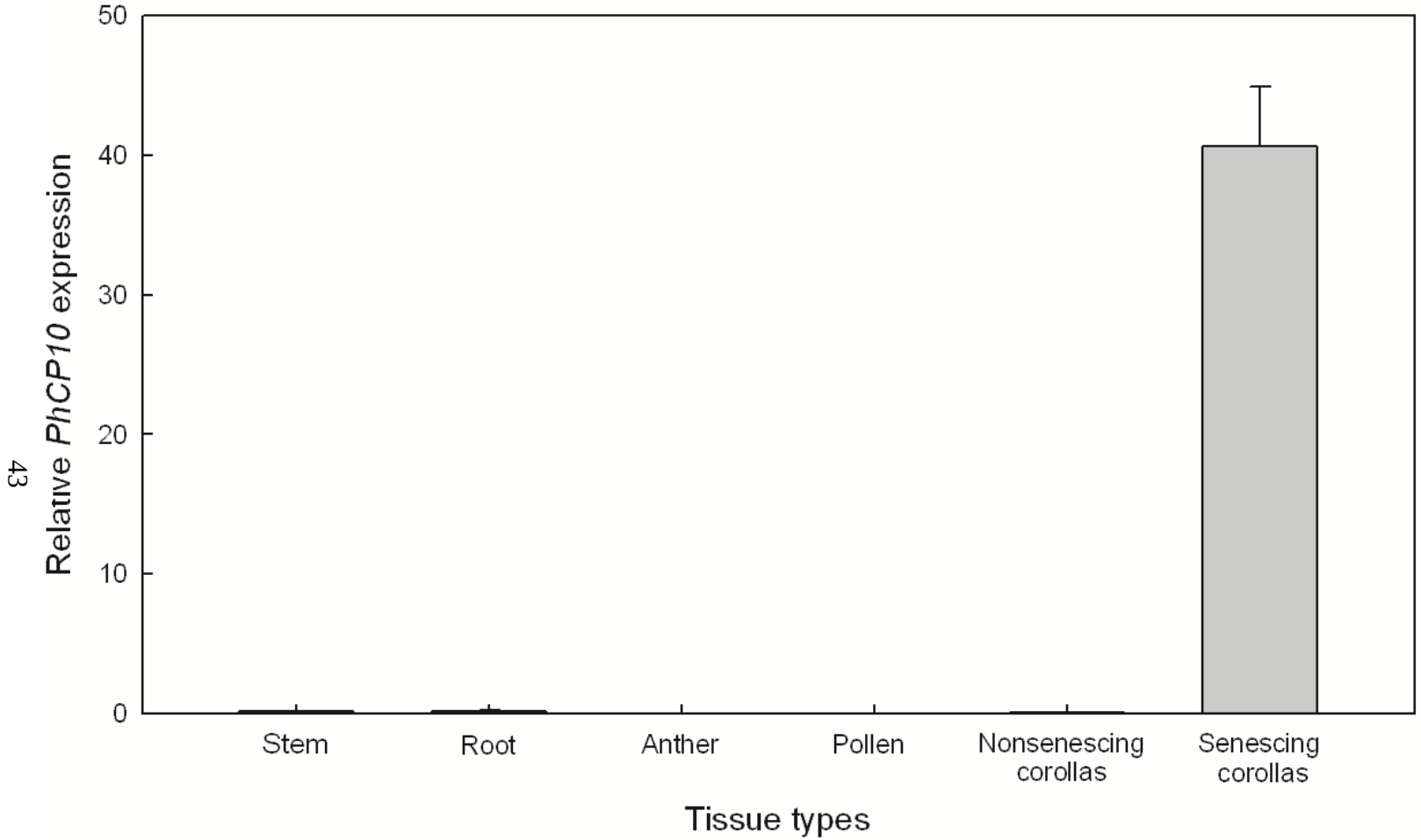
50
40
30
20
10
0
Relative PhCP10 expression
Stem
Root
Anther
Pollen
Nonsenescing corollas
Senescing corollas
Tissue types

Fig. 2.5.

Fig. 2.6. Relative expression of *PhCP10* in unpollinated flowers during flower senescence in *Petunia* ×*hybrida* 'Mitchell Diploid' (petunia). Corollas were collected 0, 2, 4, 6, 7, 8, 9, and 10 days after anthesis (daa). Flowers were visibly wilted at Day 7 (A). Images are representative of the corollas collected at the corresponding time (A). RNA was extracted from six corollas.

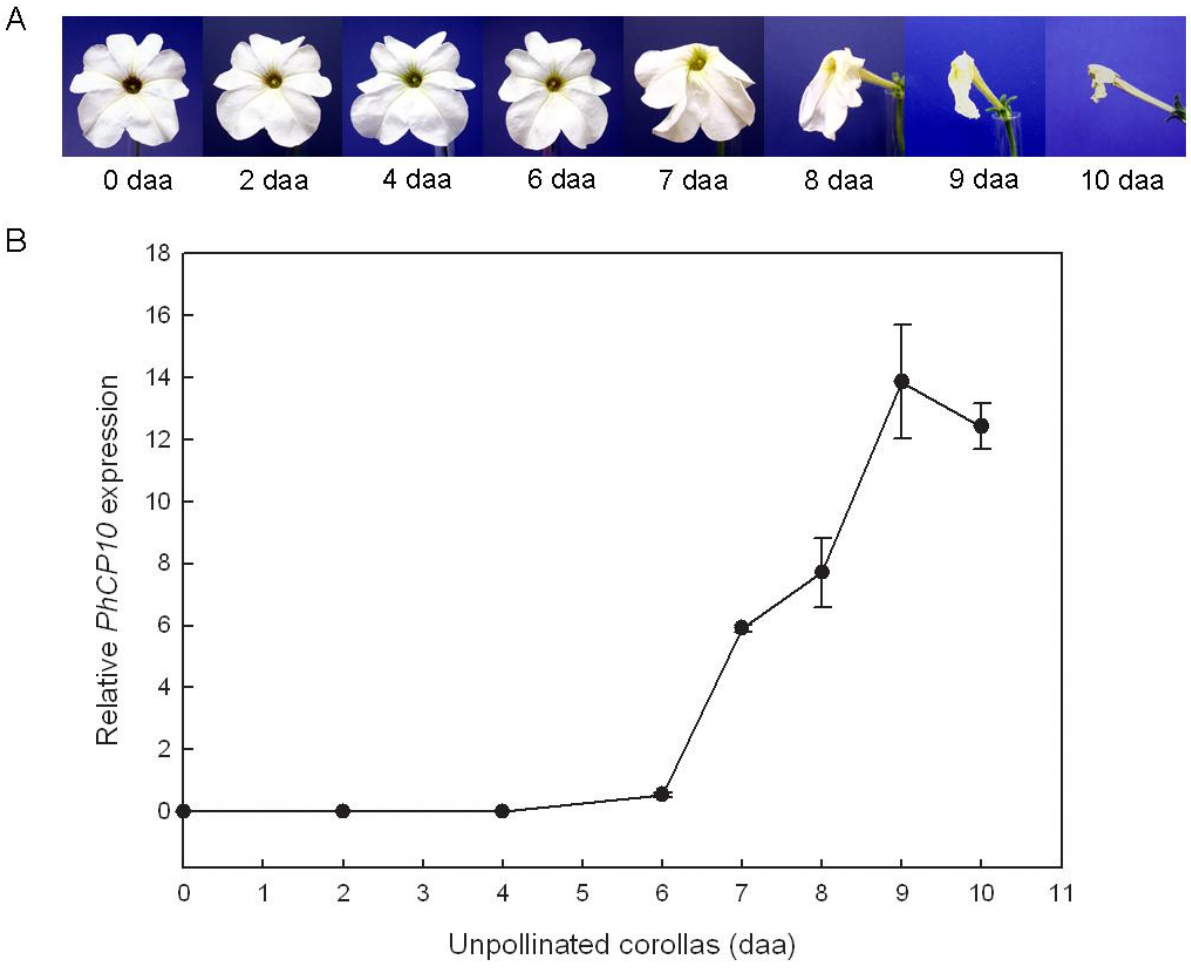
A
0 daa
2 daa
4 daa
6 daa
7 daa
8 daa
9 daa
10 daa
B
Relative PhCP10 expression
Unpollinated corollas (daa)
0
2
4
6
8
10
12
14
16
18
0
1
2
3
4
5
6
7
8
9
10
11

Fig. 2.6.

Fig. 2.7. Relative expression of *PhCP10* in *Petunia ×hybrida* ‘Mitchell Diploid’ (petunia) pollinated flowers. Corollas were collected every 12 h from 0 hours after pollination (0 hap, anthesis) until 96 hap. Flowers were visibly wilted at 48 hap (A). Images are representative of the corollas collected at corresponding time (A). RNA was extracted from six corollas.

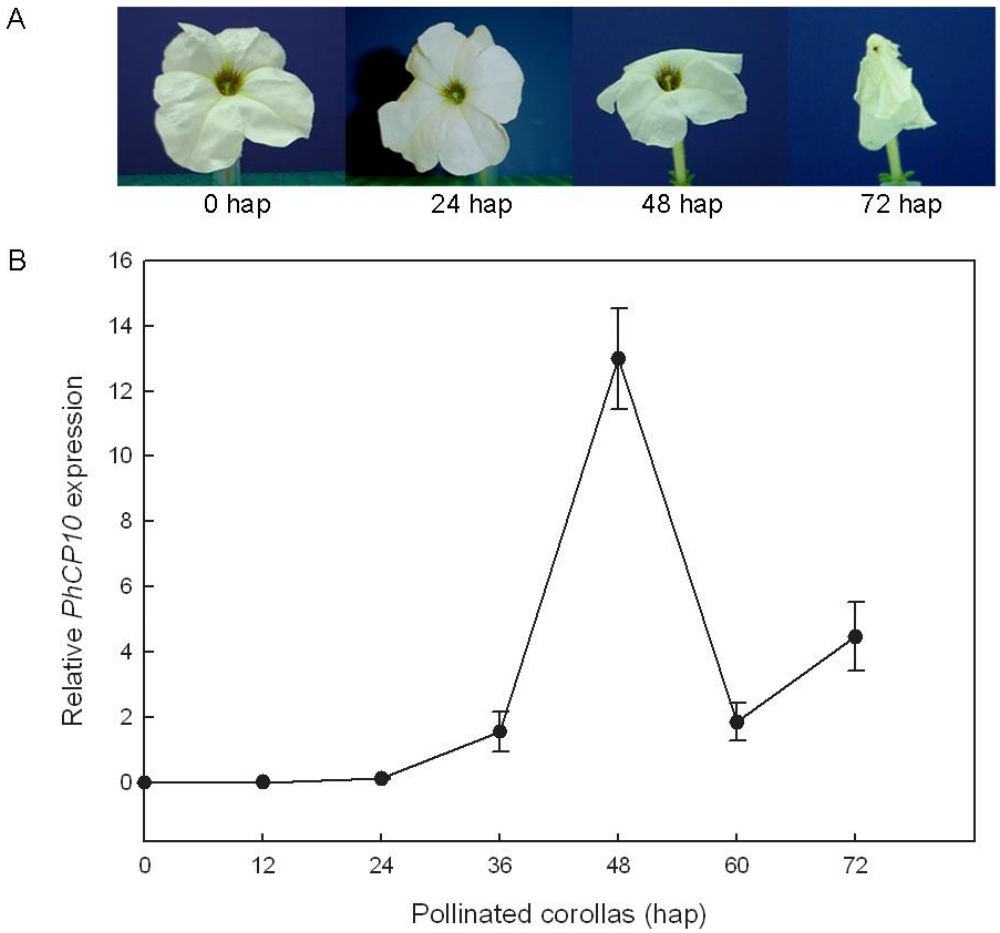
A
0 hap
24 hap
48 hap
72 hap
B
Relative PhCP10 expression
0
2
4
6
8
10
12
14
16
0
12
24
36
48
60
72
Pollinated corollas (hap)

Fig. 2.7.

Fig. 2.8. Relative expression of *PhCP10* in ethylene treated flowers of *Petunia* ×*hybrida* 'Mitchell Diploid' (petunia). Unpollinated flowers detached at anthesis were placed in deionized water. Test tubes containing one flower each were placed in a 24-L chamber and treated with 0 or 2 $\mu L \cdot L^{-1}$ C_2H_4 for four h. Control flowers were place in an ethylene-free chamber for 36 h. RNA was extracted from six corollas (n=6).

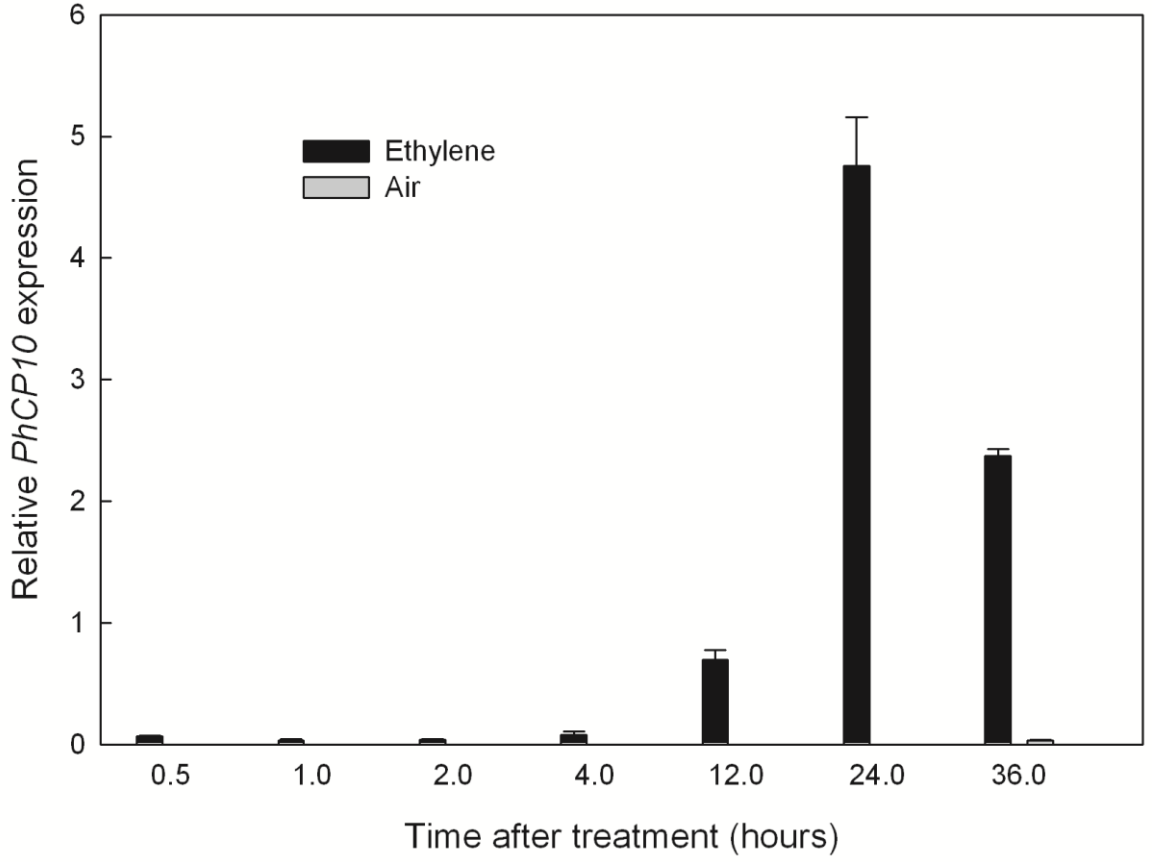
Relative PhCP10 expression
Ethylene
Air
0
1
2
3
4
5
6
0.5
1.0
2.0
4.0
12.0
24.0
36.0
Time after treatment (hours)

Fig. 2.8.

Fig. 2.9. Relative expression of *PhCP10* in ethylene and cyclohexamide treated flowers of *Petunia ×hybrida* ‘Mitchell Diploid’ (petunia). Detached unpollinated flowers prior to ethylene exposure were treated with the protein synthesis inhibitor, cycloheximide (50 mM) or received no cycloheximide (0 mM). These flowers were then divided into two groups and they were treated with ethylene (2 $\mu L \cdot L^{-1}$ for 4 h) or air (0 $\mu L \cdot L^{-1}$ for 4 h). All flowers were treated in 24-L sealed chambers. RNA was extracted from six corollas.

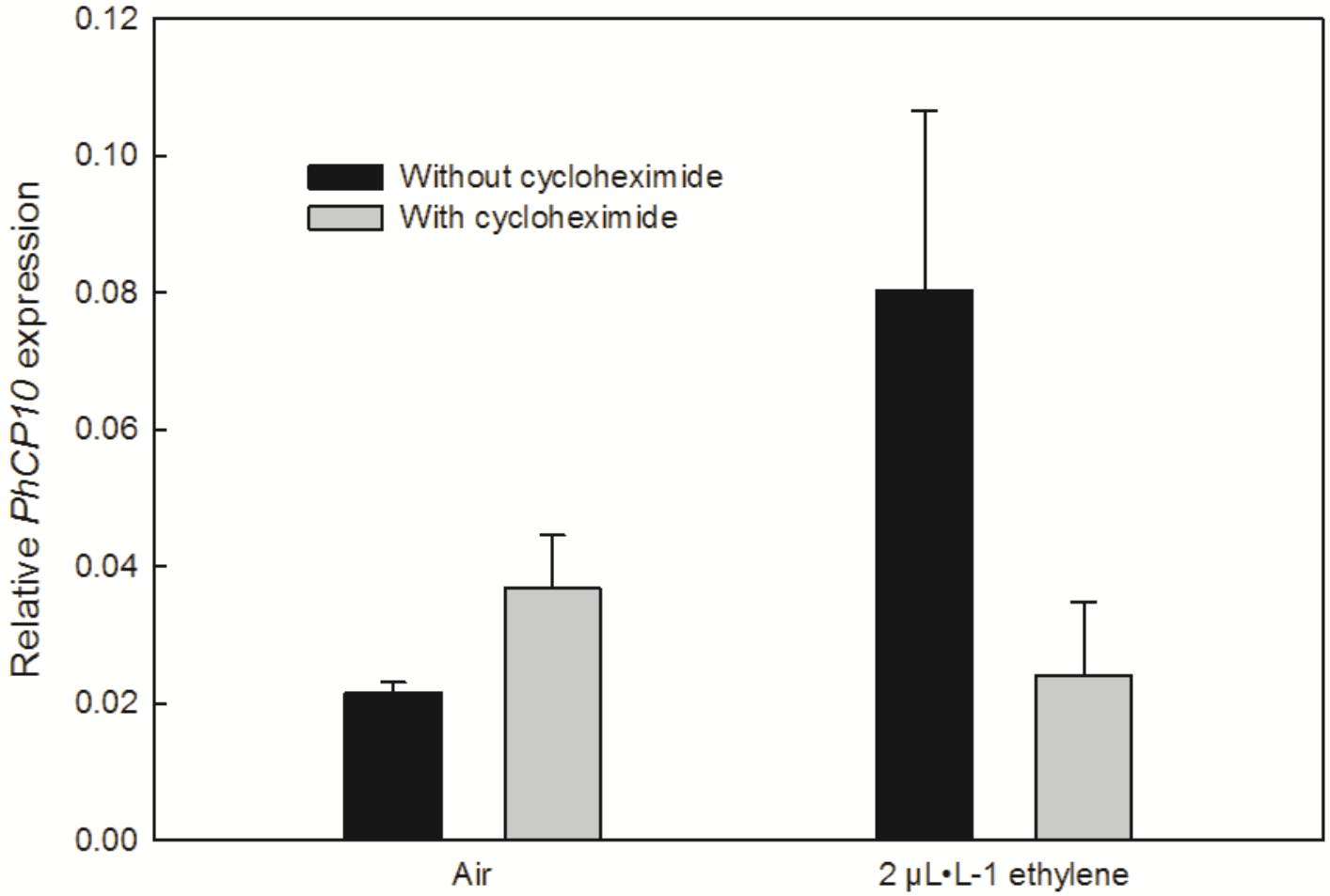
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Relative PhCP10 expression
Without cycloheximide
With cycloheximide
Air
2 µL•L-1 ethylene

Fig. 2.9.

Fig. 2.10. Relative expression of *PhCP10* and *PhASR* (drought stress marker gene) in drought-stressed *Petunia ×hybrida* 'Mitchell Diploid' (petunia) leaves. Prior to drought stress experiments plants were watered to container capacity, then the plants were allowed to dry-down until the plants showed visible symptoms of leaf wilting. Plants remained wilted for twenty-four hours and were then rewatered. Images were representative of leaves or plants prior to drought stress (left), during the first cycle of drought stress (3 photos in the middle) and of chlorotic leaves at the end of three cycles of drought stress (right) (A). The cycle was repeated a total of three times until visual and measureable leaf chlorosis (i.e. senescence) had occurred (A). Leaf chlorosis was measured with a handheld chlorophyll content (CC) meter ± SD. RNA was extracted from three basal leaf halves.

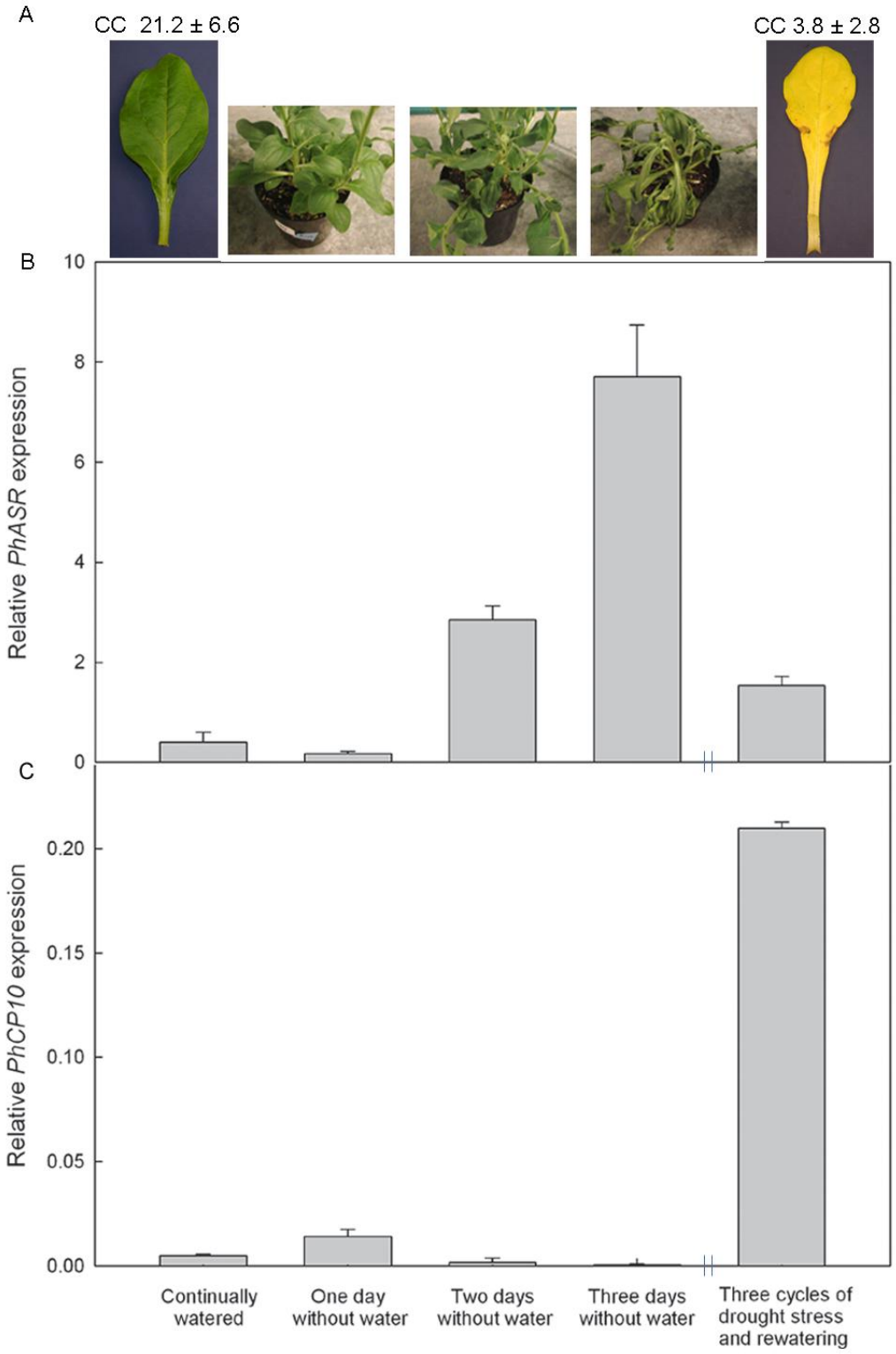
A
CC 21.2 ± 6.6
CC 3.8 ± 2.8
B
Relative PhASR expression
10
8
6
4
2
0
C
Relative PhCP10 expression
0.20
0.15
0.10
0.05
0.00
Continually watered
One day without water
Two days without water
Three days without water
Three cycles of drought stress and rewatering

Fig. 2.10.

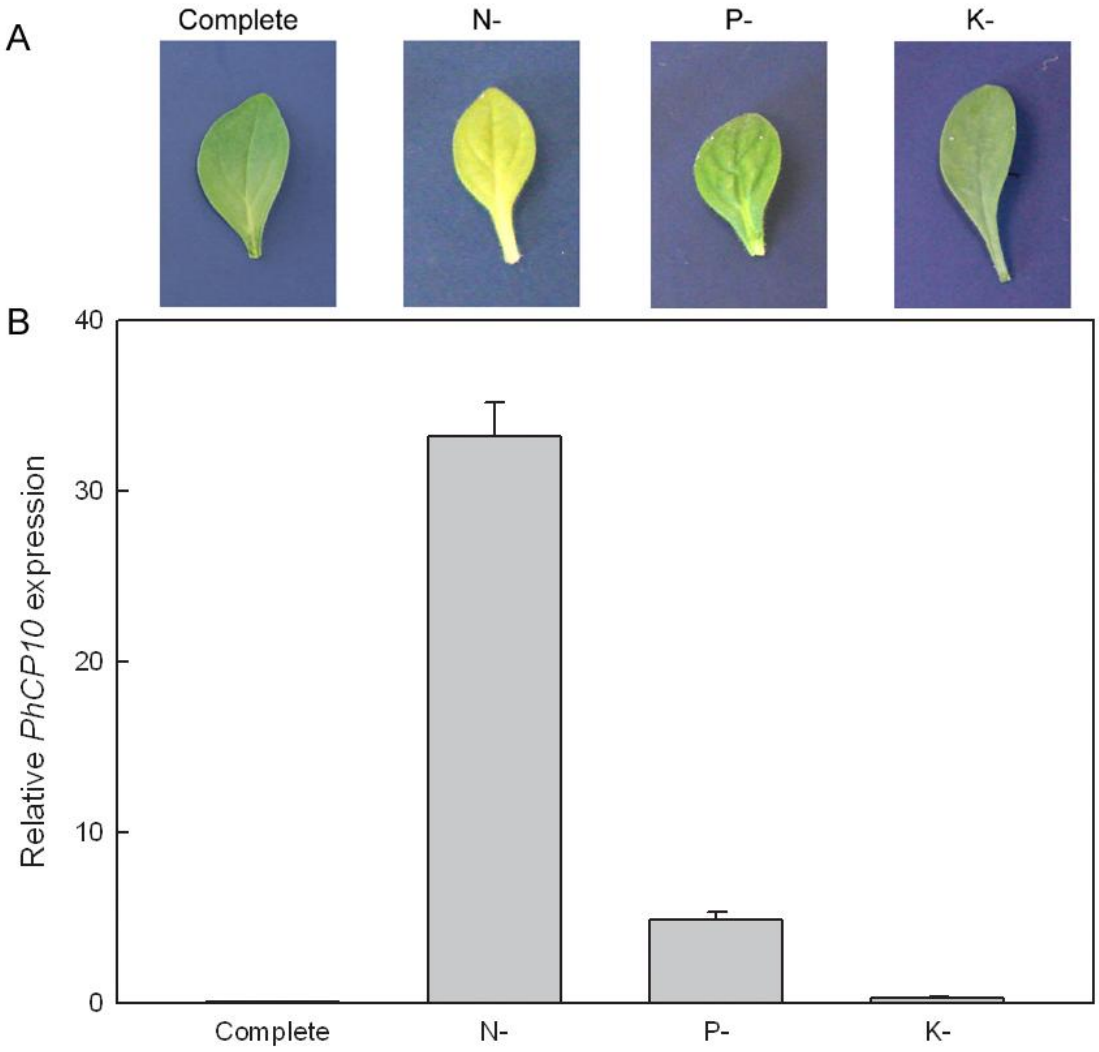

Fig. 2.11. Relative expression of *PhCP10* in nutrient deprived leaves of *Petunia* ×*hybrida* 'Mitchell Diploid' (petunia) plants. Plants were deprived of nitrogen (-N), phosphorus (-P), or potassium (-K) for 28 d. Control plants received complete nutrient solution (Complete) (A). The components for the nutrient solutions were formulated according to Machlis and Torrey (1956). All nutrient deprived plants had the complete nutrient solution except for nitrogen deprived plants which had 5.3 mM $CaCl_2$ and 5.3 mM KCl instead of $Ca(NO_3)_2$ and KNO_3; phosphorus deprived plants had 1.1 mM KCl instead of KH_2PO_4 and potassium deprived plants which had 5.3 mM $NaNO_3$ and 1.1 mM NaH_2PO_4 instead of KNO_3 and KH_2PO_4. Leaves were collected three weeks after application nutrient deprivation treatments began. Plants were arranged in a randomized block design with 2 pots per treatment. RNA was extracted from five leaves.

Fig. 2.12. Relative expression of *PhCP10* in *Petunia ×hybrida* 'Mitchell Diploid' *PhCP10* RNAi corollas. Three lines with lowest *PhCP10* expression in the T_0 were selected for further evaluations in T_1 (lines 75, 68, and 81). Senescing corollas at 48 hour after pollination were collected for relative *PhCP10* expression measurements. Expression levels were calculated as a percentage of expression compared to expression level of 'WT' corollas at 48 hap. RNAi lines 75, 68 and 81 showed a 96.8, 92.8 and 67.1% reduction of *PhCP10* expression, respectively. Average expression ± SD was determined based on ten plants for line 75, seven plants for line 68 and two plants for line 81.

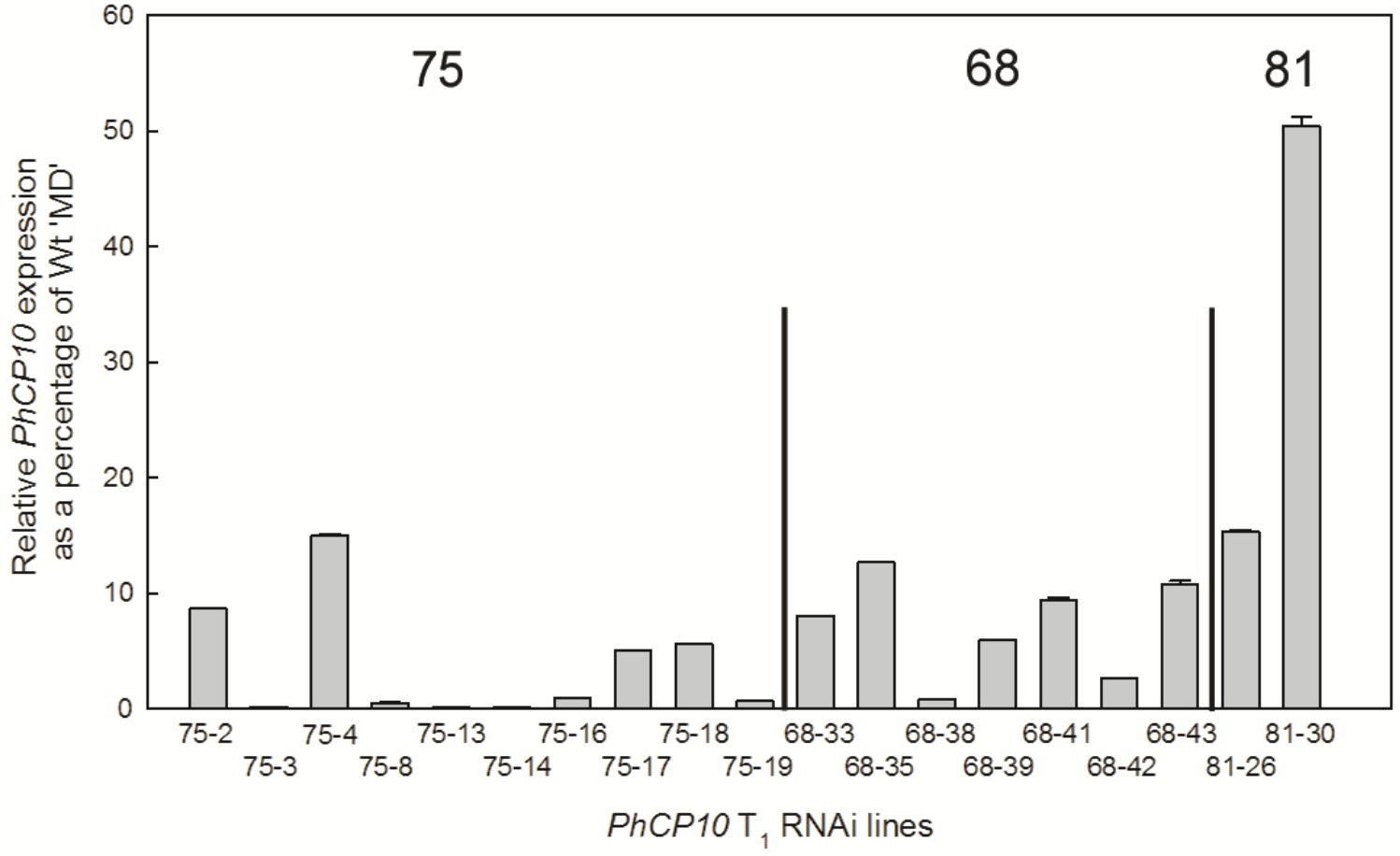
75
68
81
Relative PhCP10 expression as a percentage of Wt 'MD'
0
10
20
30
40
50
60
75-2
75-3
75-4
75-8
75-13
75-14
75-16
75-17
75-18
75-19
68-33
68-35
68-38
68-39
68-41
68-42
68-43
81-26
81-30
PhCP10 T1 RNAi lines

Fig. 2.12.

Fig. 2.13. Multiple amino acid alignment of five senescence associated cysteine proteases. The five cysteine proteases were *Arabidopsis thaliana (*SAG12, AAC49135*)*, *Brassica napus* (rape) (BnSAG12.1, AAD53011and BnSAG12.2, AAD53012), *Nicotiana tabacum* (tobacco) (NtCP1, AAW78661) and *Petunia ×hybrida* (petunia) (PhCP10, AAU81596*)* . Gene names and GenBank accession numbers are in parenthesis. Amino acid sequences were aligned with Clustal W multiple sequence alignment. Sequence comparison showed conserved amino acid residues necessary for cysteine protease activity (arrows) and conserved cysteine residues involved in disulfide bridges (boxes).


B. napus (BnSAG12.1) MALEHIKIFLIVSLVSS----FCFSTTLS-RLLDDELIMQKKHDEWMAEH
A. thaliana (SAG12) MALKHMQIFLFVAIFSS----FCFSITLS-RPLDNELIMQKRHIEWMTKH
B. napus (BnSAG12.2) MALTQIQIFLIVSLVSS----FSLSITLS-RPLLDEVAMQKRHAEWMTEH
N. tabacum (NtCP1) MAFANLSQYLCLALFFICLGLWSSQVALS-RPINYEATMRARHDQWIVHH
P. hybrida (PhCP10) MTFVNPSHYPSLALFFIVLGLWSSHLVSSIRPFNYEETLRASHEEWITHH
B. napus (BnSAG12.1) GRTYADMNEKNNRYVVFKRNVERIERLNNVPAGRTFKLAVNQFADLTNDE
A. thaliana (SAG12) GRVYADVKEENNRYVVFKNNVERIEHLNSIPAGRTFKLAVNQFADLTNDE
B. napus (BnSAG12.2) GRVYADANEKNNRYAVFKRNVERIERLNDVQSGLTFKLAVNQFADLTNEE
N. tabacum (NtCP1) EKVYKDLNEKEVRFQIFKENVERIEAFN-AGEDKGYKLGFNKFSDLTNEE
P. hybrida (PhCP10) GKIYKDVNEKEMRFQIFNENVKRIEAFN-AGEDKGYKLSVNKFADLTNEE
B. napus (BnSAG12.1) FRFMYTGYKGDFVLFSQSQTKSTSFRYQNVFFGALPIAVDWRKKGAVTPI
A. thaliana (SAG12) FRSMYTGFKGVSALSSQSQTKMSPFRYQNVSSGALPVSVDWRKKGAVTPI
B. napus (BnSAG12.2) FRSMYTGFKGNSVLSSR--TKPTSFRYQNVSSDALPVSVDWRKKGAVTPI
N. tabacum (NtCP1) FRVLHTGYKRSHPKVMTSSKGKTHFRYTNVTD--IPPTMDWRKKGAVTPI
P. hybrida (PhCP10) FRVLHTGYKRRDPKVMSSSKPKTHFRYVNVTD--IPRTMDWRKKGAVTPI
B. napus (BnSAG12.1) KNQGSCGCCWAFSAVAAIEGATQIKKGKLISLSEQQLVDCDTN--DFGCS
A. thaliana (SAG12) KNQGSCGCCWAFSAVAAIEGATQIKKGKLISLSEQQLVDCDTN--DFGCE
B. napus (BnSAG12.2) KDQGLCGSCWAFSAVAAIEGVAQIKKGKLISLSEQELVDCDTN--DGGCM
N. tabacum (NtCP1) KDQKECGCCWAFSAVAAMEGLHQLKTGELIPLSEQELVDCDVEGEDEGCS
P. hybrida (PhCP10) KDQKDCGCCWAFSAVAAIEGLHQLKTGKLIPLSEQELVDCDVHGEDEGCS
B. napus (BnSAG12.1) GGLMDTAFEHIMATGGLITESNYPYKGEDANCKIKSTKPSAASITGYEDV
A. thaliana (SAG12) GGLMDTAFEHIKATGGLITESNYPYKGEDATCNSKKTNPKATSITGYEDV
B. napus (BnSAG12.2) GGLMDTAFNYTITIGGLTSESNYPYKSTNGTCNFNKTKQIATSIKGFEDV
N. tabacum (NtCP1) GGLLDTAFDFILKNKGLTTEVNYPYKGEDGVCNKKKSALSAAKITGYEDV
P. hybrida (PhCP10) GGLLDTAFTFIMKNKGLTTEANYPYKGEDGVCNKEKSALSAAKIG-YEDV
B. napus (BnSAG12.1) PVNDENALMKAVAHQPVSVGIEGGGFDFQFYSSGVFTGECTTYLDHAVTA
A. thaliana (SAG12) PVNDEQALMKAVAHQPVSVGIEGGGFDFQFYSSGVFTGECTTYLDHAVTA
B. napus (BnSAG12.2) PANDEKALMKAVAHHPVSIGIAGGDIGFQFYSSGVFSGECTTHLDHGVTA
N. tabacum (NtCP1) PANSEKALLQAVANQPVSVAIDGSSFDFQFYSSGVFSGSCSTWLNHAVTA
P. hybrida (PhCP10) PADSEKALLKAVANQPVSVAIDGSSFDFQFYSSGVFSGSCSTWLNHAVTA
B. napus (BnSAG12.1) VGYSQSSAGSKYWIIKNSWGTKWGEGGYMRIKKDIKDKEGLCGLAMKASY
A. thaliana (SAG12) IGYGESTNGSKYWIIKNSWGTKWGESGYMRIQKDVKDKQGLCGLAMKASY
B. napus (BnSAG12.2) VGYGRSKNGLKYWILKNSWGPKWGERGYMRIKKDIKPKHGQCGLAMNASY
N. tabacum (NtCP1) VGYGATTDGTKYWIIKNSWGSKWGDSGYMRIKRDVHEKEGLCGLAMDASY
P. hybrida (PhCP10) VGYGATTDGIKYWIIKNSWGSNWGDNGYIRMKRDIHDKEGLCGLATEASY
B. napus (BnSAG12.1) PTI
A. thaliana (SAG12) PTI
B. napus (BnSAG12.2) PTM
N. tabacum (NtCP1) PTA
P. hybrida (PhCP10) PVA

Fig. 2.13.

Fig. 2.14. Transient GFP expression of *PhCP10* promoter deletion constructs in lima bean cotyledons (*Phaseolus lunatus* 'Henderson-Bush'). Lima bean cotyledons were bombarded with tungsten particles coated with promoter DNA by Particle Inflow Gun (PIG). Images of lima beans expressing GFP were taken hourly for 75 hours. Images were registered and aligned utilizing ImageJ software (Rasband 1997-2007). An area of image (400 x 300 pixel) was selected for quantifying GFP expression. Images were separated into red, green and blue channels to remove background fluorescence. Total expression was determined as area multiplied by the mean gray scale value of red and green channels. CaMV35s (Cauliflower Mosaic Virus 35s) driving GFP expression was shown in top graph and the bottom graph showed GFP expression without CaMV35s. Means are the average of three replications (n=3).

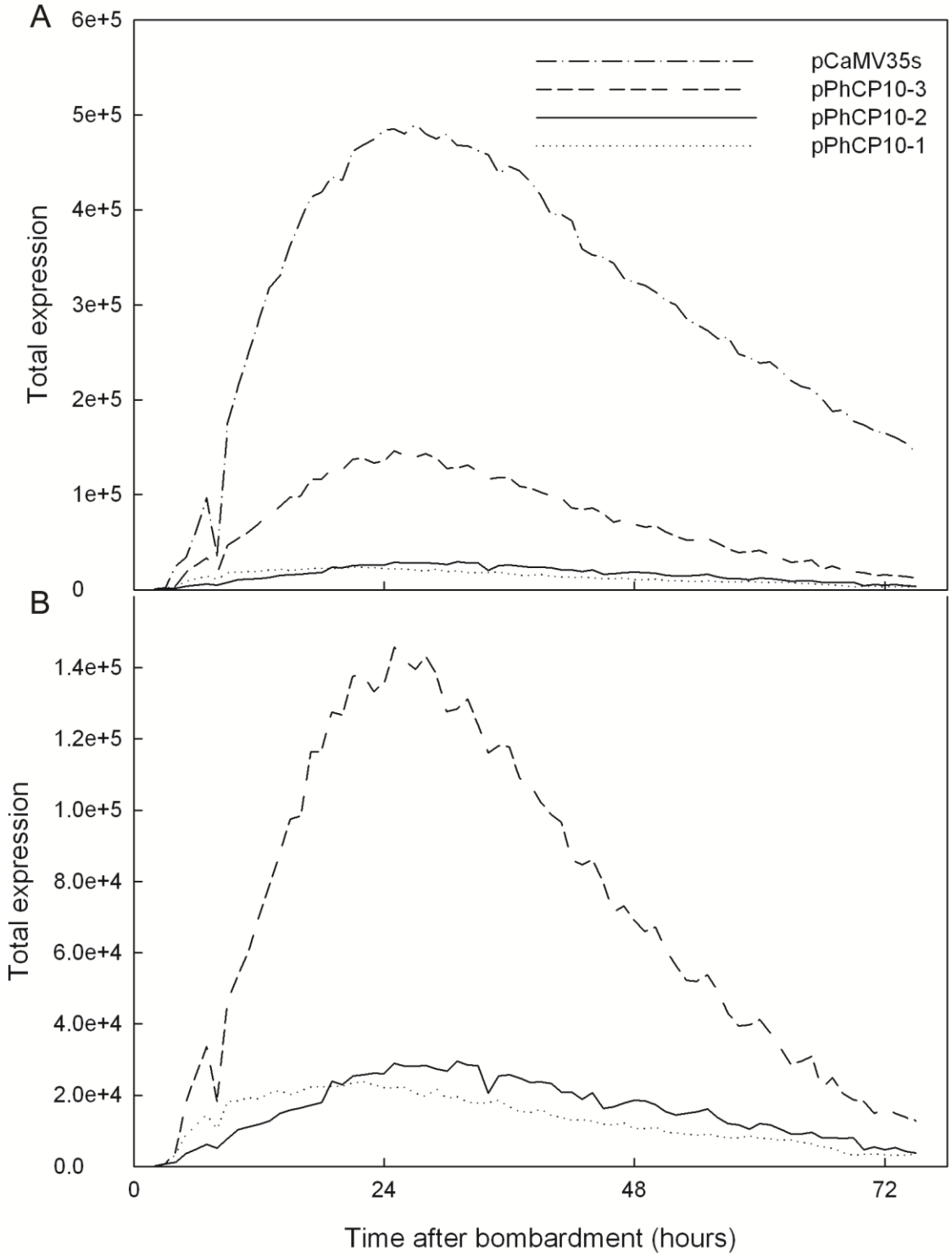
A
B
pCaMV35s
pPhCP10-3
pPhCP10-2
pPhCP10-1
Total expression
6e+5
5e+5
4e+5
3e+5
2e+5
1e+5
0
1.4e+5
1.2e+5
1.0e+5
8.0e+4
6.0e+4
4.0e+4
2.0e+4
0.0
0
24
48
72
Time after bombardment (hours)

Fig. 2.14.

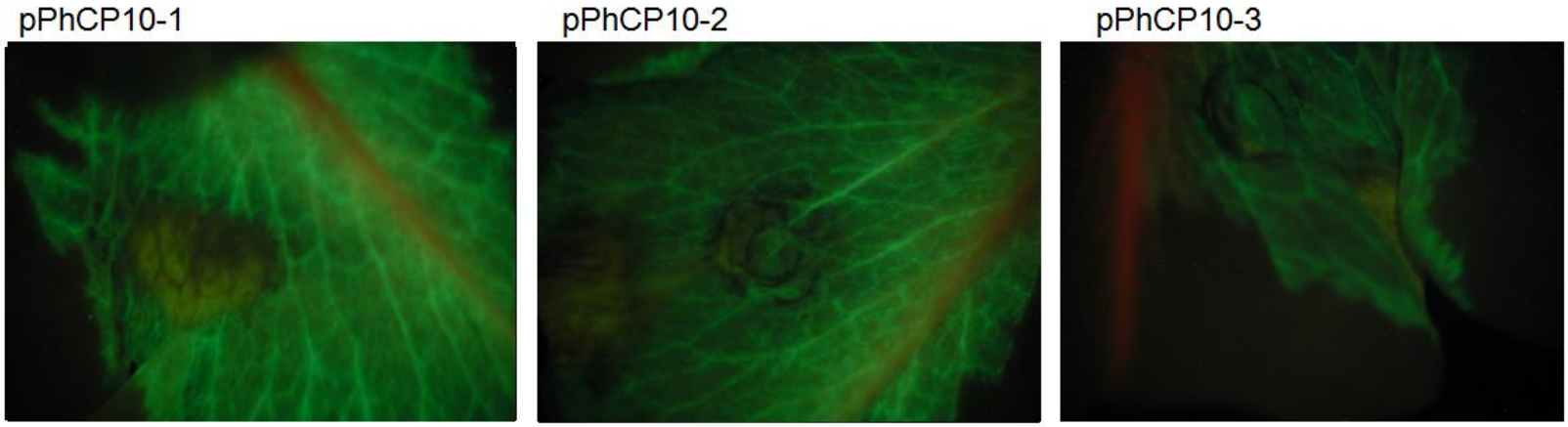

Fig. 2.15. Transient GFP expression of *PhCP10* promoter constructs in *Petunia ×hybrida* (petunia) flowers. Images of GFP expression in agroinfiltrated petunia corollas were taken 72 hours after pollination. Agroinfiltrations were preformed with a needleless syringe on the adaxial side of petunia flowers. Flowers were detached from the plants one day before anthesis and pollinated at anthesis.

Fig. 2.16. Images of GFP expression in a transgenic *Petunia ×hybrida* (petunia) transformed with the pPhCP10-3 promoter construct (transgenic plant pPhCP10-3 #46). Images of GFP expression in petunia corollas are photographed 0 hours after pollination (hap) and 48 hap. Photos on the right are white light images.

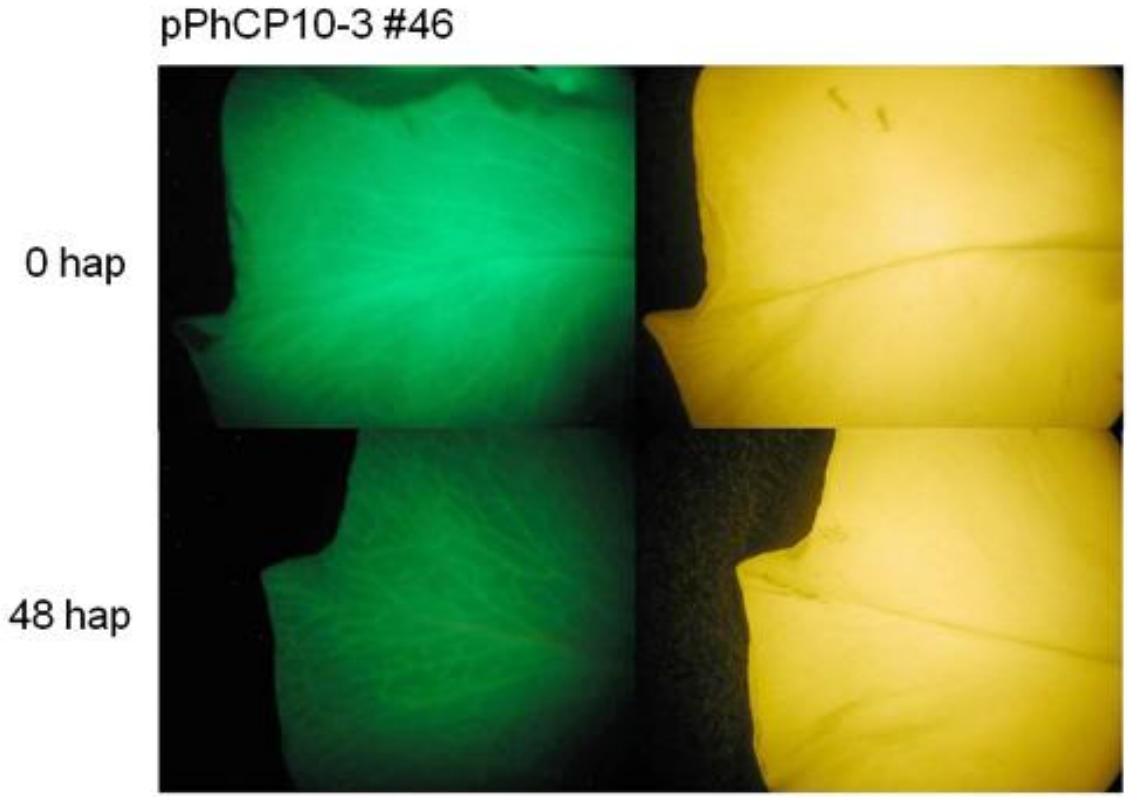

Fig. 2.16

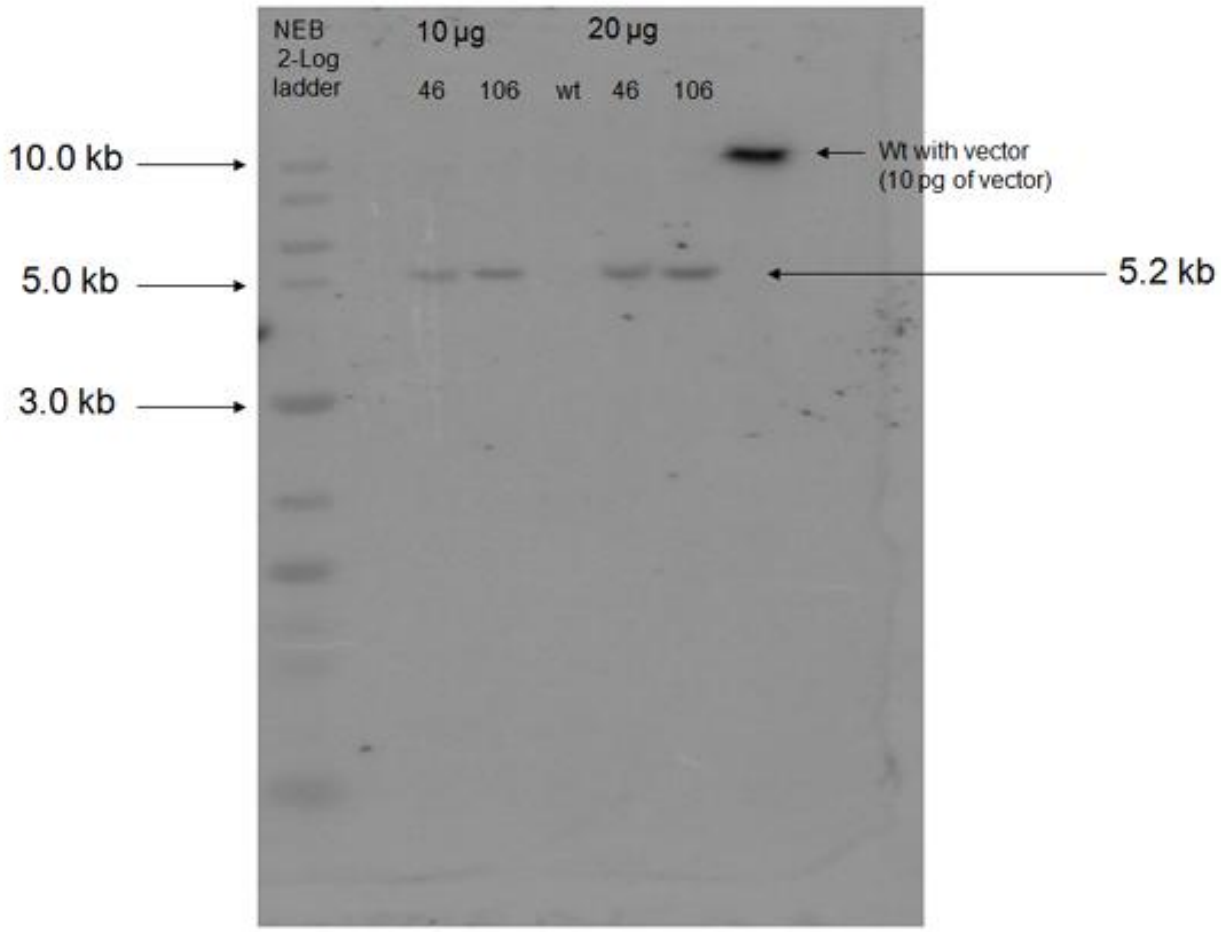

Fig. 2.17: Southern blot analysis of two stably transformed transgenic plants expressing GFP driven by pPhCP10-3 (plant # 46 and 106). Ten and 20 micrograms of DNA were digested with *Xba*I. Ten picograms of pPhCP10-3 plasmid DNA was used as a positive control. NEB 2-Log ladder was used as a molecular weight marker. Minimum expected size of fragment is ≈ 2.7 kb from right broader (RB) to *Xba*I site at the 3'end of GFP.

CHAPTER 3:

ABSCISIC ACID APPLICATION ENHANCES DROUGHT STRESS TOLERANCE IN BEDDING PLANTS

Abstract.

Drought stress is a major cause of postproduction decline in bedding plants. The plant hormone abscisic acid (ABA) regulates drought stress responses by mediating stomatal closure, thereby reducing transpirational water loss. Exogenous ABA applications delay wilting and allow plants to survive short periods of severe drought. The effectiveness of the ABA biochemical, s-ABA (ConTego™, Valent Biosciences Corp.), at delaying wilting and extending shelf life during drought stress was evaluated in six bedding plant species. Spray and drench applications of 0 or 500 $mg \cdot L^{-1}$ s-ABA were applied to *Impatiens walleriana* (impatiens), *Pelargonium* ×*hortorum* (seed geranium), *Petunia* ×*hybrida* (petunia), *Tagetes patula* (marigold), *Salvia splendens* (salvia) and *Viola* ×*wittrockiana* (pansy). Water was subsequently withheld and wilting symptoms were compared between treated and control plants. s-ABA applications delayed wilting in all crops by 1.7 to 4.3 d. Leaf chlorosis was observed following s-ABA application in drought-stressed seed geraniums, marigolds, and pansies. In seed geraniums and marigolds the drought stress itself resulted in leaf chlorosis that was equivalent to or more

severe than the s-ABA application alone. In pansies, s-ABA applications induced leaf chlorosis that was more severe than the drought treatment. Overall, s-ABA was consistently effective at reducing water loss and extending shelf life for all species treated. Applications of s-ABA to bedding plants before shipping and retailing would allow plants to maintain marketability even under severe drought stress conditions.

Floriculture crops represent a $4.2 billion dollar industry in the United States, with bedding plants accounting for approximately 44% of their total wholesale value (USDA, 2009). In the last seventeen years, there has been a change in the retailing of floriculture crops, as customers purchase more bedding and garden plants from big-box retailers (mass merchandisers and club centers) and general retailers (department stores and supermarkets) and less from traditional garden centers and florists (Yue and Behe, 2008). Subsequently, the shipping and retailing of these plants may occur farther from the site of production, making proper postproduction care and handling increasingly important to insure that customers receive the best quality plants (Starman et al., 2007). During shipping and retailing, plants may be exposed to harsh environmental conditions, including high temperatures and irregular irrigation, which cause rapid substrate drying and plant wilting. These drought-stressed plants quickly become undesirable to the customer. It is estimated that 5% of the plant material retailing at independent garden centers may become unsalable, while postproduction losses at the big-box retailers may be as high as 10 to 15% (Healy, 2009).

Very little research has been conducted on the postproduction care and handling of floriculture crops. While environmental conditions during greenhouse production are optimized for plant growth and development, postproduction environments are often less than ideal. Floriculture crops suffer stress from high temperature, low light, nutrient deficiencies, and water deprivation during shipping and retailing (Armitage, 1993; Starman et al., 2007). Toning by reducing fertility and available water during the last two weeks of greenhouse production has been promoted as a means of "hardening off" plants so that they are less susceptible to the environmental stresses experienced after production (Armitage, 1993; Starman et al., 2007). Reduced end-of-production fertilization effectively increases the quality and shelf life of a number of containerized vegetative annuals (Beach et al., 2009). While this production practice enhances the value of many floriculture crops under standard shipping and retailing conditions, there is no experimental evidence to indicate whether this shelf life extension would be observed if plants experienced severe drought stress.

Plant wilting can be caused by a combination of enhanced water loss and inadequate watering, and it is a major cause of postproduction decline in greenhouse crops (Barrett and Campbell, 2006). The plant hormone abscisic acid (ABA) plays a role in plant responses to environmental stresses, and ABA applications decrease water loss and enhance drought tolerance (Leskovar and Cantliffe, 1992; Yamazaki et al., 1995). The use of chemicals to reduce water loss in floriculture crops has shown variable results, but new formulations of abscisic acid are now available for commercial growers to use as a plant growth regulator (Barrett and Campbell, 2006).

Recent research has focused on the use of concentrated ABA or ABA analogs as a means of maintaining the marketability of horticulture crops by reducing drought stress symptoms (Blanchard et al., 2007; Kim and van Iersel, 2008; Monteiro et al., 2001; Sharma et al., 2006). ABA analogs have been used to effectively reduce water use and extend the shelf life of tomato (*Solanum lycopersicon*), snapdragon (*Antirrhinum majus*) and nasturtium (*Tropelaum majus*) transplants (Sharma et al., 2006). The application of exogenous ABA during spring/summer production periods also reduces respiration and water loss in potted miniature rose (*Rose hybrida* L.) and results in increased flower longevity (Monteiro et al., 2001). Experiments with a new ABA formulation (s-ABA; ConTego™, Valent Biosciences Corp.) have shown that sprench (spray to runoff) applications of 125 or 250 $mg \cdot L^{-1}$ delay drought induced wilting symptoms in seven of nine species of bedding plants that were evaluated (Blanchard et al., 2007). Shelf life extensions for the seven species varied from 1.1 to 5.8 d (Blanchard et al., 2007). Overall, exogenous ABA applications have the potential to enhance drought tolerance in plants, but many questions remain about the optimal application methods and effectiveness across different plant species.

The goal of this research was to investigate the effectiveness and utility of s-ABA for reducing postproduction decline due to water stress. The specific research objectives were 1) to determine whether the application of s-ABA could effectively reduce drought induced wilting in a variety of bedding plants, 2) to determine if the method of application influences effectiveness and 3) to document and quantify any side effects that result from exogenous applications of ABA.

Materials and Methods

Sources and handling of plant material. Six species of the most popular bedding plants were selected to investigate the effectiveness of s-ABA application for enhancing drought tolerance. Bedding plants were obtained from Green Circle Growers Inc. (Oberlin, OH) and The Greenhouse Shoppe (Wooster, OH). Species included *Impatiens walleriana* 'Xtreme Lavender', *Pelargonium* ×*hortorum* 'Maverick Red', *Petunia* ×*hybrida* 'Ultra Red', *Tagetes patula* 'Bonanza Orange', *Salvia splendens* 'Picante Scarlet', and *Viola* ×*wittrockiana* 'Bingo Rose Frost'. All bedding plants were obtained as 508 cell packs (111.6 cm^3/ cell) except for salvia (72 plug tray; 41.6 cm^3/cell) and seed geraniums (finished 10-cm pots). All plants except the seed geraniums were transplanted to 11-cm pots containing soilless greenhouse media (Promix BX, Premier Horticulture, Quebec, Canada). Plants were grown under normal greenhouse conditions in Wooster, OH from 27 May to 19 June 2009. Plants were maintained using natural irradiance with supplemental irradiance provided by high-pressure sodium and metal halide lamps (GLX/GLS e-systems GROW lights, PARSource, Petaluma, CA) with average photosynthetic photon flux of ≈200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ from 0500 to 1900 HR daily. Greenhouse temperatures were 23.4/17.5 ºC day/night with daytime relative humidity of 66.7 ± 10.5 %. Plants were irrigated daily with Peters Excel® All-purpose 15N-2.15P-12.5K (Scotts-Sierra Horticulture Products, Marysville, OH) at 200 mg $N \cdot L^{-1}$. This was reduced to 100 mg $N \cdot L^{-1}$ one week prior to flowering. All species were treated with s-ABA when they reached a marketable stage of one to two open flowers per plant. Plants

were watered to field capacity 12 h prior to an application of s-ABA. Drench applications were applied based on manufacturer's recommendations (volume of 60 mls) at the rate of 0 or 500 mg·L^{-1} s-ABA and sprays (volume of ≈ 28 mls) at the rate of 0 or 500 mg·L^{-1} s-ABA with the addition of 0.05% CapSil® (Aquatrols Corporation of America, Inc., Cherry Hill, NJ). Spray applications were applied with a Regulator Bak-pak® sprayer (H.D. Hudson Manufacturing Company, Chicago, IL). All plants were held in the greenhouse during subsequent evaluations. Half of the bedding plants were watered daily (continually watered) and the other half were held without water either for 7 d (impatiens, marigolds, seed geraniums and pansies), 8 d (salvia), or 10 d (petunia). Continually watered plants were irrigated with 100 mg N·L^{-1}. s-ABA treated plants had water withheld until they showed visible symptoms of wilt. After the drought stress, plants were rewatered daily to determine if the stressed plants would recover. Visual observations were taken daily and data are the average of four replications.

Evaluations of wilt status and leaf chlorosis. Whole plant wilt status and leaf chlorosis ratings were collected daily starting just prior to application of s-ABA. Wilt status ratings were from 1 to 5 with 5 being completely turgid, 4 soft to touch, 3 starting to wilt, 2 wilted with complete loss of turgor and 1 wilted to the point that leaves are dry and brittle. Leaf chlorosis ratings were from 1 to 11 with a rating of 11 being completely green with no sign of chlorosis, 10: having ≤ 10% leaf chlorosis, 9: 11-20%, 8: 21-30%, 7: 31- 40%, 6: 41-50%, 5: 51- 60%, 4: 61-70%, 3: 71-80%, 2: 81-90%, and 1: 91-100% chlorosis.

Statistical analysis. Reported values are means of four plants in a completely randomized block design. Eleven-cm bedding plants were blocked by watering regiment, continually watered verses drought-stressed, and by replication. Values obtained from visual observations were analyzed by using Proc GLM (generalized linear model) with LSD means separation (least significant difference test). Values were analyzed by SAS (Statistical Analysis System, SAS Institute, Inc., Cary, NC).

Results

Wilt status and visual observations of bedding plants. The application of a 500 mg·L^{-1} s-ABA spray or drench to drought-stressed plants delayed visible wilting in all treated bedding plants and resulted in a subsequent increase in shelf life of 1.7 to 4.3 d depending on the species (Table 3.1, Fig. 3.1, and Fig. 3.2). In drought-stressed plants of all species, the control plants wilted before those sprayed or drenched with 500 mg·L^{-1} s-ABA ($P \leq 0.05$) (Fig. 3.1 and Fig. 3.2). At the end of the drought stress treatment, impatiens, petunia, and marigold maintained higher wilt status ratings (i.e. more turgid) with drench versus spray applications of 500 mg·L^{-1} s-ABA ($P \leq 0.05$) (Fig. 3.2). Pansy maintained higher turgor with spray applications ($P \leq 0.05$) (Fig. 3.2). However, in all species except for marigold there was no difference in the shelf life extension between spray or drench applications (Table 3.1). Drench applications of s-ABA on marigolds resulted in a longer shelf life extension than spray applications ($P \leq 0.05$) (Table 3.1). Overall, the s-ABA applications were most effective at delaying visual wilting symptoms in petunia, resulting in a shelf life extension of from 4.0 d (spray) to 4.3 d (drench) over the controls

(0 mg·L^{-1} s-ABA) (Table 3.1 and Fig. 3.2). s-ABA application was the least effective on marigold, extending shelf life by 1.7 d (spray) and 3.0 d (drench) over the controls (0 mg·L^{-1} s-ABA) (Table 1 and Fig. 3.2). Seed geraniums showed curling and graying of the leaves, and wilting symptoms were not as obvious as those observed in the other species (Fig. 3.1B). s-ABA application resulted in a 3.0- (spray) to 3.7- (drench) day extension in the shelf life of seed geraniums. All other species had a similar 2- to 3- d increase in shelf life due to delays in drought-induced wilting (Table 3.1 and Fig. 3.2). s-ABA treated plants that were rewatered after the drought stress recovered and in many cases were indistinguishable from the continually watered control plants.

Leaf chlorosis of 11-cm finished bedding plants. Although applications of s-ABA were effective at delaying wilting, they also resulted in leaf chlorosis (i.e. yellowing) on seed geranium, marigold, and pansy (Fig. 3.3). Continually watered seed geraniums had more severe leaf chlorosis with drench application of s-ABA, while spray applications resulted in more severe chlorosis for pansies ($P \leq 0.05$) (Fig. 3.4). Continually watered marigolds treated with 500 mg·L^{-1} s-ABA spray or drench resulted in 10 to 15% more leaf chlorosis, with no differences observed between spray or drench applications (Fig. 3.4). Seed geraniums and marigolds under drought stress without s-ABA treatment also developed leaf chlorosis (Fig. 3.4). Drought-stressed seed geraniums developed more severe leaf chlorosis without s-ABA (0 mg·L^{-1} s-ABA spray and drench) than with s-ABA treatment (500 mg·L^{-1} s-ABA spray and drench) (Fig. 3.4). For drought-stressed marigolds, there was no difference in the severity of leaf chlorosis between control (0 mg·L^{-1} s-ABA spray and drench) or s-ABA treated (500 mg·L^{-1} s-ABA spray and

drench) plants (Fig. 3.4). Drought-stressed and continually watered pansies treated with 500 mg·L^{-1} s-ABA had 10% to 25% more leaf chlorosis then non-treated controls (0 mg·L^{-1} s-ABA spray and drench) ($P \leq 0.05$) (Fig 3.4).

Discussion

Drought stress is a major cause of postproduction shrinkage in the floriculture industry (Barrett and Campbell, 2006). ABA applications resulted in reduced water loss under severe drought stress for all bedding plants treated. This reduction in water loss allowed impatiens, seed geraniums, petunias, marigolds, salvia, and pansies to tolerate temporary periods of drought stress similar to what they might experience during shipping and retailing. The ABA-induced delay in visual wilting symptoms resulted in shelf life extensions from 1.7 to 4.3 d depending on the species and method of application. s-ABA applications delayed wilting in impatiens, petunias and salvia without the development of any negative side effects, and after rewatering, the treated plants were indistinguishable from continually watered plants. The application of s-ABA also enhanced the drought tolerance of seed geraniums, marigolds and pansies, but symptoms of leaf chlorosis were observed that may reduce their salability.

Under our experimental conditions, s-ABA applications were most effective at delaying wilting symptoms in petunias and least effective in marigolds. Responses to ABA applications have been shown to vary based on the species (Blanchard et al., 2007; Sharma et al., 2006). Blanchard et al. (2007) reported that sprench applications of 125 and 250 mg·L^{-1} s-ABA were effective in extending the shelf life of New Guinea

impatiens (*Impatiens hawkeri* 'Harmony Grape') for 4.3 to 5.8 d, but were not effective in extending the shelf life of impatiens (*Impatiens walleriana* 'Tempo Lavender') or bacopa (*Sutera cordata* 'Cabana'). In all of the species that we evaluated, including impatiens (*Impatiens walleriana* 'Xtreme Lavender'), both spray and drench s-ABA applications resulted in shelf life extensions in drought-stressed plants. Differences in environmental conditions, s-ABA concentrations and cultivar selection may explain the differences between the present study and reports published previously.

The method of s-ABA application affected the shelf life and the wilt status of some but not all species. At the end of our drought stress treatments there were some differences in wilt status based on method of application for impatiens, petunias and pansies, but this did not result in a significant difference in shelf life extension. Only in marigolds did one application method (drench) result in an extension in shelf life over the other method. In pepper seedlings, ABA applications to the leaves reduced plant growth more than applications to the roots (Leskovar and Cantliffe, 1992). Drought stress, at the whole plant level, is initially sensed in the roots where ABA is synthesized and translocated to the leaves via the xylem (Malladi and Burns, 2007). In the leaves, free ABA results in stomatal closure and reduced water loss. Recently it was reported that under severe drought conditions plants also synthesize ABA in their leaves (Malladi and Burns, 2007). These findings suggest that ABA applications to the leaves may result in quicker and more effective stomatal closure than root applications. This response may be very species specific. Only in drought-stressed pansies were the wilting symptoms less severe when plants were sprayed with s-ABA.

Along with some variability in wilt status and shelf life extension based on species and method of application, we also noticed variability in the occurrence of side effects. The only side effect we noticed during our experiment was leaf chlorosis, which occurred on three of the six species tested. Blanchard et al., (2007) reported that leaf chlorosis was observed on s-ABA treated pansies, but there was no quantification of the severity of the response nor was any data presented for non-drought-stressed plants treated with s-ABA. Kim and van Iersel (2008) observed leaf abscission following applications of s-ABA to drought-stressed salvia (*Salvia splendens* 'Bonfire'). We did not, however, observe s-ABA or drought induced leaf abscission in salvia or any other species that were evaluated. Drought stress induced leaf chlorosis was not prevented in seed geraniums and marigolds treated with s-ABA, and chlorosis symptoms were the same or more severe in drought-stressed plants than well watered plants treated with s-ABA. Only in pansies was it clear that the s-ABA treatment itself induced leaf chlorosis, as the drought-stressed plants in the absence of s-ABA (0 $mg \cdot L^{-1}$controls) were not yellow. The leaf chlorosis observed in pansies may have resulted from increased synthesis or sensitivity to other senescence related hormones including ethylene. Exogenous applications of ABA have been shown to increase ethylene production and decrease cytokinins, which would induce leaf senescence (Nilsen and Orcutte, 1996; Taiz and Zeiger, 2002). Further evaluation of the ABA-induced leaf chlorosis in pansies is needed to determine the involvement of other hormones.

Applications of s-ABA were effective at delaying symptoms of wilting in severely drought-stressed plants. The use of s-ABA by floriculture producers would

allow bedding plants to temporarily avoid drought stress induced wilting during shipping and the first few days of retailing. Overall, this should increase the quality and aesthetic value of the plants so that postproduction shrinkage can be reduced and profits can be maximized for both retailers and growers.

Acknowledgements

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Table 3.1. Days until visual symptoms of wilt and extension of shelf life of six species of bedding plants treated with s-ABA and drought-stressed.[z]

Species and cultivar	Concentration and method of application of s-ABA ($mg \cdot L^{-1}$) Time before wilt (days)					
	Drench			Spray		
	0	500	Shelf life extension	0	500	Shelf life extension
Impatiens walleriana 'Xtreme Lavender'	4.3b	7.0a	2.7	4.0b	6.7a	2.7
Pelargonium × *hortorum* 'Maverick Red'	3.3b	7.0a	3.7	3.7b	6.7a	3.0
Petunia × *hybrida* 'Ultra Red'	5.7b	10.0a	4.3	5.3b	9.3a	4.0
Tagetes patula 'Bonanza Orange'	3.7c	6.7a	3.0	3.3c	5.0b	1.7
Salvia splendens 'Picante Scarlet'	5.3b	8.0a	2.7	5.3b	7.7a	2.4
Viola × *wittrockiana* 'Bingo Rose Frost'	4.3b	6.7a	2.4	4.0b	7.0a	3.0

[z]Values are an average of the number of days from Day 0 that it took each plant to show visible symptoms of wilt which was a rating of 3 (starting to wilt) or less on the wilt status rating scale of 1-5. Values are means of four replications (n=4). Values in rows followed by different letters are statistically different at $P \leq 0.05$ (least significant difference test).

Fig. 3.1. Images of six species of bedding plants treated with either 0 or 500 mg·L^{-1} s-ABA drench or spray (from left to right in each photo). Plants were drought-stressed until s-ABA treated plants began to wilt. Images are of *Impatiens walleriana* 'Xtreme Lavender' (impatiens) (A), *Pelargonium* ×*hortorum* 'Maverick Red' (seed geranium) (B), *Petunia* ×*hybrida* 'Ultra Red' (petunia) (C), *Tagetes patula* 'Bonanza Orange' (marigold) (D), *Salvia splendens* 'Picante Scarlet' (salvia) (E), and *Viola* ×*wittrockiana* 'Bingo Pink Frost' (pansy) (F). Images are representative of all replications and were taken at 5 d after application of s-ABA and drought stress for all crops except seed geranium (B) which was taken at 6 d, petunia (C) which was at 7 d and marigolds which was at 4 d (n=4).

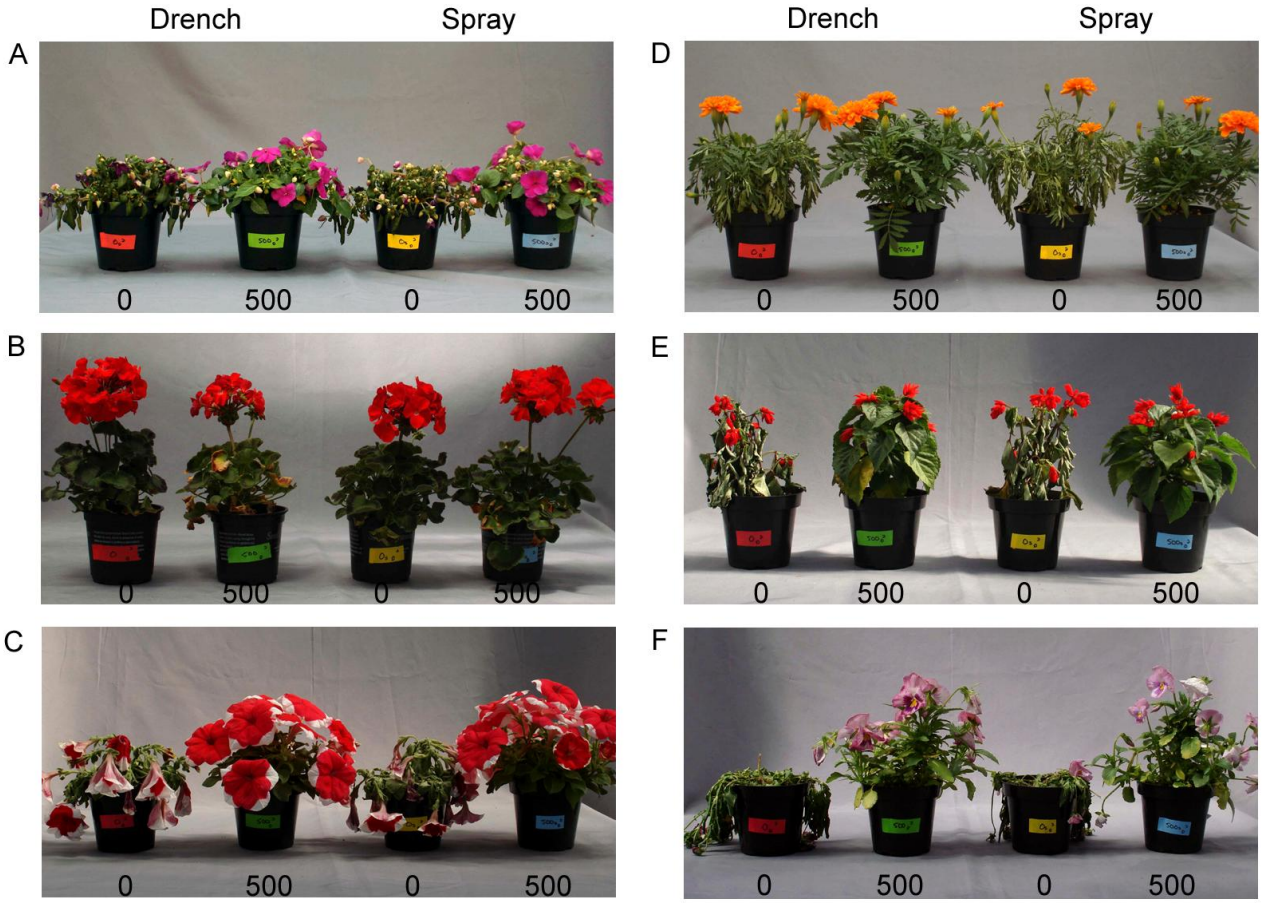
Drench
Spray
A
0
500
0
500
B
0
500
0
500
C
0
500
0
500
Drench
Spray
D
0
500
0
500
E
0
500
0
500
F
0
500
0
500

Fig. 3.1.

Fig. 3.2. Wilt status ratings of six species of bedding plants that received either a drench or spray application of 0 or 500 $mg \cdot L^{-1}$ s-ABA. Wilt status ratings were taken daily over the course of the drought stress. Plants were drought-stressed until all s-ABA treated plants began to show visual symptoms of wilt. Day 0 observations were taken just prior to s-ABA application and at the start of the drought stress. Water was withheld for 7 d for *Impatiens walleriana* 'Xtreme Lavender' (impatiens), *Pelargonium ×hortorum* 'Maverick Red' (seed geranium), *Tagetes patula* 'Bonanza Orange' (marigold) and *Viola ×wittrockiana* 'Bingo Pink Frost' (pansy), 8 d for *Salvia splendens* 'Picante Scarlet' (salvia) and 10 d for *Petunia ×hybrida* 'Ultra Red' (petunia). Visual wilt status ratings were from 1 to 5 with 5-being completely turgid, 4-soft to touch, 3-starting to wilt, 2-wilted with complete loss of turgor and 1-wilted to the point that leaves are dry and brittle. Values are the mean of four plants (n=4).

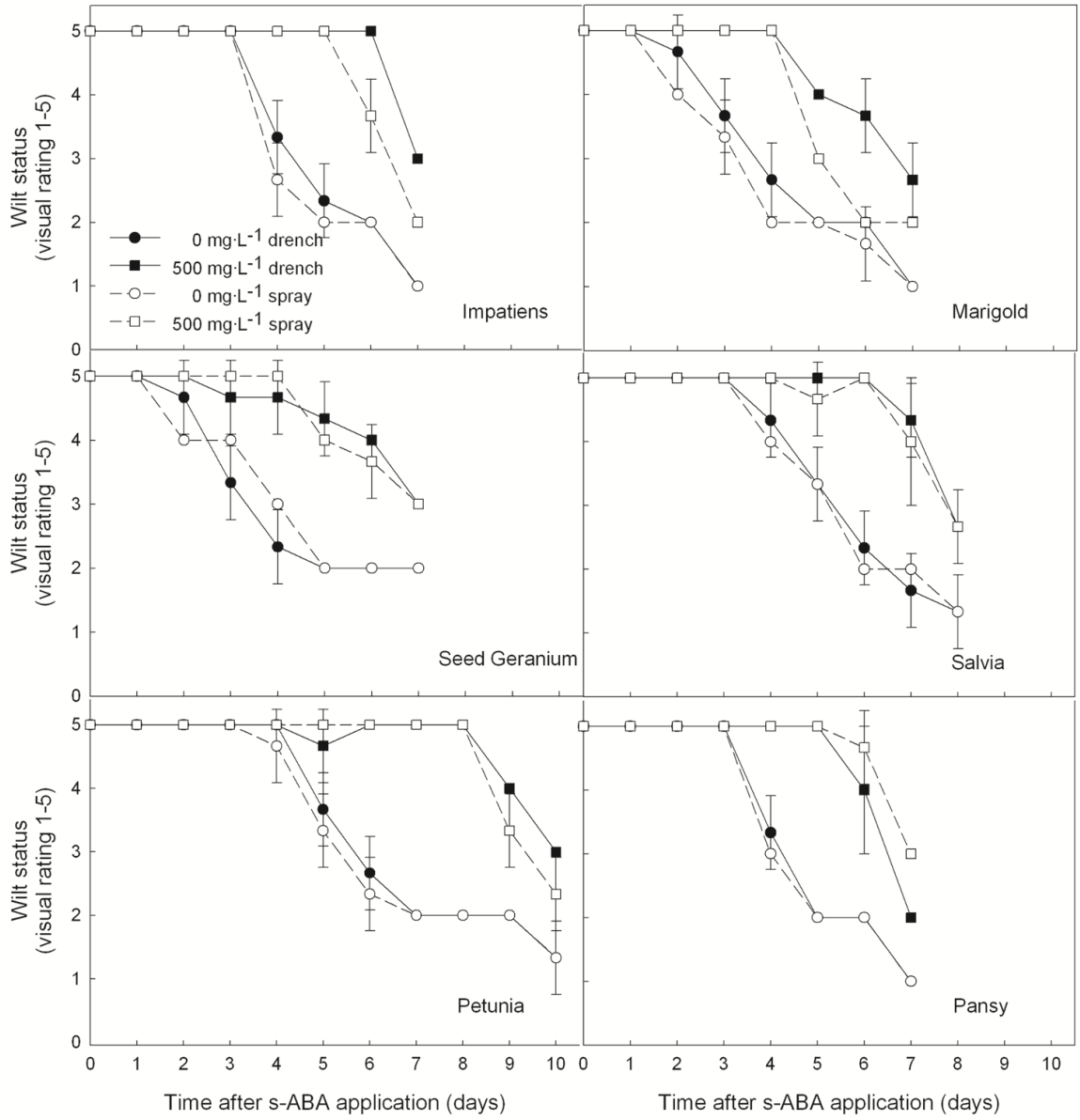
Wilt status (visual rating 1-5)
0 mg·L-1 drench
500 mg·L-1 drench
0 mg·L-1 spray
500 mg·L-1 spray
Impatiens
Marigold
Seed Geranium
Salvia
Petunia
Pansy
Time after s-ABA application (days)
Time after s-ABA application (days)

Fig. 3.2.

Fig. 3.3. Symptoms of leaf chlorosis were observed on *Pelargonium* ×*hortorum* 'Maverick Red' (seed geraniums) (A), *Tagetes patula* 'Bonanza Orange' (marigolds) (B) and *Viola* ×*wittrockiana* 'Bingo Rose Frost' (pansies) (C) following s-ABA application. Plants were treated with 500 mg·L^{-1} s-ABA spray and continually watered. Images are representative of leaf chlorosis symptoms observed after s-ABA applications. Leaf chlorosis ratings were from 1 to 11. A rating of 11 being completely green with no sign of chlorosis, 10: having ≤ 10% leaf chlorosis, 9: 11-20%, 8: 21-30%, 7: 31- 40%, 6: 41-50%, 5: 51- 60%, 4: 61-70%, 3: 71-80%, 2: 81-90%, and 1: 91-100% chlorosis. Leaf chlorosis ratings of these images would be a seven for seed geraniums (A), an eight for marigolds (B) and a seven for pansies (C).

Fig.3.4. Leaf chlorosis ratings of drought-stressed and continually watered *Pelargonium* ×*hortorum* 'Maverick Red' (seed geraniums), *Tagetes patula* 'Bonanza Orange' (marigolds) and *Viola* ×*wittrockiana* 'Bingo Rose Frost' (pansies). Plants were treated with 0 or 500 $mg \cdot L^{-1}$ s-ABA drench or spray and were drought-stressed for 7 d. Water was withheld until s-ABA treated plants began to show visible symptoms of wilt. Leaf chlorosis ratings were from 1 to 11. A rating of 11 being completely green with no sign of chlorosis, 10: having ≤ 10% leaf chlorosis, 9: 11-20%, 8: 21-30%, 7: 31- 40%, 6: 41-50%, 5: 51- 60%, 4: 61-70%, 3: 71-80%, 2: 81-90%, and 1: 91-100% chlorosis. Visual ratings were taken daily with Day 0 being just prior to s-ABA application (n=4).

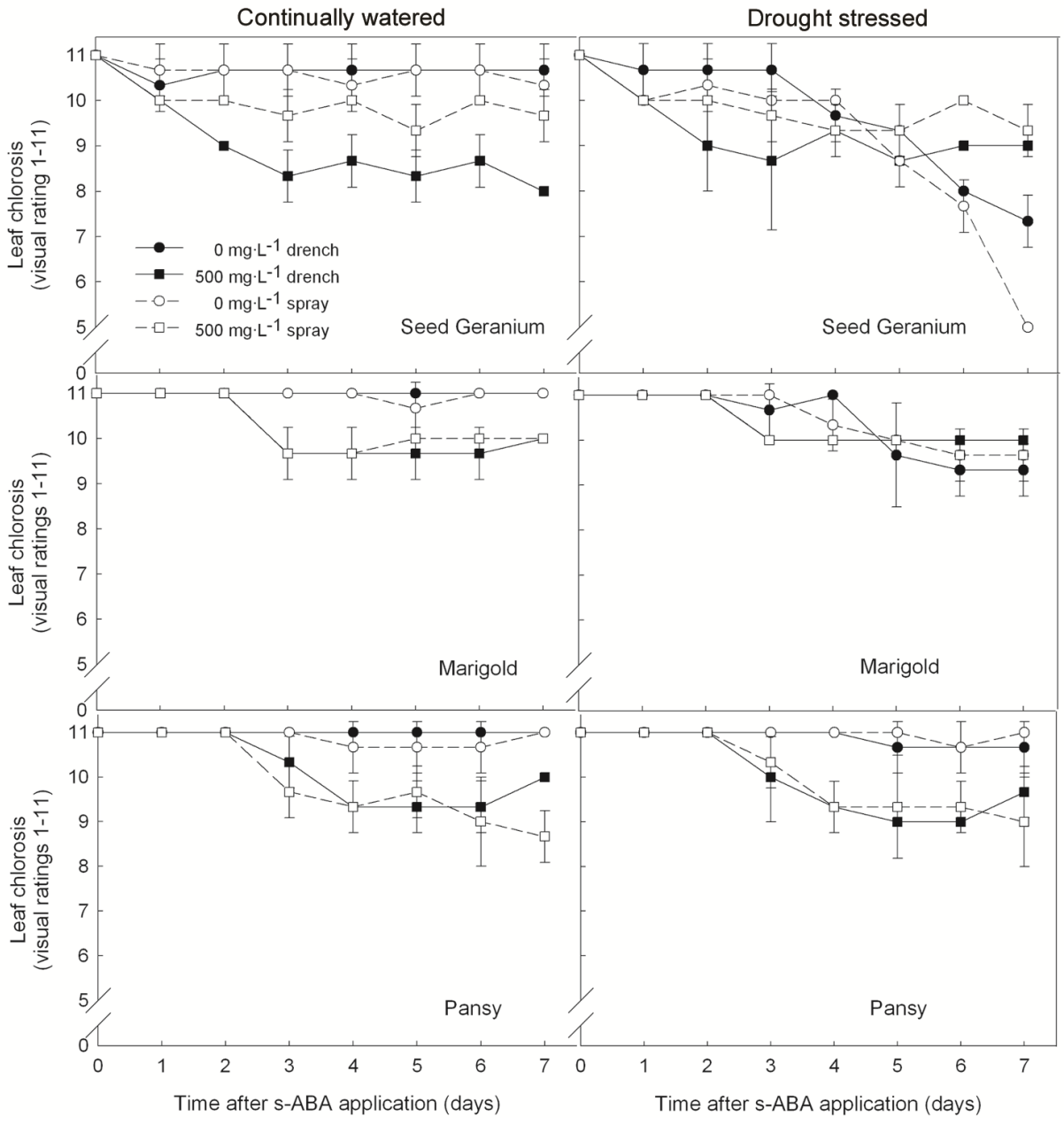
Continually watered
Drought stressed
Leaf chlorosis (visual rating 1-11)
Leaf chlorosis (visual ratings 1-11)
0 mg·L-1 drench
500 mg·L-1 drench
0 mg·L-1 spray
500 mg·L-1 spray
Seed Geranium
Marigold
Pansy
Time after s-ABA application (days)

Fig. 3.4.

CHAPTER 4:

BENZYLADENINE AND GIBBERELLIC ACID APPLICATION PREVENTS ABSCISIC ACID-INDUCED LEAF CHLOROSIS IN PANSY AND VIOLA

Abstract.

Drought stress during the shipping and retailing of floriculture crops can reduce postproduction shelf life and marketability. The plant hormone abscisic acid (ABA) mediates drought stress responses by closing stomata and reducing water loss. Applications of exogenous s-ABA effectively reduce water loss and allow a variety of species to survive temporary periods of drought stress. Unfortunately, s-ABA application can also lead to leaf chlorosis, which reduces the overall quality of economically important bedding plant species, including *Viola ×wittrockiana* (pansy). The goal of this research was to determine how to prevent s-ABA- induced leaf chlorosis in pansy and a closely related species, *Viola cornuta* (viola). All concentrations of both spray (250 or 500 $mg \cdot L^{-1}$) and drench (125 or 250 $mg \cdot L^{-1}$) s-ABA applications induced leaf yellowing. Young plants at the plug stage and 11-cm finished plants with one to two open flowers were further evaluated to determine if the developmental stage of the plants influenced s-ABA effectiveness or the development of negative side-effects. Both plugs and finished pansies and violas developed leaf chlorosis following s-ABA applications, but symptoms

were generally more severe in finished plants. The individual application of benzyladenine (BA), gibberellic acid (GA_{4+7}) or the ethylene perception inhibitor, 1-methylcyclopropene (1-MCP), prior to s-ABA application, had no effect on the development of s-ABA-induced leaf chlorosis. However, applications of 5 or 10 $mg \cdot L^{-1}$ benzyladenine and gibberellic acid as a mixture (BA + GA_{4+7}) prior to a drench or spray application of s-ABA prevented leaf chlorosis. The application of s-ABA and BA + GA_{4+7} would allow floriculture crops to tolerate temporary periods of drought stress without any loss of postproduction quality.

Floriculture crops may encounter harsh environmental conditions during shipping and while on display in retail stores. These poor postproduction environments can result in losses of 5 to 20% (Armitage, 1993; Healy, 2009). A major cause of postproduction decline is drought stress resulting from infrequent or inconsistent watering (Barrett and Campbell, 2006). Plants respond to water deficit by closing their stomata and decreasing transpirational water loss. This drought stress response is regulated by the plant hormone abscisic acid (ABA) (Malladi and Burns, 2007).

The exogenous application of ABA or ABA analogs delays drought stress-induced wilting in a variety of bedding and potted plants (Blanchard et al., 2007; Kim and van Iersel 2008; Monteiro et al., 2001; Sharma et al., 2005; Sharma et al., 2006; Waterland et al., 2010). While ABA applications allow plants to survive temporary periods of drought stress, negative side-effects including leaf necrosis, chlorosis or abscission have been observed in some species (Blanchard et al., 2007; Kim and van

Iersel, 2008; Waterland et al., 2010). We observed leaf chlorosis on drought-stressed *Tagetes patula* (marigolds), *Pelargonium* ×*hortorum* (seed geraniums) and pansies treated with drench or spray applications of s-ABA. Leaf chlorosis in pansies was not directly induced by drought treatment, as symptoms were observed in both watered and drought-stressed plants that were treated with s-ABA (Waterland et al., 2010). It is unclear why s-ABA-induced leaf chlorosis and senescence in pansies or how this side-effect might be prevented.

ABA is the main plant hormone associated with drought stress, but most stress responses are the result of cross-talk between hormone signaling pathways (Huang et al., 2008). While the application of a plant hormone may result in a specific response, these applications can also modify the synthesis and perception of other hormones. The resulting hormone modifications or the interactions between multiple hormones may cause the observed response. To understand a plant's response to an exogenous hormone application, it is therefore necessary to understand these potential hormone interactions. In most cases, drought stress results in an increase in the synthesis of ABA and ethylene, and a decrease in the synthesis of auxin, gibberellins and cytokinins (Nilsen and Orcutte, 1996). ABA applications enhance leaf senescence and abscission by increasing ethylene production and/or increasing ethylene sensitivity (Zacarias and Reid, 1990). Under severe and moderate drought stress, *Petunia* ×*hybrida* (petunia) show an increase in ethylene production as well as an increase in s-ABA, the biologically active form of ABA (Vardi and Mayak, 1989). Changes in the balance of ABA, ethylene, gibberellins and cytokinins within the plant may be responsible for the development of leaf senescence

(Blanchard et al., 2007; Waterland et al., 2010) and leaf abscission (Kim and van Iersel, 2008) following s-ABA applications.

The application of other plant hormones including cytokinins and gibberellins, or the inhibition of ethylene perception may provide a means of preventing ABA-induced leaf chlorosis and abscission. Ethylene perception inhibitors, such as 1-MCP, have been used to prevent senescence and abscission in a variety of floriculture crops including cut flowers and potted flowering plants (Blackenship and Dole, 2003). Cytokinins reduce leaf yellowing and senescence by delaying chlorophyll degradation (Gan and Amasino, 1995; Gan and Amasino, 1997). Transgenic petunia and *Nicotiana tabacum* (tobacco) plants that over-produce cytokinins show delayed leaf senescence and reduced symptoms of leaf chlorosis following drought stress (Clark et al., 2004; Gan and Amasino, 1995). In some species, the application of gibberellic acids (specifically GA_{4+7}) reduces postproduction losses by preventing leaf senescence (Han, 1997; Ranwala et al., 2003; Ranwala and Miller, 1998). Even the use of cytokinins and gibberellins in combination is effective at reducing leaf chlorosis in a variety of floriculture crops (Funnell and Heins, 1998; Han, 2001; Kim and Miller, 2009; Ranwala et al., 2000). It is not known why senescence is prevented or delayed in some species by cytokinins and in others by gibberellins, nor is it possible to predict which species will respond to a particular hormone or combination with delayed senescence. Plant responses to PGRs are highly variable, therefore all PGRs must be examined in different species, cultivars, and even at various developmental stages before useful recommendations can be developed (Gent and McAvoy, 2000).

The goal of this research was to determine how to prevent s-ABA-induced leaf chlorosis in pansy and viola. While s-ABA applications allow pansies to survive temporary periods of drought stress, negative side-effects like leaf chlorosis decrease overall crop quality (Waterland et al., 2010). Our specific objectives were 1) to determine if cultivar, developmental stage, method of application and concentration influence the effectiveness of s-ABA and the occurrence of leaf chlorosis symptoms, 2) to identify an application method and/or concentration of s-ABA that would not induce leaf chlorosis, and 3) to determine whether s-ABA-induced leaf chlorosis can be prevented by the application of other PGRs including cytokinins, gibberellins or ethylene perception inhibitors.

Materials and Methods

Plant material. *Viola* ×*wittrockiana* (pansy) 'Dynamite Purple' (Kieft-Pro-Seeds), 'Karma Rose Fire' (Goldsmith Seeds), or 'Matrix Blue Frost' (PanAmerican Seed) and *Viola cornuta* (viola) 'Penny Deep Blue' (Goldsmith Seeds), 'Skippy Lavender' (Kieft-Pro-Seeds), 'Sorbet Yellow Delight' or 'Sorbet Babyface Purple' (PanAmerican Seed) were used in the experiments as described below. Plugs (young plants in the vegetative stage) were obtained from Green Circle Growers Inc., (Oberlin, OH) or Bob's Market (Mason, WV). Cell-packs (1204s, 96.1 cm^3/cell) of viola 'Sorbet Babyface Purple' and pansy 'Matrix Blue Frost' were obtained from Green Valley Growers Inc., (Ashland, OH) for all finished plant experiments (Expt. 1and 2).

Expt. 1: s-ABA treatment of 11-cm finished pansies and violas. Pansy 'Dynamite Purple', 'Karma Rose Fire', and 'Matrix Blue Frost' and viola 'Penny Deep Blue', 'Skippy Lavender' and 'Sorbet Babyface Purple' plugs or cell-packs were transplanted to 11-cm pots with soilless greenhouse media (Pro-Mix-BX-Mycorise-Pro®, Premier Horticulture, Quebec, Canada). Plants were grown under natural irradiance with supplemental lighting provided as needed by high-pressure sodium and metal halide lamps (GLX/GLS e-systems GROW lights, PARSource, Petaluma, CA). The average photosynthetic photon flux (*PPF*) was 330 $\mu mol \cdot m^{-2} \cdot s^{-1}$ from 0700 to 1900 HR daily [mean daily light integral (DLI) = 18.4 $mol \cdot m^{-2} \cdot d^{-1}$]. Mean greenhouse temperatures were 22.4/15.8 ºC ± 2.0/1.6 day/night with daytime relative humidity of 51.6 ± 7.5%. Plants were irrigated daily with 200 mg $N \cdot L^{-1}$ Peters Excel® Cal-Mag 15N-2.15P-12.5K (Scotts-Sierra Horticulture Products, Marysville, OH), which was reduced to 100 mg $N \cdot L^{-1}$ two weeks prior to flowering. s-ABA treatments were applied to finished pansies and violas when they reached a marketable stage of one to two open flowers per plant and the foliage and roots had reached the edge of the pot. Applications were on 26 April, 2008 for pansy 'Matrix Blue Frost' and viola 'Sorbet Babyface Purple' and 6 May, 2008 for the other four cultivars. All plants were watered to container capacity 12 h prior to s-ABA (ConTego™, Valent BioSciences Corp., Libertyville, IL) application. s-ABA was applied according to manufacturer's recommendations as a drench at 0, 125 or 250 $mg \cdot L^{-1}$ (60 ml per container) or as a spray at 0, 250 or 500 $mg \cdot L^{-1}$ (approximately 6.6 ml per plant) with the addition of 0.05% CapSil® (Aquatrols Corporation of America, Inc., Cherry Hill, NJ). Spray applications were applied with a Regulator Bak-pak® sprayer (H.D. Hudson

Manufacturing Company, Chicago, IL). Half of the plants from each treatment were irrigated daily with 100 mg $N \cdot L^{-1}$. The other half (i.e. drought-stressed) had water withheld until the s-ABA treated plants wilted, and then they were rewatered. Plants were held in the greenhouse under the environmental conditions described previously for all subsequent evaluations.

Expt. 2: PGR applications to 11-cm finished pansies and violas. PGRs were applied to 11-cm finished pansy 'Matrix Blue Frost' and viola 'Sorbet Babyface Purple' prior to s-ABA application. Cell-packs were transplanted and plants were grown in the greenhouse as described above. Treatments for Expt. 2A began on 13 May, 2008. Average *PPF* was 340 $\mu mol \cdot m^{-2} \cdot s^{-1}$ from 0500 to 1900 HR daily (DLI = 17.6). Mean greenhouse temperatures were 22/15°C ± 2.1/0.9 day/night with daytime relative humidity of 51.2 ± 5.6%. PGR treatments included spray applications (approximately 4.4 ml per plant) of benzyladenine (BA) (MaxCel®, Valent BioSciences Corp., Libertyville, IL), gibberellic acid (GA_{4+7}) (ProVide®, Valent BioSciences Corp., Libertyville, IL), or BA + GA_{4+7} (Promalin®/Fascination®, Valent BioSciences Corp., Libertyville, IL) were applied at the rates of 0, 1, 2, 5 or 10 $mg \cdot L^{-1}$ (plus 0.05% CapSil®) 12 h prior to 0 or 250 $mg \cdot L^{-1}$ s-ABA. Treatments also included 1-methylcyclopropene (1-MCP) (EthylBloc®, Floralife®, Inc., Walterboro, SC), an ethylene perception inhibitor, at the manufacturer's recommended rate of 300 $nL \cdot L^{-1}$ for 10 h prior to a drench application of 0 or 250 $mg \cdot L^{-1}$ s-ABA. All plants in this experiment were irrigated daily with 100 mg $N \cdot L^{-1}$.

Based on the observations from the first PGR experiment outlined above (Expt. 2A), a concentration of 5 $mg \cdot L^{-1}$ BA + GA_{4+7} was selected for the treatment of all six

pansy and viola cultivars in Expt. 2B. Treatments began on 13 June, 2008 under the greenhouse conditions described previously. Average *PPF* was 359 $\mu mol \cdot m^{-2} \cdot s^{-1}$ from 0500 to 1900 HR daily (DLI = 19.8). Mean greenhouse temperatures were 24.3/18°C ± 3.0/2.5 day/night with daytime relative humidity of 60.9 ± 8.7%. BA + GA_{4+7} at 0 or 5 $mg \cdot L^{-1}$ (plus 0.05% CapSil®) was applied 12 h prior to either a drench application of 0 or 250 $mg \cdot L^{-1}$ s-ABA or a spray application of 0 or 500 $mg \cdot L^{-1}$ s-ABA (plus 0.05% CapSil®). Half of the plants in this experiment were irrigated daily and the other half had water withheld for 5 d before being rewatered. Chlorophyll changes in the leaves were quantified with a chlorophyll content meter (SPAD-502, Konica Minolta Sensing, Inc., Osaka, Japan). The lowest basal leaf on each plant was measured at 5 d after s-ABA application on both drought-stressed and watered plants.

Expt. 3: s-ABA treatment of pansy and viola plugs. Viola 'Penny Deep Blue', viola 'Skippy Lavender', pansy 'Dynamite Purple' and pansy 'Karma Rose Fire' plugs were received in 512-cell trays (5.4 cm^3/cell) and viola 'Sorbet Yellow Delight' and pansy 'Matrix Blue Frost' were in 288-cell trays (7 cm^3/cell). Plugs were randomly assigned to empty plug trays with equal spacing among all replicates and treatments. All plugs were grown and evaluated in the greenhouse under natural irradiance with supplemental lighting (as stated above) and an average *PPF* of 250 $\mu mol \cdot m^{-2} \cdot s^{-1}$ from 0700 to 1900 HR daily (DLI= 11.6). Mean greenhouse temperatures were 23.4/16.7 ºC ± 1.2/3.7 day/night with a daytime relative humidity of 49.5 ± 8.5%. Five week old pansy and viola plugs were treated on 9 April or 18 April, 2008 for initial determination of s-ABA effectiveness and leaf chlorosis. Plugs were sprenched (spray to drench, approximately 43 ml per tray)

with 0, 75, 125, 250, 500 or 1000 mg·L^{-1} s-ABA (plus 0.05% CapSil®). Half of the plugs had water withheld for 2 d and were then rewatered. The other half were irrigated daily throughout the experiment with 100 mg N·L^{-1}.

Expt. 4: Application of BA + GA_{4+7} and s-ABA on pansy and viola plugs. Viola 'Penny Deep Blue', viola 'Sorbet Yellow Delight', pansy 'Dynamite Purple' and pansy 'Karma Rose Fire' plugs were received in 288 cell trays (7 cm^3/cell). Replications and treatments were randomly assigned to empty plug trays as described for Expt. 3. Nine week old plugs were treated with BA + GA_{4+7} prior to s-ABA application. Treatments began on 8 Jan. 2010 and plugs were grown and evaluated under the greenhouse conditions described previously. The average *PPF* during this experiment was 269 µmol·m^{-2}·s^{-1} from 0700 to 1800 HR daily (DLI = 11.6). Mean greenhouse temperatures were 21.9/15.6 ºC ± 1.1/0.9 day/night with a daytime relative humidity of 29.0 ± 8.1%. Plugs were sprenched (approximately 29 ml per tray) with 0 and 5 mg·L^{-1} BA + GA_{4+7} 12 h prior to an application of 0 or 1000 mg·L^{-1} s-ABA (plus 0.05% CapSil®). All plugs were irrigated daily with 100 mg N·L^{-1}.

Evaluations of wilt status and leaf chlorosis. Visual observations of wilt status and leaf chlorosis were taken daily for all plugs and finished plants. Day 0 was the day of s-ABA treatment. All observations on Day -1 or Day 0 were made just prior (1-2 h) to the application of PGRs. Wilt status ratings were from 1 to 5; 5 = completely turgid, 4 = soft to touch but still upright, 3 = starting to wilt and no longer upright, 2 = wilted with complete loss of turgor and 1 = wilted to the point that leaves are dry and desiccated. The shelf life of drought-stressed plants was calculated as the number of days that it took

plants to reach a wilt status rating of 3 or less, after which they were considered unmarketable. Leaf chlorosis ratings for the whole plant were from 1 to 11; 11 = completely green with no sign of chlorosis, 10 = ≤ 10% leaf chlorosis, 9 = 11-20%, 8 = 21-30%, 7 = 31-40%, 6 = 41-50%, 5 = 51-60%, 4 = 61-70%, 3 = 71-80%, 2 = 81-90% and 1 = 91-100% leaf chlorosis.

Statistical analysis. Reported values are the means ± SD of four replications, which is four plants in Expt. 1 and 2. For Expt. 3 and 4 six individual plugs were used as one replication. Data are the average of three replications for pansy 'Matrix Blue Frost' and viola 'Sorbet Yellow Delight' and four replications for all other pansies and violas in Expt. 3. Expt. 4 values are the average of four replications. All experiments were conducted in a completely randomized block design with Expt. 1 and 2 blocked by watering regiment, watered vs. water withheld, and by replication. Expt. 3 and 4 were blocked by treatment. All values were analyzed using Proc GLM (generalized linear model) with LSD means separation (least significant difference test) ($P \leq 0.05$). Values were analyzed by SAS Version 9.1.3 (Statistical Analysis System, SAS Institute, Inc., Cary, NC).

Results

Expt. 1: Wilt status and shelf life extension in s-ABA treated 11-cm finished pansies and violas. s-ABA treatments delayed wilting symptoms in drought-stressed pansies and violas (Fig. 4.1 and Table 4.1). Treated plants had increased shelf life compared to control plants (0 $mg \cdot L^{-1}$ s-ABA), and shelf life extensions ranged from 0.5 to 7.7 d

depending on the concentration of s-ABA, method of application and cultivar (Table 4.1). The effectiveness of the s-ABA treatment increased at higher concentrations, and differences were observed between cultivars (Fig. 4.1B and Table 4.1). Plants treated with 125 mg·L^{-1} s-ABA were the first to show symptoms of wilting when compared to the 250 or 500 mg·L^{-1} treatments (Fig. 4.1B). Application of 500 mg·L^{-1} s-ABA resulted in the greatest delay in wilting (Fig. 4.1B) and led to shelf life extensions of 5.0 to 7.7 d beyond those of the non-ABA treated controls (Table 4.1). The longest shelf life extension was observed in pansy 'Dynamite Purple' and the shortest was in viola 'Skippy Lavender'. Three d after rewatering, all the pansies and violas treated with s-ABA recovered from drought stress, but 71% of control plants (0 mg·L^{-1} s-ABA) died (data not shown).

Expt. 1: Leaf chlorosis of 11-cm finished pansies and violas treated with s-ABA.

Although s-ABA was effective at delaying wilting, basal leaf chlorosis was induced in both finished pansies and violas (Fig. 4.2 and Fig. 4.3). The degree of leaf yellowing was similar in drought-stressed and non-stressed plants (data not shown), therefore leaf chlorosis was further investigated in watered plants to directly evaluate the effect of s-ABA applications. Control plants (0 mg·L^{-1} drench or spray) had little or no leaf chlorosis throughout the experiment (Fig. 4.2). ABA-induced leaf chlorosis was first observed on Day 1 in pansy 'Dynamite Purple' and viola 'Penny Deep Blue', Day 3 in viola 'Sorbet Babyface Purple' and Day 2 for all other cultivars (Fig. 4.2). The severity of leaf chlorosis (as determined by the percentage of the plant that had yellow leaves) continued to increase until Day 5 or later depending on the cultivar. By Day 6 all

cultivars treated with 500 mg·L^{-1} s-ABA (except 'Sorbet Babyface Purple') had a rating of 7 or lower, indicating that 30% or more of the plant was yellow (Fig. 4.2).

Expt. 2A: Preventing s-ABA-induced leaf chlorosis with pre-application of other PGRs. Pretreatment with the ethylene perception inhibitor, 1-MCP, did not reduce the development of leaf chlorosis in s-ABA treated plants (Fig. 4.3). Spray applications of cytokinin (BA at 1, 2, 5 or 10 mg·L^{-1}) or gibberellins (GA_{4+7} at 1, 2, 5, or 10 mg·L^{-1}) also did not reduce leaf chlorosis (Fig. 4.3 and data not shown). In contrast, spray applications of BA + GA_{4+7}, at either 5 or 10 mg·L^{-1}, completely prevented s-ABA-induced leaf chlorosis based on visual observations (Fig. 4.3 and data not shown).

Expt. 2B: Preventing s-ABA-induced leaf chlorosis with pre-application of BA + GA_{4+7}. SPAD meter readings indicated that s-ABA reduced the chlorophyll content in basal leaves by 25 to 85% depending on cultivar, concentration, and application method (Fig. 4.4). Spray applications of 500 mg·L^{-1} s-ABA caused more leaf yellowing than drench applications of 250 mg·L^{-1} in two of the six cultivars (viola 'Penny Deep Blue' and pansy 'Matrix Blue Frost') (Fig. 4.4). When plants were pretreated with 5 mg·L^{-1} BA + GA_{4+7}, the chlorophyll content of leaves at 5 d after s-ABA application was the same as non s-ABA treated controls (Fig. 4.4).

Expt. 3: Wilt status of pansy and viola plugs treated with s-ABA. Sprench applications of s-ABA delayed visible wilting in drought-stressed pansy and viola plugs. One d after withholding water, control plugs (0 mg·L^{-1} s-ABA) were more wilted than all s-ABA treatments (Fig. 4.5B). All control plugs were severely wilted and had reached a wilt status rating of 1 by Day 2. The 1000 mg·L^{-1} s-ABA treated plugs remained turgid

longer than all other treatments. Plugs of pansy 'Matrix Blue Frost' and viola 'Sorbet Yellow Delight' treated with 1000 mg·L^{-1} s-ABA did not show any wilting symptoms when water was withheld for 2 d. Concentrations of 75, 125, 250 and 500 mg·L^{-1} of s-ABA were equally effective at preventing wilting, except in pansy 'Dynamite Purple' where 500 mg·L^{-1} was as effective as 1000 mg·L^{-1} (Fig. 4.5B). Plugs were rewatered after they had been drought-stressed for 2 d. The survival rate of rewatered plugs was dependent on the concentration of s-ABA. With s-ABA sprench applications of 75, 125, 250, 500 and 1000 mg·L^{-1}, pansy plugs had survival rates of 53.7, 40.7, 81.9, 81.0 and 95.8%, respectively and viola plugs had survival rates of 49.1, 56.5, 75.0, 75.0 and 100%, respectively. The survival rate in all the control (0 mg·L^{-1} s-ABA) plugs was 0% after rewatering (data not shown).

Expt. 3: Leaf chlorosis of pansy and viola plugs treated with s-ABA. Watered plugs that were treated with s-ABA developed lower leaf chlorosis similar to what was observed on 11-cm finished plants (Fig. 4.6). In some instances, this leaf chlorosis was less severe at the plug stage. ABA-induced leaf chlorosis was observed on five of the six cultivars by Day 1 (Fig. 4.6B). On Day 3, viola 'Sorbet Yellow Delight' and pansy 'Matrix Blue Frost' had more severe leaf chlorosis at higher concentrations of s-ABA (500 and 1000 mg·L^{-1}) compared to the lower concentrations (75, 125, and 250 mg·L^{-1}) (Fig. 4.6B). Applications of s-ABA did not induce leaf yellowing in viola 'Skippy Lavender', while the most severe symptoms were observed in pansy 'Matrix Blue Frost' (Fig. 4.6B). Similar leaf chlorosis symptoms were observed in drought-stressed plugs following s-ABA application (data not shown).

Expt. 4: Preventing s-ABA-induced leaf chlorosis with pre-applications of BA + GA_{4+7} in pansy and viola plugs. Pansy and viola plugs sprenched with 1000 $mg \cdot L^{-1}$ s-ABA and 0 $mg \cdot L^{-1}$ BA + GA_{4+7} developed leaf chlorosis 2 d after application (Fig. 4.7). Pretreatment with 5 $mg \cdot L^{-1}$ BA + GA_{4+7} prevented the development of leaf chlorosis on all plugs that received 1000 $mg \cdot L^{-1}$ s-ABA (Fig. 4.7). All BA + GA_{4+7} treated plugs were visually indistinguishable from the control plugs (0 $mg \cdot L^{-1}$ s-ABA plus 0 $mg \cdot L^{-1}$ BA + GA_{4+7}) (Fig. 4.7).

Discussion

All applications of s-ABA regardless of cultivar, developmental stage, method of application or concentration were effective at delaying wilting symptoms in pansies and violas exposed to severe drought stress. However, we were unable to identify a combination of method of application and concentration of s-ABA that would effectively enhance drought tolerance in any of these plants without inducing leaf chlorosis. Leaf chlorosis in general was less severe in young plants (plugs) than in finished plants that were flowering. s-ABA treated viola 'Skippy Lavender' developed little or no leaf chlorosis at the plug stage, but finished plants had 20 to 50% leaf chlorosis (leaf chlorosis rating of 8 to 6) depending on the s-ABA treatment. This indicates that responses to s-ABA will be highly dependent on both cultivar and developmental stage. Plant responses to PGRs are known to vary based on both of these factors (Gent and McAvoy, 2000).

A plant's response to hormones is controlled by both the hormone concentration and the exposure time. In pansies and violas, higher concentrations of s-ABA resulted in

more severe symptoms of leaf chlorosis. These exogenous applications can also increase or decrease the endogenous production of other plant hormones and alter tissue sensitivity to hormones. In some plants, exogenous ABA applications stimulate ethylene production (Sharp et al., 2000; Zhang et al., 2009). Ethylene is a gaseous plant hormone known to regulate leaf and flower senescence, and it has been associated with postproduction/postharvest leaf chlorosis and senescence in a variety of crops (Blankenship and Dole, 2003; Bleecker and Kende, 2000). Leaf senescence and abscission in ethylene-sensitive crops can be prevented by treatment with the ethylene perception inhibitor, 1-MCP (Blackenship and Dole, 2003; Celikel et al., 2002). In some species, including Easter lilies (*Lilium longiflorum*), leaf senescence is not associated with ethylene production and it is prevented by the application of cytokinins (BA) and/ or gibberellins (GA_{4+7}) (Franco and Han, 1997). In our experiment, 1-MCP did not prevent s-ABA- induced leaf chlorosis, indicating that ethylene may not be associated with leaf senescence in pansy and viola.

The application of PGRs that include cytokinins or gibberellins reduces or prevents leaf yellowing in a number of species. Cytokinins such as BA delay the development of leaf yellowing in a variety of floriculture crops including lilies (*Lilium* sp.), tulips (*Tulipa gesneriana*), geraniums, gerbera daisy (*Gerbera jamesonii*), goldenrod (*Solidago canadensis*), zinnia (*Zinnia elegans*) and miniature roses (*Rosa ×hybrida*) (Franco and Han, 1997; Kim and Miller, 2009; Padhye and Runkle, 2008; Philosoph-Hadas et al., 1996; Pinto et al., 2005; Tjosvold et al., 1994). The application of only gibberellic acid, specifically GA_{4+7}, also effectively enhances postproduction quality by

reducing the occurrence of leaf chlorosis and senescence. Basal leaf yellowing is reduced or prevented by GA_{4+7} application in Easter and Oriental (*Lilium* sp.) lilies (Han, 1997; Ranwala and Miller, 2000; Ranwala et al., 2000; Ranwala et al., 2003). Neither BA nor GA_{4+7} alone was effective at preventing, or even reducing, the s-ABA-induced leaf chlorosis that we observed in pansy and viola.

PGR applications that included a combination of both cytokinins and gibberellic acids (BA + GA_{4+7}) were the only treatment that effectively reduced s-ABA-induced leaf chlorosis. The application of PGRs that contain both BA and GA_{4+7} also prevent postproduction cold-induced leaf chlorosis and senescence in many species of lilies including potted Easter lilies, potted Oriental lilies (*Lilium* sp. 'Stargazer'), and cut Asiatic and Oriental lilies (*Lilium* sp.) (Funnell and Heins, 1998; Han, 1995; Han, 1997; Han, 2001; Ranwala and Miller, 1998; Ranwala and Miller, 2005; Ranwala et al., 2000; Ranwala et al., 2003; Whitman et al., 2001). Postproduction leaf yellowing in tulips is reduced following the application of BA and GA_{4+7} (Kim and Miller, 2009). It remains unclear why BA + GA_{4+7} are effective in combination, but not individually in these species.

Application of BA + GA_{4+7} prior to the application of s-ABA could be used by growers to prevent leaf chlorosis. One potential problem with using BA + GA_{4+7} to reduce s-ABA-induced leaf chlorosis is increased internode elongation. The exogenous application of GA independently or in combination with cytokinins results in increased stem elongation in some species including poinsettia (*Euphorbia pulcherrima*) and Easter lily (Blanchard and Runkle, 2008; Han, 2000; Ranwala et al., 2003; Ranwala and Miller,

1999; Whitman et al., 2001). In Easter lilies, undesirable stem elongation was minimized by determining the optimum concentration of GA_{4+7} and utilizing only foliar spray applications (Ranwala et al., 2003). At 21 d after treatment we did not measure any differences in plant height resulting from BA + GA_{4+7} applications (data not shown). Under our experimental conditions, the low concentrations of GA did not induce stem elongation in violas or pansies. Nineteen of 20 cultivars of tulips treated with BA + GA_{4+7} also displayed no stem elongation (Kim and Miller, 2009). The pre-application of a PGR that contains low concentrations of BA + GA_{4+7} provides a simple and practical way for growers to effectively utilize s-ABA to delay visual symptoms of wilting in drought-stressed crops without inducing leaf chlorosis.

Acknowledgements

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Table 4.1. Shelf life of drought-stressed 11-cm finished *Viola cornuta* (viola) and *Viola* ×*wittrockiana* (pansy) treated with s-ABA.[z]

	Application method and s-ABA concentration ($mg \cdot L^{-1}$) Time until visually wilted (days)					
Species and cultivar	Drench			Spray		
Viola cornuta	0	125	250	0	250	500
'Penny Deep Blue'	2.5d	5.0c	5.8c	2.5d	7.3b	8.3a
'Skippy Lavender'	2.3e	3.3d	5.0c	2.3e	6.5b	7.3a
'Sorbet Babyface Purple'	4.5d	6.8c	7.8b	4.3d	8.3b	9.5a
Viola ×*wittrockiana*						
'Dynamite Purple'	3.3e	5.0d	5.8c	1.3f	6.5b	9.0a
'Karma Rose Fire'	3.5cd	4.0c	5.5b	2.5d	8.3a	9.0a
'Matrix Blue Frost'	4.3d	5.8c	7.0b	3.5d	7.5b	8.8a

[z]Values are means of four replications (n=4). Values in rows followed by different letters are statistically different at $P \leq 0.05$ (least significant difference test). Shelf life is the average number of days from Day 0 that it took each plant to show visible symptoms of wilt. This is equivalent to a rating of 3 (starting to wilt) or less on the wilt status rating scale of 1 to 5; where 5 = completely turgid, 4 = soft to touch but still upright, 3 = starting to wilt and no longer upright, 2 = wilted with complete loss of turgor and 1 = wilted to the point that leaves are dry and desiccated. Shelf life extension is the number of additional days from the time in which the control plants wilted until the s-ABA treated plants wilted.

Fig. 4.1. Visual symptoms of wilting and daily wilt status ratings of 11-cm finished *Viola cornuta* (viola) and *Viola* ×*wittrockiana* (pansy) after withholding water. Images are of viola 'Sorbet Babyface Purple' 5 d after the application of s-ABA and subsequent drought stress (A). The left image is of drench applications of 0, 125, and 250 $mg \cdot L^{-1}$ s-ABA and the right image is of spray applications of 0, 250, and 500 $mg \cdot L^{-1}$ s-ABA (A). Water was withheld until s-ABA treated plants were wilted (wilt status rating of 3 or below), and wilt status ratings were determined daily (B). Day 0 observations were taken just prior (1 to 2 h) to s-ABA application and withholding of water. Wilt status ratings were from 1 to 5; 5 = completely turgid, 4 = soft to touch but still upright, 3 = starting to wilt and no longer upright, 2 = wilted with complete loss of turgor and 1 = wilted to the point that leaves are dry and desiccated. Values are means ± SD of four replications (n=4).

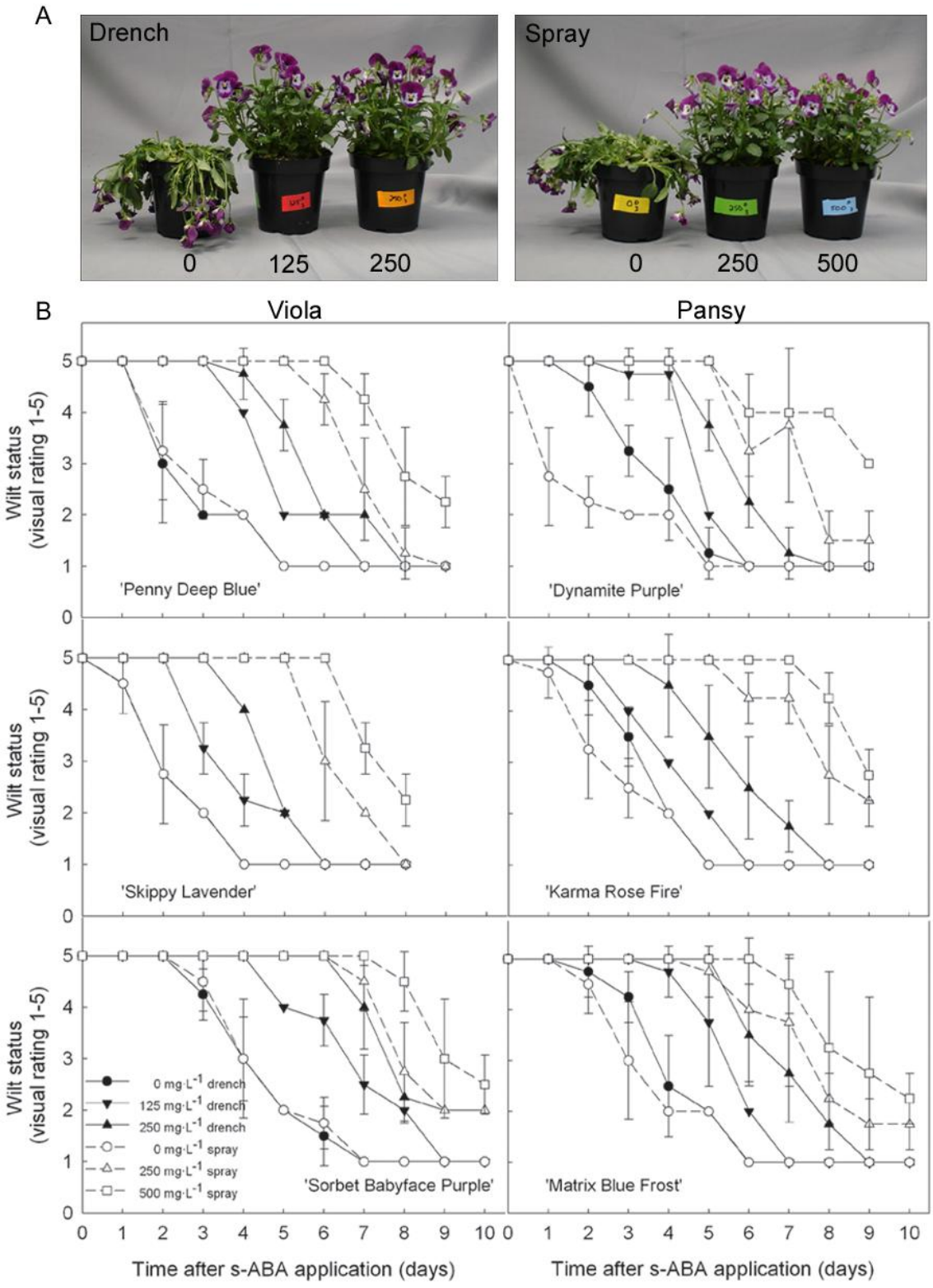
A
Drench
0
125
250
Spray
0
250
500
B
Viola
Pansy
Wilt status (visual rating 1-5)
'Penny Deep Blue'
'Dynamite Purple'
'Skippy Lavender'
'Karma Rose Fire'
'Sorbet Babyface Purple'
'Matrix Blue Frost'
0 mg·L-1 drench
125 mg·L-1 drench
250 mg·L-1 drench
0 mg·L-1 spray
250 mg·L-1 spray
500 mg·L-1 spray
Time after s-ABA application (days)

Fig. 4.1.

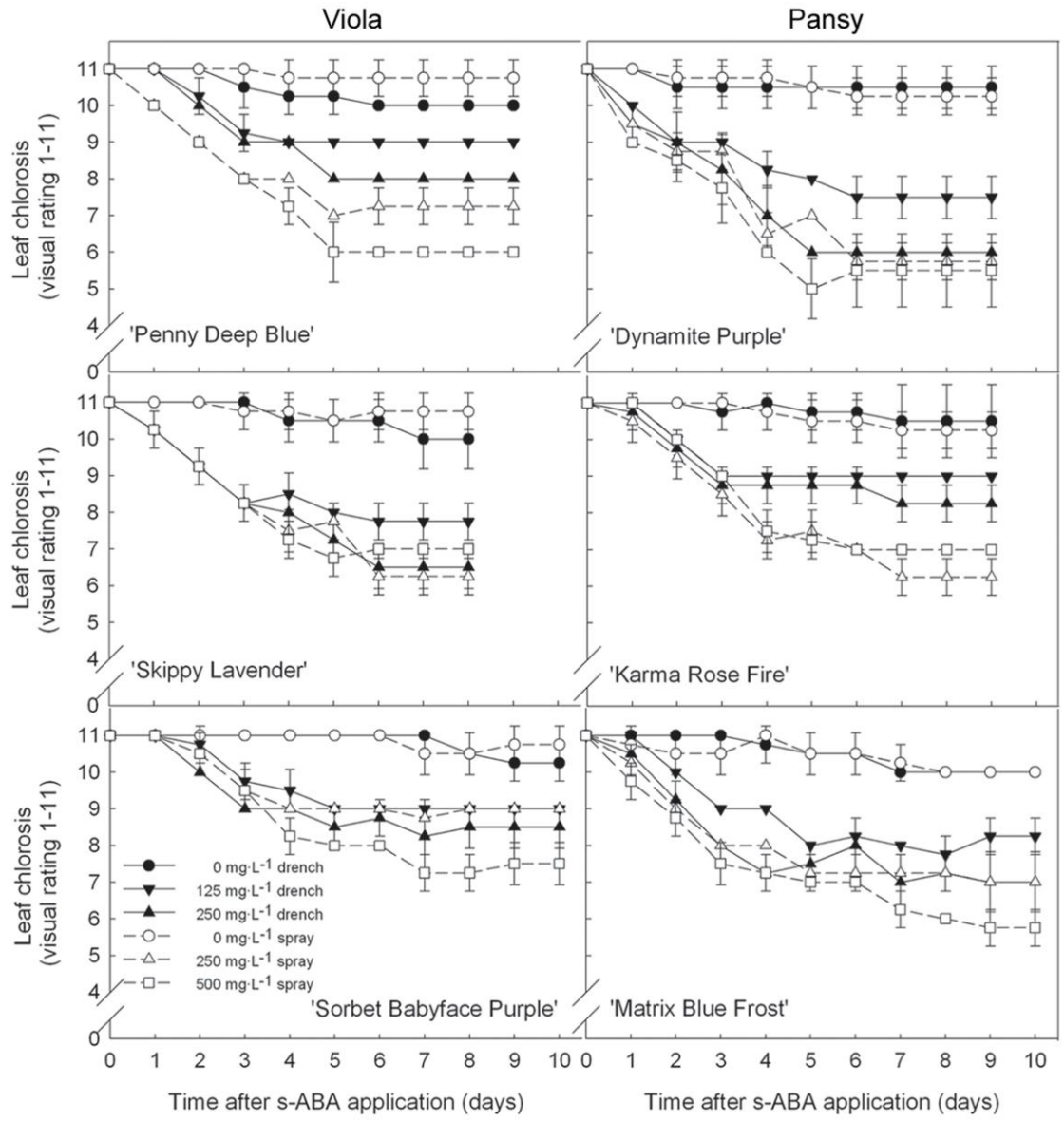

Fig. 4.2. Leaf chlorosis ratings of finished 11-cm *Viola cornuta* (viola) and *Viola* ×*wittrockiana* (pansy) after a drench or spray application of s-ABA. Finished plants were irrigated daily with 100 mg $N \cdot L^{-1}$. Leaf chlorosis ratings for the whole plant were from 1 to 11; 11 = completely green with no sign of chlorosis, 10 = ≤ 10% leaf chlorosis, 9 = 11-20%, 8 = 21-30%, 7 = 31-40%, 6 = 41-50%, 5 = 51-60%, 4 = 61-70%, 3 = 71-80%, 2 = 81-90% and 1 = 91-100% leaf chlorosis. Visual ratings were recorded daily with Day 0 observations taken just prior (1 to 2 h) to s-ABA application. Values are means ± SD of four replications (n=4).

Fig. 4.3. *Viola cornuta* (viola) and *Viola* ×*wittrockiana* (pansy) treated with other plant growth regulators (PGRs) to prevent s-ABA-induced leaf chlorosis. Violas and pansies were pretreated with BA, GA_{4+7}, BA + GA_{4+7} or 1-MCP. BA, GA_{4+7} and BA + GA_{4+7} were applied at the rate of 5 or 10 mg·L^{-1}. Plants were treated with 300 nL·L^{-1} 1-MCP for 10 h. Plants were treated 12 h later with drench applications of 0 or 250 mg·L^{-1} s-ABA. Images are of viola 'Sorbet Babyface Purple' and are representative of these experiments. Photos were taken 2 d after s-ABA application. All plants were irrigated daily with 100 mg N·L^{-1}.

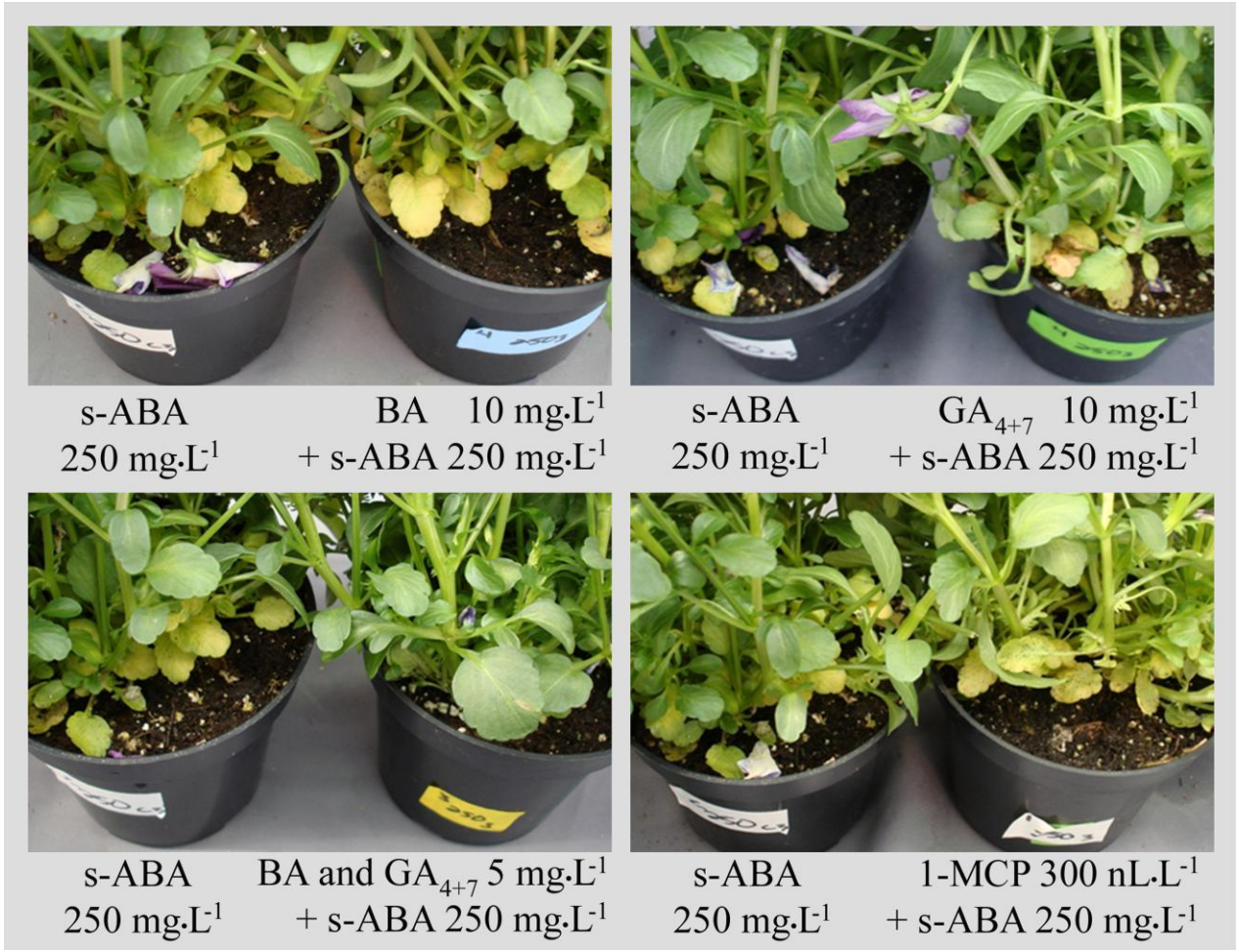
s-ABA
250 mg.L-1
BA 10 mg.L-1
+ s-ABA 250 mg.L-1
s-ABA
250 mg.L-1
GA4+7 10 mg.L-1
+ s-ABA 250 mg.L-1
s-ABA
250 mg.L-1
BA and GA4+7 5 mg.L-1
+ s-ABA 250 mg.L-1
s-ABA
250 mg.L-1
1-MCP 300 nL.L-1
+ s-ABA 250 mg.L-1

Fig. 4.3.

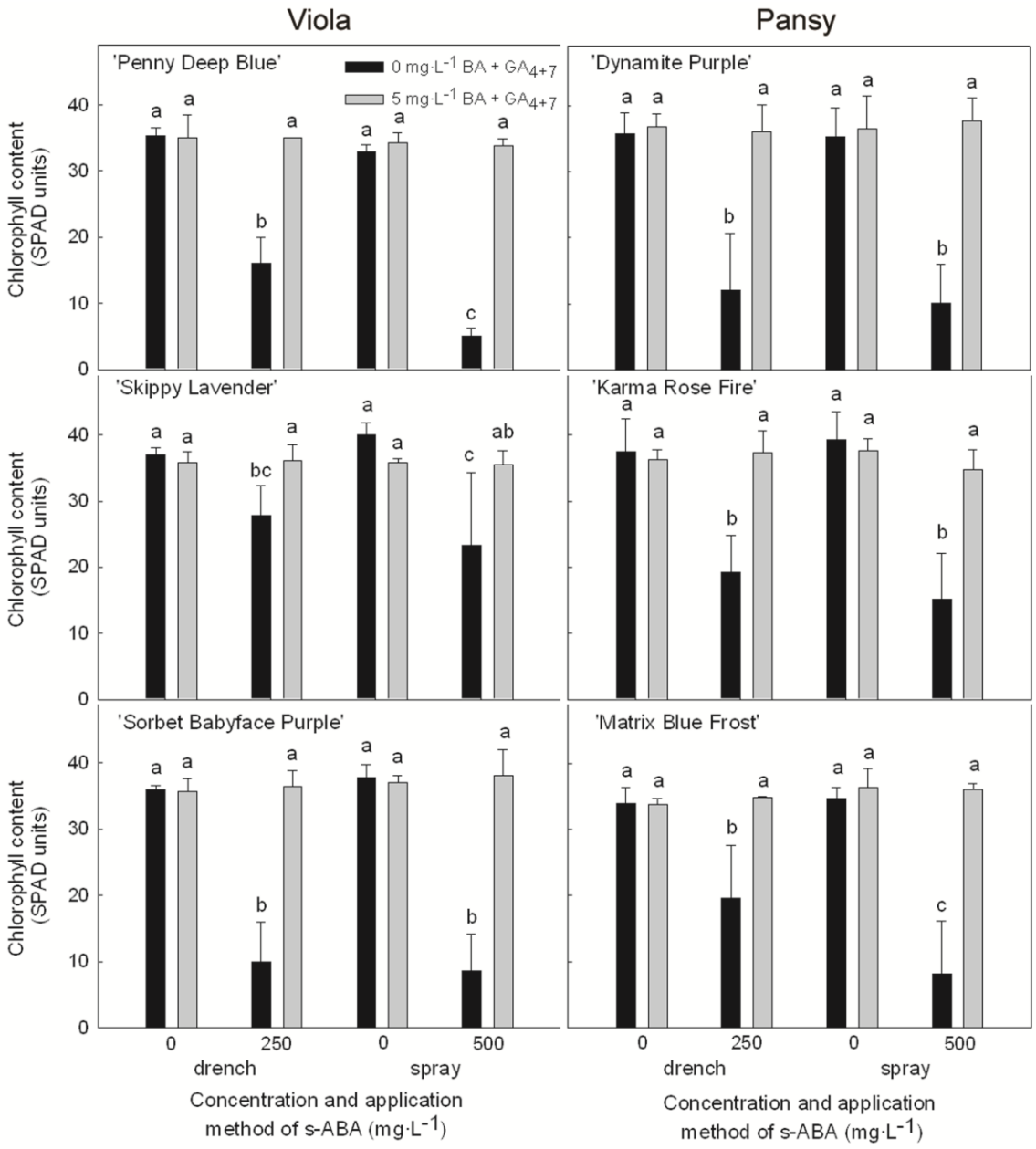

Fig. 4.4. Chlorophyll content readings of *Viola cornuta* (viola) and *Viola* ×*wittrockiana* (pansy) 5 d after s-ABA application in watered plants. Finished plants were irrigated daily with 100 mg $N \cdot L^{-1}$. BA + GA_{4+7} (0 or 5 $mg \cdot L^{-1}$) was applied 12 h prior to either a drench (0 or 250 $mg \cdot L^{-1}$) or spray (0 or 500 $mg \cdot L^{-1}$) application of s-ABA. Data was analyzed using Proc GLM (generalized linear model) with means separated by letters. Values are means ± SD of four replications (n =4).

Fig. 4.5. Wilt status ratings of *Viola cornuta* (viola) and *Viola* ×*wittrockiana* (pansy) plugs after a sprench application of s-ABA and 2 d of withholding water. Wilt status ratings were from 1 to 5; 5 = completely turgid, 4 = soft to touch but still upright, 3 = starting to wilt and no longer upright, 2 = wilted with complete loss of turgor and 1 = wilted to the point that leaves are dry and desiccated. (A). Visual ratings were taken daily with Day 0 being a completely turgid plug just prior (1 to 2 h) to s-ABA application. Values are the mean ± SD of three replications (n=3) for viola 'Sorbet Yellow Delight' and pansy 'Matrix Blue Frost' and four replications (n=4) for all other cultivars (B). Each replication consisted of six plugs.

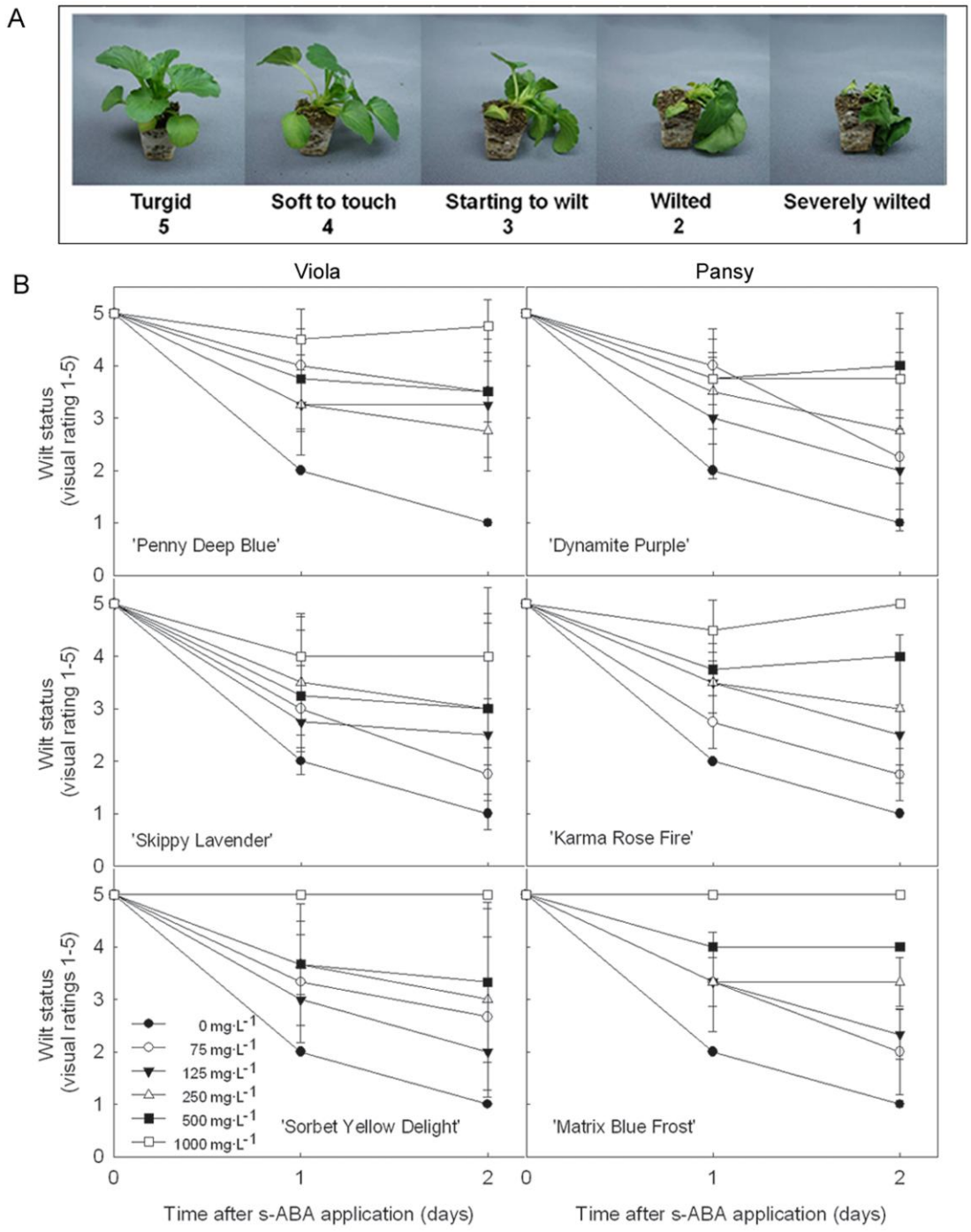
A
Turgid
5
Soft to touch
4
Starting to wilt
3
Wilted
2
Severely wilted
1
B
Viola
Pansy
Wilt status
(visual rating 1-5)
'Penny Deep Blue'
'Dynamite Purple'
'Skippy Lavender'
'Karma Rose Fire'
'Sorbet Yellow Delight'
'Matrix Blue Frost'
0 mg·L-1
75 mg·L-1
125 mg·L-1
250 mg·L-1
500 mg·L-1
1000 mg·L-1
Time after s-ABA application (days)

Fig. 4.5.

Fig. 4.6. Leaf chlorosis ratings of *Viola cornuta* (viola) and *Viola* ×*wittrockiana* (pansy) plugs after a sprench application of s-ABA. Plugs were irrigated daily with 100 mg $N \cdot L^{-1}$. Leaf chlorosis ratings for the whole plant were from 1 to 11; 11 = completely green with no sign of chlorosis, 10 = ≤ 10% leaf chlorosis, 9 = 11-20%, 8 = 21-30%, 7 = 31-40%, 6 = 41-50%, 5 = 51-60%, 4 = 61-70%, 3 = 71-80%, 2 = 81-90% and 1 = 91-100% leaf chlorosis. (A). Visual ratings were recorded daily with Day 0 observations taken just prior (1 to 2 h) to s-ABA application. (B). Values are the mean of three replications (n=3) for viola 'Sorbet Yellow Delight' and pansy 'Matrix Blue Frost' or four replications (n=4) ± SD for all other cultivars (B). Each replication contained six plugs.

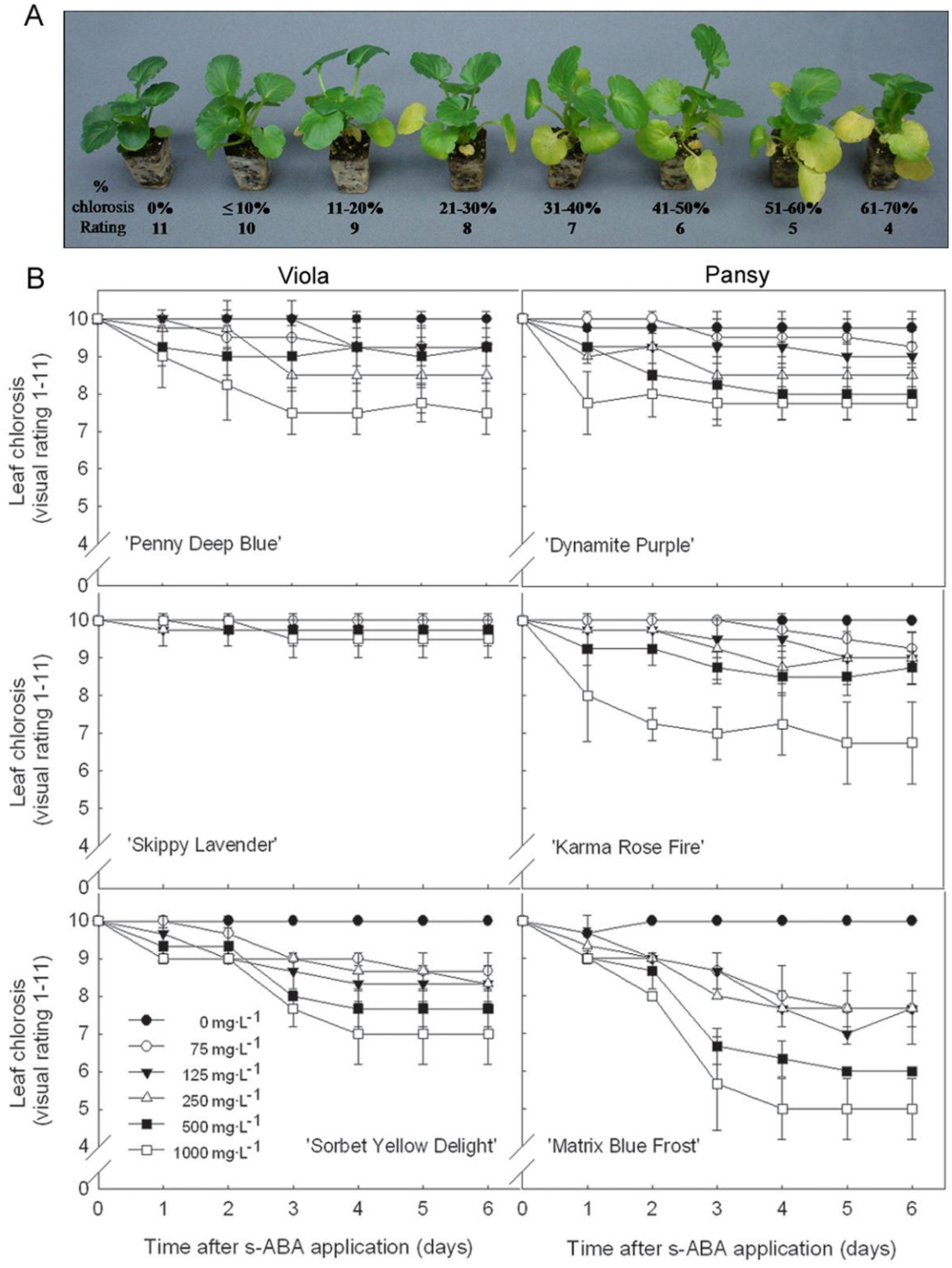
A
% chlorosis Rating
0% 11
≤10% 10
11-20% 9
21-30% 8
31-40% 7
41-50% 6
51-60% 5
61-70% 4
B
Viola
Pansy
Leaf chlorosis (visual rating 1-11)
'Penny Deep Blue'
'Dynamite Purple'
'Skippy Lavender'
'Karma Rose Fire'
'Sorbet Yellow Delight'
'Matrix Blue Frost'
0 mg·L-1
75 mg·L-1
125 mg·L-1
250 mg·L-1
500 mg·L-1
1000 mg·L-1
Time after s-ABA application (days)

Fig. 4.6.

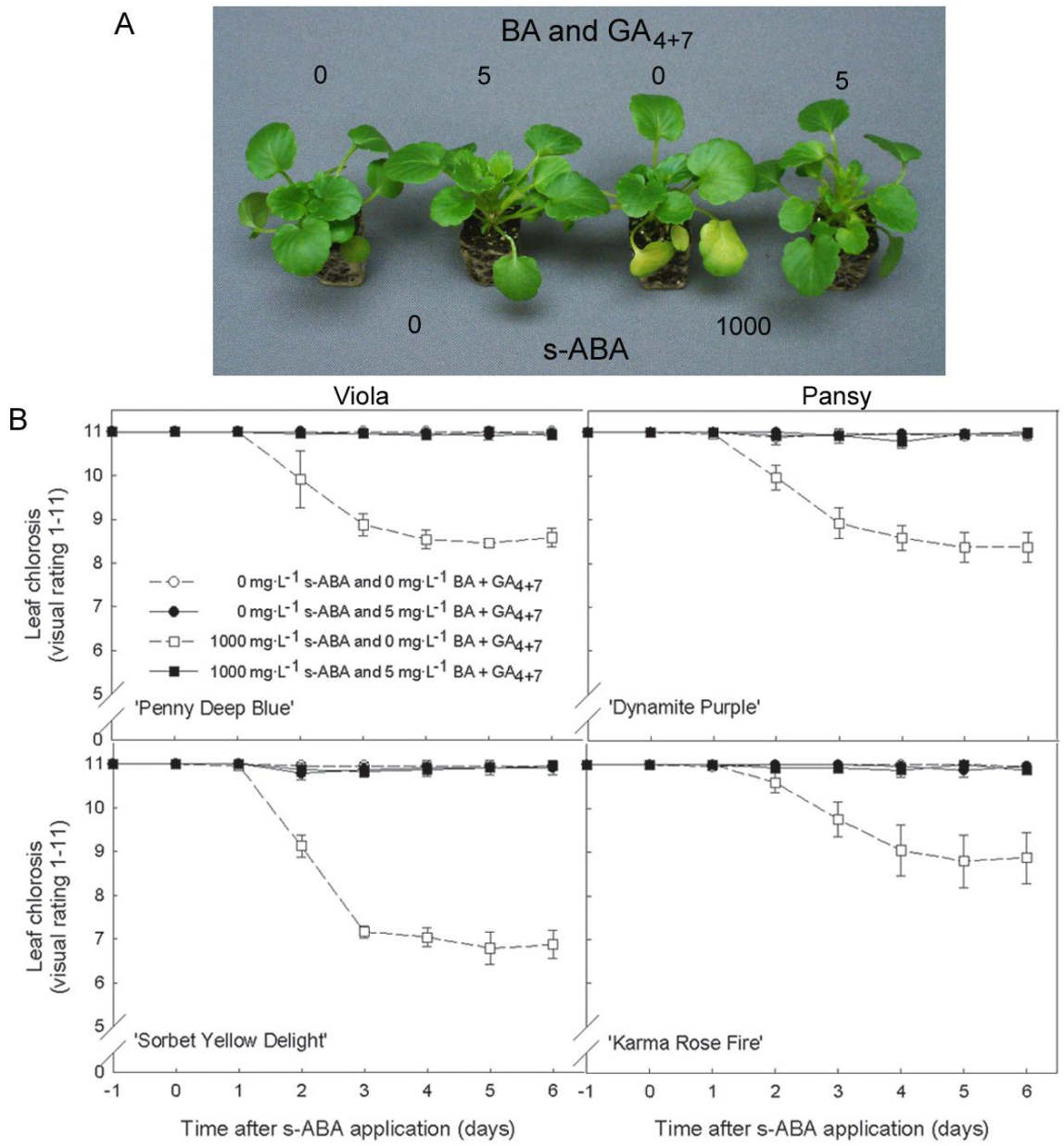

Fig. 4.7. Visual observations and leaf chlorosis ratings of *Viola cornuta* (viola) and *Viola* ×*wittrockiana* (pansy) plugs after applications of 0 or 5 mg·L^{-1} BA + GA$_{4+7}$ and 0 or 1000 mg·L^{-1} s-ABA. Images are of viola 'Sorbet Yellow Delight' 3 d after s-ABA application (A). Plugs were irrigated daily with 100 mg N·L^{-1}. Leaf chlorosis ratings for the whole plant were from 1 to 11; 11 = completely green with no sign of chlorosis, 10 = ≤ 10% leaf chlorosis, 9 = 11-20%, 8 = 21-30%, 7 = 31-40%, 6 = 41-50%, 5 = 51-60%, 4 = 61-70%, 3 = 71-80%, 2 = 81-90% and 1 = 91-100% leaf chlorosis. (B). Visual ratings were taken daily. Day minus one was immediately prior (1 to 2 h) to BA and GA$_{4+7}$ applications (B). Values are the mean of four replications ± SD with each replication consisting of six plugs (n=4) for all pansies and violas (B).

CHAPTER 5:

ABSCISIC ACID APPLICATIONS DECREASE STOMATAL CONDUCTANCE AND DELAY WILTING IN DROUGHT-STRESSED CHRYSANTHEMUMS

Abstract.

Drought stress during shipping and retailing reduces the postproduction quality and marketability of potted plants. Plants respond to drought stress by closing their stomata and reducing transpirational water loss. This stress response is mediated by the plant hormone abscisic acid (ABA). Exogenous applications of s-ABA, the biologically active form of the hormone, can enhance drought tolerance and extend shelf life in a variety of bedding plants. However, little is known about the effectiveness of s-ABA at enhancing drought tolerance in high-value potted perennial crops like *Chrysanthemum ×morifolium* (chrysanthemum). Chrysanthemums 'Festive Ursula' were drenched (0, 125, 250 or 500 $mg \cdot L^{-1}$) or sprayed (0, 500 or 1000 $mg \cdot L^{-1}$) with s-ABA. All applications containing s-ABA were effective at delaying wilting by reducing stomatal conductance and decreasing water loss. Shelf life was extended from 1.2 to 4.0 d depending on the concentration of s-ABA. Spray applications of 500 $mg \cdot L^{-1}$ s-ABA to six additional chrysanthemum cultivars increased shelf life from 1.6 to 3.8 d following drought stress. s-ABA treatment also allowed severely drought-stressed chrysanthemums to recover and remain

marketable after rewatering. Growers can treat chrysanthemums with s-ABA to reduce water use during shipping and to delay wilting if plants are not adequately watered during retailing.

Plants may be exposed to high temperatures and irregular or infrequent irrigation during shipping and retailing. These poor postproduction environments cause rapid substrate drying, plant wilting and accelerated senescence. Drought stress is a major cause of postproduction decline in greenhouse crops, and plants quickly become unsalable (Armitage, 1993; Barrett and Campbell, 2006; van Iersel et al., 2009). The plant hormone abscisic acid (ABA) helps plants survive drought stress by closing stomata to reduce transpirational water loss and prevent wilting (Malladi and Burns, 2007).

Antitranspirants can be used by floriculture producers to prevent wilting during shipping and retailing (Goreta et al., 2007; Martin and Link, 1973). These products enhance drought tolerance by providing a physical barrier to water loss or by inducing stomatal closure. Physical antitranspirants contain resins, polymers or waxes that coat the leaves and physically block the stomata. Physiological antitranspirants reduce transpiration rates by inducing the plant to close their own stomata. These products may contain ABA or other chemicals that cause the plant to produce ABA. Extended stomatal closure and reduced transpiration can lead to heat stress under high temperatures, and antitranspirants may also cause phytotoxicity. Research is therefore needed to determine how to effectively use antitranspirants to enhance the postproduction quality of greenhouse and nursery crops.

Comparative research has shown that antitranspirants that contain ABA are more effective at reducing water loss and delaying drought-induced wilting than physical antitranspirants (Goreta et al., 2007). A new commercial product containing s-ABA, the biologically active form of ABA, (ConTego™, Valent BioSciences Corp.), delays wilting in a variety of bedding plants under severe drought stress (Blanchard et al., 2007; Kim and van Iersel, 2008; Waterland et al., 2010a; Waterland et al., 2010b). Unfortunately, s-ABA applications also cause leaf chlorosis, necrosis and abscission in some species (Blanchard et al., 2007; Kim and van Iersel, 2008; Waterland et al., 2010a; Waterland et al., 2010b). Although plants treated with s-ABA exhibit enhanced drought tolerance, they are often unmarketable due to these negative side-effects. Little is known about the effectiveness or phytotoxicity of s-ABA on high-value potted crops like *Chrysanthemum ×morifolium*. Chrysanthemums are an important fall crop that accounts for about 20% of the total potted perennial market in the United States (USDA, 2009).

The goal of this research was to determine if s-ABA could be used to enhance the drought tolerance of potted chrysanthemums without any phytotoxicity. Our objectives were 1) to determine if exogenous application of s-ABA delays wilting in finished chrysanthemums exposed to drought stress, and 2) to identify any symptoms of phytotoxicity that would negatively affect the marketability of treated chrysanthemums.

Materials and Methods

Plant materials and experimental treatments

Expt. 1. Chrysanthemum ×morifolium 'Festive Ursula' in 15-cm pots were obtained from Green Circle Growers Inc. (Oberlin, OH), and Expt. 1 was conducted in Wooster, OH from 28 Sept. to 9 Oct. 2007. Average greenhouse temperatures were 25.8/21 ± 2.1/2.6ºC day/night with daytime relative humidity of 59.7 ± 7.3%. Plants were irrigated daily with Peters Excel® Cal-Mag 15N-2.15P-12.5K (Scotts-Sierra Horticulture Products, Marysville, OH) at 200 mg $N \cdot L^{-1}$ and were grown under natural irradiance with supplemental lighting provided by high-pressure sodium and metal halide lamps (GLX/GLS e-systems GROW lights, PARSource, Petaluma, CA). An average photosynthetic photon flux (*PPF*) of 176 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (maximum *PPF* of 600 $\mu mol \cdot m^{-2} \cdot s^{-1}$) was provided from 0700 to 1800 HR daily with a mean daily light integral (DLI) of 7.6 $mol \cdot m^{-2} \cdot d^{-1}$. Chrysanthemums were treated with either a spray or drench application of s-ABA (ConTego™, Valent BioSciences Corp., Libertyville, IL) at the half open flower stage (stage 3) (Syngenta Flowers Inc., 2010). Chrysanthemums were watered to container capacity (until leaching occurred) 12 h prior to an application of s-ABA. s-ABA was applied as a drench at 0, 125, 250 or 500 $mg \cdot L^{-1}$ (60 ml per container) or as a spray at 0, 500 or 1000 $mg \cdot L^{-1}$ (approximately 22.3 ml per plant) with the addition of 0.05% CapSil® (Aquatrols Corporation of America, Inc., Cherry Hill, NJ). Spray applications were performed with a Regulator Bak-pak® sprayer (H.D. Hudson Manufacturing Company, Chicago, IL). Half of the plants from each ABA treatment had water withheld (drought-stressed), and the other half were irrigated daily. After 6 d, the

drought-stressed plants were rewatered to evaluate plant recovery. Rewatered plants were then irrigated for 3 d. Stomatal conductance readings were taken using a LI-1600 steady state porometer (LI-COR Inc., Lincoln, NE). Stomatal conductance was measured one d before s-ABA treatment (-1 d), one d (1 d), three d (3 d), and nine d after treatment (9 d for drought-stressed chrysanthemums was equivalent to 6 d of withholding water followed by 3 d of rewatering). Data are the average of three replications with three leaves measured per replication (n=3).

Expt. 2. Finished 20-cm pots of chrysanthemum 'Brandi', 'Colina Red', 'Flashy Gretchen', 'Golden Cheryl', 'Regina' and 'Wilma' were obtained from Green Valley Growers (Ashland, OH) for Expt. 2. Plants were grown under normal greenhouse conditions, as described above, in Wooster, OH from 21 Sept. to 15 Oct. 2009. Average greenhouse temperatures were 23.1/16.4 ± 2.2/2.0 ºC day/night with daytime relative humidity of 49.8 ± 11.6 %. The average *PPF* was 259 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (maximum *PPF* of 924 $\mu mol \cdot m^{-2} \cdot s^{-1}$) from 0600 to 1800 HR daily (DLI of 12.9 $mol \cdot m^{-2} \cdot d^{-1}$). Plants were irrigated daily with 100 mg $N \cdot L^{-1}$ as described previously. All plants were watered to container capacity 12 h prior to an application of s-ABA. Spray applications of s-ABA were applied at 0 or 500 $mg \cdot L^{-1}$ (plus CapSil®) (approximately 33.3 ml per plant). Chrysanthemums were treated with s-ABA when they were at the marketable stage of a few open flowers per plant (stage 2) (Syngenta Flowers Inc., 2010). The application rate of 500 mg L^{-1} s-ABA was selected based on the results from Expt. 1. Half of the chrysanthemums were irrigated daily and the other half were severely drought-stressed by completely withholding water until the s-ABA treated plants wilted. Irrigated plants

were watered daily with 100 mg $N \cdot L^{-1}$. After the s-ABA plants showed visual symptoms of wilting, all drought-stressed plants were rewatered daily for 3 d. The initial rewatering was with clear RO water and all subsequent irrigations were with 100 mg $N \cdot L^{-1}$. Data are the average of four replications with one plant per replication (n=4).

Evaluations of wilt status. Visual observations were taken daily. All visual observations were based on whole plant wilt status. Wilt status ratings were from 1 to 5; 5 = completely turgid, 4 = soft to the touch and starting to wilt, 3 = wilted, 2 = severely wilted and 1 = wilted to the point that leaves were dry and desiccated.

Statistical analysis. Experiments were blocked by watering treatment (irrigated daily verses drought-stressed) and by replication. Values obtained from stomatal conductance and visual observations were analyzed by Proc GLM (generalized linear model) with LSD means separation (least significant difference test) ($P \leq 0.05$) using SAS Version 9.1.3 (Statistical Analysis System, SAS Institute, Inc., Cary, NC).

Results and discussion

Expt. 1. Determination of effective s-ABA concentration and application method. All concentrations of s-ABA and both application methods were effective at delaying wilting in drought-stressed chrysanthemums 'Festive Ursula' with no apparent phytotoxicity (Fig. 5.1 and Fig. 5.2). s-ABA was more effective at higher concentrations, and the plants drenched with 500 $mg \cdot L^{-1}$ or sprayed with 500 or 1000 $mg \cdot L^{-1}$ s-ABA had the longest shelf life extension (Table 5.1). These plants remained turgid (above a wilt status rating of 3) for 5 to 6 d before visual wilting was observed (Fig. 5.2). Chrysanthemums

that were drenched with 125 and 250 mg·L^{-1} s-ABA had a shorter shelf life extension, but showed delayed wilting by at least 1 d compared to control (0 mg·L^{-1} s-ABA) plants (Fig. 5.2 and Table 5.1).

Spray, drench and sprench (spray to drench) applications of s-ABA have been shown to delay drought-induced wilting in bedding plants and woody ornamentals (Blanchard et al., 2007; Kim and van Iersel, 2008; Waterland et al., 2010a; Waterland et al., 2010b; van Iersel et al., 2009). Unfortunately, s-ABA applications cause leaf chlorosis, necrosis, and abscission in some species (Blanchard et al., 2007; Kim and van Iersel, 2008; Waterland et al., 2010a; Waterland et al., 2010b). Symptoms of phytotoxicity were most severe following the application of high concentrations of ABA (500, 1000 or 2000 mg·L^{-1} s-ABA) (Kim and van Iersel, 2008; Waterland et al., 2010a; Waterland et al., 2010b). In chrysanthemums, s-ABA applications delayed wilting without causing any apparent damage to leaves or flowers. This product may therefore be useful for extending the postproduction shelf life of potted chrysanthemums that encounter drought stress during shipping and/or retailing.

Stomatal conductance decreased at 1 d after s-ABA treatment, indicating that both spray and drench applications effectively induced stomatal closure in treated chrysanthemums. Drought-stressed chrysanthemums treated with s-ABA had a more rapid decrease in stomatal conductance than non-ABA treated control plants (0 mg·L^{-1} s-ABA), and the largest differences were measured at 1 d after application (Fig. 5.3A). By Day 3, stomatal conductance was the same in all drought-stressed plants regardless of s-ABA treatment (Fig. 5.3A). Both spray and drench applications of s-ABA also resulted

in a decrease in stomatal conductance in all irrigated plants (Fig. 5.3B). In contrast to the drought-stressed plants, stomatal conductance in s-ABA treated plants that were irrigated remained lower than controls on Day 3.

In salvia, drench applications of s-ABA resulted in rapid stomatal closure, and stomatal conductance decreased within 3 h of application (Kim and van Iersel et al., 2008). Stomatal conductance in chrysanthemums may have decreased as rapidly, but our earliest measurement was taken at 1 d after application. While shelf life extension was clearly rate-dependent, rate-dependent differences in stomatal conductance at 1 and 3 d after treatment were not observed (Table 5.1, Fig. 5.2 and Fig. 5.3). If chrysanthemum stomata responded within hours of the s-ABA application, these differences may have allowed the plants receiving higher concentrations of s-ABA to close their stomata faster and conserve more soil moisture earlier in the stress response than those treated with lower concentrations. This suggests that the timing of s-ABA application, immediately prior to any anticipated drought stress, may provide optimal protection.

After 6 d of drought stress, plants were rewatered to evaluate their recovery. s-ABA treated plants were visually indistinguishable from the irrigated plants within 3 d of rewatering (Day 9), while the control plants (0 $mg \cdot L^{-1}$ s-ABA drench and spray) developed necrosis on the margins of lower leaves (data not shown). Stomatal conductance increased on Day 9 in all rewatered plants except those that were sprayed with 1000 $mg \cdot L^{-1}$ s-ABA (Fig. 5.3A). At 9 d, the stomatal conductance of irrigated plants that had been drenched with s-ABA had returned to pre-ABA treatment rates (Fig. 5.3B). In contrast, stomatal conductance was still lower in all irrigated plants that had

been sprayed with s-ABA (Fig. 5.3B). This suggests that spray applications had a longer efficacy than that of drench applications. In chrysanthemums, the s-ABA may be absorbed and metabolized more quickly from the roots than from the leaves. It is also possible that daily irrigation may have leached some of the s-ABA from the soil before it was taken up by the plant. Previous experiments with bedding plants indicate that spray applications are more effective in pansies, while drench applications are more effective in marigolds (Waterland et al., 2010a).

Expt. 2. Evaluations of cultivar differences. s-ABA delayed visual symptoms of wilting in all six chrysanthemum cultivars treated in Expt. 2. Chrysanthemums treated with 500 $mg \cdot L^{-1}$ s-ABA remained turgid longer and had a higher wilt status rating than control plants (0 $mg \cdot L^{-1}$ s-ABA) within a few d after treatment and withholding of water (Fig. 5.4). The delay in wilting and subsequent shelf life extension was cultivar dependent. ABA treatment extended the shelf life of all drought-stressed chrysanthemums except 'Brandi' (Table 5.2). ABA was most effective in 'Flashy Gretchen' and 'Wilma' (3.8 d shelf life extension) and least effective in 'Golden Cheryl' (1.6 d shelf life extension) (Table 5.2). Cultivar variations could be associated with differential sensitivity to ABA or differences in growth habit, exposed media surface area, or water usage. The 'Brandi' chrysanthemums that had no shelf life extension when treated with s-ABA were larger and had a more open growth habit than the other cultivars. The larger leaf surface area and increased air movement within the canopy could have resulted in more water loss from these plants. The larger surface area of exposed media could also have resulted in greater water evaporation from the substrate.

All s-ABA treated chrysanthemums recovered (regained turgor and a rating of 5) within 6 h of rewatering and were indistinguishable from the irrigated controls (Fig. 5.5). These plants were considered marketable and had no symptoms of leaf or flower damage. Drought-stressed chrysanthemums that received no s-ABA (0 $mg \cdot L^{-1}$ s-ABA) had leaf chlorosis or necrosis and some of the plants did not regain turgor (Fig. 5.5). All non-ABA treated chrysanthemums 'Regina', 'Wilma', and 50% of 'Flashy Gretchen' did not recover after rewatering and remained at a wilt status rating of 1. All flowers from these plants were wilted. Rewatered 'Brandi', 'Colina Red', and 'Golden Cheryl' that received no s-ABA regained turgor, but were less marketable than ABA-treated plants because they developed basal leaf chlorosis or necrosis on the leaf margins (Fig. 5.5).

Conclusion

Applications of s-ABA delayed symptoms of drought stress in all seven chrysanthemum cultivars evaluated. ABA-treated plants maintained turgor by rapidly closing stomata and reducing transpirational water loss. While s-ABA effectively delays drought-induced wilting in a variety of floriculture crops, it's use can result in leaf abscission and senescence symptoms that reduce the marketability and overall quality of the plants (Blanchard et al., 2007; Kim and van Iersel, 2008: Waterland et al., 2010a; Waterland et al., 2010b). In this research, no phytotoxicity was observed and shelf life was extended in all drought-stressed chrysanthemums except the cultivar Brandi. The greatest shelf life extension and delay in wilting was observed with higher concentrations (500 or 1000 $mg \cdot L^{-1}$) of s-ABA. s-ABA is a very promising growth regulator that growers can use to

protect chrysanthemums from drought stress during shipping and retailing. Decreasing postproduction shrinkage will increase product sell through and increase profitability for both growers and retailers.

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Table 5.1. Days until the appearance of visual symptoms of wilting and shelf life extension of drought-stressed *Chrysanthemum* ×*morifolium* 'Festive Ursala' treated with s-ABA[z].

Application method and s-ABA concentration ($mg \cdot L^{-1}$)	Time until visually wilted[z] (days)	Shelf life extension[y] (days)
Drench		
0	1.7c	
125	2.9b	1.2
250	2.9b	1.2
500	5.7a	4.0
Spray		
0	2.2c	
500	5.3a	3.1
1000	6.0a	3.8

[z]Values are means of three replications (n=3). Values in rows followed by different letters are statistically different at $P \leq 0.05$ (least significant difference test). Values are the average number of days from Day 0 that it took each plant to show visible symptoms of wilting, which was a rating of 3 (wilted) on the wilt status rating scale of 1-5. Wilt status ratings were from 1 to 5; 5 = completely turgid, 4 = soft to the touch and starting to wilt, 3 = wilted, 2= severely wilted, and 1 = wilted to the point that leaves were dry and desiccated. [y]Shelf life extension refers to the number of additional days from the time the control plants wilted until the s-ABA treated plants wilted.

Table 5.2. Days until the appearance of visual symptoms of wilting and shelf life extension of six cultivars of *Chrysanthemum* ×*morifolium* treated with s-ABA and drought-stressed[z].

	Time until visually wilted[z] (days)		Shelf life extension[y] (days)
Cultivar	0 mg·L^{-1} s-ABA	500 mg·L^{-1}s-ABA	
'Brandi'	1.7a	2.5a	0.8
'Colina Red'	3.8b	5.5a	1.8
'Flashy Gretchen'	5.5b	9.3a	3.8
'Golden Cheryl'	2.2b	3.8a	1.6
'Regina'	3.8b	7.0a	3.3
'Wilma'	4.8b	8.5a	3.8

[z]Values are means of four replications (n=4). Values in rows followed by different letters are statistically different at $P \leq 0.05$ (least significant difference test). Values are the average number of days from Day 0 that it took each plant to show visible symptoms of wilting, which was a rating of 3 (wilted) on the wilt status rating scale of 1-5. Wilt status ratings were from 1 to 5; 5 = completely turgid, 4 = soft to the touch and starting to wilt, 3 = wilted, 2 = severely wilted, and 1 = wilted to the point that leaves were dry and desiccated. [y]Shelf life extension refers to the number of additional days from the time the control plants wilted until the s-ABA treated plants wilted.

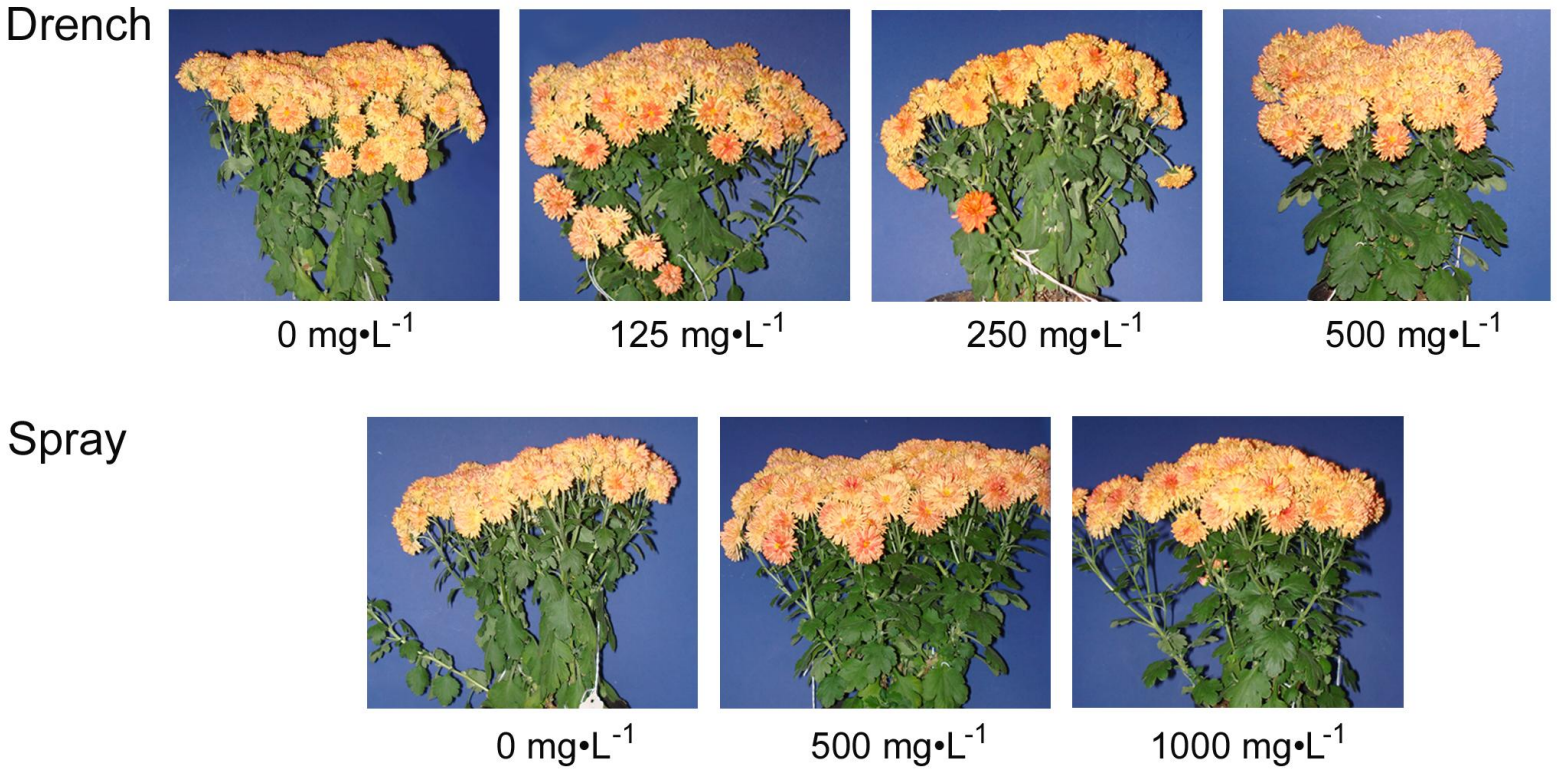

Fig. 5.1. Visual observation of drought-stressed *Chrysanthemum* ×*morifolium* 'Festive Ursula' (chrysanthemums) after treatment with s-ABA as a drench at 0, 125, 250, or 500 mg·L^{-1} or as a spray at 0, 500, or 1000 mg·L^{-1}. Images are after 3 d of drought stress and s-ABA treatment.

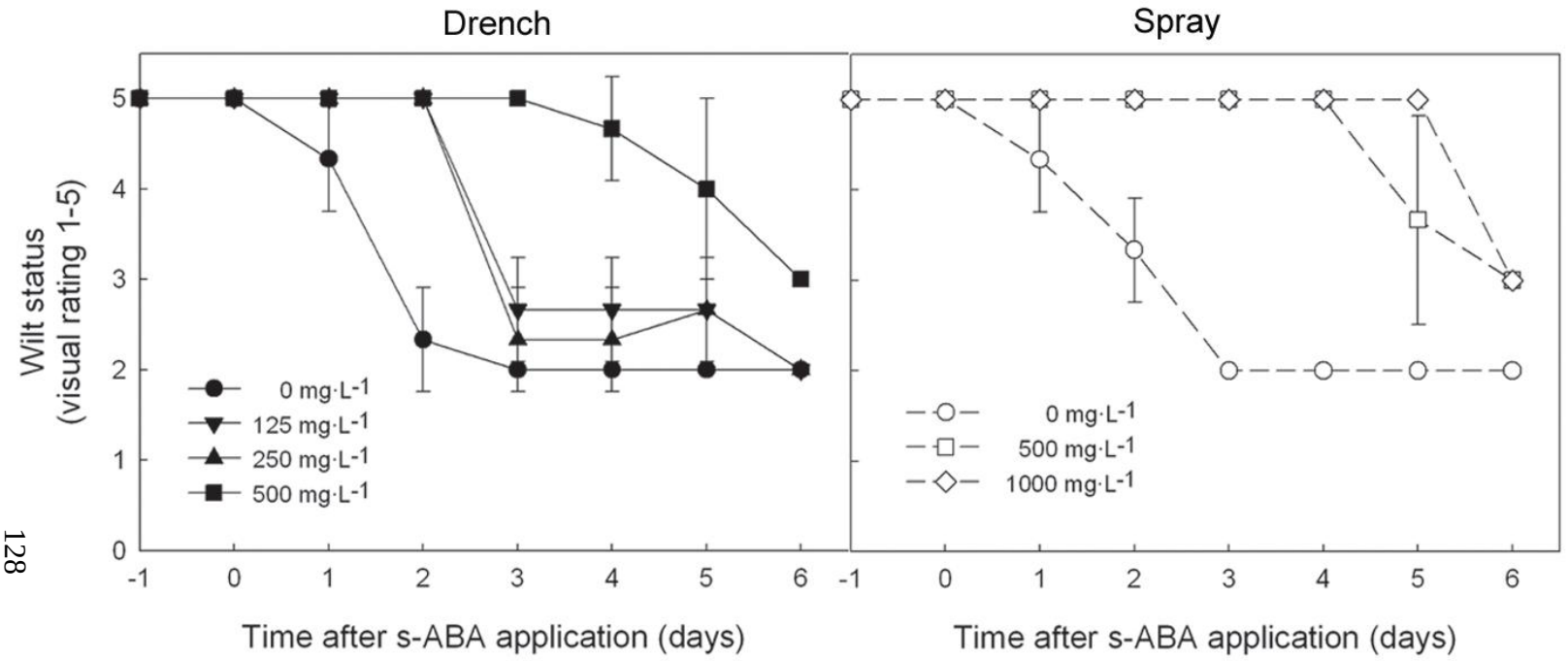

Fig. 5.2. Wilt status ratings of drought-stressed *Chrysanthemum* ×*morifolium* 'Festive Ursula' (chrysanthemums) treated with s-ABA as a drench at 0, 125, 250, or 500 mg·L^{-1} or as a spray at 0, 500, or 1000 mg·L^{-1}. Chrysanthemums were drought-stressed for 6 d after s-ABA application. Wilt status ratings were from 5 to 1; 5 = completely turgid, 4 = soft to touch and starting to wilt, 3 = wilted, 2 = severely wilted and 1 = wilted to the point that leaves are dry and desiccated. Visual ratings were taken daily. Day -1 is one d prior to s-ABA application. Values are the means ± SD of three replications (n=3).

Fig. 5.3. Stomatal conductance readings of *Chrysanthemum* ×*morifolium* 'Festive Ursula' (chrysanthemums) treated with s-ABA as a drench at 0, 125, 250, or 500 mg·L^{-1} or as a spray at 0, 500, or 1000 mg·L^{-1}. Drought-stressed chrysanthemums had water withheld for 6 d and were then rewatered for 3 d (A). Stomatal conductance was measured on drought-stressed and subsequently rewatered chrysanthemums (A) and irrigated daily (B) chrysanthemums after spray or drench applications of s-ABA. Stomatal conductance was measured at -1 d (1 d prior to an application of s-ABA), 1, 3 and 9 d after s-ABA treatment. Irrigated chrysanthemums were watered daily with 200 mg N·L^{-1}. Values are the mean ± SD of three replications with three leaves per replication (n=3).

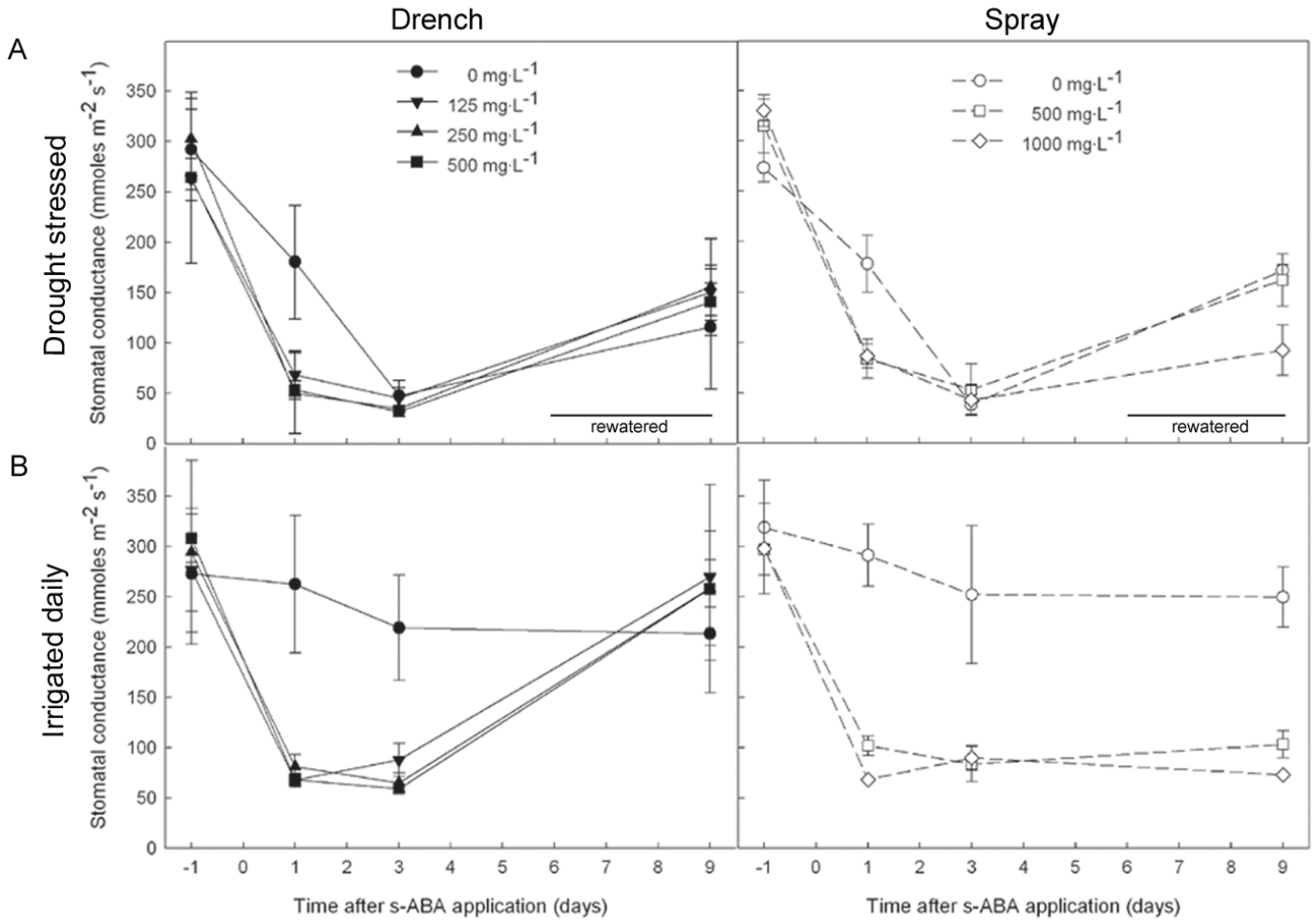
Drench
Spray
A
B
Drought stressed
Irrigated daily
Stomatal conductance (mmoles m-2 s-1)
0 mg·L-1
125 mg·L-1
250 mg·L-1
500 mg·L-1
1000 mg·L-1
rewatered
Time after s-ABA application (days)

Fig. 5.3.

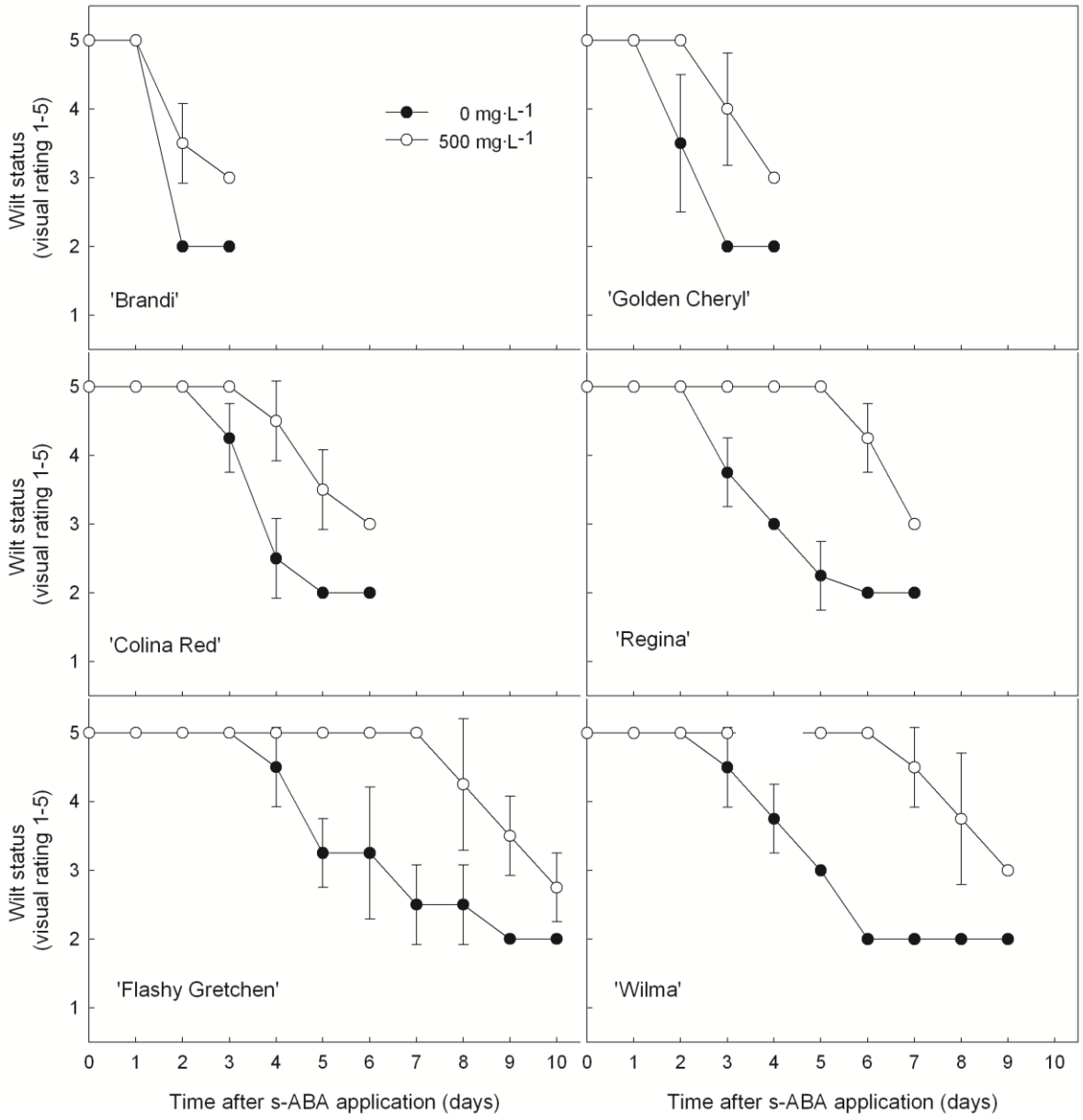

Fig. 5.4. Wilt status ratings of six cultivars of *Chrysanthemum ×morifolium* (chrysanthemums) after s-ABA application and withholding of water. Chrysanthemums were sprayed with 0 or 500 mg·L^{-1} s-ABA. Water was withheld until 500 mg·L^{-1} s-ABA treated plants wilted. Wilt status ratings were from 5 to 1; 5 = completely turgid, 4 = soft to touch and starting to wilt, 3 = wilted, 2 = severely wilted and 1 = wilted to the point that leaves are dry and desiccated. Visual ratings were taken daily with Day 0 just prior to the s-ABA application (1 to 2 h). Values are the means ± SD of four replications (n=4).

Fig: 5.5. Visual observations of six cultivars of *Chrysanthemum ×morifolium* (chrysanthemums) after drought stress and subsequent rewatering. Plants were sprayed with 0 or 500 mg·L^{-1} s-ABA. Water was withheld until 500 mg·L^{-1} s-ABA treated plants were visibly wilted (wilt status rating of 3), then plants were rewatered for three days to evaluate recovery. Images are representative of all replications (n=4) and were taken after 3 d after rewatering.

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