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Protein degradation and peptidase activity during petal senescence in *Dendrobium* cv. Khao Sanan

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ABSTRACT

During ethylene-induced petal senescence in *Dendrobium* cv. Khao Sanan flowers, the levels of water-insoluble protein in petals decreased but the levels of water-soluble proteins were not affected. Total peptidase activity in the petals increased from day 1 of ethylene treatment and showed a peak by day 4. Treatment of excised flowers with E-64, a specific inhibitor of cysteine peptidases, prior to ethylene exposure, almost doubled the time to visible petal senescence. Since the membrane-impermeable form was used, the results might suggest an effect on an extracellular peptidase. Treatment of the flowers with 1,10-phenanthrolin, a general metal chelator, also doubled the time to ethylene-induced visible petal senescence. This compound might have several effects other than on metallopeptidases. A partial cDNA encoding a cysteine peptidase gene (*Den-Cys1*) in senescent petals was identified. Its transcript abundance in petals showed a large increase, within one day of the onset of ethylene treatment. Treatment of the flowers with ethylene also resulted in an increase in *DenCys1* abundance, an increase in total peptidase activity, and a decrease of soluble protein, in the column (the organ consisting of fused anthers, filaments, stigma, and style). The results show that senescence, both in petals and the column, is accompanied by increased peptidase activity and degradation of water-insoluble protein. This is the first report to show that a specific inhibitor of cysteine peptidases delays the time to petal senescence.

1. Introduction

Protein degradation during petal senescence has been characterized in several plants, including the monocotyledonous *Alstroemeria* (Wagstaff et al., 2002), *Hemerocallis* (daylily; Stephenson and Rubinstein, 1998), *Iris* (Pak and van Doorn, 2005) and *Gladiolus* (Azeez et al., 2007), and dicotyledons such as carnation (*Dianthus*; Sugawara et al., 2002) and *Petunia* (Jones et al., 2005). The data generally show a decrease in protein levels, but in some species this decrease was much smaller than in others. For example, Eason et al. (2002) reported only a small decrease in protein levels in *Sandersonia* petals.

The above mentioned data refer to water-soluble proteins. Little is known about levels of water-insoluble proteins. An exception are the data on petals in plant-attached and detached Geraldton waxflower (*Chamelaucium uncinatum*). In attached flowers a small decrease was found in water-soluble protein levels, accompanied by a small increase in water-insoluble proteins. In detached flowers a large decrease was found in water-insoluble proteins, and no clear change in water-soluble proteins (Olley et al., 1996).

In most species studied an increase occurs in peptidase activity, just before the onset of visible senescence symptoms. For example, *Sandersonia* (Eason et al., 2002) and *Iris* (van Doorn et al., 2003) petals showed a large increase in peptidase activity. In contrast, in *Ipomoea* petals no increase in peptidase activity was observed, although the water-soluble protein levels decreased drastically (Winkenbach, 1970a,b; Matile and Winkenbach, 1971). We hypothesized that in senescing petals of *Dendrobium* an increase in peptidase activity, if it occurs, might affect the level of water-soluble and water-insoluble proteins.

Several inhibitors of peptidase have been shown to delay the time to petal senescence, indicating a prominent role of peptidases in its regulation or execution. For example, 2,2′-dipyridyl, which inhibited the activity of cysteine peptidases, delayed senescence in *Sandersonia* petals (Eason et al., 2002), and 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) and diisopropyl-fluorophosphate (DFP), both serine peptidase inhibitors, delayed senescence in *Iris* (Pak and van Doorn, 2005). However, these compounds are not specific. 2,2′-Dipyridyl is a general metal chelator, thus also inhibits the activity of several enzymes other than peptidases (Horky et al., 1998; Watkins et al., 2003), and both AEBSF and DFP are also not

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specific for serine peptidases or even peptidases in general (Pak and van Doorn, 2005).

Preliminary experiments with *Dendrobium* flowers indicated that the time to visible petal senescence was considerably delayed after treatment with E-64, a specific inhibitor of cysteine peptidases, or with EDTA, a general metal chelator (unpublished results). As the effect of EDTA might be due, among many other possible causes, to inhibition of metallopeptidases, we also tested the effect of 1,10-phenanthrolin, another metal chelator which is used, *in vitro*, as an inhibitor of metallopeptidases (Herman et al., 1998).

In orchids such as *Dendrobium*, the time to visible petal senescence of unpollinated flowers is often more than 30 d after anthesis. Petal senescence can be rapidly induced, in a predictable manner, either by pollination or by treatment with ethylene. Here we chose ethylene treatment to induce petal senescence. The present paper reports on the levels of soluble and insoluble protein, total peptidase activity, and the isolation and expression of a cysteine peptidase gene.

In orchids, the column is the organ consisting of fused anthers, filaments, stigma, and style. Experiments with *Dendrobium* flowers indicated that pollination-induced petal senescence requires an increase in ethylene production in the column, and that the column is the main source of the increase in ethylene production (unpublished data). It is known that pollination in *Dendrobium* hastens petal senescence through the action of ethylene (Ketsa et al., 2001). Exogenous ethylene induces senescence in many cells in the stylar tissue of species other than orchids (Wu and Cheung, 2000). If this is also true for orchids, we hypothesized, we would expect an increase in peptidase activity and a decrease in protein level in the column. We therefore also investigated the effect of exogenous ethylene on protein degradation, peptidase activity, and the expression of a cysteine peptidase gene in the column, in relation to the effects found in the petals.

2. Materials and methods

2.1. Plant materials

Inflorescences of *Dendrobium* cv. Khao Sanan (also called cv. Wanna) were obtained from a commercial grower near Bangkok. The inflorescences were brought to the laboratory within 2 h after harvest. The second, third and fourth open flowers, counted from the end of the stem, were excised from the inflorescences. The pedicels were placed in 10 mL vials containing distilled water. All flowers were held in an air-conditioned room, at 25 °C and 80% RH. The photon flux density was ca. 15 μmol m^{-2} s^{-1}.

2.2. Ethylene treatment, effect of 1-MCP

Detached flowers were placed in airtight plastic chambers (37 cm × 47 cm × 35 cm). An appropriate volume of ethylene was applied through an injecting port, and brought to a final concentration of 0.4 μL L^{-1}. Flowers were exposed to ethylene at 25 °C for 24 h. Control flowers were placed in identical chambers, without ethylene.

If applied, 1-methylcyclopropene (1-MCP) treatment was given prior to ethylene. 1-MCP was generated by adding water to EthylBloc® (Smartfresh, Springhouse, PA) powder, placed in a vial in a chamber similar to the ones used for ethylene treatment. Allowing water into the vial with Ethylbloc® powder resulted in a final concentration of 500 nL L^{-1} of 1-MCP gas in the chamber. Flowers were exposed to 1-MCP for 3 h. Control flowers were placed in identical chambers, without 1-MCP.

2.3. Protein extraction and quantification

The method was similar to that of Olley et al. (1996). Individual petals and columns were separately ground in liquid nitrogen and mixed with 5 mL of buffer containing 50 mmol L^{-1} Tris–HCl, pH 7.6, 2 mmol L^{-1} disodium ethylenediamine tetraacetate, 2 mmol L^{-1} dithiothreitol and 10 mmol L^{-1} $MgCl_2$, for 30 min at 25 °C. The homogenates were centrifuged at 17,000 × *g* for 20 min at 4 °C. The supernatant was retained and used as a source of soluble proteins. Insoluble proteins were extracted by resuspending the pellets in 7 mL of 0.1 mol L^{-1} NaOH and incubating at 80 °C overnight. The protein contents were measured using the standard Bradford method and bovine serum albumin.

2.4. Extraction and assay of peptidase activity

Assay of peptidase activity was performed based on the method of Nieri et al. (1998). Petals were homogenized in 3 mL of ice-cold extraction buffer (50 mmol L^{-1} Tris–HCl, pH 7.4), whereas 5 mL of extraction buffer was added for columns. The homogenates were centrifuged at 10,000 × *g* for 10 min at 4 °C and the supernatant was collected and kept at −70 °C until peptidase assay. Duplicates of each sample in 1.5-mL microcentrifuge tubes containing 400 μL incubation buffer (50 mmol L^{-1} Na-acetate, pH 5.0 with 0.5% azocasein, from Fluka), and 200 μL crude extract were used for the assay. Preliminary measurements at various pHs showed that pH 5.0 was close to optimal in the present assay. All sample tubes were incubated at 37 °C for 24 h. Reactions were stopped by adding 100 μL of 50% TCA to sample tubes. Sample and reference tubes were then incubated on ice for 1 h and centrifuged at 10,000 × *g* for 3 min at 4 °C. The supernatants were alkalinized by adding 100 μL of 10 mol L^{-1} NaOH and absorbance was then measured at 492 nm. Peptidase activity was defined using arbitrary units; one unit is equal to a change of 0.01 absorbance units per hour at 492 nm.

2.5. Effects of inhibitors on the time to petal senescence

All inhibitors used were from Sigma, except pepstatin A, which was purchased from Fluka. Pedicels of excised flowers were placed in 10-mL vials containing solutions of inhibitors, for 24 h at 25 °C, prior to the 24 h 0.4 μL L^{-1} ethylene treatment. Compounds tested were the specific cysteine peptidase inhibitor L-*trans*-epoxysuccinyl-leucyl amido(4-guanidio) butane (E-64), at 0.001, 0.01, and 0.1 mmol L^{-1}; the metal chelators ethylenediaminetetraacetic acid (EDTA) and 1,10-phenanthrolin, both at 0.1, 1, and 10 mmol L^{-1}, phenylmethylsulphonyl fluoride (PMSF), an inhibitor of serine peptidases, at 0.1, 1.0 and 10 mmol L^{-1}, and pepstatin A, an inhibitor of aspartic peptidases, at 0.005, 0.05 and 0.5 mmol L^{-1}. 1,10-Phenanthrolin, PMSF, and pepstatin A were first dissolved in ethanol, before inclusion in aqueous solution. A control for low ethanol levels was included in these tests.

2.6. RNA extraction, cDNA synthesis and cloning of a cysteine peptidase

Petals of *Dendrobium* cv. Khao Sanan flowers, five days after treatment with ethylene, were homogenised and frozen. Total RNA was isolated from the frozen material using the CTAB method (Chang et al., 1993). Before reverse transcription reactions, contaminated DNA in total RNA at various stages of senescence of flowers was eliminated using DeoxyribonucleaseI (DNaseI, RNase-free, Fermentus, Canada). The first strand cDNA was reverse transcribed from DNase-treated RNA of *Dendrobium* using M-MLV reverse Transcription System Kit (Promega, USA). A pair of forward and reverse primers of cysteine peptidase gene (5′-GATCATGGGGTTGCAATCGT-

Table 1
Effect of ethylene and 1-MCP on the time to petal senescence in *Dendrobium* cv. Khao Sanan. Results are means of 10 replicate flowers ± SE.

Treatment	Time to petal senescence (days)
Distilled water	10.1 ± 0.9
0.4 μL L^{-1} ethylene	4.9 ± 0.7
500 nL L^{-1} 1-MCP	29.3 ± 1.0
500 nL L^{-1} 1-MCP + 0.4 μL L^{-1} ethylene	28.9 ± 1.1

3′ and 5′-ATCCCTGCTCTTGTGCGAAG-3′) was designed from a sequence of cysteine peptidase gene from *Phalaenopsis* in the NCBI database (accession number U34747). Partial cDNAs were cloned and sequenced. A new pair of specific primers for the gene encoded for cystene peptidase from *Dendrobium* was designed (forward primer 5′-GGCTACGGGAAAACACAG-3′) and reverse primer 5′-AGGATAAGAAGCCTCCATTGC-3′) and used for semi-quantitative RT-PCR and quantitative real time RT-PCR.

2.7. Gene expression

Semi-quantitative reverse transcriptase (RT)-PCR was conducted to analyze gene expression. The *18S-rRNA* primers (5′-CATGGCCGTTCTTAGTTGGTGGAG-3′ and 5′-AAGAAGCTGGCCGCG-AAGGGATAC-3′) were used as an internal control to normalize all sample loadings. PCR products (5 μL) were removed from the reaction tubes after 33 cycles and were analyzed on a 1.2% agarose gel. PCR conditions were as follows: one cycle of denaturing at a temperature of 94 °C for 3 min and followed by 40 cycles of 95 °C for 30 s, 53 °C for 30 s, 72 °C for 30 s and then one cycle at 72 °C for 10 min, for both genes. Data were confirmed by quantitative RT-PCR, using an ABI7000 real-time PCR machine (Applied Biosystems, USA) and SYBR green (QuantiTect^{TM} SYBR® Green PCR Kit, Qiagen, Germany). The primers of *Den-Cys1* and *18S* were the same as above. Data were analyzed by using $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

2.8. Statistical analysis

Means were compared using least signifcant difference (LSD) and *t* test. All experiments were repeated at least once at a later date. The results were similar. Only one set of experiments is shown, and only this set was included in the statistical analysis.

3. Results

3.1. Effect of exogenous ethylene on petal senescence

When excised flowers were treated with 1-MCP and left in water at 25 °C, the time to petal senescence was approximately 30 d (Table 1). If the flowers were not treated with 1-MCP the time to senescence was about 10 d. Treatment with ethylene, for 24 h at 25 °C, reduced the time to senescence, counted from the end of ethylene treatment, to 5 d (Table 1). After ethylene treatment and in controls, the first visible symptoms were showing of the vascular bundles as darker coloured against the background colour of the petals. This is called venation. It was observed within 24 h after the end of ethylene treatment. Folding of the petals, known as drooping, occurred within 18 h after the ethylene treatment had ended. Subsequently, the petals showed water soaking, at day 5. This was considered the first symptom of senescence. Water soaking was followed, within about a day, by browning, and then by desiccation. In untreated flowers the same senescence symptoms were found, occurring in the same order, but at a later point in time (water soaking after 10 d, browning after about 12 d, and desiccation after about 14 d). After 1-MCP treatment, the petals showed desiccation rather than the senescence symptoms that were caused by ethylene treatment.

3.2. Petal protein levels

The levels of water-soluble protein in the petals of control flowers increased between day 2 and 3 (Fig. 1A), although the values on day 1 and day 5 were not statistically different (statistical not shown). The levels of water-insoluble proteins in the controls slightly decreased (Fig. 1B). At the end of the experimental period the levels were statistically different from the levels at the beginning (statistical not shown). Ethylene treatment slightly altered the levels of water-soluble proteins, but levels were the same at the beginning and at the end of the experimental period (Fig. 1A). Ethylene treatment, in contrast, resulted in a sharper decrease of water-insoluble protein, compared to controls (Fig. 1B). Levels of total protein in the petals were also slightly lower after ethylene treatment (Fig. 1C)

3.3. Petal peptidase activity

The total peptidase activity in the petals of ethylene-treated flowers was significantly higher than those of the control flowers, already by the end of the 24 h ethylene treatment (Fig. 2). A significant difference was also observed on days 2–5 after ethylene treatment (Fig. 2). An increase in peptidase activity was found in petals of ethylene-treated flowers, between day 3 and 4 (Fig. 2; statistics not shown).

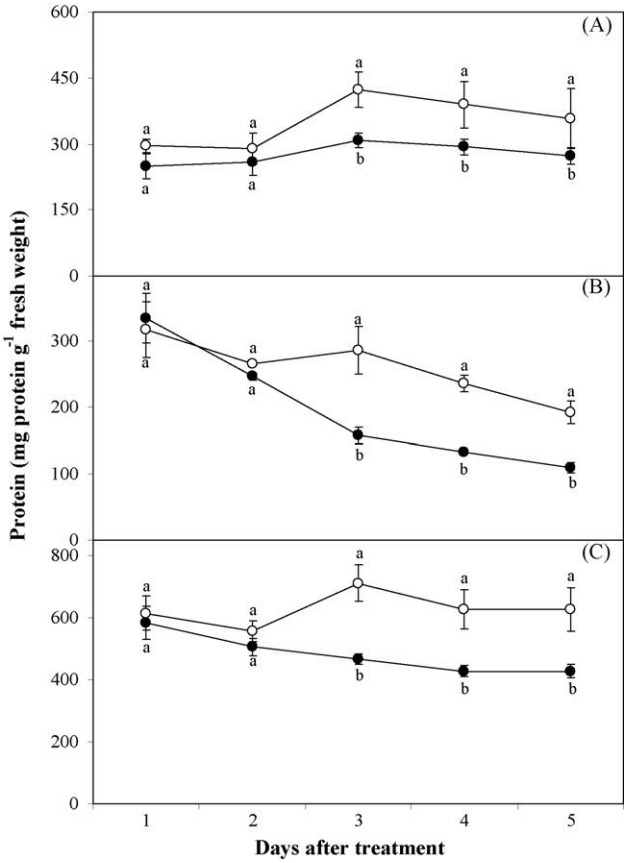

Fig. 1. Protein levels in petals of *Dendrobium* cv. Khao Sanan flowers. (A) soluble protein, (B) insoluble protein, and (C) total protein. Flowers were treated with air (○) or 0.4 μL L^{-1} ethylene (●). Protein content was determined using the standard Bradford assay. Values are means ± SE (n = 3). Statistical differences between treatments (P < 0.05) for each day are indicated by a different letter.

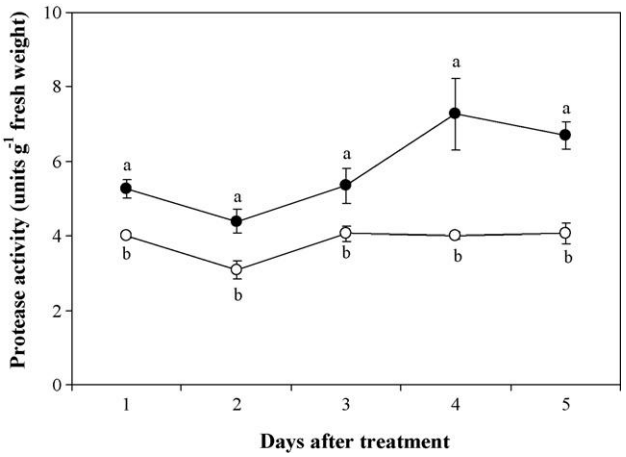

Fig. 2. Peptidase activity in petals of *Dendrobium* cv. Khao Sanan. Flowers were treated with air (○) or 0.4 μL L^{-1} ethylene (●). Peptidase activity was measured using azocasein as a substrate. Values are means ± SE (n = 3). Statistical differences between treatments (P < 0.05) for each day are indicated by a different letter.

3.4. Effects of inhibitors on the time to petal senescence

E-64 is a specific inhibitor of cysteine peptidase. If applied for 24 h in the vase solution of cut flowers, prior to the 24 h ethylene treatment, it delayed the time to petal senescence. A considerable effect was found at 0.001 and 0.01 mmol L^{-1}, and a smaller effect at 0.1 mmol L^{-1} (Table 2). As the lower level was more effective, a toxic effect was apparent. A similar treatment with EDTA, a metal chelator, had no effect at 0.1 mmol L^{-1}, but slightly delayed the time to petal senescence when applied at 1 or 10 mmol L^{-1} (Table 2). A large effect was found after application of 1,10-phenanthrolin, which is also a metal chelator. At 0.1 mmol L^{-1} it almost doubled the time to petal senescence. At 1 or 10 mmol L^{-1} its effect became increasingly smaller, possibly due to a toxic effect.

Also tested were phenylmethylsulphonyl fluoride (PMSF), an inhibitor of serine peptidases, at 0.1, 1.0 and 10 mmol L^{-1}, and pepstatin A, an inhibitor of aspartic peptidases, at 0.005, 0.05 and 0.5 mmol L^{-1}. At each of the concentrations tested, these two compounds resulted in toxicity symptoms in the petals, even by the end of the ethylene treatment (results not shown).

Table 2
Effect of E-64, a specific cysteine peptidase inhibitor, and some other chemicals, on the time to visible petal senescence of ethylene-treated flowers of *Dendrobium* cv. Khao Sanan. Detached flowers were placed in inhibitor solutions for 24 h at 25 °C prior to treatment with 0.4 μL L^{-1} ethylene for 24 h. The time to senescence was counted from the end of ethylene treatment. Results are means of 7 replications ± SE.

Treatments	Senescence (days after treatment)
Distilled water	8.4 ± 1.6
0.4 μL L^{-1} ethylene	4.7 ± 0.5
Cysteine peptidase inhibitor	
0.001 mM E-64 + 0.4 μL L^{-1} ethylene	8.0 ± 1.0
0.01 mM E-64 + 0.4 μL L^{-1} ethylene	9.0 ± 0.7
0.1 mM E-64 + 0.4 μL L^{-1} ethylene	6.1 ± 1.1
Metal chelators	
0.1 mM EDTA + 0.4 μL L^{-1} ethylene	4.7 ± 0.4
1 mM EDTA + 0.4 μL L^{-1} ethylene	5.7 ± 1.1
10 mM EDTA + 0.4 μL L^{-1} ethylene	6.0 ± 1.5
0.1 mM 1,10-Phenanthrolin + 0.4 μL L^{-1} ethylene	9.6 ± 1.3
1 mM 1,10-Phenanthrolin + 0.4 μL L^{-1} ethylene	7.6 ± 1.4
10 mM 1,10-Phenanthrolin + 0.4 μL L^{-1} ethylene	5.4 ± 0.3

3.5. Sequence analysis of the partial cDNA fragment encoding a petal cysteine peptidase

A 711 bp cDNA fragment, from a gene encoding a cysteine peptidase was identified in the cDNA of senescent petals of *Dendrobium* cv. Khao Sanan flowers. The isolated fragment was called *Den-Cys1.* Its accession number is EU152214.

The deduced amino acid sequence (171 aa) contained two catalytic active sites: the cysteine-peptidase histidine active site (amino acid sequence: LDHGVAIVGYG, starting at residue 97 from the onset of the isolated fragment) and the cysteine-peptidase asparagine active site (amino acid sequence: YWIVRNSWGPEWGEKGYIRM, starting at residue 117 from the onset of the fragment). Seven amino acids were present between the two active sites. The third active site (cysteine active site, amino acid sequence QGQCGSCWAFST) of the typical catalytic triad in cysteine peptidases was not found in the present fragment. The deduced amino acid sequence of the present fragment did have an endoplasmic reticulum retaining targeting sequence (amino acid sequence KDEL) at its C terminal end. The sequenced fragment shared 85% of its nucleotide sequence with a *Phalaenopsis* cysteine peptidase (full cds, accession PSU34747).

3.6. Transcript abundance of Den-Cys1 in petals

Semi-quantitative RT-PCR was used to study the accumulation of *Den-Cys1* mRNA. The transcript remained below the detection limit in controls not treated with ethylene (Fig. 3A). At the end of the 24 h treatment with ethylene, the transcript showed high abundance (Fig. 3A). A further increase in abundance was noted on day 5 after the end of ethylene treatment (Fig. 3A). Quantitative RT-PCR confirmed these data (Fig. 3C).

3.7. Effects of ethylene on the column: protein levels, total peptidase activity, and the transcript abundance of Den-Cys1

From day 3 after the end of the ethylene treatment, the levels of water-soluble and water-insoluble protein in the column were lower than in the untreated controls, although the differences were rather small (Fig. 4). A decrease in protein levels, comparing day 1 and day 5, was only found in water-insoluble protein in the column of ethylene-treated flowers (Fig.4; statistical not shown). A large rise was observed in peptidase activity, on day 4 after the end of ethylene treatment (Fig. 5). Using semi-quantitative RT-PCR, an increase in the abundance of the *DenCys1* transcript was observed in the column, by the end of ethylene treatment. The transcript abundance, as found on gels, seemed to have lowered by day 2, but had clearly increased again by day 5 (Fig. 3B). Quantitative RT-PCR showed little increase in transcript abundance on day 1 and 2, but showed a large increase on day 5 (Fig. 3D).

4. Discussion

Flowers treatment with 1-MCP lasted for about 30 d. If treated with 0.4 μL L^{-1} ethylene, for 24 h after the end of 1-MCP treatment, there was no effect of ethylene on the parameters measured, indicating that 1-MCP effectively inhibited ethylene perception. Previously, it was found that 1-MCP treatment prevented ethylene-induced floral abscission in *Dendrobium* (Uthaichay et al., 2007), also showing effective protection against exogenous ethylene.

If 0.4 μL L^{-1} ethylene was applied for 24 h, the petals showed visible senescence symptoms 5 d after the end of the ethylene treatment. Interestingly, the controls in the present experiments showed petal senescence about 10 d after 24 h treatment with air rather than ethylene. If there would be no exogenous ethylene and

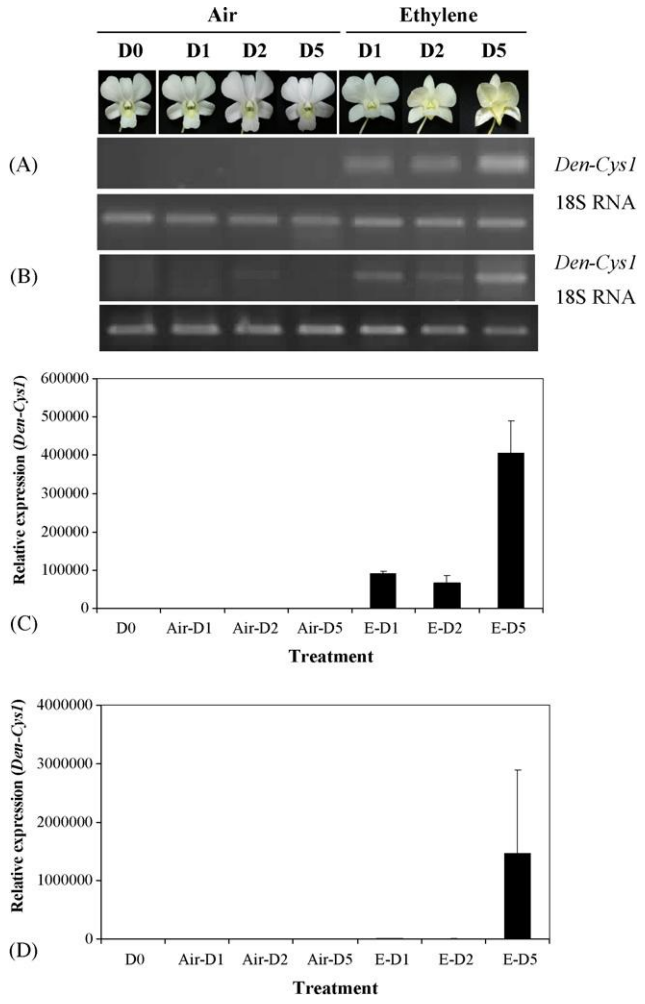

Fig. 3. Expression of *Den-Cys1* in *Dendrobium* cv. Khao Sanan flowers. Total RNA was extracted from petals (A and C) or column (B and D). (A and B) Semi-quantitative reverse transcriptase PCR analysis. Controls (lane 1–4) and after 0.4 $\mu L L^{-1}$ ethylene (lane 5–7) at days 0 (end of treatment), 1, 2 and 5. (C and D) Quantitative reverse transcriptase PCR analysis. Legend as shown above A and B.

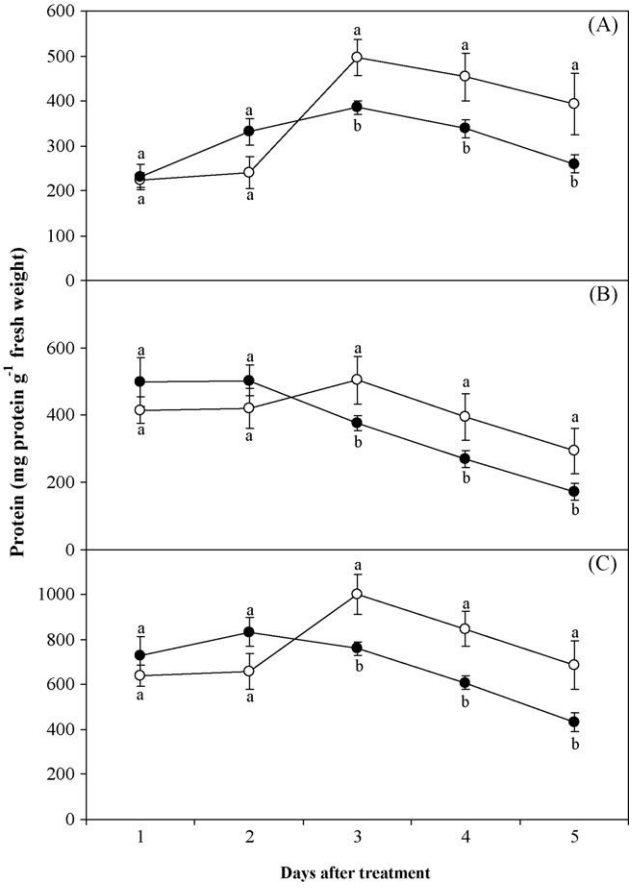

Fig. 4. Protein levels in the column of *Dendrobium* cv. Khao Sanan flowers. (A) Soluble protein, (B) Insoluble protein, (C) total protein. Flowers were treated with air (○) or 0.4 $\mu L L^{-1}$ ethylene (●). Values are means ± SE ($n = 3$). Statistical differences ($P < 0.05$) for each day are shown by a different letter.

no endogenous ethylene production, one would expect these controls to last at least as long as the flowers treated with 1-MCP, that is about 30 d. Flowers that remain attached to the plant and do not become pollinated, can last for more than two months (unpublished observations). The difference between these treatments is suggestive of the idea that the little ethylene that is in the air during vase life, and/or the ethylene that was produced by the flower due to various types of stress, during vase life, reduced the time to petal senescence in the controls to 10 d. However, other possible causes of the relatively short life of the controls can at present not be excluded.

The visible senescence symptoms were preceded by a decrease in water-insoluble protein, whereas the levels of water-soluble protein were not affected. As water-insoluble proteins are predominantly localized to membranes (Srivastava and Srivastava, 2003; Hooshdaran et al., 2004), the data might suggest net degradation mainly of membrane proteins. The pool of water-soluble proteins, including enzymes needed for degradation of various compounds, seems stable. Proteins in this pool might well be degraded, but this might be compensated by new synthesis and, possibly, by transfer of insoluble proteins into the soluble fraction. A similar distinction between water-soluble and water-insoluble protein was made previously in cut Geraldton waxflower. The results were similar to those found here: a decrease of insoluble protein accompanied by no change in soluble protein (Olley et al., 1996). Until more species have been tested, it is not clear if the degradation of insoluble protein tends to occur more rapidly than that in soluble protein during

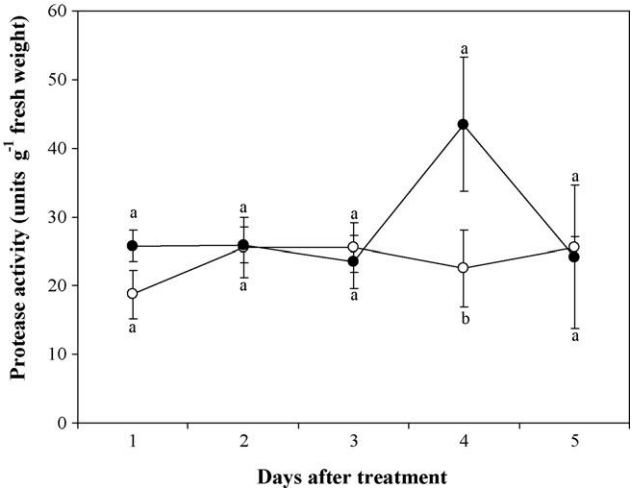

Fig. 5. Peptidase activity in the column of *Dendrobium* cv. Khao Sanan. Flowers were treated with air (○) or 0.4 $\mu L L^{-1}$ ethylene (●). Peptidase activity was measured using azocasein as a substrate. Values are means ± SE ($n = 3$). Statistical differences ($P < 0.05$) for each day are shown by a different letter.

petal senescence. The senescence of *Sandersonia* petals was accompanied by a rather small decrease in soluble protein levels, despite a large increase in peptidase activity (Eason et al., 2002). In *Sandersonia*, just as in Geraldton waxflowers and *Dendrobium*, more net degradation might have occurred in the water-insoluble protein fraction than in the water-soluble one.

In *Dendrobium* an increase in total peptidase activity was found by the end of the ethylene treatment, and a peak in activity was noted just before the onset of the visible senescence symptoms. The decrease in water-insoluble protein occurred gradually, thus is not directly related with peptidase activity. Nonetheless, the decrease in soluble protein can likely be accounted for by the measured increase in total peptidase activity.

Treatment with 0.01 mmol L^{-1} E-64, a specific inhibitor of cysteine peptidases, for 24 h prior to ethylene treatment, almost doubled the time to ethylene-induced petal senescence. This suggests that cysteine peptidases are important in determining the time to visible senescence. This is apparently the first experimental evidence for a delay of petal senescence by E-64. The effect is rather unexpected. It is known that E-64 does not move through membranes (Yasuda et al., 2004; Inoue et al., 2006; Leung-Toung et al., 2006). When E-64 was tested *in vitro*, by including it in a homogenate of *Iris* tepals that were about to senescence, E-64 reduced total peptidase activity to 66% of that in the controls (Pak and van Doorn, 2005). When freshly isolated tepals were continuously placed, until just before visible senescence, in a solution of this compound, no effect was found on peptidase activity (unpublished results). Similar treatment of *Iris* tepals with a solution containing E-64d, the membrane-permeable form of E-64, reduced total peptidase activity to 32% of controls (Pak and van Doorn, 2005). These data suggest that little E-64 enters the petal cells. Nonetheless, even if E-64 does not enter the cells, the effect of E-64 on the time to visible petal senescence in *Dendrobium* might be accounted for. The cell walls of most cells contain several peptidases. Programmed cell death induced by victorin in oat leaves was inhibited by E-64 and by inhibitors of serine peptidases and inhibitors of caspases. It was concluded that a cell wall cysteine peptidases was required for programmed cell death induced by victorin (Coffeen and Wolpert, 2004). It might be tempting to hypothesize that E-64 has a similar effect on a peptidase in the cell walls of senescing *Dendrobium* cv. Khao Sanan petals.

E-64, and some other peptidases tested (PMSF, a serine peptidase inhibitor, and pepstatin A, an aspartic peptidase inhibitor) had toxic side effects. PMSF and pepstatin A were toxic at the lowest concentration tested. The potential role of serine peptidases or aspartic peptidases could therefore as yet not be assessed. The toxic effect was apparently not due to ethanol, the solvent, as controls for ethanol did not have the effect, and 1,10-phenanthrolin, which was dissolved in the same amount of ethanol, considerably delayed the time to visible petal senescence.

Interestingly, treatment with 0.1 mmol L^{-1} 1,10-phenantrolin doubled the time to ethylene-induced petal senescence. This chelator is used in *in vitro* tests to discriminate between metallo- and other peptidases. 1,10-Phenantrolin binds various metal ions. It is utilized, for example, to assess the levels of Fe^{2+} in solutions but also binds zinc, cobalt, and calcium ions (Naseri et al., 2004). Zinc or cobalt ions are required for the catalytic activity of many metalloendopeptidases, whilst some serine endopeptidases require calcium ions (Fontés et al., 2005). As many enzymes other than peptidases require binding to metal ions for their activity, the effect of 1,10-phenantrolin, when tested *in vivo* (by placing flowers in a solution), cannot be assigned to a specific class of proteins. EDTA, another metal chelator, when included in the water in which the flowers were stood, increased the time to senescence by 1.0-1.3 d. EDTA is also used to inhibit metallopeptidase activity *in vitro* (Qi et al., 1999), but again when it is absorbed by the plant it can have numerous effects other than inhibition of metallopeptidases. Similarly, visible senescence in *Petunia* (Knee, 1996) and *Sandersonia* (Eason et al., 2002) was delayed by 2,2′-dipyridyl, which is a liposoluble metal chelator (Horky et al., 1998; Watkins et al., 2003; Demougeot et al., 2004). The activity of peptidases secreted a by parasitic worm was inhibited to the same degree with the metal chelating compounds 1,10-phenantrolin and 2,2′-dipyridyl, whereas the cysteine peptidase E-64 had no effect (Rhoads et al., 1997). Eason et al. (2002) claimed that 2,2′-dipyridyl is an inhibitor of cysteine peptidases. However, the literature apparently does not describe such an effect. This is in contrast to the effect of 2,2′-dipyridyl disulfide, a well-known inhibitor of cysteine peptidase activity (Willenbrock and Brocklehurst, 1984; Otto and Schirmeister, 1997). Treatment of *Sandersonia* flowers with 2,2′-dipyridyl inhibited the petal-senescence-associated increase in the mRNA abundance of at least three petal cysteine peptidases (Eason et al., 2002), indicating a relatively early role of this metal chelator in the senescence process.

A partial cDNA putatively encoding a cysteine peptidase gene (*Den-Cys1*) was identified in senescent petals of *Dendrobium* cv. Khao Sanan. This is similar to the increased abundance of cysteine protease mRNA during petal senescence in several other species (van Doorn and Woltering, 2008). Amino acid alignment of the sequenced part of *Den-Cys1* showed the typical His and Asp catalytic cysteine peptidase active sites, but not the expected Cys active site, which usually comes before the His and Asp catalytic site (Grudkowska and Zagdańska, 2004). Nonetheless, the high homology of the sequence with known proteins strongly suggests that the sequenced EST represents a cysteine peptidase. The KDEL motif of *Den-Cys1* was present at the C-terminus of the deduced amino acid sequence, where it is also found in many other cysteine peptidases (Schmid et al., 1998). The KDEL motif functions as a retrieval signal for soluble proteins of the endoplasmic reticulum (ER) lumen and of proteins in vesicles that are derived from the ER. The latter include vesicles that deposit proteins to vacuoles (Schmid et al., 1998). *Den-Cys1*is therefore similar to several other cysteine peptidases that have been implicated in senescence (Eason et al., 2002; Jones et al., 2005). At the end of the ethylene treatment the transcript of *Den-Cys1* was highly expressed in the petals. This expression pattern was consistent with a small rise in total peptidase activity, also by the end of ethylene treatment.

The column of flowers treated with ethylene also showed an increase in the transcript abundance of the isolated cysteine peptidase, accompanied by an increase in peptidase activity. The ethylene treatment was also associated with a decrease in water-insoluble protein levels, whereas no decrease was observed in the level of water-soluble protein, similar to the effects in petals. The time lines of these processes were similar in both columns and petals (although a large increase in *DenCys1* transcript abundance occurred later in columns than in petals, and the increase in protease activity also came later in the column). In several species ethylene treatment induced senescence and death in many cells of the style (Wu and Cheung, 2000). The same might be true for the stylar part of the column in *Dendrobium* flowers. If so, the column might participate, just as the petals, in nourishing other cells, after pollination. As noted in Section 1, our unpublished data indicated that the column of *Dendrobium* flowers is the main source of the increase in ethylene production following pollination. The present data suggest the idea that this increase in column ethylene production might induce senescence (cell death) in column cells. Wu and Cheung (2000) described that in many plant species pollination induces programmed death in cells of the central stylar tissue. Thus after pollination cell death was restricted to the area where

the pollen tubes were transmitted. The cell death process apparently facilitated the passage of the pollen tubes, and nourished the pollen tubes. Whether or not there is senescence (cell death) in the *Dendrobium* column, following pollination, and whether or not this aids in nourishing the pollen tubes and/or the ovary, remains to be elucidated.

In conclusion, treatment with ethylene resulted in an early increase in the transcript abundance of a senescence-associated cysteine peptidases in the petals, in an early rise in peptidase activity, and in a later decrease in water-insoluble protein levels. The delay of petal senescence by a specific inhibitor of cysteine peptidases (E-64) indicates that the activity of this class of peptidases is limiting the processes leading to visible petal senescence. As E-64 does not permeate membranes, the results might suggest an effect on an extracellular cysteine peptidase. Treatment of the flowers with 1,10-phenanthrolin, a metal chelator, also doubled the time to ethylene-induced visible petal senescence. However, this compound might have several effects other than on metallopeptidases.

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