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Enhanced expression of serine proteases during floral senescence in Gladiolus

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Abstract

Programmed cell death during senescence in plants is associated with proteolysis that helps in remobilization of nitrogen to other growing tissues. In this paper, we provide one of the few reports for the expression of specific serine proteases during senescence associated proteolysis in *Gladiolus grandiflorus* flowers. Senescence in tepals, stamens and carpels results in an increase in total protease activity and a decrease in total protein content. Of the total protease activity, serine proteases account for about 67–70% while cysteine proteases account for only 23–25%. In-gel assays using gelatin as a substrate and specific protease inhibitors reveal the enhanced activity of two trypsin-type serine proteases of sizes 75 kDa and 125 kDa during the course of senescence. The activity of the 125 kDa protease increases not only during tepal senescence but also during stamen and carpel senescence indicating that it is responsive to general senescence signals.

Keywords: Gladiolus; Senescence; Flower; Serine protease; Cysteine protease; Protease inhibitor; In-gel assay; Pollen-specific protease

1. Introduction

Floral senescence is a developmentally controlled process associated with the large scale degradation of cellular and macromolecular components (Rubinstein, 2000). It is influenced by the developmental stage of the organ, the interaction of various hormones as well as by environmental stresses. Once initiated, it continues irreversibly leading either to floral abscission, wilting or both (Woltering and van Doorn, 1988; Rogers, 2006). Floral senescence includes senescence of petals or tepals, anthers and carpels, each of which proceeds independently with their own organ specific programme. While senescence in anthers occurs soon after anther dehiscence, senescence in petals and carpels may take place several hours to a few days after successful fertilization. Petal senescence (wilting) has been classified by Woltering and van Doorn (1988) as either ethylene sensitive (type I) or ethylene insensitive (type II). Both types undergo programmed cell death that is associated with several biochemical changes such as increase in membrane permeability, leakage of ions, lipid peroxidation, increase in reactive oxygen species, nuclear fragmentation as well as expression/activation of nucleases, proteases, lipases and other cell wall hydrolases leading to loss of cell structure (Paliyath and Droillard, 1992; Bieleski and Reid, 1992; Rubinstein, 2000; Xu and Hanson, 2000; Wagstaff et al., 2002, reviewed by Thomas et al., 2003 and Rogers, 2006 and references therein). Proteolysis is one of the most important components of programmed cell death and helps in remobilization of nitrogen in the form of amino acids to other growing organs (Bieleski, 1995; Soudry et al., 2005). Proteolysis during senescence may occur from the release of proteases from the vacuole into the cytosol as well as by *de novo* synthesis of proteases during senescence. Mostly, cysteine proteases have been shown to play a role in senescence in leaves as well as flowers (Jones et al., 1995,

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2005; Valpuesta et al., 1995; Wagstaff et al., 2002; Coupe et al., 2003; Eason et al., 2002). However, the degradation of the large proteome in the cell may require not just cysteine proteases but other types of proteases as well for efficient and rapid proteolysis. Yet, apart from cysteine proteases, there is very little information available on the role of other classes of proteases during senescence in flowers.

Gladiolus is an important ornamental flower that provides an excellent model for the study of tepal senescence since flowers in different stages of senescence are present on the same spike. Programmed cell death during senescence in Gladiolus tepals has been shown to be associated with nuclear fragmentation and DNA laddering (Yamada et al., 2003). In this paper, we show that proteolysis in Gladiolus in different floral tissues is predominantly associated with the expression of serine proteases. The paper provides evidence on the up-regulation of specific senescence-associated serine proteases of 75 kDa and 125 kDa in floral tissues.

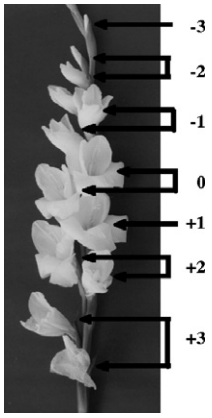

Fig. 1. Stages of the flowers that were used for study. −2 = bud stage; 0 = open flower; +2 = senescent flower. The flowers were marked as described in the text. Flowers in stage −2 were expanded buds at a stage just before petals begin to unfurl (stamen length was 1.4–1.8 cm, carpel length was 3.5–4.5 cm), flowers in stage 0 were fully open with no visible symptoms of petal drooping (stamen length 3–3.5 cm carpel length 7.8–8.2 cm), flowers in +2 stage showed extensive senescence (drooping) especially in upper tepal lobes (anthers had lost pollen grains).

2. Results

Three different floral tissues viz. tepals, stamen and carpels at three different stages of flower development and senescence viz. stage −2 (opening bud), stage 0 (fully open flower) and stage +2 (senescent stage) were chosen for analysis of total protein content and proteolytic activity (Fig. 1). It was observed that progression of development from bud to senescence was associated with a decrease in total protein content in all the tissues (Fig. 2). The protein content in the senescent stage was about 50% of that of the bud stage in tepals and stamens and 60% of the bud stage in carpels. When protease activity was measured in these stages, an increase in total protease activity was observed with the onset and progression of senescence in all the three tissues. The increase was almost 6-fold in tepals, 2.5-fold in stamens and 1.5-fold in carpels. In order to estimate the relative contribution of the different classes of proteases towards the total protease activity in different tissues of Gladiolus, the activity was tested in presence of different class specific inhibitors. As shown in Fig. 3, the relative contribution by serine proteases ranged from 67–70% (~30% of the total protease activity observed in presence of APMSF) in all the tissues in all stages of senescence. In contrast cysteine proteases accounted for only about 21–25% of the total protease activity (~75–79% of the total protease activity observed in presence of E-64) while metallo-proteases accounted for 1–5% of the total activity (~95–99% of the total protease activity observed in presence of EDTA) in all the tissues. Thus, although there was an increase in the total protease activity during the course of senescence the relative contribution by the various classes of proteases did not change much during senescence in all the three floral tissues investigated for the three different stages. We investigated if the increase in protease activity during senescence was associated with the synthesis/activation of some senescence associated proteases. An in-gel protease assay was performed using total soluble proteins from tissues of all the three different stages. As shown in Fig. 4 several distinct bands (observed as clearing in the gel) could be visualized. The bulk of the visible protease activity was associated with a 75 kDa protease that was present in all the three floral tissues. The amount of this protease activity increased in the later stages of senescence

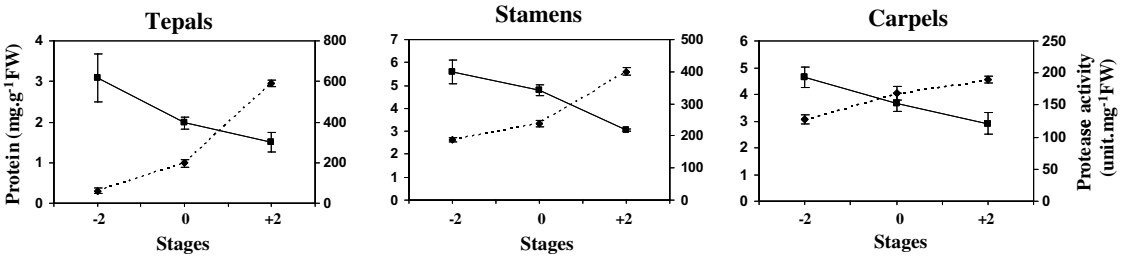

Fig. 2. Total protein content and total protease activity in tepals, stamens and carpels of Gladiolus at three different stages of floral development and senescence. Assays were carried out as described in methods. −2 = bud stage; 0 = open flower; +2 = senescent flower. Square boxes – protein content; diamonds with broken line – protease activity.

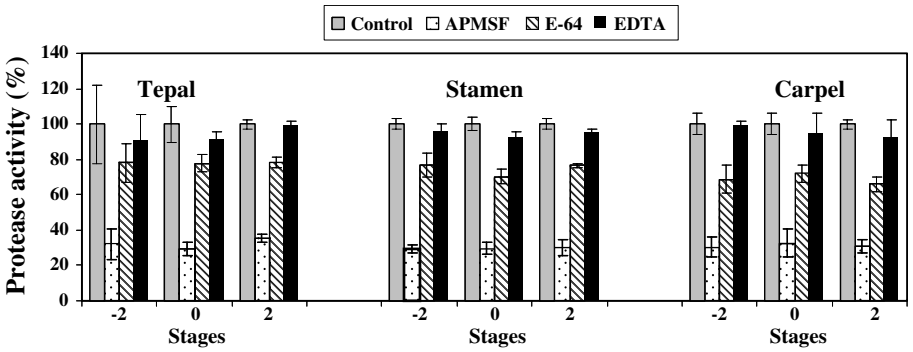

Fig. 3. Inhibition of total protease activity with class specific protease inhibitors. Inhibitors were pre-incubated with the enzyme for 30 min prior to assay as described in materials and methods. The activities of the respective controls at each stage for each tissue were taken as 100% to determine percent activity left after inhibition. −2 = bud stage; 0 = open flower; 2 = senescent flower.

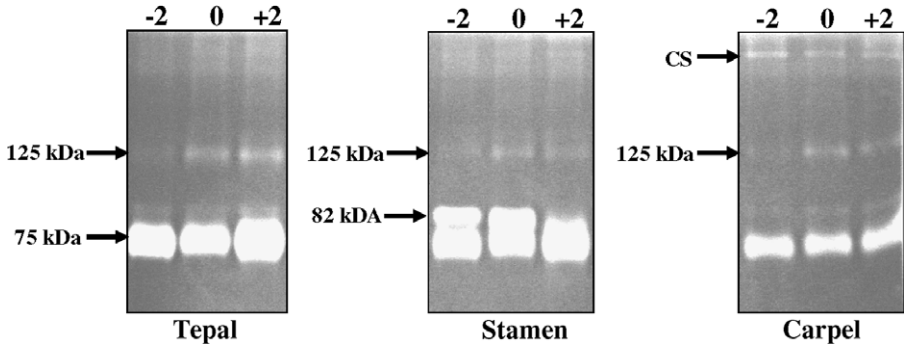

Fig. 4. Protease in-gel assay of proteins from three different floral developmental stages in tepals, stamens and carpels. Samples were electrophoresed on a 10% polyacrylamide gel containing 0.1% gelatin as described along with a standard molecular weight marker. Sizes of the proteases that are enhanced during senescence are marked. CS, carpel specific.

(+2 stage) in tepals and stamens but not in carpels. In addition to this protease, activity of another protease in the size range of 125 kDa, showed a progressive increase from the −2 stage to the +2 stage during the course of tepal senescence. Interestingly, this 125 kDa protease was also present in stamens and carpels and in these tissues too the amount/activity of this protease increased with the progression of senescence of the tissue with maximum levels present in the 0 stage for anthers and carpels. The results clearly indicated that the protease was responsive to senescence signals not only in tepals but also in stamens (which senesce earlier at the 0 stage) and carpels and thus represented a general senescence associated protease. Apart from this protease there were other proteases that were specific to these tissues and distinct from those of the tepals. In addition to the protease of 75 kDa, stamens also contained another major protease of 82 kDa just above this band that accounted for almost 35% of the total protease activity visible in the gel (as estimated by densitometry, data not shown). This protease was either absent or present in very low levels in tepals and carpels. The activity of this protease was high in anthers from the bud stage and fully open stage but decreased rapidly during anther senescence. Since anthers from the +2 stage have undergone dehiscence and lost most pollen, we investigated if the drastic reduction in levels of the 82 kDa protease was associated with the release of pollen. Protein preparations from pollen grains (collected from anthers of stages 0 and +1) were tested for protease activity. As shown in Fig. 5, the 82 kDa protease was specifically associated with the pollen grains but not present in anthers that had lost their pollen. Carpels were also associated with a high molecular weight protease that was barely visible in stamens and tepals. All proteases showed

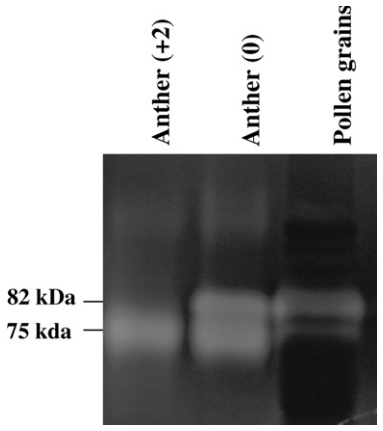

Fig. 5. Protease in-gel assay of proteins from anthers and pollen grains to study the location of the 82 kDa protease. Anthers from stage +2 and 0 were used as controls along with protein samples from pollen grains (collected from stages 0 and +1).

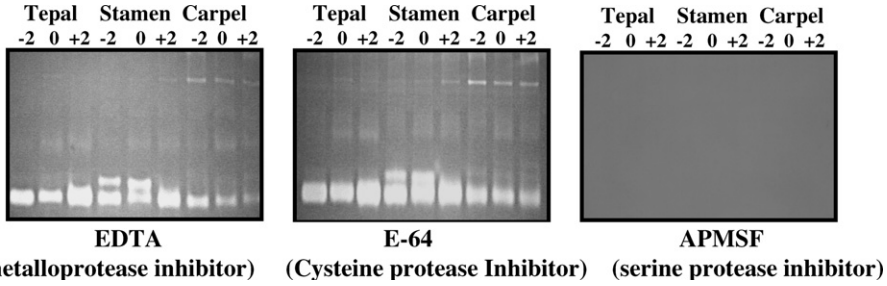

Fig. 6. Inhibition of the proteases in an in-gel assay with class specific protease inhibitors.

optimal activity at pH 7.5 although they were active over a pH range of 5–9 (data not shown).

In order to study the nature of these proteases, their activity was monitored in the presence of inhibitors that are specific for different classes of proteases. Samples were pre-incubated with the inhibitors which were also added subsequently during renaturation. As shown in Fig. 6, no inhibition could be observed with EDTA and E-64 indicating that the proteases visible on the gel did not belong to the metallo-protease and cysteine protease class. Interestingly, all the proteases could be inhibited not only by PMSF (a broad serine protease inhibitor; data not shown) but also with APMSF (an inhibitor of trypsin-type serine proteases). No bands were visible on the gel upon incubation with these two inhibitors indicating that all the proteases from tepals, stamens and carpels, including the 125 kDa senescence-associated protease belonged to the serine protease subfamily with specificity towards Arg–Lys bond.

3. Discussion

One of the most important features of programmed cell death during senescence is the remobilization of nitrogen in the form of amino acids to other growing tissues (Bieleski, 1995; Soudry et al., 2005). The large scale degradation of proteins is brought about by the activation or release of proteases from the tonoplast as well as *de novo* synthesis of other proteases. In this paper, we show that there is a progressive decrease in total protein of Gladioli during the course of senescence as observed by others (Arora and Singh, 2004) and that the proteolytic activity in Gladiolus flowers during senescence is predominantly associated with trypsin-type serine proteases (proteases with a Lys–Arg cleavage specificity). We also provide evidence for the senescence enhanced expression of a trypsin-type 75 kDa protease in tepals and stamens and a 125 kDa protease in tepals, stamens and carpels. Several previous reports have shown the predominance of cysteine proteases and their cDNAs during senescence in flowers (Jones et al., 1995, 2005; Valpuesta et al., 1995; Guerrero et al., 1998; Stephenson and Rubinstein, 1998; Wagstaff et al., 2002; Eason et al., 2002; Coupe et al., 2003; Wang et al., 2004). In most of these studies, serine proteases accounted for only a small fraction of the total protease activity. So far only few reports have shown the involvement of serine proteases in senescence. While Jiang et al. (1999) showed the appearance of a 70 kDa serine protease during senescence of parsley leaves, Pak and van Doorn (2005) had suggested that about 40% of the total protease activity in *Iris* could be governed by serine proteases. Our results show that the proteolytic processes in Gladiolus are mainly brought about by serine proteases (accounting for about 67–70% of the total protease activity) while cysteine proteases account for about 23–25% of the total protease activity in all the tissues. Given that the total protease content in pre-senescent and late senescent stages as visualized by in-gel assays does not differ dramatically, the observed decrease in total protein content in all tissues may actually point towards regulation of proteolysis at the level of protease release from vacuoles or their post-translational modification in cytoplasm for activation. Due to limitations of the in-gel assays as well as measurement of differential activity of class specific proteases by current methods, a clear distinction between protease activity during early growth (when the protease is enclosed in the vacuole and therefore not available for protease activity in the cytoplasm), and late senescence (when the protease may be released in the cytosol and actively involved in proteolysis) is not possible. In both cases the total amount of proteases may not increase much but may greatly influence proteolysis due to controlled release/activation of protease in the cytosol (Hayashi et al., 2001). Nevertheless, our results clearly demonstrate that proteolysis during floral senescence in Gladiolus is associated with the increased activity/amount of two trypsin type serine proteases of sizes 75 kDa and 125 kDa. In particular, the 125 kDa protease is specifically responsive to senescence signals as evident from the fact that its level increases not only during tepal senescence but also during stamen and carpel senescence. Thus it represents a general senescence specific protease. The total protease activity of the 125 kDa protease during senescence in spite of its several fold increase is, however, less than the increase in total protease activity of the 75 kDa protease. The levels of 125 kDa protease begin to increase in the fully open flower. The fully open flower actually represents a stage where senescence has already started, leading to the first visible symptoms of senescence at the +1 stage whereas senescence in anthers is already visible at the 0 stage. What is rather surprising is the presence of only serine proteases and the absence of

detectable cysteine type proteases in our in-gel assays even after long incubation times and in spite of the fact that they account for 23–25% of the total protease activity. This is similar to results obtained by Stephenson and Rubinstein (1998) where only serine proteases were visible on gelatin containing SDS–polyacrylamide gels in *Hemerocallis* although they accounted for less than 10% of the total protease activity. Using the same gelatin based system we have been able to detect the activity of commercial papain, a well known cysteine protease, under identical conditions (data not shown). The transcriptional up-regulation of a cysteine protease gene during senescence in Gladiolus has already been reported by Arora and Singh (2004). Although the expression and activity of the encoded protease was not demonstrated, the possibility of expression of low levels of protease not detectable by our methods cannot be ruled out. Alternatively, since this gel based assay system requires that proteases that are denatured by SDS be renatured for detection of activity, only those proteases that have the ability to renature are detected. It is possible that the cysteine proteases in Gladiolus may not regain their active conformation and are hence not detected on gelatin–SDS gels.

Another interesting feature was the presence of a distinct 82 kDa anther-specific protease that accounted for almost 35% of the total protease activity in anthers with little or no expression in other tissues. This protease was highly active in the bud stage and in the fully open flower but decreased dramatically to less than 5% of the bud levels in the later stages of anther senescence. Since the protease appears to be present before anther dehiscence, it could represent a protease that is involved and highly active during tepal degradation and therefore not observed in later stages after pollen release. Conversely, it may be a constituent part of the pollen grains and may be lost once pollen are released. The latter explanation appears to hold true as evident from Fig. 5, where the protease is associated with pollen grains and is not observed in anthers that have lost pollen. A high molecular weight protease specific to carpels was also detected. However, the level of this protease appeared to be the same in all stages of carpel development and senescence.

4. Conclusion

In conclusion, we show that proteolysis in floral organs in Gladiolus is predominantly associated with trypsin-type serine proteases and involves the senescence enhanced expression of 75 kDa and 125 kDa proteases.

5. Experimental

5.1. Plant material

Spikes of the white variety of Gladiolus (*Gladiolus grandiflorus* var Snow Princess) were chosen for study. The spikes, containing about 12–14 flowers in different stages of growth and senescence, were cut with a sharp blade and immediately placed in water. The fully open flowers were designated as 0 stage. Flowers above this stage in different stages of opening were marked as −1, −2 and −3 in an ascending order with −3 indicating the bud stage. Flowers below the 0 stage were marked progressively as +1, +2 and +3 in a descending order and represented flowers in different stages of senescence with +3 indicating completely wilted flowers (Fig. 1).

5.2. Protein extraction and assay

Samples of tepals, stamens and carpels (400 mg tissue) from various developmental and senescent stages were ground in liquid nitrogen. Pollen grains were collected from anthers of stages 0 and +1 by gently tapping the anthers against the walls of a micro-centrifuge tube. The ground tissue was suspended in 2 ml buffer containing 50 mM Tris–Cl pH 7.5, 1 mM EDTA pH 8.0, 0.1% SDS, mixed and centrifuged at 12,000*g* for 5 min. The supernatant was used for total protease assay using azocasein as a synthetic substrate as described and also for in-gel assays (Holwerda and Rogers, 1992). Briefly, 20 μl extract was mixed with 300 μl 100 mM sodium phosphate buffer (pH 7.5) containing 50 μl 0.6% (w/v) azocasein (Sigma) supplemented with 100 μl 0.1% Triton X-100, and the mixture was incubated at 37 °C for 3 h. The reaction was terminated by adding 200 μl 10% TCA, incubated at 4°C for 30 min, centrifuged at 10,000*g* for 10 min and the absorbance of the supernatant was determined at 366 nm (Ultrospec® 3000, Pharmacia Biotech). One unit of protease activity was defined as the amount of enzyme that gave an increase of 0.01 absorbance per minute. To study the contribution of each class of protease (serine, cysteine and metallo-proteases), different protease inhibitors were selected (Beynon and Salvesen, 1993). The inhibitors used were 50 mM EDTA (metallo-protease-specific); 1 mM PMSF (phenylmethylsulfonyl fluoride; serine protease specific); 100 μM APMSF ((4-Amidino-Phenyl)-Methane-Sulfonyl Fluoride; specific for serine proteases with Lys/Arg cleavage specificity) and 2 μM E-64 ((2*S*,3*S*)-3-(*N*-{(*S*)-1-[*N*-(4-guanidinobutyl)carbamoyl]3-methylbutyl}carbamoyl) oxirane-2-carboxylicacid; cysteine protease specific). The enzyme aliquot (10 μg protein) was first incubated with the requisite concentration of the inhibitor (without substrate) for 30 min and then assayed as described above. All assays were carried out in triplicates. Proteins were estimated as described by Peterson (1977).

5.3. Zymography

Proteases were resolved on a 10% SDS–gelatin polyacrylamide gel containing 0.10% gelatin (Hellmich and Schauz, 1988). Protein samples (10 μg protein) were mixed with an equal volume of a non-reducing sample buffer (0.1 M Tris–HCl, pH 6.8; 2% (w/v) SDS, 10% (v/v) glyc-

erol, 0.01% bromophenol blue), incubated at 37°C for 30 min and electrophoresed at room temperature. After electrophoresis, the gel was washed in renaturing buffer (2.5% Triton X-100, 10 mM EDTA 50 mM Tris–HCl, pH 7.5) for 30 min with shaking. Protease activities were monitored by incubating the gel in 10 mM Ca^{++}, 10 mM Mg^{++}, 50 mM Tris–HCl, pH 7.5, at 37 °C for 12–16 h in an incubator cum shaker. The gel was stained with Coomassie Blue-R250 (0.1% R250 in 50% methanol/10% glacial acetic acid) and destained with a solution containing 50% methanol/10% glacial acetic acid. Clear bands observed in a blue background represented the sites of protease activity.

To identify the class of the proteases observed on the gel, different protease inhibitors (as described above) were added to the protein samples (10 μg protein) prior to electrophoresis and incubated for 30 min at 37 °C along with the loading buffer (non-reducing buffer). Following renaturation, gels were incubated in presence of inhibitors in 10 mM Ca^{++}, 10 mM Mg^{++} (except when EDTA was used), 50 mM Tris–HCl, pH 7.5 at 37 °C for 12–16 h.

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