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A Putative Proline-rich Protein of *B. napus*

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ABSTRACT

Proline-rich proteins are among the major protein components of plant cell walls. So far, two different proline-rich cell wall proteins have been described in *Brassica napus*. This paper reports a study on expression and sequence analysis of a novel class of a proline-rich putative protein, tentatively designated Ae4. The largest ORF of Ae4 encodes 166 amino acid residues without the start and stop codons. Ae4 is a partial length cDNA. The Ae4 gene expression was investigated and the results demonstrate that it accumulates in all vegetative tissues tested as well as in the embryogenic culture of *Brassica napus*. However, expression of Ae4 was undetectable in the non-embryogenic and cytokinin-treated embryogenic tissues. These results indicate that the Ae4 gene might play a role in somatic embryo formation.

Keywords: Proline-rich protein, PRP, *Brassica napus*, oilseed rape, somatic embryogenesis

INTRODUCTION

Proline-rich proteins (PRPs) are one class of structural cell wall protein members (Showalter, 1993). All the PRPs are characterised by the repeating occurrence of Pro-Pro repeats contained within a variety of larger repeat units. These proteins lack the SerPro4 repetitive element defined for extensins (Jose-Estanyol and Puigdomenech, 2000). The most extensively studied PRPs are from soybean that contains the PPVYK motif or variations (Hong *et al.*, 1987, 1989; Datta *et al.*, 1989; Wyatt *et al.*, 1992). Members of the PRP gene family were shown to be developmentally regulated and their expression tissue/organ specific (Hong *et al.*, 1989; Lindstrom and Vodkin 1991; Jose-Estanyol *et al.*, 1992). In general, PRPs are thought to have a structural role in the cell wall (Cassab and Varner, 1988). They have been also implicated in plant defence reactions (Chen and Varner, 1985; Ebener *et al.*, 1993), nodule morphogenesis (Franssen *et al.*, 1987; Wilson *et al.*, 1994), and are expressed during somatic embryogenesis (Aleith and Reichter, 1990; Gyorgyey *et al.*, 1997; Yasuda *et al.*, 2001).

To date, two different proline-rich cell wall proteins have been described in *Brassica napus* (*B. napus*). In this paper, we report the sequence and expression analysis of an additional proline-rich putative protein, tentatively designated as *Ae*4. The amino acid sequence of *Ae*4 has been deduced from the nucleic acid sequence of a copy DNA (cDNA), isolated previously from a subtracted library of *B. napus* embryogenic culture (Namasivayam *et al.,* 2006b).

MATERIALS AND METHODS

Sources of Plant Materials

Plants of *Brassica napus* ssp. *oleifera* cv. Primor were grown from seeds, in pots with soil in the Botanic Garden, Cambridge. Sources and preparation of plant materials for the pre-embryogenic, mature embryogenic and non-embryogenic *Brassica napus* ssp. *oleifera* cv. Primor culture was as described in Namasivayam *et al.* (2006a,b). The cytokinin-treated embryogenic tissue was generated from hypocotyls of embryoids grown for 20 days on MS media containing 10^{-4} M kinetin, 2% (w/v) sucrose and

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0.8% (w/v) agar. Various organs/tissues such as young leaves, stem, buds, flowers, siliques, roots, stamens, carpels, petals and sepals from mature *B. napus* plants were harvested, immediately frozen in liquid nitrogen and stored at–80°C until isolation of total RNA.

Sequence Analysis

The *Ae*4 cDNA sequence has been submitted to the GenBank under the accession number AY570239. Sequence analysis was carried out using BLAST 2.0 (Basic Local Alignment Search Tool; Altschul *et al.*, 1997), accessible from the internet (http://www.ncbi.nlm.nih.gov/BLAST). Alignments of the protein sequence with several closely related genes was carried out using the CLUSTAL W program from the Biology Workbench version 3.2, accessible from the internet (http://biowb.sdsc.edu/CGI/BW.cgi). Other sequence analyses were performed using Biology Workbench version 3.2 to compute molecular weight (MW), hydrophobicity and isoelectric point (pI) determination.

Total RNA Isolation

Total RNA from various frozen tissues/organs of the mature plant and tissue culture materials were extracted using the acid guanidium thiocyanate-phenol-chloroform extraction method described by Chomczynski & Sacchi (1987). The concentration of RNA in each sample was determined spectrophotometrically (Sambrook *et al.*, 1989).

Northern Blot Analysis

Equal amounts of total RNA (10 (g per lane) were resolved on 1.3% (w/v) agarose-formaldehyde denaturing gel and blotted onto Hybond™–XL nylon membrane (Amersham Biosciences). Hybridisation was carried out at 65°C using standard techniques (Sambrook et al., 1989). The entire Ae4 sequence was used as probe labelled with [^{32}P-α]-dCTP using the Prime-IT® II Random Primer Labeling Kit (Stratagene). Washes were carried out at room temperature in the first wash buffer (40 mM sodium phosphate pH 7.2, 1% (w/v) SDS and 1 mM EDTA) for 10 min and followed by second wash in 40 mM sodium phosphate pH 7.2, 5% (w/v) sodium dodecyl sulphate (SDS) and 1 mM ethylenediaminetetraacetic acid (EDTA) at 65°C for 15 min. The hybridisation signals were captured by a Phosphorimager Typhoon 8600 (Amersham Pharmacia Biotech). After removal of probe, the same blot was hybridised with radiolabelled Arabidopsis *Actin*2/7 cDNA probe as a loading control.

RT-PCR

Equal amounts of DNase-treated total RNA (200 ng) from each tissue sample was added individually to a sterile 0.2 ml polymerase chain reaction (PCR) tube and the volume adjusted to 13.5 µl with DEPC-treated sterile deionised water (SDW). Oligo (dT_{18}) (1 µl of 20 pmoles/µl) was added to the tube and the reaction mix incubated for 10 min at 70°C. Following brief centrifugation, the following reagents were added: 4 µl 5 x first strand buffer (Promega), 0.5 µl 'RNase Out' ribonuclease inhibitor (40 U/µl) (Invitrogen), 0.5 µl 10 mM Bioline dNTPs mix and 0.5 µl MMLV-RT RNase H minus (200 U/µl) (Promega) and incubated at 37°C for 1 h. Later, the reaction mix was heat deactivated before using for PCR reactions. PCR reactions were performed in 12.5 µl reactions with the following components: 2 µl of the RT product, 1 x Bioline PCR buffer (Mg^{2+}- free), 1.5 mM $MgCl_2$, 0.4 mM dNTP mix, 2.5 pmoles of forward primer (5′ GGACTATAAATTGGTGTTGGAGGTTTCA 3′) and reverse primer (5′- TATTTATAGT CCTCCCGTAATGCCA - 3() respectively, and 1.5 U BioTaq DNA polymerase. An internal control was prepared using actin2 primers (forward primer: 5′-CCATTCTTGCTTCCCTCAG-3′ and reverse primer: 5(-GACGTAAGTAAAAACCCAG-3′) and containing all the components as above to test for equal loading of the template. Also, a negative control without template was included. Amplification was performed as follows: 95°C for 3 min; followed by 35 cycles at 94°C for 30 s, 65°C for 1 min, 72°C for 1 min; and a final extension at 72°C for 3 min. The annealing temperature used for *Actin*2 was 60°C. Reverse transcription polymerase chain reaction (RT-PCR) products were separated on a 2% (w/v) agarose gel and the gel was photographed. The agarose gel containing the PCR amplified products was blotted (Southern, 1975) and hybridised with a labelled specific probe (*Ae*4 cDNA).

RESULTS

Sequence Analysis

The BNPE AE4 clone contains a cDNA insert of 678 bp, excluding a poly A tail. The longest open reading frame (ORF) encodes 166 amino

acids starting from nucleotide no. 3 and there is no start and stop codons, as shown in *Fig. 1.* Therefore, it is unlikely to be a full length clone. The predicted amino acid sequence is rich in proline (44.89 %), lysine (10.2 %), threonine (10.22 %), valine (8.89 %), serine (6.67 %), tyrosine (5.78 %) and glutamine (5.78 %). The predicted protein fragment represents a calculated molecular weight of 17.7 kDa and has a predicted pI value of 10.5. Hydropathy analysis indicated that 13 residues at the N terminal end of the predicted protein fragment are hydrophobic and the other regions are highly hydrophilic. The polypeptide is primarily composed of two repeat units: a 10-mer repeat unit (P P I/V K/M P P P V Q K/Q) and a 7-mer repeat unit (P P T P I/S/T Y S). Protein database search revealed that there is no significant similarity between the predicted amino acid sequence and protein sequences deposited in the GenBank. However, comparison of the *Ae*4 nucleotide sequence with nucleotide sequences in the GenBank showed that it is homologous (82% identical) to a genomic fragment of Arabidopsis in chromosome 2 (*At2g27380*) which encodes a putative proline-rich protein. Also, a few hits to *B. napus* seed EST sequences with a homology of 80% to 95% were found in the database. A comparison of the amino acid sequence of this clone with the Arabidopsis putative proline-rich protein and translated EST sequences is depicted in *Fig. 2.*

Expression Analysis of Ae4

The pattern of expression of *Ae*4 in organs and tissues was investigated using Northern analysis and RT-PCR. The Northern analysis on the organ/tissue specific-blot failed to detect a distinct band for *Ae*4 transcripts except for a very faint smear observed in each sample lane

```
3   caacaccaacttatagccctcctatcaaaccaccacccagtgcaaaagcctccaactccc   62
    Q  H  Q  L  I  A  L  L  S  N  H  H  P  V  Q  K  P  P  T  P

63  acttatagtcctccgataaagccaccaccagtgcagaagcctccaacaccgacctacagt  122
    T  Y  S  P  P  I  K  P  P  P  V  Q  K  P  P  T  P  T  Y  S

123 ccaccagttaaaccaccaccagtgaagcctccaacacctatttatagtcctcccgtaatg  182
    P  P  V  K  P  P  P  V  K  P  P  T  P  I  Y  S  P  P  V  M

183 ccaccaccagtgcaacaacctccgacaccatcttatagtcctcctgtaaaaccaccacca  242
    P  P  P  V  Q  Q  P  P  T  P  S  Y  S  P  P  V  K  P  P  P

243 gtgcaaaaacctccaacacccacttatagtccacctgttaaaccaccacctgtgcaaaag  302
    V  Q  K  P  P  T  P  T  Y  S  P  P  V  K  P  P  P  V  Q  K

303 cctccaactccaacttacagtcctcctatcaaaccaccacccgtgcaaaaacctccaaca  362
    P  P  T  P  T  Y  S  P  P  I  K  P  P  P  V  Q  K  P  P  T

363 ccaacttatagccctcctatcaaaccaccacctgtgcaaaagcctccgacgcccacttat  422
    P  T  Y  S  P  P  I  K  P  P  P  V  Q  K  P  P  T  P  T  Y

423 agtccacctgttaaaccaccacccgtccagaagcctcccacaccaacttacagtcctcct  482
    S  P  P  V  K  P  P  P  V  Q  K  P  P  T  P  T  Y  S  P  P

483 atcaaaccacctccagtgaaacctccaacaccaatttatagtccgccagtgaaaccacca  542
    I  K  P  P  P  V  K  P  P  T  P  I  Y  S  P  P  V  K  P  P

543 cccgtgcaaaagcctccaacaccaacgtacagcccaccaattaaaccacctccagtaaaa  602
    P  V  Q  K  P  P  T  P  T  Y  S  P  P  I  K  P  P  P  V  K

603 cctccgacaccaacttatagtcctcctgtaaaaccacctccagtgcaaaagcctccgacg  662
    P  P  T  P  T  Y  S  P  P  V  K  P  P  P  V  Q  K  P  P  T

663 cccacttatagtccac  678
    P  T  Y  S  P
```

Fig. 1: Nucleotide and deduced amino acid sequence of clone Ae4 (Genbank accession no. AY570239). The ORF is underlined. This is a truncated clone without the start and stop codons.

```
CD825663 Translated          ---------------------------------------------------
CD830533 Translated          ---------------------------------------------------
NP_180307                1   MRVPLIDFLRFLVLILSLSGASVAADATVKQNFNKYETDSGHAHPPPIYG  50
Ae4                          ---------------------------------------------------

CD825663 Translated          ---------------------------------------------------
CD830533 Translated          ---------------------------------------------------
NP_180307               51   APPSYTTPPPPIYSPPIYPPPIQKPPTYSPPIYPPPIQKPPTPTYSPPIY 100
Ae4                          ---------------------------------------------------

CD825663 Translated          ---------------------------------------------------
CD830533 Translated          ---------------------------------------------------
NP_180307              101   PPPIQKPPTPTYSPPIYPPPIQKPPTPTYSPPIYPPPIQKPPTPSYSPPV 150
Ae4                          ---------------------------------------------------

CD825663 Translated          ---------------------------------------------------
CD830533 Translated          ---------------------------------------------------
NP_180307              151   KPPPVQMPPTPTYSPPIKPPPVHKPPTPTYSPPIKPPVHKPPTPIYSPPI 200
Ae4
                             --------------------------------------------------

CD825663 Translated          ---------------------------------------------------
CD830533 Translated          ---------------------------------------------------
NP_180307              201   KPPPVHKPPTPIYSPPIKPPPVHKPPTPTYSPPVKPPPVHKPPTPIYSPP 250
Ae4                          ---------------------------------------------------

CD825663 Translated          ----------------------------------------------------P
CD830533_Translated          ----------------------------------------------------
NP_180307              251   IKPPPVHKPPTPIYSPPVKPPPVQTPPTPIYSPPVKPPPVHKPPTPTYSP 300
Ae4
                         1   ----------------------QHQLIALLS-------------------   9

CD825663 Translated      1   PVKPPPVQKPPTPTYSPPVKPPPVQKPPTPTYSPPIKPPPVQKPPTPTYS  50
CD830533_Translated      1   --MPPPVQQPPTPSYSPPVKPPPVQKPPTPTYSPPVKPPPVQKPPTPTYS  48
NP_180307              301   PVKSPPVQKPPTPTYSPPIKPPPVQKPPTPTYSPPIKPPPV-KPPTPIYS 350
Ae4                     10   --NHHPVQKPPTPTYSPPIKPPPVQKPPTPTYSPPVKPPPV-KPPTPIYS  57

CD825663 Translated      51   PPIKPPPVQKPPTPTYSPPIKPPPVQKPPTPTYSPPVKPPPV-NPPAPIY  99
CD830533 Translated     100   PPIKPPPVQKPPTPTYSPPIKPPPVQKPPTPTYSPPVKPP---------- 139
NP_180307               351   PPVKPPPVHKPPTPIYSPPVKPPPVHKPPTPIYSPPVKPPPIQKPPTPTY 400
Ae4                      58   PPVMPPPVQQPPTPSYSPPVKPPPVQKPPTPTYSPPVKPPPVQKPPTPTY 107

CD825663 Translated     100   SPPVKPPPVQQPPTPSYSP----------------PVKPPPVQKPPTPTY  133
CD830533 Translated     140   ----------------------------------------PVQKPPTPTY  149
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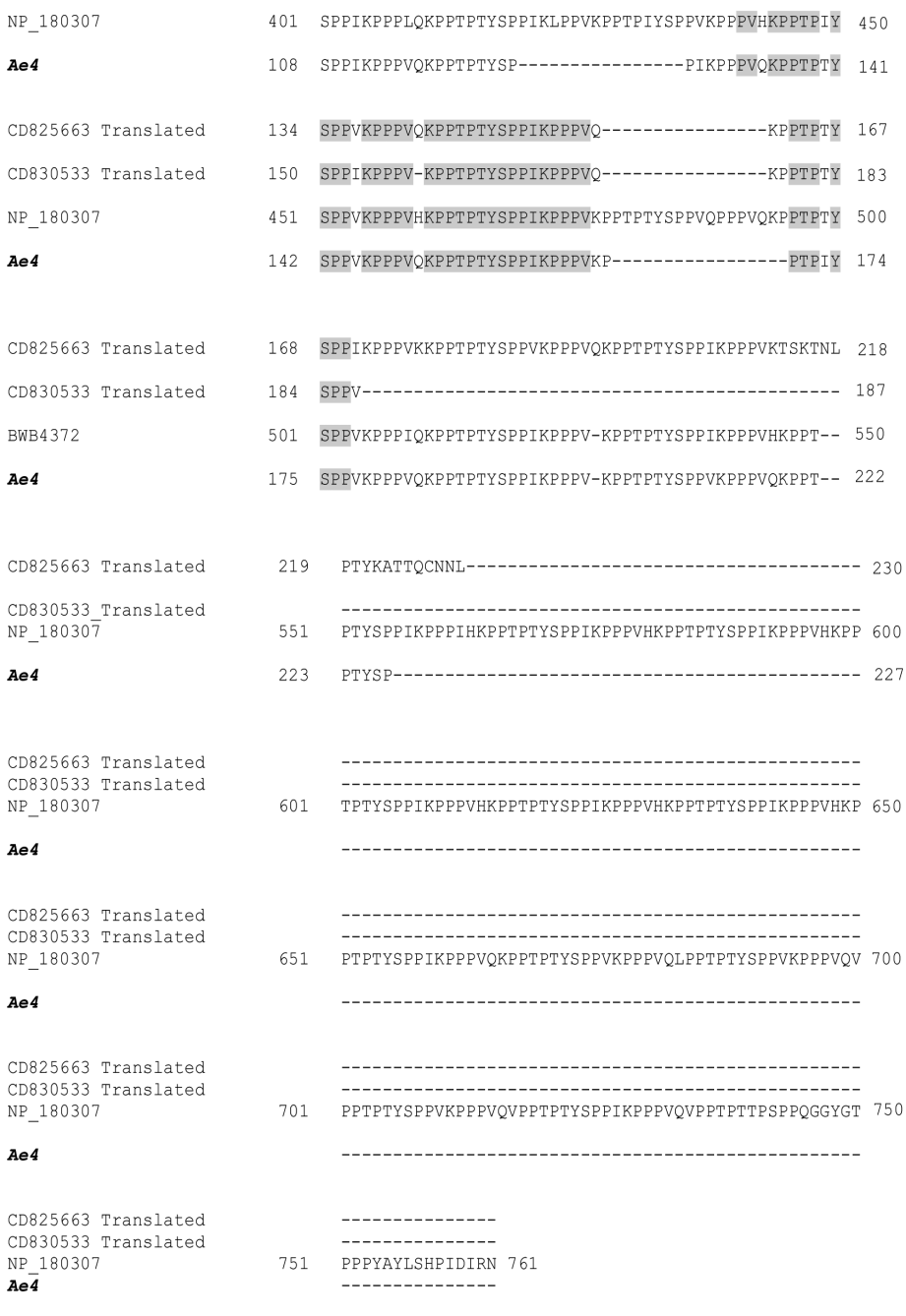

Fig. 2: Alignment of predicted amino acid sequence of Ae4 with the Arabidopsis putative proline rich protein and translated EST sequences

Shaded sequences denote identical amino acids and gaps introduced in the alignment are marked with dashes. The amino acid sequences were obtained from GenBank: Arabidopsis At2g27380 (GenBank accession no. NP_180307), Brassica seed EST clone BN25061G23 (GenBank accession no. CD825663) and Brassica napus seed EST clone BN40045N17 (GenBank accession no. CD830533)

(*Fig. 3A,* top panel). However, hybridisation with the *Actin*2/7 cDNA probe (*Fig. 3A*, bottom panel) showed clear signals, suggesting that the RNA was not degraded. To verify these results, a RT-PCR approach was employed using *Ae*4 gene-specific primers and equal amount of cDNA from leaves, stems, buds, flowers, siliques, roots and carpels. After 30 amplification cycles, no product was visible on the ethidium bromide stained gel (*Fig. 3C*, top panel). Therefore, the gel was blotted and the RT-PCR gel blot was hybridised with a ^{32}P-labelled *Ae*4 cDNA. An autoradiograph of this blot showed the presence of an approximately 370 bp band (as expected) in all lanes with various intensities of hybridisation (*Fig. 3C*, bottom panel), suggesting differential expression of *Ae*4 in different organs/tissues of the mature *B. napus* plant. Also, there was an additional faint band at approximately 600 bp in lane 2, suggesting the presence of another isoform of the *Ae*4 gene or possibly an unspliced *Ae*4 transcript.

Northern analysis of the tissue culture blot (*Fig. 3B*, top panel) detected expression of *Ae*4 transcripts in the embryogenic culture, both in the pre-embryogenic (lane PEC) and mature embryogenic tissue (lane MEC). Relative to the actin control, expression in both these tissues

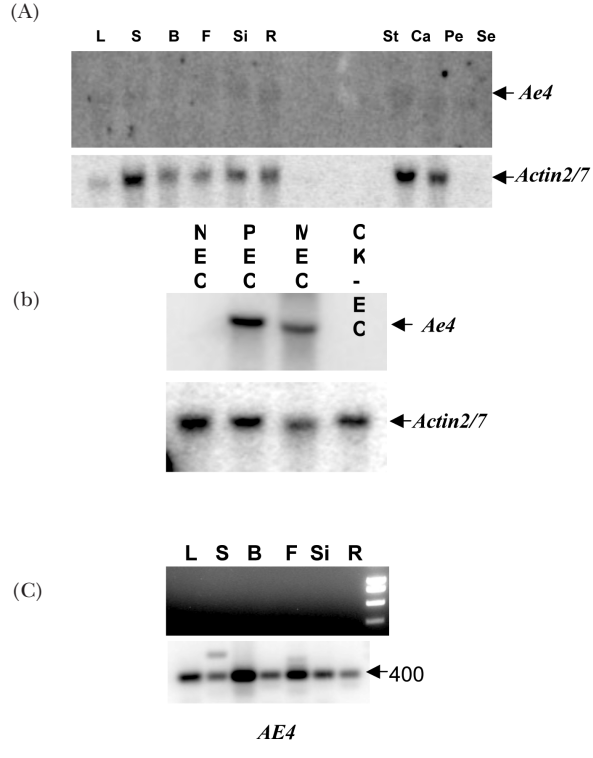

Fig. 3: Expression analysis of Ae4 in Brassica napus

(A) Tissue/organ-specific and (B) tissue culture RNA gel blots containing 10 µg of total RNA per lane were first hybridised to ^{32}P-labelled Ae4 and then to an Arabidopsis Actin2/7 cDNA (control). Lanes: L, leaves; S, stem; B, buds; F, flowers; S, siliques; R, roots; St, stamens; Ca, carpels; Pe, petals; Se, sepals; NEC, non-embryogenic tissue; PEC, pre-embryogenic tissue; MEC, mature embryogenic tissue; CK-EC, cytokinin-treated tissue.
(C) Top panel shows results of RT-PCR analysis of Ae4 gene expression. (C) Bottom panel is the RT-PCR gel blot probed with ^{32}P-labelled Ae4 cDNA. Lanes: M, 1 kb Bioline DNA ladder; L, leaves; S, stems; B, buds; F, flowers; Si, siliques; R, roots; Ca, carpels.

seems to be approximately at the same level. By contrast, *Ae*4 transcripts were not expressed at detectable levels in the non-embryogenic tissue (lane NEC) and the cytokinin-treated embryogenic tissue (lane CK-EC).

DISCUSSION

Ae4 Encodes a Partial Length Protein with Proline-rich Domain

The *Ae*4 cDNA encodes a partial length protein having a proline-rich domain. Both the amino acid composition and the presence of repeating motifs of proline are characteristics of proline-rich cell wall proteins (Jose-Estanyol and Puigdomenech, 2000). The 10-mer motif of the putative AE4 protein is unique in its sequence and belongs to a group of long repeat elements (Gyorgyey *et al.*, 1997). The repeating motifs do not correspond to any of the common motifs previously identified in proline-rich proteins and extensins (Showalter, 1993, Gyorgey *et al.*, 1997; Jose-Estanyol and Puigdomenech, 2000). Therefore, *Ae*4 may encode a novel class of proline-rich proteins (PRPs). Two PRP genes have already been isolated from *Brassica napus*, one that accumulated during pod development (Coupe *et al.*, 1993) and the other one induced by cold treatment (Goodwin *et al.*, 1996). However, the *Ae*4 sequence is not similar to either of them.

Expression Analysis of Ae4

The expression of *Ae*4 transcripts in all vegetative and floral tissues examined corresponds to the observations by Fowler *et al.* (1999) in Arabidopsis. They reported that *AtPRP2* and *AtPRP4* transcripts were most abundant in the aerial parts of the plant, namely in leaves, stems, flowers and siliques. Also, *AtPRP4* expression was detected in the early stages of lateral root formation. Since most PRPs are members of a multigene family, it is likely that the same will be true for those of *B. napus*.

Based on Northern analysis, *Ae*4 was detected in the pre-embryogenic but not non-embryogenic tissue and this suggests that the encoded protein may be associated with somatic embryogenesis. There have been a few PRP transcripts that have been shown to accumulate during somatic embryogenesis, such as in carrot (Aleith and Richter, 1990; Holk *et al.*, 1996; Yasuda *et al.*, 2001) and *Medicago sativa* (Gyorgey *et al.*, 1997). A proline-rich protein encoded by the *DC 2.15* gene was identified as one of the genes that is differentially expressed during induction of somatic embryogenesis in carrot cell suspension culture (Aleith and Richter, 1990). The expression of this gene during somatic embryogenesis was detectable from 3 days after induction, and transcript abundance increased until the heart-shape stage (Aleith and Richter, 1990). This observation was further supported by promoter studies of the *DC 2.15* gene (Holk *et al.*, 1996). Also, Gyorgyey *et al.* (1997) reported that *MsPRP5,* a cDNA clone encoding a small proline-rich protein is preferentially expressed in alfalfa dedifferentiated callus cells. They proposed that the proline-rich protein may cause structural changes of the cell wall required for certain switches in function by plant cells.

He *et al.* (2002) reported that expression of a soybean PRP gene was inhibited by treatment with kinetin. This is consistent with our observation from the Northern analysis of the tissue culture blot that *Ae*4 transcripts could not be detected in cytokinin-treated tissue. This suggests that cytokinin treatment has suppressed *Ae*4 transcription to undetectable levels, which correlates with the suppression of secondary embryogenesis (Loh *et al.*, 1983). Alternatively, *Ae*4 transcripts could be down regulated due to the low rate of secondary embryogenesis in the cytokinin-treated tissue.

Potential Roles of Ae4 in Brassica napus

Proline-rich proteins have been thought to provide strength and rigidity in the cell wall by forming covalently cross-linked networks with cell wall components (Showalter, 1993). PRPs have a relatively high content of tyrosine and lysine residues which have been implicated as the substrate for the peroxidase-mediated insolubilisation of PRPs in soybean via isodityrosine crosslinks (Kleis-San Francisco and Tierney, 1990; Bradley *et al.*, 1992; Brisson *et al.*, 1994). Insolubilisation of PRPs in the cell wall occurs as a rapid response to wounding and treatment with fungal elicitors. PRPs are rapidly insolubilized within the cell wall in response to physical damage, treatment with fungal elicitors, and pathogen infection (Kleis-San Francisco and Tierney, 1990; Bradley *et al.*, 1992; Brisson *et al.*, 1994), indicating an active role in plant defence reactions. Thus, it was proposed that the PRPs

contribute to the cell wall structure of specific cell types based on their patterns of gene expression during plant development and induction by biotic and abiotic stresses. The enhanced expression of *Ae*4 transcripts in the pre-embryogenic and mature embryogenic tissues suggests that *Ae*4 may have a role during embryo formation. In carrot cultures, it was suggested that two PRPs encoded by No.93 and *DC 2.15* might act as extracellular signal factors during the development of somatic embryos (Yasuda *et al.*, 2001). However, there is no clear evidence for PRPs as signalling molecules that induce somatic embryogenesis.

Assuming that *Ae*4 encodes a proline-rich protein, we propose that the expression of *Ae*4 transcripts preferentially in embryogenic tissue could be possibly to provide mechanical strength to the embryonic cells that will protect the cell during later phase of embryo development in vitro. More experiments such as isolation of the full-length sequence of *Ae*4, immunolocalization and transgenic studies, especially promoter analysis, are required to provide more information on possible biological functions of the *Ae*4 gene. Also, the inducibility of the *Ae*4 gene in response to abiotic and biotic stresses in *B. napus* should be tested to explore its regulation.

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