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RESEARCH PAPER

Transcriptional activation of a 37 kDa ethylene responsive cysteine protease gene, *RbCP1*, is associated with protein degradation during petal abscission in rose

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Abstract

Cysteine proteases play an important role in several developmental processes in plants, particularly those related to senescence and cell death. A cysteine protease gene, *RbCP1*, has been identified that encodes a putative protein of 357 amino acids and is expressed in the abscission zone (AZ) of petals in rose. The gene was responsive to ethylene in petals, petal abscission zones, leaves, and thalamus. The expression of *RbCP1* increased during both ethylene-induced as well as natural abscission and was inhibited by 1-MCP. Transcript accumulation of *RbCP1* was accompanied by the appearance of a 37 kDa cysteine protease, a concomitant increase in protease activity and a substantial decrease in total protein content in the AZ of petals. Agro-injection of rose petals with a 2.0 kb region upstream of the *RbCP1* gene could drive GUS expression in an abscission zone-specific manner and was blocked by 1-MCP. It is concluded that petal abscission is associated with a decrease in total protein content resulting from rapid transcription of *RbCP1* and the expression of a 37 kDa protease.

Key words: Abscission, agro-infiltration, cysteine protease, ethylene, petal, programmed cell death, *RbCP1*, rose, senescence.

Introduction

Abscission is a natural event that involves cell separation in response to developmental or environmental cues. Organs like flowers, petals, sepals, and leaves may be shed once they have served their purpose (Addicot, 1982, van Doorn and Stead, 1997). Several physiological studies have been performed to understand the factors involved in abscission and these have helped in identifying a major role for ethylene in regulating organ abscission (Abeles and Gahagan, 1968; reviewed by Roberts *et al.*, 2002). Further studies on ethylene-insensitive mutants in *Arabidopsis* and tomato have led to the identification of key components required for the transduction of the ethylene signal during abscission (Lanahan *et al.*, 1994; Bleecker and Patterson, 1997; Whitelaw *et al.*, 2002). In addition, the role of various enzymes involved in cell separation and the genes encoding these have been elucidated (Tucker *et al.*, 1988; del Campillo and Bennett, 1996; Kalaitzis *et al.*, 1997; Lashbrook *et al.*, 1998; Gonzalez-Carranza *et al.*, 2002; Belfield *et al.*, 2005; Sane *et al.*, 2007). In recent years, the roles of novel components of abscission such as *HAESA*, *IDA*, and *IDL* genes that may function and interact in an ethylene-independent manner have also been studied (Jinn *et al.*, 2000; Butenko *et al.*, 2003; Stenvik *et al.*, 2006, 2008).

Proteases play an important role in various developmental processes in plants such as seed germination, xylogenesis, and tapetum degradation as well as organ senescence and programmed cell death (reviewed by Schaller, 2004). Several cysteine protease genes have been found to be up-regulated during programmed cell death in senescing floral tissues of many plants while protease in-gel assays have revealed the appearance of novel proteases during the senescence of flowers (Jones *et al.*, 1995, 2005; Valpuesta *et al.*, 1995; Guerrero *et al.*, 1998; Stephenson and Rubinstein, 1998; Eason *et al.*, 2002; Wagstaff *et al.*, 2002;

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Wang *et al.*, 2004; Pak and van Doorn, 2005, Azeez *et al.*, 2007). However, so far, none have been reported during abscission.

We are interested in understanding the molecular basis of petal abscission and have been using the highly ethylene-sensitive fragrant rose (*Rosa bourboniana* var. Gruss an Teplitz) as a model for petal abscission. Since abscission zone cells have previously been shown to undergo features of cell death (Evensen *et al.*, 1993), we were interested in finding out if cysteine proteases may play an important role in the process of abscission as well. In this paper, it is reported that the progression of petal abscission in ethylene-treated and field-abscising flowers of rose is associated with the rapid transcription of a cysteine protease gene, *RbCP1*, the enhanced expression of a 37 kDa cysteine protease, and a decrease in total protein content.

Materials and methods

Plant material

Flowers of the fragrant variety of rose (*Rosa bourboniana* var. Gruss an Teplitz) were used in the present study. They were picked early in the morning (before sun rise), cut with a sharp blade and the stalks immediately placed in water. Care was taken to ensure that flowers were of the same developmental stage (unpollinated and with only one or two of the outer most petals open).

Ethylene and 1-MCP treatments

Cut flowers were kept in water in a closed air-tight chamber, treated with 0.5 $\mu l\ l^{-1}$ ethylene for 16–18 h (until abscission occurred) and petal abscission zones collected at 0 h (ethylene untreated), 4 h, 8 h, and 12 h from the time of initiating the ethylene treatment as described earlier (Sane *et al.*, 2007). For 1-methyl cyclopropene (1-MCP) treatment, cut flowers were exposed to 1 $\mu l\ l^{-1}$ 1-MCP (Ethylbloc from Biotechnologies for Horticulture Inc, Walterboro, SC, USA) for 12 h. For samples that underwent natural/developmental abscission in the field (time of abscission, 38–45 h), flowers were marked at the time of opening of the outermost whorl and abscission zones collected at time intervals of 8 h, 12 h, 24 h, and 36 h. For early ethylene responsive accumulation of *RbCP1*, cut flowers were exposed to 0.5 μl^{-1} ethylene for short time intervals of 30, 60, and 120 min and abscission zones collected and frozen immediately. For each sample 30–40 flowers were chosen and abscission zones (2 mm^2) were collected from 10–15 petals per flower and pooled. For the ethylene treatment of other floral and vegetative tissues, these were exposed to 0.5 μl^{-1} ethylene for 8 h, as described earlier and samples collected before and after ethylene treatment.

Isolation of RNA and cloning of cysteine protease cDNA

RNA was isolated from frozen petal abscission zones of different samples as described by Asif *et al.* (2000). RNA was also isolated from different tissues such as petal, sepal, stamen, carpel, thalamus, pedicel, and leaf before and after 8 h ethylene treatment.

DNA-free RNA from 8 h ethylene-treated samples was reverse transcribed using the Superscript II reverse transcriptase from Invitrogen (Palo Alto, USA) and primed with the 3′ RACE adapter primer (5′-GGCCACGCGTC-GACTAGTACTTTTTTTTTTTTTTT-3′). The rose cysteine protease gene, *RbCP1*, was obtained initially as an artefact of PCR while performing 3′ RACE for rose *ETR1*. The amplified 450 nt fragment was cloned in pBluescript-tIISK (Stratagene, USA) and sequenced on an automated DNA sequencer (ABI 373A from Applied Biosystems Inc, USA) using the thermosequenase dye terminator cycle sequencing kit from Amersham-Pharmacia. The cloned fragment showed similarity to papain like cysteine proteases and contained a 3′ UTR of 285 nucleotides. Based on the sequence, specific primers CyPro-R1 5′-ACA AGT TGC AAC ACC ACA CAT GTT C-3′, CyPro-R2 5′-ACA TCT TGA AGT AGC CAT TGT CAC C-3′, CyPro-R3 5′-AGC AAG AAC AGC ATG GTT CAC ATC C-3′ and CyProR4P1 5′-GTT CCT GTT GGT GGA TCG AAT CAG CTT C-3′ were designed for 5′ RACE and genome walking. For 5′ RACE, cDNA was prepared using the SMART cDNA synthesis kit (Clontech Laboratories Inc, Palo Alto, USA) from 8 h ethylene-treated petal abscission zone RNA while a genome walking library was prepared from total rose petal DNA using the Genome walker kit (Clontech). Both 5′ RACE and genome walking were performed to obtain the complete cysteine protease ORF. Although 5′ RACE extended the *RbCP1* cDNA partially, it failed to provide the complete cDNA sequence. Further extension towards the 5′ end was carried out using the primer CyproR4P1 in combination with the genome walker adapter primer, to obtain a fragment of 2.4 kbp that contained the 5′ region and 2.0 kb of the sequence upstream of the initiation codon. Based on the sequence, a forward primer containing the initiation codon (RCypro-OF 5′-ACA GGA TCC CAT GGC TCC TCC TCG TTT G-3′) was designed and used in combination with the 3′ adapter primer to amplify the cDNA containing the complete open reading frame of 1077 bases and the 285 bases 3′ UTR (GenBank Accession No. EU057180).

Northern analyses and semi-quantitative RT-PCR

Total RNA (30 μg) from the ethylene-treated abscission zones (0–12 h) and the 12 h 1-MCP treated abscission zones was resolved on a 1.2% denaturing formaldehyde-agarose gel as described by Sambrook *et al.* (1989) and modified in the Qiagen Oligotex handbook. RNA was transferred to nylon membranes (Hybond N, Amersham-Pharmacia Biotech, Uppsala, Sweden) by vacuum transfer using the vacugene apparatus (Pharmacia) and cross-linked by baking for 2 h. Radiolabelling of probes for hybridization was performed using α-^{32}PdCTP in an asymmetric PCR of the full-length cysteine protease cDNA. Hybridization and washings of blots were performed as described (Sambrook *et al.*, 1989). Signals obtained on the blots were visualized

on an X-ray film (Fujifilm SuperRX) or quantified on a phosphorimager (Molecular imager FX, BioRad) using the software QuantityOne-4.2.3 version.

For the comparison of expression between ethylene-treated and untreated carpel, sepal, petal, pedicel, thalamus, and leaf tissues, and for studying early ethylene responsive expression (30–120 min), 2 μg RNA from these samples was reverse transcribed as described and amplified using the primers Cypro-OF and CyproR4P1 to give an amplification product of 300 nt. Actin was used as an internal control for normalization using the primers RActF1 5′-ATG ACA TGG AGA AGA TCT GGC ATC A-3′ and RActR2 5′-AGC CTG GAT GGC AAC ATA CAT AGC-3′. PCR was carried out for 27 cycles using the cycling parameters 94 °C, for 3 min (first window, one cycle) followed by 27 cycles of 94 °C for 5 s; 55 °C for 10 s, and 72 °C for 20 s. At least three independent reactions were run to confirm the results.

Protein extraction and total protease activity

Frozen tissue samples from different stages of ethylene-treated (0 h, 4 h, and 8h) and naturally abscising petal abscission zones (0 h, 12 h, 24 h, and 36 h) were ground in liquid nitrogen. The powder was suspended in 2 ml buffer containing 50 mM TRIS-Cl pH 7.5, 1 mM EDTA pH 8.0, 0.1% SDS and centrifuged at 12 000 *g* for 5 min. The supernatant was used for the estimation of total proteins from all samples (Peterson, 1977) and total protease assay (0 h, 8 h ethylene-treated and 24 h natural abscission zone samples) using azocasein as a synthetic substrate as described earlier (Holwerda and Rogers, 1992; Azeez *et al.*, 2007). For the total protease assay, 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). A duplicate reaction for each sample was prepared where TCA was added at the start to act as a control. One unit of protease activity was defined as the amount of enzyme that gave an increase of 0.01 absorbance units min^{-1}. To study the contribution of different proteases, protease inhibitors such as E-64 [L-*trans*-epoxysuccinylleucylamide-(4-guanidino)-butane, specific for cysteine proteases] and PMSF (phenylmethylsulphonylfluoride, specific for serine proteases) were added separately at a concentration of 2 μM and 1 mM, respectively, to the reactions. The enzyme aliquot was first incubated with the requisite concentration of the inhibitor (without substrate) for 30 min and then assayed as described above. All extractions and assays were carried out in triplicates.

Protease in-gel assay

Protein samples were prepared by mixing the protein aliquot (10 μg each) 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) glycerol, 0.01% bromophenol blue], incubated at 37 °C for 60 min and then electrophoresed in cold on a 12% SDS-gelatin polyacrylamide gel embedded with 0.10% gelatin (Hellmich and Schauz, 1988). 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 45 min with shaking and then incubated for 12–16 h in a buffer containing 10 mM Ca^{2+}, 10 mM Mg^{2+}, and 50 mM TRIS-HCl, pH 7.5, at 37 °C in an incubator-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 differentiate between cysteine and serine proteases, protease inhibitors, namely 2 μM E-64 and 1 mM PMSF, specific against each class, were added to the protein samples prior to electrophoresis and incubated for 30 min at 37 °C along with non-reducing loading buffer. Following renaturation, gels were incubated in the presence of inhibitors in 10 mM Ca^{2+}, 10 mM Mg^{2+}, 50 mM TRIS-HCl, pH 7.5 at 37 °C for 12–16 h.

Statistical analysis

Data from the total protein content and protease activity in the abscission zones from three independent experiments were analysed and expressed as mean ±standard deviation. Data were subjected to analysis of variance at an alpha level of 0.05 using the Microsoft Excel software. Values that exhibited significant differences at $P < 0.05$ were considered.

Isolation and analysis of the RbCP1 *promoter*

To obtain the proximal promoter of the *RbCP1* gene, a reverse primer RbCP1-PRO2 – GGATCCGTCAAAC-GAGGAGGAGCCAT containing the initiation codon (underlined), with a *Bam*HI site was used to amplify a 2.0 kb fragment of the putative promoter from the 2.4 kb fragment that had been obtained by genome walking. This was cloned at the *Bam*HI site of pBI101.2 in translational fusion with the GUS gene. The plasmid was introduced into *Agrobacterium* strain GV3101. The *HAESA* gene promoter was cloned from *Arabidopsis* using the primers HAE-Pro-F1 5′-GAG TAA CGA AAC AAG AAA TAG GAG-3′ and HAE-Pro-F2 5′-ACT CTG TCA CCG TTT CCT AAG AAG-3′ sequentially in combination with the primer HAE-R 5′-ATC TAG ATT TTT TGG AAA AGG AAT CG-3′ to obtain a fragment of 1708 nt. This was digested with *Xba*I (present in the reverse primer and at a position 1596 nt upstream of the start codon) to obtain the promoter fragment. This was cloned in pBI101.1 to obtain pHAESA.

Promoter analysis through agroinjection of the RbCP1 *promoter in rose buds*

Recombinant agrobacteria containing the promoter of *RbCP1* (in pBI101.2) were grown in Luria broth in a 25 ml

culture overnight and harvested at 3000 rpm (5 min) on an SS34 rotor (RC-5C, Dupont-Sorvall). The pellet was resuspended in LB to an OD of 1. Acetosyringone was added to the suspension at a final concentration of 0.1 mg ml^{-1}. For agroinjection, young buds that would open in two days time were chosen. A needle (size 23, dimensions 0.63×25 mm) fitted on to a 2 ml syringe (filled with the agrobacterial suspension) was lightly inserted in the centre of the petals. The agrobacterial suspension (0.5 ml, containing 0.01% acetosyringone), was slowly forced into the petal and allowed to infiltrate all through the petal up to the point of attachment of the petal with the thalamus. Agroinjection was performed with three flowers per construct and three petals per flower. The excess suspension was wiped out and the buds were kept for 2 d on the plant. As a control, agrobacteria containing pBI101 (no promoter), pBI121 (GUS driven by CaMV 35S promoter), and pHAESA (GUS driven by the 1.6 kb abscission-specific promoter of the *Arabidopsis HAESA* gene) were also used for agroinfiltration of independent flowers. After 2 d from agroinfiltration, flowers were carefully cut under water and treated with ethylene for 8 h in a closed chamber as described before. After 8 h of ethylene treatment, the petals were detached and stained for GUS expression. Staining was carried out for 16 h at 37 °C as previously described (Gattolin *et al.*, 2006). The tissue was destained in 70% ethanol at 37 °C until examination. Light microscopy was performed on a Leica Wild M3Z microscope (Leica Germany).

Results

Isolation of the RbCP1 *gene*

The full-length cDNA sequence of the cysteine protease gene, *RbCP1*, was obtained by a combination of RACE and genome walking, followed by cloning of the entire cDNA to yield a sequence of 1359 bp that included an ORF of 1074 nt and a 3' UTR of 285 nt (GenBank Accession No. EU057180). The gene encoded a protein of 357 amino acids that included an N-terminal signal peptide of 22 amino acids as deduced by TargetP analysis (http://www.cbs.dtu.dk/services/TargetP/). The protein had a predicted molecular mass of 39.5 kDa (for the pre-protein) and 37.2 kDa (for the mature protein) and a pI of 6.1 as determined using the ProtParam tool of ExPASy (http://www.expasy.org/tools/protparam.html). BLAST analysis of the predicted protein (http://www.ncbi.nlm.nih.gov/BLAST/) showed 75–82% amino acid identity and 80–90% amino acid similarity to various cysteine proteases from *Prunus armeniaca*, *Vitis vinifera*, *Actinidia deliciosa* etc. and also revealed a conserved Peptidase C1A domain from amino acids 141 to 354 (Fig. 1). The peptidase C1A subfamily consists of cysteine peptidases (CPs) such as papain and also includes animal CPs. The conserved interspersed ERFNIN motif [**E**-X3-**R**-X2-(I/V)-**F**-X2-**N**-X3-**I**-X3-**N**] that is a characteristic feature of most papain-type cysteine proteases was

M A P P R L T L V L S L L A V V L S T C A A↓S S F D E S S P I R L
V P D G L R E L E D Q V V Q V L G Q V C H V R S F A R F A Y R Y E
K R Y E S V E **E** M G R **R** F E I **F** A E **N** K K L **I** R S T **N** R K G L S Y
K L G V N R F A D W T W E E F Q R H R L G A A Q N C S A T T K G N
H K L T D A V P P L T K N W R D E G I V T P V K D Q G H C G S C W
T F S T T G A L E A A Y V Q A F G K Q I S P S E Q Q L V D C A G A
F N N F ***G C S G G*** L P S Q A F E Y I K Y N G G L D T E Q A Y P Y T
A V D G A C K F S S E N V G V R V L D S V N I T L N D E E E L K H
A V A F V R P V S V A F Q V V Q D F R L Y K S G V Y T S E T C G N
T P M D V N H A V L A V G Y G V E N G V P Y W L I K N S W G Q S W
G D N G Y **F K M E** Y G K N M C G V A T C A S Y P V V A *

Fig. 1. Complete amino acid sequence of the predicted rose cysteine protease gene, *RbCP1* (Accession No. EU057180). The signal peptide is shown in italics with the arrow marking the site of the likely cleavage. The C1A peptidase domain has been underlined. The ERFNIN motif residues are shown in bold while the active site residues C^{164} H^{304} N^{324} have been boxed. The GCXGG motif is shown in bold and italics. The putative SUMO modification site FKME has been shown in bold and is boxed. An asterisk indicates the termination of protein.

conserved in RbCP1 as was the GCXGG motif (Karrer *et al.*, 1993). The predicted active site catalytic residues C^{164} H^{304} N^{324} as well as other features, such as the presence of a lysine before the catalytic asparagine, were also conserved. The predicted protein possessed a site for possible SUMO modification (FKME) at the C-terminal end of the polypeptide. SUMO modification in tomato cysteine protease LeCP was shown to be important for its localization in the nucleus and subsequent activation of the *LeACS2* gene (Matarasso *et al.*, 2005).

Transcript accumulation of RbCP1 *during petal abscission*

In order to investigate if *RbCP1* is involved during abscission, its transcript accumulation pattern was examined in petal abscission zones obtained from ethylene-treated and naturally abscising flowers. In ethylene-treated flowers (time of abscission, 16–18 h; Sane *et al.*, 2007), a rapid increase in transcript accumulation was observed from 4 h after ethylene treatment. The increase continued up to 8 h and remained steady until 12 h after ethylene treatment (Fig. 2A). In flowers treated with 1-MCP (an inhibitor of ethylene perception) a significant delay in petal abscission was observed (time of abscission, 55–60 h, Sane *et al.*, 2007) and very low levels of the *RbCP1* transcript accumulated even after 12 h. Transcript accumulation was also studied by semi-quantitative RT-PCR during natural/developmental abscission (time of abscission, 38–45 h). Under these conditions, transcripts of *RbCP1* could be detected from the 8 h stage onwards and their levels remained steady until 24 h. Thereafter transcript levels increased substantially at 36 h prior to abscission (Fig. 2B). In view of the rapid increase in transcript levels of *RbCP1* in abscission zones of ethylene-treated flowers within 4 h of ethylene treatment, the ethylene responsiveness of *RbCP1* in abscission zones was studied by treating flowers with ethylene for shorter time intervals (30, 60, and 120 min). A significant increase in transcript accumulation was detected within 30 min of ethylene treatment, indicating that the

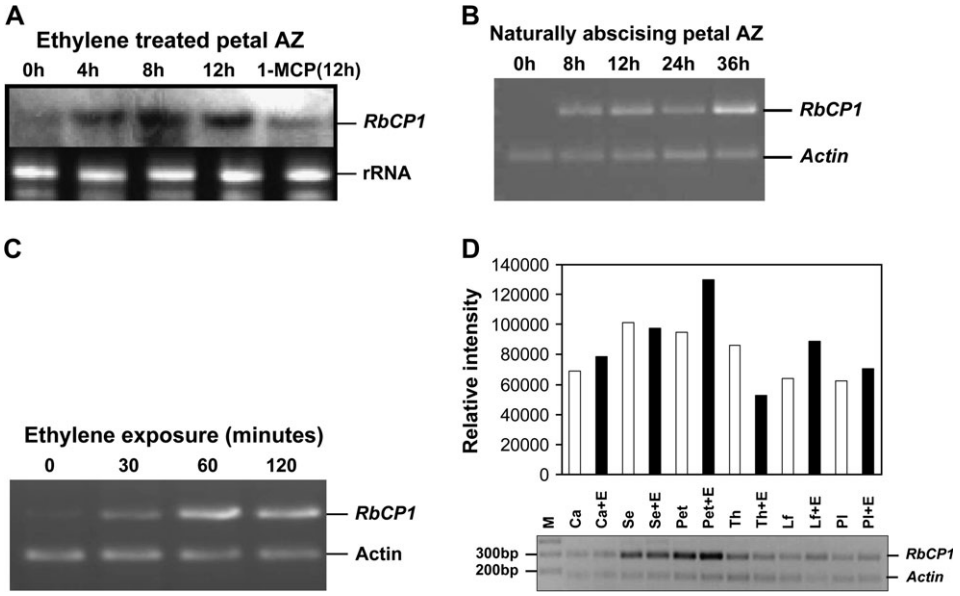

Fig. 2. (A) Transcript accumulation of *RbCP1* during the course of petal abscission in rose as determined on a northern blot. RNA from petal abscission zones of ethylene-treated (0–12 h) and 1-MCP treated (12 h) flowers was used for the study. The lower panel shows ribosomal RNA as a loading control. AZ, abscission zone. (B) Semi-quantitative RT-PCR of RNA from petal abscission zones of field-abscising flowers at different time periods (0–36 h). Reverse transcribed RNA from different samples was PCR amplified to give an amplified fragment of 300 nt using RbCP1 specific primers CyproOF and CyproR4P1. Rose actin primers that amplified a fragment of 180 bp were included in the reaction mix as an internal control for normalization. (C) Semi-quantitative RT-PCR to show early ethylene responsive accumulation (30, 60, and 120 min post-ethylene treatment) of *RbCP1* transcripts during petal abscission in rose using the same primers as described (B). Actin was used as an internal control in the same reactions for normalization. (D) Comparison of expression of *RbCP1* in various tissues before and after 8 h ethylene treatment by semi-quantitative RT-PCR (negative image). Reverse transcribed RNA from different tissues was PCR amplified using the same primers as described in (B) with actin as an internal control. The intensity of the actin band for each tissue set (with and without ethylene) was normalized and the relative intensity of the cysteine protease band calculated accordingly. White bars, ethylene untreated samples; black bars, 8 h ethylene-treated samples; Ca, carpel; Se, sepal; Pet, petal; Th, thalamus; Lf, leaf ; Pl, pedicel.

gene was rapidly up-regulated in response to ethylene in abscission zones (Fig. 2C). The expression of *RbCP1* and its response to ethylene in other tissues such as sepal, petal, carpel, pedicel, thalamus, and leaves was also studied. An increase in transcription of the gene upon ethylene treatment was observed only in petals and leaves while a decrease was observed in the thalamus (Fig. 2D).

Protein content and protease activity in petal abscission zones

In view of the increasing levels of transcript accumulation of *RbCP1* during the course of abscission in petals, the total protein content in 0 h, 4 h, and 8 h ethylene-treated and 0 h, 12 h, 24 h, and 36 h field-abscising petal abscission zones was estimated. As shown in Fig. 3A, there was a decrease in total protein content in abscission zones of both ethylene-treated and field-abscising flowers. In 4 h ethylene-treated petal abscission zone tissues, the protein content decreased to about 50% of the control, while in 8 h ethylene-treated abscission zones it went further down to about 35% of the control. In flowers undergoing natural (field) abscission, the protein content decreased to 60% of the control within 12 h of natural abscission and went further down to about 35–37% in 24 h and 36 h natural abscission zones samples. When total protease activity was measured in 0 h, 8 h ethylene-treated and 24 h naturally abscising petal abscission zones, a concomitant increase in total protease activity was observed during the course of abscission. There was a 4.3-fold increase in total protease activity in abscission zones of 8 h ethylene-treated flowers and a 2-fold increase in total protease activity in abscission zones of 24 h field-abscising flowers over the control (Fig. 3B). Interestingly, most of the proteolytic activity responsible for the observed increase during abscission (both ethylene-induced and field-abscising samples) could be inhibited by E-64, a specific inhibitor of cysteine proteases, but not by PMSF, an inhibitor of serine proteases. This indicated that the increase in protease activity during abscission was due to the expression of one (or more) cysteine proteases.

Detection of a 37 kDa cysteine protease in abscission zones by in-gels assays

It was tested if the increase in proteolytic activity was associated with the expression/activation of specific cysteine protease(s) by an in-gel assay. As shown in Fig. 4, low levels of a protease of about 37 kDa (seen as clearing in the gel) could be visualized in control (0 h) abscission zone samples. The levels of this protease increased substantially in the 8 h

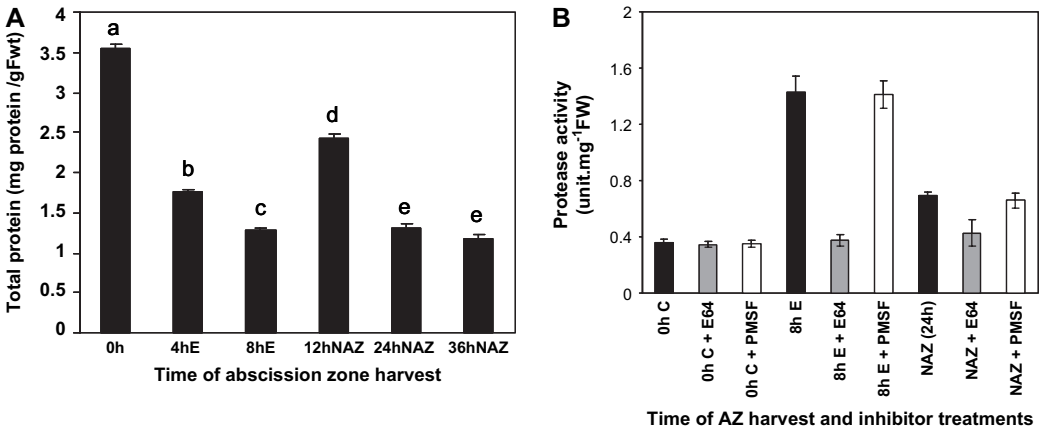

Fig. 3. Total protein content in abscission zones after ethylene treatment and during field abscission. 0 h, control samples; 4hE, 8hE, 4 h and 8 h ethylene-treated abscission zone samples; 12 h, 24 h, 36 h NAZ, 12 h, 24 h, and 36 h natural abscission zone samples. Data from three independent experiments were analysed and expressed as mean ±standard deviation. Letters (a, b, c, d, and e) over the bars indicate significant differences at P <0.05. (B) Total protease activity and contribution of cysteine proteases in abscission zone samples (after ethylene treatment and in field-abscising samples). Black bars, protease activity in the absence of any protease inhibitor; Grey bars, protease activity in the presence of the cysteine protease inhibitor E-64; White bars, protease activity in the presence of the serine protease inhibitor PMSF. 0 h, 0h control; 8hE, 8 h ethylene-treated AZ samples; NAZ, 24 h natural abscission zone (field-abscising) samples. Data from three independent experiments were analysed and expressed as mean ±standard deviation.

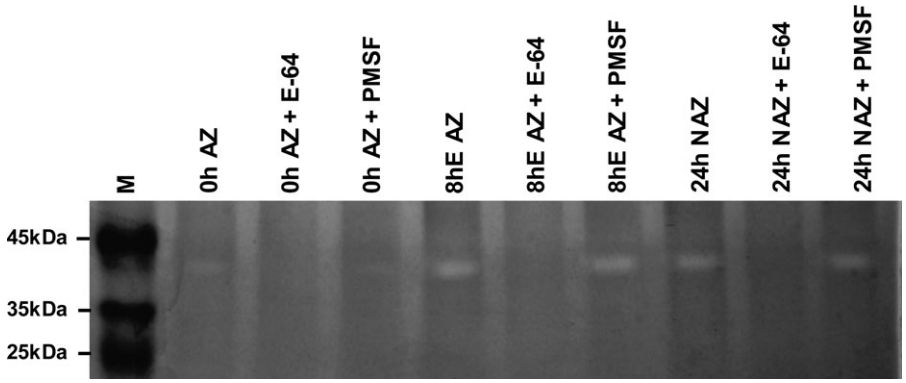

Fig. 4. Detection of proteases in petal abscission zones (after ethylene treatment and in field-abscising samples) by in-gel protease assay on a 12% SDS-polyacrylamide gel. Samples were loaded without any protease inhibitor or with a cystein protease inhibitor (+E-64) or with a serine protease inhibitor (+PMSF). The ~37 kDa cysteine protease induced after ethylene treatment and in naturally abscising samples can be seen as clearing in the gelatin containing gel. 0 h, 0 h control; 8hE, 8 h ethylene-treated abscission zone samples; 24hNAZ, 24 h natural abscission zone (field-abscising) samples.

ethylene-treated abscission zone samples and could also be seen in abscission zones of flowers undergoing natural/developmental abscission albeit at a slightly reduced level. The 37 kDa protease could be completely inhibited by E-64, but not by PMSF, indicating that it was a cysteine protease. No other protease could be seen in these samples either in the high molecular weight range or in the low molecular weight range except for a very faint band at 45 kDa.

Isolation of RbCP1 *promoter and analysis of its expression* in planta

A 2.0 kb region upstream of the *RbCP1* initiation codon was isolated using genome walking. Sequence analysis of the promoter using the software programmes PlantCare (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) and PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html) revealed the presence of ethylene responsive *cis* elements such as AT/ATTCAAA as well as closely matching sequences with changes in one or the other nucleotide of the eight nucleotide motif. Most of these were clustered between 930–1550 nt upstream of the start codon. The promoter was cloned into pBI101.2 as a translational fusion with GUS and introduced into petals of intact rose buds by agro-injection at a single point in the centre of the petal. After 2 d, the buds were treated with ethylene and tested for GUS expression. As shown in Fig. 5, no expression was observed in plants infiltrated with agrobacteria containing pBI101 (containing no promoter to drive GUS). In plants where pBI121 (containing the CaMV 35S promoter to drive GUS expression) was used, GUS expression could be observed all over the petal. Interestingly, flowers infiltrated

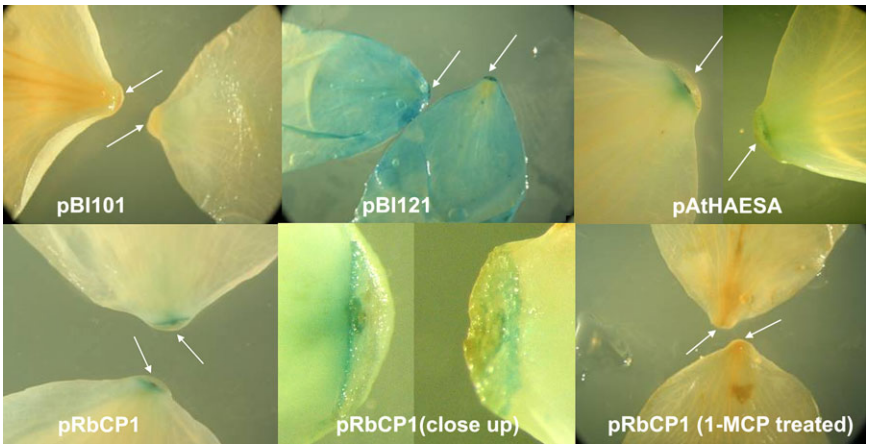

Fig. 5. Histochemical staining to test the activity of the *RbCP1* promoter using a translational promoter–GUS fusion construct by agroinfiltration. Petals in intact buds were infiltrated at the centre of the petal with agrobacteria containing different constructs (three flowers per construct and three petals per flower) using a syringe. Buds were kept for 2 d on the plant and then cut under water as described and treated with ethylene for 8 h before GUS staining. The constructs used for GUS expression study were pBI101 (promoterless), pBI121 (GUS driven by the CaMV 35S promoter), pHAESA (GUS driven by the 1.6 kb abscission-specific promoter of the *Arabidopsis HAESA* gene), and pRbCP1 (GUS driven by the 2.0 kb rose *RbCP1* promoter). GUS expression in the tips of the petals (at the point of separation from the thalamus) is shown. White arrows indicate the point of separation of the petal from the thalamus.

with the *RbCP1* promoter:GUS fusion repeatedly showed GUS expression only in the tips of the excised petals in spite of the fact that the point of agro-injection lay much above the abscission zone at the centre of the petal. A closer look revealed expression in cells lining the point of separation at the junction of the petal and the thalamus. In flowers that were treated with 1-MCP, no expression was visible. The 1.6 kbp *Arabidopsis HAESA* gene promoter was used, which is known to be abscission specific in *Arabidopsis* (Jinn *et al.*, 2000), to study if this abscission zone specificity was also observed in rose. The expression of GUS under the *HAESA* promoter was only observed in the tip of the petals at the point of separation of the petals from the thalamus, indicating that abscission zone specificity of the *HAESA* promoter was maintained even in rose.

Discussion

Abscission and senescence of flower parts are important processes in the developmental cycle of plants. While there have been numerous studies to understand floral senescence, the molecular basis of abscission in flowers remains to be elucidated. Since abscission involves cell separation in a small specialized zone of cells, there has been a focus on the role of cell wall hydrolases that lead to floral abscission (Lashbrook *et al.*, 1994, 1998; del Campillo and Bennett, 1996; Gonzalez-Carranza *et al.*, 2002; Sane *et al.*, 2007). However, the role of several other genes that could be important during the progression of abscission within this specialized zone is still not clear. In this paper, the isolation of a cysteine protease gene, *RbCP1,* from rose petal abscission zones that is expressed during the course of abscission has been described. Its transcription appears to be responsive to ethylene and is accompanied with the appearance of a 37 kDa cysteine protease. The predicted protein encoded by *RbCP1* is a typical papain-type protease based on the presence of the C1A peptidase domain and other features such as the ERFNIN motif. Cysteine proteases are involved in a variety of developmental processes, particularly related to organ death such as nucellar degradation, aleurone layer degradation, xylogenesis etc (Schaller, 2004). They have been isolated from senescing flowers such as carnations (Jones *et al.*, 1995) and petunia (Jones *et al.*, 2005) that are ethylene-sensitive as well as *Alstroemeria* (Wagstaff *et al.*, 2002), *Hemerocallis* (Valpuesta *et al.*, 1995; Guerrero *et al.*, 1998), *Sandersonia* (Eason *et al.*, 2002), and *Gladiolus* (Arora and Singh, 2004) that are ethylene-insensitive. None, so far, have been reported to be involved in organ abscission.

Our studies reveal that transcription of *RbCP1* is rapidly up-regulated during ethylene induced abscission and the earliest increase in transcript accumulation is seen within 30 min of ethylene treatment. Ethylene regulation of *RbCP1* was also apparent from reduced expression in 1-MCP-treated samples where there is a delay in petal abscission. Sensitivity of *RbCP1* to ethylene could be seen in petals as well as leaves but not in all tissues. The activation of cysteine protease genes by ethylene has previously been observed during senescence in carnations (Jones *et al.*, 1995) and petunia (Jones *et al.*, 2005) and may be one of the means by which ethylene triggers cell death. In contrast to other tissues, there was a decrease in expression of *RbCP1* in ethylene-treated samples of the thalamus. Since the thalamus has to develop later into the fruit, it is possible that the same ethylene-related signal(s) that triggers senescence/abscission in other floral tissues may, by some as yet unknown mechanism, serve to inhibit expression of *RbCP1*

in the thalamus so as to enable fruit formation. Expression of *RbCP1* is also visible in undetached field-abscising flowers where abscission is under developmental control. Interestingly, their levels undergo a substantial increase at 36 h just prior to abscission. The late increase in expression of *RbCP1* in field-abscising flowers matches well with their time of abscission (38–45 h; Sane *et al.*, 2007). It is possible that field-abscising flowers, unlike detached flowers, undergo differential regulation of the abscission process due to a more dynamic interaction of ethylene/abscission-inducing factors with other hormones and factors of the parent plant, leading to a temporal delay in abscission. The late increase in *RbCP1* expression in these flowers indicates that, besides ethylene, other abscission-related cues may also affect *RbCP1* expression.

Another major finding of our study was the significant decrease in total protein content (down to 35–37% of control) not only during ethylene-induced abscission but also during developmental abscission. Previous studies (Abeles and Holm, 1966; Lewis and Bakshi, 1968; Valdovinos *et al.*, 1971; del Campillo and Lewis, 1992) have shown that abscission is accompanied by an increase in RNA and protein synthesis (as measured by incorporation of ^{14}C leucine in abscission zone explants and by two-dimensional gel electrophoresis) and an increase in rough endoplasmic reticulum. However, the focus in those studies was more on ethylene-induced *de novo* protein synthesis rather than total protein content. Lewis and Bakshi (1968) did detect a decrease in protein synthesis at more advanced stages of abscission. Protein synthesis would be required for *de novo* synthesis of wall hydrolases that are involved in cell separation. In rose, it may also be involved in synthesis or activation of proteases that may trigger the decrease in total protein content. Indeed, the decrease in total protein during rose petal abscission was associated with a 3–4-fold increase in total protease activity. Moreover, all the increase in protease activity could be attributed to cysteine proteases (as evident from inhibition by E-64). In-gel assays clearly revealed the abscission-related appearance of a 37 kDa cysteine protease that was present in much reduced levels in control abscission zones. Thus a large proportion (if not all) of the increase in cysteine protease activity during abscission appears be related to the appearance of the 37 kDa band. Interestingly, the size of this protease matches the size of the mature protease encoded by *RbCP1* (37.2 kDa) and its appearance during abscission follows the appearance of the transcript during abscission. Nevertheless, we cannot at present unambiguously attribute the 37 kDa band to the *RbCP1* product. Although no other proteases could be detected by in-gel assays, the assay itself is limited by its ability to detect only those proteases that have the ability to renature after denaturation on an SDS-polyacrylamide gel. Thus, the presence of other abscission-related proteases that are not able to renature cannot be ruled out entirely. Our observations of an increase in *RbCP1* expression, appearance of a 37 kDa cysteine protease, increase in total protease activity and a decrease in total protein content in abscission zones, collectively suggest that progression of abscission in rose petals may be associated with gradual cell death of the abscising cells. It has been hypothesized that abscission zone cells, especially those that line the separation point, may undergo programmed cell death (Roberts, 2000). Features of programmed cell death such as the breakdown of cellular compartmentalization have previously been observed in abscission zone cells during abscission of *Pelargonium* petals (Evensen *et al.*, 1993) while abscission in *Delphinium belladonna* was shown to be preceded by DNA degradation and chromatin condensation in whole turgid petals, although abscission zones were not studied (Yamada *et al.*, 2007). Recently, expression of the LX RNase was shown to be associated with fruit and petiole abscission in tomato (Lers *et al.*, 2005) providing further indication of the degradative processes during abscission. In fact, some cysteine protease genes such as the *SAG12* in *Arabidopsis* and its homologues in *Brassica* are known to act as developmental markers of senescence (Noh and Amasino, 1999). These data lead us to believe that signals such as ethylene that activate abscission may also activate cell death in the abscission zone and that activation of *RbCP1* and protein degradation in rose petal abscission zones may be one of the means by which this is brought about. Indeed the transcriptional activation of *RbCP1* within 30 min of ethylene treatment (at least under experimental non-physiological concentrations of ethylene) coupled with the rapid decrease in the total protein to a third of the total protein content in just half the time required for complete abscission (8 h in ethylene-treated and 24 h in naturally abscising flowers) indicate that some aspects of cell death, such as protein loss, may begin well before cell separation through hydrolysis of wall polymers is completed. The ability of *RbCP1* to respond rapidly to ethylene may not be surprising, considering the fact that abscission is highly sensitive to ethylene and high doses of ethylene can bring about complete petal abscission even within 1–2 h (Sexton *et al.*, 1983). Thus, it may be expected that at least a few components of the abscission machinery must show rapid ethylene sensitivity to be able to mount the hastened response brought about by ethylene. An alternative possibility for the role of RbCP1 could be to bring about hydrolysis of the wall-associated proteins that provide strength to the adhering cells. The 22 amino acid secretory peptide on RbCP1 (as deduced by TargetP) could direct it to the secretory pathway and target it to the cell walls for this purpose, aiding the wall hydrolases in rapidly bringing about the dissolution of the middle lamella and the progressive separation of cell walls.

Interestingly, the 2.0 kbp *RbCP1* promoter was able to drive expression of the GUS gene specifically in cells lining the point of separation of the petal from the thalamus. Expression was ethylene responsive since no expression was observed in 1-MCP-treated flowers. Analysis of the promoter revealed several known ethylene-responsive elements (ATTTCAAA and similar sequences, Itzhaki *et al.*, 1994) between 930–1550 nt upstream of the start codon that may

confer ethylene responsiveness to the promoter. Further studies with deletion of these elements may provide better information on the functionality of these elements in the *RbCP1* promoter. No expression was visible in any other part of the petal. This was surprising since *RbCP1* is expressed in several tissues including the petal. It probably indicates that the 2.0 kb promoter only contains elements to drive expression in the abscission zone with other *cis* elements for petal-related expression being present upstream of the 2.0 kbp region. The 1.6 kb *Arabidopsis HAESA* gene promoter was also used as a control for abscission zone-specific expression (Jinn *et al.*, 2000) and abscission-specific expression was observed, even in rose. The differential and reproducible expression patterns of the agro-infiltrated promoter constructs of pBI101, pBI121, and pRbCP1 and the maintenance of abscission-specific expression of pHAESA even in rose indicate that this technique may be used more widely to study gene/promoter expression in the plants of interest, in addition to heterologous model systems such as *Arabidopsis* and tobacco.

In conclusion, it is shown that petal abscission in rose is associated with the expression of an ethylene-responsive cysteine protease gene, *RbCP1,* and the appearance of a 37 kDa cysteine protease that leads to a decrease in protein content during abscission and is possibly associated with programmed cell death in the abscission zone. We believe this is the first example of a protease that has a role in organ abscission.

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