Characterization of an mRNA encoding a polygalacturonase expressed during pod development in oilseed rape (Brassica napus L.)

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Abstract

Pod shatter in oilseed rape is associated with the degradation of the pectin-rich middle lamella at the site of dehiscence. It has been reported that, accompanying pod development, there is an increase in the activity of polygalacturonase (EC 3.2.1.15) and that this rise is restricted to the tissue undergoing cell separation. Using a PCR strategy a fragment of a polygalacturonase encoding an mRNA that is up-regulated specifically in the dehiscence zone tissue during pod development has been cloned. A full length clone (SAC66) complementary to this mRNA has been isolated from a dehiscence zone cDNA library and sequenced. The mRNA encoded by SAC66 shares significant amino acid homology with endopolygalacturonases from fruit of Actinidia delicosa and Lycopersicon esculentum. The transcript size of the SAC66 mRNA is 1.7 kb. Northern analysis has revealed that expression of SAC66 mRNA increases at 30 d after anthesis (DAA), reaching a plateau at 45 DAA, and that the up-regulation is restricted to the site where dehiscence takes place.

Key words: Brassica napus, oilseed rape, dehiscence zone, pod shatter, cell separation, polygalacturonase.

Introduction

Winter oilseed rape (Brassica napus) is grown as an important source of seed for vegetable oils. Seeds are contained in siliques which, at maturity, are susceptible to dehiscence. This process of shatter, as it is commonly termed, is of agronomical importance because it can result in the premature shedding of seed before the crop can be harvested. It has been reported that seed losses can be as great as 50% (1.6 t ha\(^{-1}\)) under adverse weather conditions (Macleod, 1981).

The process of abscission that causes the shedding of a range of plant parts, including leaves, flowers and fruit (Sexton and Roberts, 1982) shares a number of features in common with pod dehiscence. Both processes occur at precise sites and involve coordinated wall breakdown and cell separation. In pods the site of separation is known as the dehiscence zone (DZ) and this has been shown to comprise a row of thin-walled parenchyma cells which remain un lignified throughout pod development (Meakin and Roberts, 1990a). Separation of the parenchyma cells has been attributable to the degradation of the middle lamella (Meakin and Roberts, 1990a).

Associated with abscission is an increase in the activity of several hydrolytic enzymes including \(\beta\)-1,4-glucanase (cellulase, EC 3.1.2.4) and polygalacturonase (PG, EC 3.2.1.15) (Taylor et al., 1990, 1993; Webb et al., 1993). A localized increase in \(\beta\)-1,4-glucanase activity has also been reported prior to the onset of dehiscence in oilseed rape (Meakin and Roberts, 1990b). Although these workers could find no correlation between the activity of PG and pod development (Meakin and Roberts, 1990b), an association was observed between the temporal and
spatial activity of the enzyme and dehiscence in pods infected with *Dasineura brassicae* (pod midge) (Meakin and Roberts, 1991). Moreover, in a recent study (Coupe, 1993), evidence of a role for PG during pod dehiscence was obtained.

The approach here to ascertain the role of PG in pod dehiscence has focused on a molecular rather than biochemical study. In this paper the isolation and characterization of PG mRNA whose expression, determined by Northern blot analysis, is spatially and temporally correlated with pod development, is reported. The possible role of this enzyme during pod dehiscence is discussed.

**Materials and methods**

*Plant material and growth conditions*

*B. napus* cv. Rafal plants were grown as described by Meakin and Roberts (1990a) with modifications detailed by Coupe et al. (1993). Flowers were tagged at anthesis to determine the age of each pod in days after anthesis (DAA). Tissue was harvested at various stages after growth and expansion of the pod was completed. These times ranged from 20 DAA until the pods were completely dry and undergoing dehiscence (50 DAA). The dehiscence zone (DZ) was excised from the non-dehiscence zone (NON-Z) material and seed using a scalpel blade as described by Meakin and Roberts (1990b) and Coupe (1993). Material was placed in liquid N₂ immediately after harvesting and stored at −70°C until required.

*RNA extraction*

Total RNA was extracted from DZ and NON-Z material of pods 20, 30, 35, 40, 45, and 50 DAA using a polysomal method as described previously (Coupe et al., 1993).

**Isolation of PG cDNA using a PCR strategy**

A PCR strategy using degenerate primers was employed to amplify PG-specific DNA from RNA extracted from the DZ of *B. napus* pods at 40, 45 and 50 DAA. Multiple sequence alignments of polygalacturonase amino acid sequences, (accession numbers in brackets), deduced from mRNAs isolated from fruit tissue—*Persea americana* (Q02096), *Actinidia delicosa* (P33336) and *Lycope-rencsis esculentum* (P05117); pollen tissue—*Brassica napus* (P33337), *Nicotiana tabacum* (Q03967), *Oenothera organana* (P24548) and *Zea mays* (P26216); and flower tissue—*A rabidopsis thaliana* (X72291) were compared. Two highly conserved regions were identified, PNTDG (P1) and GPGHG (P3), located approximately 246 and 290 amino acid residues from the 3' end of *Lycope-rincsis esculentum* protein sequence. All sequences had the GPGHG motif, but *A. thaliana* and *O. organana* lacked the proline residue in the PNTDG sequence. All sequences had the GPGHG motif, but *O. organana* lacked the proline residue in the PNTDG sequence. To normalize the interpretation of the sequence and to facilitate PCR amplification, the codon GGATCCCTCGAG T was inserted at the 5' end of the sequence.

To amplify PG mRNA whose expression, determined by Northern blot analysis, is spatially and temporally correlated with pod development, is reported. The possible role of this enzyme during pod dehiscence is discussed.

Northern blot analysis of RNA

Northern analysis was carried out using a formamide/formaldehyde denaturing system. Total RNA (10 μg of each sample) was resuspended in 5 μl sterile water and an equal volume of 2× sample buffer was added. Sample buffer (2×) contained 50% (v/v) deionized formamide (Sigma), 16.5% (v/v) formaldehyde (37% conc., Sigma), 0.01 M EDTA pH 8.0 (BDH), 0.2 M Na₂HPO₄/NaH₂PO₄ pH 6.5, 0.2 mg ml⁻¹ ethidium bromide (Sigma). The samples were denatured at 65°C for 15 min and cooled on ice. Loading buffer was added at 0.2× sample volume and the samples separated on a 1% agarose, 3% formaldehyde, 10 mM Na₂HPO₄/NaH₂PO₄ pH 6.5 gel. The RNA was transferred on to a Genescreen membrane (DuPont) by capillary transfer using 0.025 M Na₂HPO₄/NaH₂PO₄ (pH 6.5) transfer buffer and RNA UV cross-linked to the membrane using a Stratalinker (Stratagene, UK) machine. The membrane was hybridized with a radiolabelled RNA probe of the antisense strand generated from 0.5 μg of Eco RI linearized SAC66 using T7 RNA polymerase (Promega protocols and applications guide, 1991). Unincorporated label was removed by Sephadex G-50 NICK column (Pharmacia Biotech). The blot was hybridized at 65°C in 50% deionized formamide (Sigma), 1% SDS, 1 M NaCl, 10% dextran sulphate, 100 μg ml⁻¹ single stranded salmon sperm DNA.
The first round PCR reaction using the degenerate oligonucleotides P1 and OG2 amplified a smear of products in the range 750–1000 bp. Nested PCR of these DNA fragments using P1 and P3 generated a band of approximately 150 bp which on cloning and sequencing showed close homology with polygalacturonases in the EMBL database. This cloned PCR fragment was used to screen a cDNA clone designated pSAC66. Both strands of the pSAC66 cDNA insert were sequenced. The cDNA is 1657 bp in length and contains one open reading frame (ORF) of 1299 nucleotides. The initiation codon is located at position 145 and the poly(A) tail encompasses 18 nucleotides PI and OG2 amplified a smear of products at position 145 and the poly (A) tail encompasses 18(A) nucleotides PI and OG2 amplified a smear of products at position 145 and the poly (A) tail encompasses 18(A).

The expression of SAC66 was monitored in total RNA extracted from dehiscence zone (DZ) or non-dehiscence-zone (NON-Z) tissue at different stages of pod development from 20–50 DAA. From 30 DAA the expression of SAC66 mRNA increased steadily in the DZ tissue and reached a plateau by 45 DAA. In contrast, only a low level of SAC66 transcript in total RNA populations extracted from dehiscence zone (DZ) or non-dehiscence-zone (NON-Z) tissue at different stages of pod development.
level of hybridization of SAC66 RNA was observed in NON-Z tissue throughout pod development and the size of this hybridizing transcript was only 1 kb. A similar low level of hybridization to this transcript could be seen in leaf (L) and seed (S) tissue.

Discussion

Although it had been reported previously that no correlation was apparent between pod development and PG activity (Meakin and Roberts, 1990b), recent studies have revealed that an increase in the activity of the enzyme can be detected at the site of cell separation immediately prior to pod dehiscence (Coupe, 1993). As this discrepancy in observations might, in part, be a consequence of the extraction and assay of the enzyme, it was decided to study the expression of PG mRNA during the time-course of pod growth and differentiation in an attempt to ascertain whether this enzyme contributed to the cell separation process.

In order to isolate PG cDNAs from our dehiscence zone library, a PCR strategy was employed using degenerate primers based on the conserved sequences of PG enzymes within the databases. With the aid of a nested PCR approach a PG fragment was cloned successfully and used to isolate the equivalent full length cDNA, termed pSAC66, from the library. The transcript encoded by SAC66 exhibited closest homology to PGs from tomato (Grierson et al., 1986) and kiwifruit (Atkinson and Gardner, unpublished). Based on the length of its amino-terminal domain and the conserved motif F-G-A-K-G-D-G within this domain at amino acids 74–80, it is likely to act as an endoPG (Tebutt et al., 1994). Studies of the phylogenetic origin of the SAC66 mRNA indicates that it is more closely related to the fruit PGs than to the PG previously isolated from B. napus pollen.

The demonstration that the expression of SAC66 mRNA increases specifically in the DZ tissue as pod development progresses suggests that the peptide may play some role in the time-course of dehiscence. An increase in the activity of PG could contribute to the breakdown of the middle lamella that has been shown to accompany cell separation in the DZ (Meakin and Roberts, 1990a) and may be responsible for the loss of cell wall pectins reported to take place at the site of dehiscence (Josefsson, 1968). The solubilization of polyuronides is also seen at the site of leaf abscission and it has been proposed that this is brought about by elevated PG activity (Webb et al., 1993).

Northern analysis suggests that an mRNA exhibiting some homology to SAC66 is expressed in NON-Z tissue, leaves and seeds. The transcript of this mRNA is significantly smaller than SAC66, being only 1 kb and from its size would not appear to encode the PG identified from B. napus pollen (Robert et al., 1993). The expression of the 1 kb RNA transcript is maintained at a relatively constant level throughout pod development and further work will be necessary in order to determine whether it encodes an additional PG.

Genomic Southern analysis indicates that SAC66 may be encoded by a small family of genes (data not shown). However, as B. napus is an amphidiploid of B. oleracea...
and *B. rapa* its genome is likely to contain at least two copies of any gene, one from each parental genome (UN, 1935).

Studies of the molecular biology of pod development have resulted in the identification of two other gene products in addition to SAC66 whose spatial and temporal patterns of expression are closely correlated with the dehiscence process (Coupe *et al.*, 1993, 1994). Work is currently being undertaken to determine whether the manipulation of expression of these proteins affects the timing or capacity of a *B. napus* plant to undergo shatter.

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**References**


