

RESEARCH PAPER

# Changes in expansin activity and gene expression during ethylene-promoted leaflet abscission in *Sambucus nigra*

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## Abstract

During ethylene-promoted leaflet abscission in *Sambucus nigra* a 7-fold increase in expansin activity was detected specifically in tissues undergoing cell separation, whilst only low levels of activity were seen in adjacent non-abscising tissues. An RT-PCR strategy was used to amplify expansin fragments from a cDNA library generated from mRNA extracted from ethylene-treated leaflet abscission-zone tissue. Two different full-length expansin-encoding cDNAs were isolated with sizes of 1190 bp and 1169 bp and named *SniExp2* and *SniExp4*, respectively. The deduced protein sequences of *SniExp2* and *SniExp4* exhibited 67% homology and comprised 256 and 249 amino acids, respectively. Both putative proteins contained signal sequences at their N terminal ends, suggesting that they were likely to encode secreted or transmembrane proteins. Northern analyses with probes specific for each gene revealed that transcripts for both of these cDNAs accumulated specifically in abscission zone tissues in response to ethylene treatment, with no expression being apparent in either freshly excised material or non-separating tissues. These data support a role for expansins in ethylene-stimulated abscission and provide evidence that specific family members contribute to the cell separation process that takes place during organ shedding.

Key words: Abscission, cell separation, cell wall, elder, ethylene, expansins, *Sambucus nigra*.

## Introduction

Abscission is the process by which organs such as leaves, flowers, or fruit are shed during the life of a plant (Sexton and Roberts, 1982), and is the consequence of the breakdown in adhesion between specifically differentiated cells located at the site of organ shedding (Roberts *et al.*, 2000, 2002). Although the precise sequence of events that brings about wall dissolution remains unclear, there is convincing evidence from biochemical and molecular genetic studies that the action of hydrolytic enzymes such as  $\beta$ -1,4-glucanase (Taylor *et al.*, 1994; Lashbrook *et al.*, 1998; Brummell *et al.*, 1999a) and polygalacturonase (Kalaitzis *et al.*, 1995; Petersen *et al.*, 1995; Gonzalez-Carranza *et al.*, 2002) contribute to the phenomenon. Moreover, the increase in activity of these enzymes, during the shedding of organs such as leaves, flowers, and fruit, is the consequence of *de novo* gene expression. By manipulating the expression of specific genes using strategies such as antisense mRNA or T-DNA gene knockouts it has been possible to determine the role of specific enzymes in cell separation. For instance, antisense tomato plants where a specific family member of  $\beta$ -1,4-glucanase has been down-regulated exhibit a delayed time-course of flower and leaf abscission (Lashbrook *et al.*, 1998). Furthermore, the time-course of floral organ abscission is delayed in *Arabidopsis* plants where an abscission-related polygalacturonase has been 'knocked out' using a T-DNA strategy (Roberts *et al.*, 2002). In both these instances, abscission was not completely prevented, suggesting either that other family members of these enzymes are critical for cell separation to take place or that additional biochemical events are necessary.

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Expansins were first characterized as proteins that directly induce the extension of isolated plant cell walls (McQueen-Mason *et al.*, 1992) and are believed to be important regulators of wall extension during plant cell growth (reviewed in Lee *et al.*, 2001; Cosgrove *et al.*, 2002; Li *et al.*, 2003). Expansins appear to operate by disrupting hydrogen bonds between cellulose microfibrils and xyloglucans that tether them to one another in plant cell walls (McQueen-Mason and Cosgrove 1994, 1995; Whitney *et al.*, 2000). As well as this role during cell growth, expansins have been shown to play an important role in fruit softening (Rose *et al.*, 1997; Brummell *et al.*, 1999b; Anjanasree and Bansal, 2003). Similarly, expansins have been shown to be expressed at the point of radicle emergence in germinating tomato seeds (Chen and Bradford, 2000) and in the micro-*pylar* endosperm of *Datura ferox* seeds in response to red light (Mella *et al.*, 2004), suggesting that they may play a general role in promoting wall dissolution. Abscission and fruit softening both involve cell wall breakdown and many of the same types of enzymes are involved in the two processes (Rose and Bennett, 1999; Rose *et al.*, 2003). Although there is some circumstantial evidence of an association between expansins and abscission (Cho and Cosgrove, 2000), no data have previously been published showing a correlation between the activity of these proteins and organ shedding.

The abscission zone of many organs typically comprises only one or two layers of cells and this restricts opportunities for extracting and assaying the activities of the enzymes and other proteins that may contribute to cell separation. By contrast, the leaflet abscission zone of *Sambucus nigra* is composed of 40–50 rows of cells providing material amenable for biochemical analysis (Taylor *et al.*, 1994). In this paper the first demonstration that abscission is associated with elevated expansin activity is reported and a characterization of two expansin genes that may contribute to the process of organ shedding is carried out.

## Materials and methods

### Plant material

*S. nigra* leaves were obtained from hedgerows in the local vicinity to the University Of Nottingham, Sutton Bonington campus. The explants, generated as described by Coupe *et al.* (1995) were incubated for 0, 12, and 24 h in the presence or absence of ethylene ( $10 \mu\text{l l}^{-1}$ ) at 24 °C with a 16/8 h light/dark period. The explants were supported in 1% (w/v) agar (Technological No. 3) in 9 cm Petri dishes (Sterilin). Potassium permanganate was used in air-treated samples to absorb any ethylene present. AZ (abscission-zone) and NZ (non-zone) tissue was harvested, immersed in liquid nitrogen, and stored at  $-70$  °C for RNA isolations.

### Expansin activity measurements

Cell wall proteins were extracted and assayed for expansin activity using a protocol based on that described by McQueen-Mason *et al.* (1992). Portions of frozen plant tissues, weighing 5 g, were homogenized in a Waring blender with 20 ml of 25 mM HEPES, 1%

polyvinylpyrrolidone (40 000 MW), 1% Triton X100, 3 mM sodium metabisulphite, 2 mM EDTA, 2 mM dithiothreitol, pH 6.8. The homogenate was collected on a 50  $\mu\text{m}$  nylon mesh and excess liquid removed by squeezing. Wall fragments were washed three times in 20 ml of the same homogenizing buffer without Triton X100. Wall fragments were retained on the nylon mesh and then resuspended in 10 ml of 1 M NaCl, 25 mM HEPES, 3 mM sodium metabisulphite, 2 mM EDTA, 2 mM dithiothreitol, pH 6.8 and left to extract for 1 h at room temperature. The salt extract was filtered through the nylon mesh, its volume measured, and proteins precipitated by the gradual addition of  $0.39 \text{ g ml}^{-1}$  of solid ammonium sulphate. After 10 min on ice, protein precipitates were recovered by centrifugation at 10 000 g for 10 min at 4 °C. The supernatant was removed and the pellets stored at  $-20$  °C until they were assayed for expansin activity. Immediately prior to expansin assays, precipitates were resuspended in 1 ml of 50 mM sodium acetate, pH 4.5 and desalted through a 5 ml column of Sephadex G25 (Amersham Bioscience) in the same solution, and brought to a final volume of 5 ml. Expansin assays were carried out in a purpose-built extensometer using a cellulose/xyloglucan composite as described by Whitney *et al.* (2000). Briefly, 2 mm wide strips of composite were placed between the clamps of the extensometer, bathed in 50 mM sodium acetate, pH 4.5 and extended by the application of a 5 g weight to the lower clamp. After 15 min extension, the bathing solution was replaced with 100  $\mu\text{l}$  of the solution of desalted cell-wall proteins. Extension rates were measured for the 10 min prior to and 10 min after protein addition. Expansin activity was calculated as the rate of extension after protein addition minus the rate prior to protein addition.

### Isolation of expansin cDNA fragments

Expansin sequences were amplified by PCR using degenerate oligonucleotides designated Exp-sense 5'-ATGGGGGGGICNTGYGGN-3' and Exp-antisense 5'-TGCCARTTYTGNCCCCAR-3' (I=deoxy inosine; N=A, T, C or G; Y=C or T; and R=A or G) designed to highly conserved amino acid domains of known plant expansins. Amplification was performed with 3  $\mu\text{l}$  ( $1 \times 10^8$  plaque-forming units  $\mu\text{l}^{-1}$ ) of a  $\lambda$ -zap *S. nigra* AZ cDNA library (Taylor *et al.*, 1994) using *Taq* polymerase according to the manufacturer's instructions (Life Technologies). Amplification conditions were 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min followed by 1 cycle of 72 °C for 10 min. Reaction products of ~480 bp were analysed and cloned using the TOPO TA cloning kit (Invitrogen Life Technologies) following the manufacturer's recommendations. PCR products were cloned from three independent PCR reactions and 10 independent plasmid inserts from each cloning exercise sequenced to identify the cloned products.

### Library screening

The *S. nigra* abscission zone cDNA library above was probed with *SniExp* fragments 1, 2, and 3 PCR products amplified from purified plasmid DNA using the degenerate oligonucleotides and conditions described above. The amplification products were separated on 1% agarose gels stained with ethidium bromide. PCR products were gel-purified and 25 ng of the PCR products were radiolabelled using the Random Primer Labeling Kit (Stratagene, Prime It II) according to the manufacturer's instructions. Unincorporated nucleotides were separated from radiolabelled DNA using a Sephadex G-50 NICK column (Amersham Biosciences) and the eluted probe was denatured by boiling for 5 min followed by cooling on ice for 5 min before adding to the hybridization mix. The probes were used to screen 200 000 recombinant plaques plated on four plates of 50 000. Replicate lifts were probed and hybridized as described by Coupe *et al.* (1993). Plaques that hybridized to *SniExp* 1, 2, and 3 probes were rescreened at lower densities. Positive phage clones were converted to pBluescript SK<sup>-</sup>, using *in vivo* excision according to the

manufacturer's instructions (Stratagene, USA). Sequence data revealed that two full-length cDNAs had been isolated representing *SniExp2* and a novel expansin cDNA termed *SniExp4*.

### Sequence analysis

Sequence data for each clone was obtained using an Applied Biosystems automated sequencer (model 373A) employing *Taq* dideoxy terminators. Sequence data obtained were compared to other sequences in the EMBL and GenBank DNA databases. Multiple sequence alignment of the *S. nigra* expansins was performed using the Clustal W program (<http://www.ebi.ac.uk/clustalw/>). Identical and similar residues were coloured using BOXSHADE ([http://www.ch.embnet.org/software/BOX\\_form.html](http://www.ch.embnet.org/software/BOX_form.html)). To determine the phylogenetic relationship of *SniExp2* and *SniExp4* to expansins with high identity from the EMBL databank and *Arabidopsis*  $\alpha$  expansins (Li *et al.*, 2002), the predicted signal peptides were removed (<http://www.cbs.dtu.dk/services/SignalP>), a multiple sequence alignment performed using the MegAlign Clustal W (PAM 250 residue weight table) program of DNASTar, and a phylogenetic tree constructed using the same program.

### RNA isolation and northern analysis

Total RNA was extracted from leaf *S. nigra* AZ and NZ tissue by grinding in liquid nitrogen followed by the addition of 900  $\mu\text{l g}^{-1}$  extraction buffer (25 mM TRIS-HCl pH 8.0, 25 mM EDTA, 75 mM NaCl, and 1% SDS (w/v)). As the tissue and extraction buffer started to thaw, 900  $\mu\text{l}$  of phenol 2:1 (v/v) CIA (chloroform 24:1 isoamyl alcohol v/v) was added, the mixture was ground until melted and vortexed briefly. Following centrifugation at 14 000 rpm for 15 min the aqueous phase was added to an equal volume of phenol 1:1 CIA (v/v), vortexed briefly and centrifuged again at the same speed for 10 min. The aqueous phase was then extracted with an equal volume of CIA and spun at 14 000 rpm for 10 min. Drops of 10 M LiCl were added to the aqueous phase to reach a final concentration of 2 M, followed by vortexing to prevent co-precipitation of DNA and polysaccharides. After overnight precipitation at  $-20^\circ\text{C}$ , the RNA was recovered by centrifuging at 14 000 rpm for 10 min and then washed with ice-cold 2 M LiCl. The pellet was dissolved in 100  $\mu\text{l}$  of water and ethanol-precipitated with 0.1 vol. 3 M sodium acetate pH 5.2 and 2.5 vols of absolute ethanol. The RNA was recovered by centrifugation at 10 000 rpm for 15 min and resuspended in an appropriate volume of water. Ten units of RNase-free DNase (Promega) were added and the solution incubated for 15 min at  $37^\circ\text{C}$  after which the DNase protein was removed using the phenol, CIA method above. The concentration and yield of RNA extracted was determined using a UV/visible spectrophotometer (Cecil, CE 2041).

Northern analysis was carried out using 10  $\mu\text{g}$  total RNA per lane. Each sample was mixed with an equal volume of  $2\times$  sample buffer (50% v/v deionized formamide (Sigma), 16.5% v/v formaldehyde (37% conc., Sigma), 10 mM EDTA pH 8.0 (BDH), 0.2 M  $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$  pH 6.5, and 0.2  $\mu\text{g ml}^{-1}$  ethidium bromide (Sigma)). The samples were denatured at  $65^\circ\text{C}$  for 15 min, cooled on ice and 4  $\mu\text{l}$  of loading buffer (0.025% (w/v) bromophenol blue, and 8% (w/v) sucrose) added. The samples were then electrophoresed in a 1% (w/v) agarose gel containing 3% (v/v) formaldehyde, and 10 mM  $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$  pH 6.5 at 75 V, with constant buffer recirculation.

Agarose gels containing RNA samples were capillary-blotted onto Hybond  $\text{N}^+$  transfer membrane (Amersham) according to the manufacturer's instructions. The RNA was UV cross-linked to the membrane using a Stratalinker (Stratagene, UK) machine (120 000  $\mu\text{J}$  of UV energy for 30 s). DNA probes used were PCR products amplified from plasmid DNA. Probes for *SniExp2* and *SniExp4* were generated using the 3' UTRs of the full-length clones. The following gene-specific primer pairs were used to amplify part of the untranslated region (UTR) for both *SniExp2* and *SniExp4*; *SniExp2* UTR Forward

5'-GTAGTGGGTCTGTGTGGATTGC-3' and *SniExp2* UTR Reverse 5'-GTAGATTAGTACAATGCATCGC-3', and *SniExp4* UTR Forward 5'-CTATTGCTGTGGTGGCTAGC-3' and *SniExp4* UTR Reverse 5'-TAAACAACATGCCCCAGTG-3'. All PCR products were purified by agarose gel electrophoresis and labelled as described above.

RNA blots were prehybridized at  $65^\circ\text{C}$  in 50% (v/v) deionized formamide (Sigma), 1% SDS (w/v), 1 M NaCl, 10% (v/v) dextran sulphate, and 100  $\mu\text{g ml}^{-1}$  single-stranded salmon sperm DNA for a minimum of 3 h. After the period of prehybridization, a radio-labelled DNA probe was added and allowed to hybridize overnight ( $>16$  h) at  $65^\circ\text{C}$ . After hybridization the membranes were washed at  $65^\circ\text{C}$  in  $2\times$  SSC (0.3 M NaCl, and 15 mM M tri-sodium citrate), 0.1% SDS (w/v) for 20 min,  $1\times$  SSC, 0.1% SDS (w/v) for 20 min, and finally 0.1% SDS (w/v), 0.1% SDS (w/v) for 20 min. Membranes were exposed to Kodak X-OMAT.AR film at  $-70^\circ\text{C}$  overnight.

## Results

### Changes in expansin activity during leaflet abscission

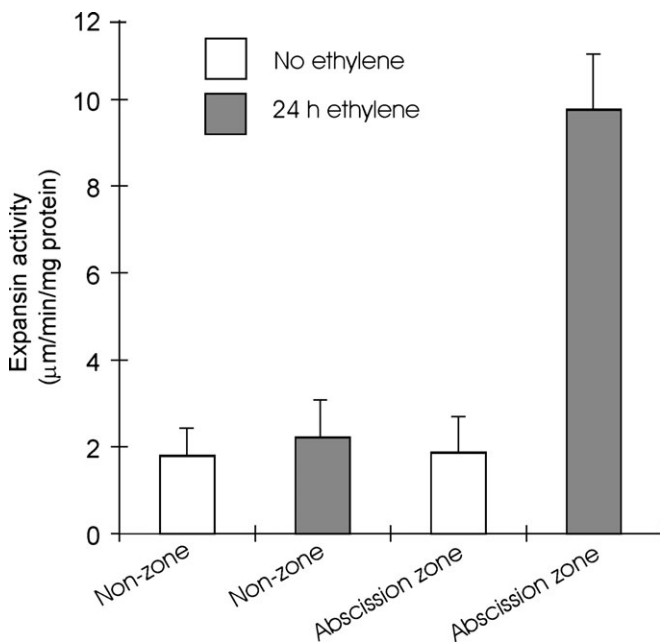
In order to investigate the potential role of expansins in abscission, cell walls from abscission zones were analysed for expansin activity before and after the induction of abscission by ethylene treatment. As a control for this experiment non-abscission zone material was harvested from neighbouring parts of the rachis. Both freshly excised abscission zone and non-zone tissues were found to possess a similar magnitude of low-level background expansin activity (Fig. 1). Exposure to ethylene for 24 h resulted in cell separation in nearly 100% of the explants, but no obvious effects on non-zone tissue were detectable. Following induction of abscission by exposure to ethylene, expansin activity rose slightly in the non-zone tissues but showed a greater than 7-fold increase in the abscission zone walls. Thus, there is a strong and tissue-specific induction of expansin activity in response to ethylene.

### Isolation of abscission-related expansin cDNAs from *S. nigra*

To investigate this induction of expansin activity further, the expression of expansin encoding genes in the abscission zone and non-zone tissues in response to ethylene was examined. Using degenerate primers designed to highly conserved regions of known plant expansins, a PCR product of expected size was amplified from a *S. nigra* ethylene-treated leaflet AZ cDNA library (Taylor *et al.*, 1994). Products from three independent PCR reactions were cloned and 10 clones from each sequenced, revealing the presence of three distinct putative expansin sequences, which were named *SniExp1*–3 (*Sambucus nigra* Expansins 1–3).

The PCR products were used as probes to screen for full-length clones from the abscission zone cDNA library. Sequence analysis of several positive clones revealed that an equivalent *SniExp2* full-length clone had been isolated, along with a novel *SniExp4* cDNA. The *SniExp2* cDNA clone was 1190 bp in length, contained a 3' UTR of 392 bp,

and an open reading frame (ORF) of 768 bp. The *SniExp4* cDNA clone was 1169 bp in length, with an ORF of 747 bp, and a 3' UTR of 388 bp. The predicted primary translation products encoded by the *SniExp2* and *SniExp4* cDNAs exhibited 67% sequence homology and comprised 256 amino acids and 249 amino acids, respectively. Full-length



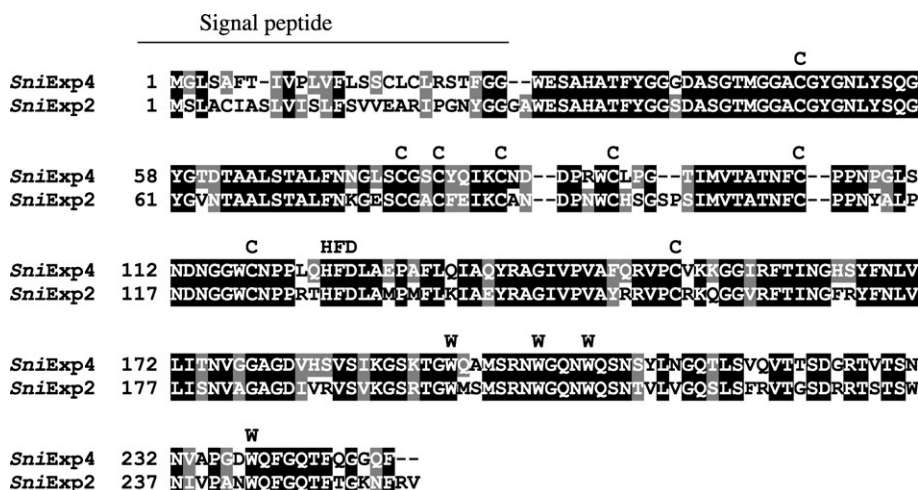
**Fig. 1.** Expansin activity extracted from *Sambucus nigra* abscission-zone and non-zone tissues undergoing ethylene-induced leaflet abscission. Leaflet abscission zones and neighbouring non-abscission-zone tissues were incubated in the presence or absence of ethylene for 24 h. Cell wall proteins were extracted from these tissues and assayed for expansin activity on a constant-load extensometer. Overall experiments were repeated three times with similar results. The results presented are averages and standard errors from six separate measurements.

cDNA clones of the *SniExp1* and *SniExp3* genes were not obtained.

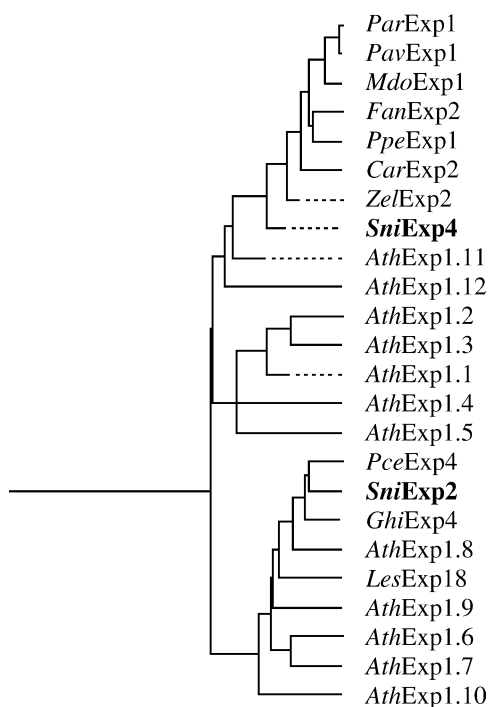
Each of the two predicted expansin polypeptides (*SniExp2* and 4) contains conserved residues that are typical of expansins (Shcherban *et al.*, 1995). These are illustrated in Fig. 2 and include a series of eight cystenyl residues found in the C-terminal half of the predicted mature proteins and the central 'HFD' box that is speculated to form part of the active site. In addition, the predicted proteins possess the four conserved tryptophanyl residues in their C-terminal half that are thought to be involved in binding to cellulose. The amino terminal regions of both expansins also encode short signal peptides for uptake into the secretory pathway as predicted by SignalP V1.1.

#### Phylogenetic analysis of isolated *S. nigra* expansins

A phylogenetic tree was generated from an alignment of the *S. nigra* deduced amino acid sequences with all annotated  $\alpha$ -expansins from *Arabidopsis* (Li *et al.*, 2002), plus representatives from the SwissProt EMBL databank (Fig. 3). Interestingly, *SniExp4* is part of a small apparent clade with two *Arabidopsis* expansins (*AthExp1.11* and 1.12) along with a number of expansins from either ripening fruit, or differentiating tracheary elements. The *SniExp4* predicted protein had highest similarity (91%) to a *Zinnia elegans* expansin gene *ZelExp2* that encodes an mRNA localized in xylem cells (Im *et al.*, 2000) and was also closely related to a clade of five expansins (>83% identity) isolated from ripening fruits of: apple, *Malus domestica* *MdoExp1*; apricot, *Prunus armeniaca* *ParExp1*; peach, *Prunus persica* *PpeExp1* (Hayama *et al.*, 2000); strawberry (*Fragaria ananassa*: *FanExp2*, Civello *et al.*, 1999); and sweet cherry, *Prunus avium* *PavExp1*. The two published expansins of this clade: the peach *PpeExp1* gene (Hayama *et al.*, 2000), and the strawberry *FanExp2* gene (Civello *et al.*, 1999)



**Fig. 2.** Alignment of the predicted protein sequences of two full-length expansin cDNAs isolated from a leaflet abscission zone cDNA library. Key conserved residues defining expansin sequences are indicated above the alignment. Shaded box indicates a region of sequence identity and similarity between the sequences.



**Fig. 3.** Phylogenetic analysis of abscission-related expansins, *SniExp* 2 and 4, as well as the highest homology sequences to *SniExp* 2 and 4 from the EMBL database. The tree was generated using the DNASTar MegAlign program from a Clustal W output, with a PAM 250 residue weight table, using the same program. Sequences include annotated *Arabidopsis*  $\alpha$ -expansins (Li *et al.*, 2002) as well as a number of high identity expansins from the SwissProt EMBL databank (including accession numbers); *Cicer arietinum*: CarExp2 (Q9FNT0), *Fragaria ananassa*: FanExp2 (Q9SBT1), *Gossypium hirsutum*: GhiExp4 (Q8LKK0), *Lycopersicon esculentum*: LesExp18 (O81999), *Malus domestica*: MdoExp1 (Q8RVK6), *Prunus armeniaca*: ParExp1 (O82093), *Prunus avium*: PavExp1 (Q9FUM3), *Prunus cerasus*: PceExp4 (Q93XP0), *Prunus persica*: PpeExp1 (Q9FS30), and *Zinnia elegans*: ZelExp2 (Q9LLB2).

are associated with cell wall disassembly during ripening. Sequence comparison of the *SniExp*2 proposed protein revealed highest identity (92%) to a cherry (*Prunus cerasus*) expansin *PceExp*4 expressed during fruit ripening, and also a tomato (*Lycopersicon esculentum*) expansin *LesExp*18 (Fleming *et al.*, 1997), which is expressed early in leaf primordium initiation.

#### Accumulation of *S. nigra* expansin transcripts during ethylene-promoted leaflet abscission

To determine the temporal and spatial expression patterns of the *SniExp*2 and 4 genes in the presence and absence of ethylene, northern analysis was performed on RNA extracted from abscission-zone and non-abscission-zone tissues before and after ethylene treatment. Figure 4 shows that transcripts for both expansins accumulated in abscission-zone tissues between 12 h and 24 h after ethylene treatment. Expression was clearly specific to the abscission-zone tissue and this observation is compatible with a role for these expansins in the cell-separation process based on the

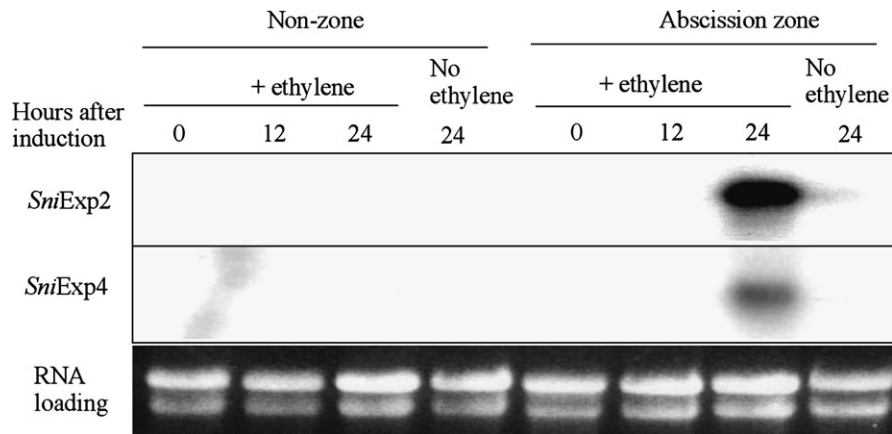
time-course for ethylene initiation of abscission and the appearance of other abscission-related transcripts in these tissues (Taylor *et al.*, 1994; Coupe *et al.*, 1997). It is intriguing that neither expansin is expressed in fresh or non-separating material, even though expansin activity could be detected in these tissues, as shown in Fig. 1.

#### Discussion

Organ shedding involves the co-ordinated breakdown of the walls of specific layers of cells in the abscission zones and a number of wall-degrading activities have been implicated in this process. It has been shown here that not only is expansin activity greatly elevated in the abscission zones of *Sambucus nigra* during ethylene-induced leaflet abscission, but also that the transcript abundance of two expansin genes is dramatically increased in a tissue-specific manner in response to ethylene treatment.

Expansin activity has generally been associated with the process of wall extension during cell growth (Li *et al.*, 2003). It has, however, become clear that expansins also make a significant contribution to the process of fruit softening, which involves wall breakdown, rather than expansion. It has been observed that fruit softening and abscission share a number of features in common (Rose *et al.*, 2003). For example, both processes involve cell wall breakdown and, in many cases, the processes underlying them are initiated by ethylene. In addition, both fruit-softening and abscission have been shown to involve a similar group of wall-degrading enzymes including, for example, polygalacturonase (Kalaitzis *et al.*, 1995) and endoglucanases (Lashbrook *et al.* 1998). Given the role of expansins in fruit-softening, it was hypothesized that these proteins might make a similar contribution to the process of abscission, and indeed it was found that expansin activity was dramatically increased in abscission zones during ethylene-induced abscission.

Two different full-length expansin cDNAs were isolated from a *S. nigra* leaflet abscission zone cDNA library and both transcripts accumulated substantially in response to ethylene with expression being restricted to abscission zones. These data represent compelling evidence for a role of expansins in abscission. A previous report by Cho and Cosgrove (2000) described how a fusion of a reporter gene to the upstream region of the *Arabidopsis* expansin *AtExp*10 resulted in the expression of GUS specifically at the base of leaf petioles and silique pedicels where they join the inflorescence stem. The authors showed that pedicels from plants overexpressing *AtExp*10 were more likely to break at the base when pressure was applied to them than were pedicels from wild-type plants. The authors proposed that this indicated a role for *AtExp*10 in abscission. Whilst these data are interesting, their interpretation is difficult because, as the authors point out, there is no recognizable abscission zone at



**Fig. 4.** Northern analysis of *SniExp 2* and *SniExp 4* during ethylene-promoted leaflet abscission. Total RNA was isolated from rachis (Non-zone) and leaflet abscission zone (Abscission zone) tissues after ethylene treatment ( $10 \mu\text{l l}^{-1}$ ) for 0, 12, or 24 h. RNA from tissues incubated in an ethylene free atmosphere for 24 h ( $-\text{C}_2\text{H}_4$ ) was included as a control.

the base of *Arabidopsis* pedicels and shedding does not naturally take place at this point. By contrast, the data presented here indicate an unambiguous involvement of expansins in abscission.

It is intriguing that both expansin genes should show the same expression pattern and that neither of them can be detected by northern analysis in fresh or non-separating material even though both these tissues have measurable expansin activity. However, it is notable that expansins are encoded by extensive multigene families with 38 expansin-like open reading frames in the *Arabidopsis* genome and 80 in that of rice (Li *et al.*, 2002, 2003) and it is likely that other family members account for the activity seen in freshly excised *S. nigra* material. In general, most tissues examined in plants appear to express several different expansin isoforms. For example, Lee and Kende (2002) reported that eight different expansin genes showed very similar expression patterns in rice leaves. The biochemistry of expansin action remains poorly understood with only one isoform (cucumber expansin 1) having been characterized to any extent at the biochemical level (McQueen-Mason and Cosgrove, 1994, 1995; Whitney *et al.*, 2000). It has been shown that  $\alpha$ - and  $\beta$ -expansins work on different types of cell wall to one another (Cosgrove *et al.*, 1997) and it is assumed that this reflects different specificity for wall polymers. It is not yet clear, however, if different  $\alpha$ -expansins (such as those described here) may work on different components of the cell wall, but such substrate specificity might explain the presence of more than one expansin isoform in these tissues.

The biochemical role of expansins in cell wall breakdown has not yet been resolved. Rose *et al.* (1997) showed that an expansin was abundantly expressed during tomato fruit softening. Brummell *et al.* (1999b) subsequently showed that the fruit of transgenic plants overexpressing this expansin soften more rapidly than those of wild-type plants and that softening was reduced in antisense plants

with reduced expansin expression. Although the impact of altered expansin levels on fruit-softening is evident, the molecular basis of these effects remains obscure. It has been noted that expansin proteins share structural features with family 45 endoglucanases and probably share a common ancestral origin (Cosgrove, 1999). Despite this structural resemblance, no hydrolytic activity has yet been associated with expansins themselves, although they have been shown to synergize the hydrolytic activity of a *Trichoderma* cellulase on crystalline cellulose (Cosgrove, 1999), suggesting that they may increase disorder in the cellulose crystals making the glucan chains more susceptible to hydrolysis. This might suggest a simple scenario whereby expansins play a role in abscission by promoting the degradation of cellulosic polymers in the wall. However, whilst decreased levels of expansins in transgenic tomatoes led to significant reductions in fruit-softening and this was accompanied by distinct changes in wall polymer metabolism (Brummell *et al.*, 1999b), these changes were complex, and, for example, involved a large decrease in the depolymerization of wall pectins, and it was concluded that the effects seen were due both to a direct decrease in expansin-catalysed wall loosening and indirect effects on wall metabolism. Whilst it is now clear that expansins play a role in tissues undergoing cell wall breakdown, the exact nature of this role remains to be determined.

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