Characterization of the cysteine protease, *PhCP10*, during the senescence of *Petunia x hybrida* flowers

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Abstract

Proteases play an important role in the degradation and remobilization of proteins during flower senescence. The majority of proteases that are upregulated during senescence and programmed cell death are from the cysteine protease class of proteases. Recently, nine putative cysteine proteases were identified from *Petunia x hybrida*. Six of the nine cysteine proteases were upregulated during petal senescence. One cysteine protease, *PhCP10*, is upregulated early in senescence, is expressed only in senescing tissues and appears to be regulated by ethylene. The *PhCP10* sequence shows high homology to *SAG12* (senescence-associated gene) from *Arabidopsis*. *SAG12* is senescence specific in *Arabidopsis* leaves, but little is known about its expression in flowers. TAIL-PCR was preformed to obtain the *PhCP10* promoter . The *PhCP10* promoter sequence also shares homology with the senescence-specific and basal promoter regions of *SAG12*. Promoter constructs driving GFP expression have been analyzed utilizing transient expression in lima bean cotyledons and in petunia flowers. Transient expression in lima beans and petunia flowers has detected a possible regulatory element that appears to enhance *PhCP10* expression in a similar manner to the enhancer region in the *SAG12* promoter. We are currently transforming petunias with the PhCP10:GFP constructs to further characterize the temporal and spatial expression of *PhCP10* during flower senescence and following ethylene treatment.

Introduction

Senescence results in severe profit losses for horticultural crops. With floriculture crops, the beauty of the floral display is

Results

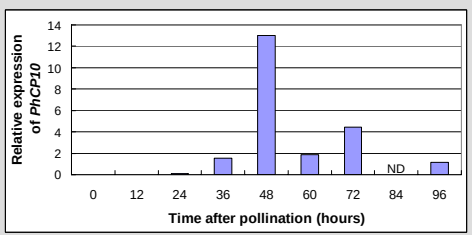


Figure 1. Relative expression of *PhCP10* in *Petunia x hybrida* 'Mitchell Diploid' (MD) after pollination. 0 hour after pollination is at anthesis (flower opening). Total RNA extracted from four flowers at each time point. A. Flowers at 0, 24, 48, 72, and 96 hours after pollination (hap). B. qRT-PCR results of *PhCP10* expression every twelve hours. Time point 84 hap has no data (ND).

PhCP10 expression is specific to senescing corollas and is highest at the first visible sign of wilting.

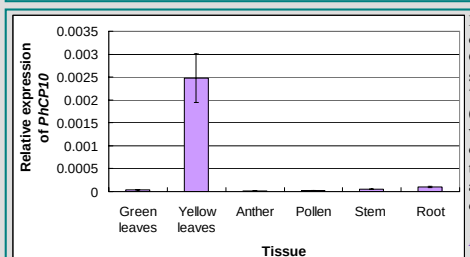
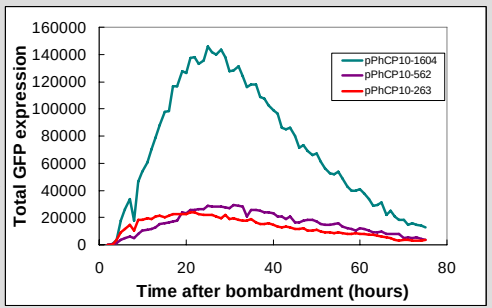


Figure 2. Relative expression of *PhCP10* in various tissues. Green leaves (non-senescing), stem and root tissue were from 7 wk old seedlings. Yellow (naturally- senescing) leaves were collected from the bottom of 6 month old plants. Anthers tissue was from non-dehisced anthers. Pollen was freshly dehisced pollen.

PhCP10 is only expressed in senescing tissues.

Figure 5. Total GFP expression of bombarded lima bean cotyledons with three PhCP10 promoter constructs. Total expression is a mean gray scale value of all pixels x area.

Based on total expression and visual observation pPhCP10-1604 contains a regulatory element that appears to enhance expression.



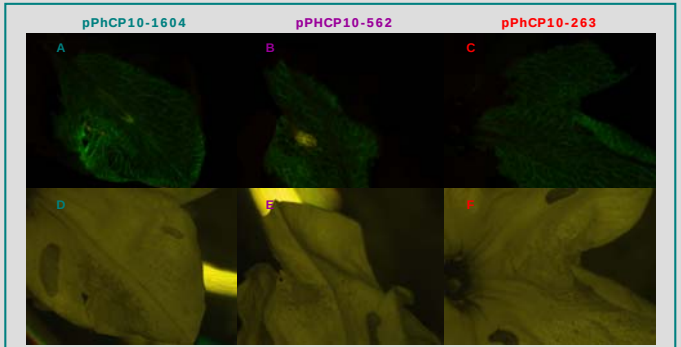


Figure 6. 'MD' flowers 48 h after pollination. 'MD' flowers were harvested at -24 h before anthesis. Flowers were co-infiltrated with an *Agrobacterium* mixture containing the PhCP10 promoter driving GFP expression and *Agrobacterium* containing suppresser of silencing p19. Flowers were pollinated at anthesis and visualized for 72 hap until completely wilted. A-C are images of GFP expression in flower depicted in D-F. D-F are white light images of infiltrated flowers. The experiment was replicated with three flowers for each construct.

Promoter constructs infiltrated into petunia flowers support the identification of a regulatory element in the largest promoter construct that appears to enhance GFP expression.

important for that product's customer appeal. One way to go about enhancing floral displays is to identify and modify genes associated with floral senescence. Microarray and subtractive hybridization methods in a variety of flowering crops have been used to identify genes associated with senescence (Breeze et al., 2004; Buchanan-Wollaston et al., 2003; and Thomoson et al., 2004). Some of the genes that are transcriptionally upregulated in senescing tissues are 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.

Materials and Methods

Gene expression experiments

RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA). qRT-PCT was performed in an iQ5 Thermocycler utilizing iQ SYBR Green Master Mix (BioRad, Hercules, CA). All calculations of relative expression are based on normalization to Actin. Each reaction was preformed in triplicate.

Promoter identification

TAIL-PCR (thermal asymmetric interlaced-PCR) was performed to obtain the 5' end of the *PhCP10* coding sequence and promoter (Liu and Whittier, 1995). 1.604 kb of the promoter regions was obtained.

Promoter constructs

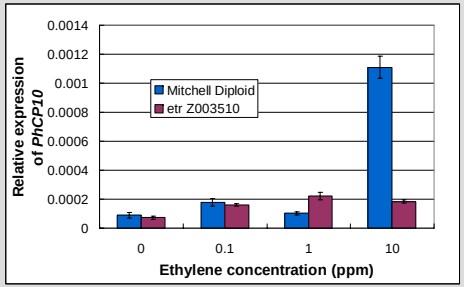
PhCP10 promoter fragments for reporter gene analysis where determined based on sequence similarity to SAG12 promoter from *Arabidopsis* (Fig. 4). Promoter constructs were placed in a transient vector pJL10 for agro-infiltration into *Petunia x hybrida* 'Mitchell Diploid' flowers. The same vector containing promoter constructs was also used to bombard lima bean cotyledons.

Transient evaluations in lima bean

Lima bean (*Phaseolus lunatus*) seeds were sown on a moist paper towel and allowed to germinate for four days. Cotyledons were excised and placed on OMS media. Three lima bean cotyledons were bombarded with tungsten particles with a total of 5 µg of DNA for each vector construct. Lima beans cotyledons where visualized every hour for 72 h after bombardment. Image J software was used to quantify GFP expression.

Figure 3. Relative expression of *PhCP10* after ethylene treatment. *Petunia x hybrida* Etr Z003510 is an ethylene insensitive line transformed in 'MD'. Flowers were harvested at anthesis and treated with 0, 0.1, 1, and 10 ppm ethylene for 4 h in an air tight chamber. Each treatment was four flowers pooled for RNA extraction.

PhCP10 is regulated by ethylene



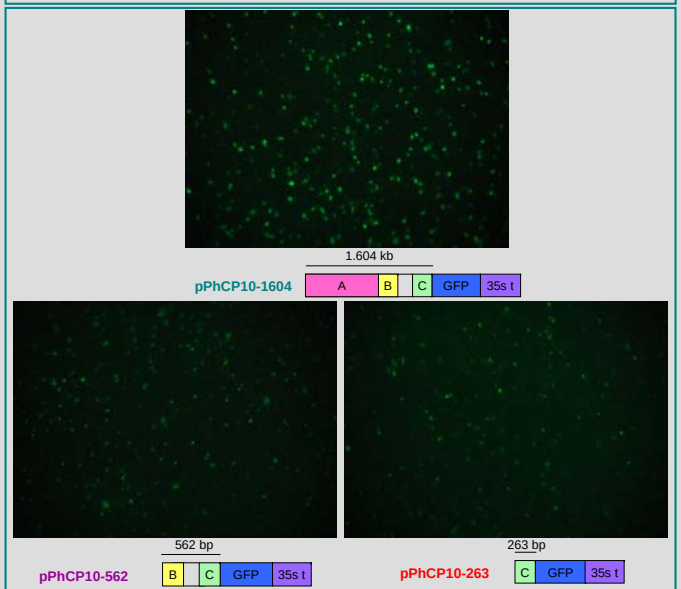


Figure 4. Transient expression of PhCP10 promoter constructs in excised lima bean cotyledons. Images were taken 32 h after bombardment with PhCP10 promoter constructs driving GFP (Green Fluorescent Protein) expression. Promoter regions in the individual constructs included A = enhancer region, B = senescence-specific region, C = basal promoter region. Region B and C have 59.7% and 58.3% nucleic acid similarity to SAG12 senescence-specific and basal promoter regions, respectively. No enhancer region was identified from the PhCP10 promoter based on sequence comparisons.

GFP fluorescence was most intense in pPhCP10-1604 that includes a putative enhancer region.

Discussion

The use of transient expression systems such as the infiltration of petunia flowers and bombardment of lima bean cotyledons have resulted in the identification of a putative regulatory element in the PhCP10 promoter. This regulatory element appears to enhance *PhCP10* expression. Further studies of the PhCP10 promoter in transgenic petunia will be conducted to determine senescence-specific expression characterization. By driving GFP expression we will be able to visualize when and where *PhCP10* is expressed in senescing and ethylene treated flowers.

Conclusions

- A senescence-specific cysteine protease, *PhCP10* that is upregulated early in senescence and is controlled by ethylene has been identified.
- The promoter and coding regions of *PhCP10* have been obtained.
- A putative regulatory element in the PhCP10 promoter that enhances *PhCP10* expression in a similar manner as an enhancer identified in the senescence-specific cysteine protease, *SAG12* has been identified.

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Acknowledgements

This research was supported by the USDA Floriculture and Nursery Research Initiative. We would like to thank Dr. John Lindbo for providing the pJL10 vector.