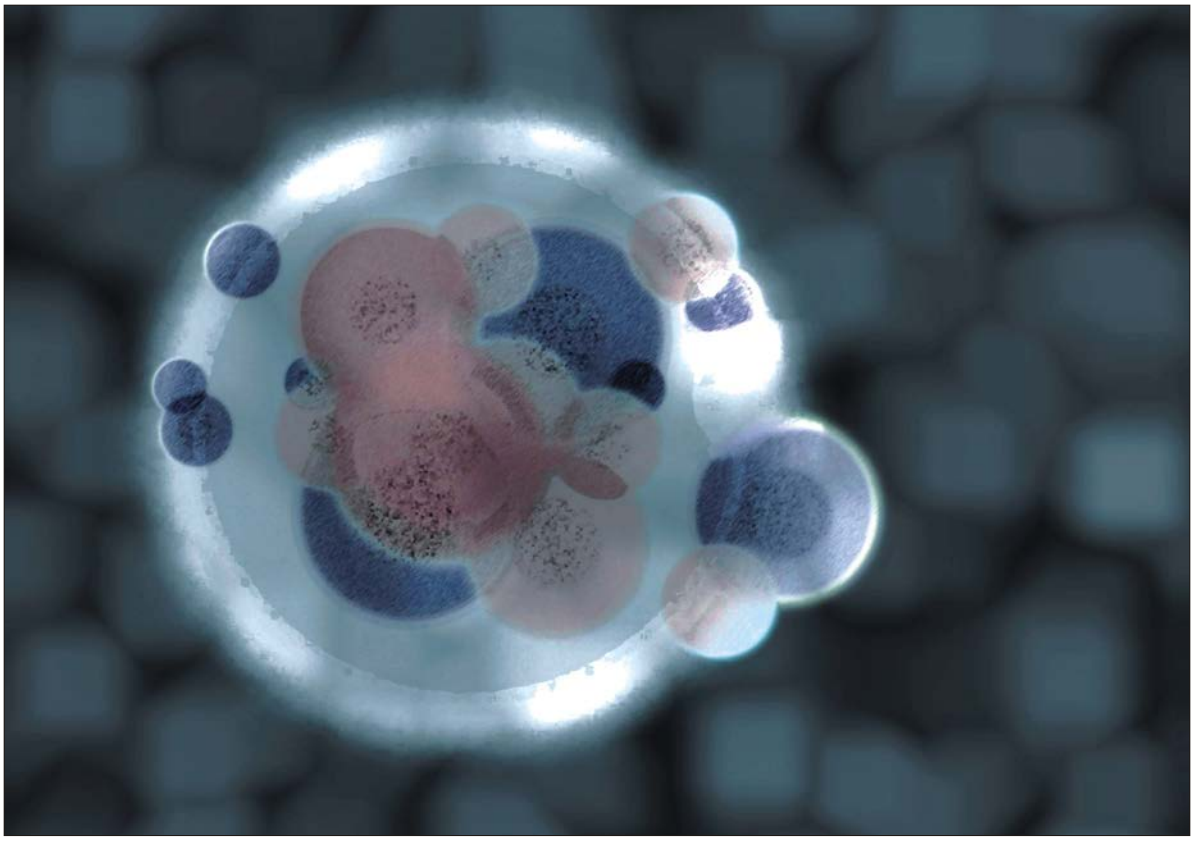

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Molecular Characterization of *Dg3*, a cDNA that Encodes a Novel Lipid Transfer Protein in *Brassica napus*

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Abstract: In this study, we have analysed the sequence of Dg3 clone using bioinformatic tools, determined copy number of this transcript in the genome of *B. napus* and expression levels at various tissues/organ. The cDNA contained 307-base pair open reading frame encoding 102 amino acid residues, 60-base pair 5'-untranslated region and 127-base pair 3'-untranslated region. The predicted mature protein has a molecular weight of 9.2 kDa and is acidic, with a predicted isoelectric point (pI) of 6.2. The Dg3 sequence has all the conserved structural characteristics of plant LTPs and showed highest homology to LTPs from other plant species. The transcripts of Dg3 were detected in all tested tissues but highest expression was in siliques and *in vitro* embryogenic cultures. Possible roles of Dg3 during somatic embryogenesis and normal plant development are discussed.

Key words: LTP, embryogenic tissue, embryogenic competence, embryoid culture, oilseed rape

INTRODUCTION

The process of acquisition of embryogenic competence by somatic cells must involve reprogramming of gene expression patterns as well as changes in the morphology, physiology and metabolism. These alterations reflect dedifferentiation, activation of cell division and a change in cell fate (Feher *et al*., 2003). Also, the changes are dependent on the down regulation of some functioning genes in differentiated cells and up regulation of genes which are required for the transition to happen. A logical first step to understand the molecular mechanisms responsible for the induction of somatic embryogenesis will be to isolate and identify genes that are differentially regulated at the transcriptional level.

In recent years, various attempts have been made to unravel the molecular mechanism involved in the acquisition of embryogenic competence. This was achieved by profiling and comparing gene expression between somatic embryos and callus cells (Sato *et al*., 1995; Kairong *et al*., 1999; Dong and Dunstan, 1999) or between embryogenic cells and non-embryogenic cells (Giroux and Pauls, 1997; Yasuda *et al*., 2001; Quiroz-Figueroa *et al*., 2002; Bishop-Hurley *et al*., 2003). Most of the identified genes were structural genes (Sterk *et al*., 1991; Sato *et al*., 1995; Gyorgyey *et al*., 1997; Helleboid *et al*., 2000a; Bishop-Hurley *et al*., 2003), early or late embryogenesis genes (Lin *et al*., 1996; Dong and Dunstan, 1999; van Zyl *et al*., 2003), hormone responsive genes (Kitamiya *et al*., 2000; Yasuda *et al*., 2001)and wound or stress induced genes (Rojas-Herrera and Loyola-Vargas, 2002; Thibaud-Nissen *et al*., 2003). These identified genes may not play a direct role in the induction of somatic embryogenesis. The exception is the Serk gene whose expression appears to mark the vegetative to embryogenic transition clearly (Schmidt *et al*., 1996). In spite of now having *Serk* as a molecular marker for competent cells, we are still far from understanding the key events underlying the transition of differentiated somatic cells to the totipotent and embryogenic cell state.

To address this problem, we have isolated and identified genes that are up regulated specifically in the pre-embryogenic stage using the long term *Brassica napus* embryogenic culture established in our laboratory (Namasivayam and Hanke, 2006b). The pre-embryogenic stage, is important as the phase in which the cells acquire embryogenic competence, the ability to become an embryo at any time later. Unlike many other embryogenesis systems, the *B. napus* secondary embryogenesis system requires no exogenous hormone to stimulate embryo development (Loh *et al*., 1983), hence it is an appropriate system for identification of genes truly involved during induction of somatic embryogenesis and not the response to an external stimulus.

In this study, we have attempted to characterise the expression pattern of Dg3, a cDNA of a novel lipid

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transfer protein, one of the previously isolated sequences from the pre-embryogenic tissue of *B. napus* (Namasivayam and Hanke, 2006). Studies of gene expression were carried out by Northern analysis and RT-PCR using various tissues of mature *B. napus* plants and at different developmental stages of the embryogenic tissue during tissue culture. To complement the gene expression analysis, Southern analysis was performed to check gene copy number in the *B. napus* genome. Based on the detailed gene expression pattern and nucleotide sequence analysis, some hypotheses on its possible functions were made.

MATERIALS AND METHODS

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

Sequence analysis: The sequence of Dg3 has been submitted to the GenBank and the database accession number is AY570248. 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, signal sequences were predicted using signalP (version 2.0, Nielsen *et al*. (1997), http://www.cbs.dtu.dk/services/SignalP/) and prediction of motifs using PROSITE program from ExPASy Molecular Biology Server (http://ca.expasy.org/prosite).

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 and 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 μg 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 Dg3 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) SDS and 1 mM 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 Actin2/7* cDNA probe as a loading control.

Extraction of plant genomic DNA: Genomic DNA of *B. napus* was prepared from young leaves using a modified CTAB (cetyltrimethyl ammonium bromide) method and is detailed below. Two to five grams of frozen young leaves were ground into a fine powder, added with 25 mL of CTAB buffer (140 mM sorbitol, 220 mM Tris-HCl pH 8.0, 22 mM EDTA, 800 mM NaCl, 1% (w/v) N-lauryl sarcosine, 0.8% (w/v) CTAB) and then incubated at 65°C for 20 min. Ten milliliter of chloroform/isoamylalcohol (24:1) was added, inverted for 20 min at room temperature and centrifuged at 2,800 g for 5 min to recover the aqueous phase. The nucleic acid was then precipitated from the aqueous phase by addition of 17 mL isopropanol, placed on ice for 10 min and centrifuged again as described above. The drained pellet was dissolved in 2 mL TE pH 8.0 and high molecular weight RNA was precipitated with an equal volume of 4 M lithium acetate, incubated on ice for 20 min. After centrifugation (as before), the DNA in the supernatant was precipitated by adding 2 volumes of 100% (v/v) ethanol, left on ice for 20 min and centrifugated as before. The pellet was dissolved in 1 mL of TE, 100 μL of 3 M sodium acetate pH 5.2 added, was extracted with an equal volume of Tris-saturated phenol and centrifuged (as before). The recovered aqueous phase was extracted with

an equal volume of phenol/chloroform/isoamylalcohol (25:24:1), followed by final extraction of the aqueous phase with an equal volume of chloroform/isoamylalcohol (24:1). The DNA was precipitated from the aqueous phase by addition of 2 volumes of ethanol and dissolved in 250 μL of TE.

Southern analysis: Three microgram of genomic DNA was digested, electrophoresed on a 0.8% (w/v) agarose gel and transferred to a positively charged Hybond™-XL nylon membranes (Amersham Biosciences) by the method of Southern (1975). Hybridization with ^{32}P-labeled DNA probes were carried out at 65°C according to the standard procedure by Sambrook *et al.* (1989). Low stringency washes were performed as follows: twice in 2×SSC, 0.1% (v/v) SDS at room temperature for 10 min and a final wash in 1×SSC, 0.1% (v/v) SDS at 55°C for 15 min. For high stringency washes, additional washes with 1×SSC, 0.1% (v/v) SDS at 55°C for 30 min and a final wash in 0.5×SSC, 0.1% (v/v) SDS at 55°C for 30 min were performed.

RT-PCR: Equal amounts of DNase-treated total RNA (200 ng) from each tissue sample was added individually to a sterile 0.2 mL PCR tube and the volume adjusted to 13.5 μL with diethylpyrocarbonate-treated sterile deionised water. One microliter of 20 pmoles μL^{-1} oligo (dT_{18}) 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×first strand buffer (Promega), 0.5 μL 'RNase Out' ribonuclease inhibitor (40U μL^{-1}) (Invitrogen), 0.5 μL 10 mM Bioline dNTPs mix and 0.5 μL MMLV-RT RNase H minus (200U μL^{-1}) (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: 2 μL of the RT product, 1×Bioline PCR buffer (Mg^{2+}- free), 1.5 mM $MgCl_2$, 0.4 mM dNTP mix, 2.5 pmoles of forward primer (5′-GTTCCAATTAGAGCAACCAGGGTTGAAAG-3′) and reverse primer (5′-CGAGCGTTTGG AGAAGTAACATACATACT-3′), respectively and 1.5U 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 as follows: 95°C for 3 min; followed by 35 cycles at 94°C for 30 sec, 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 *Actin2* was 60°C. 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 (Dg3 or *Actin2* cDNAs).

RESULTS

Sequence analysis of Dg3: The cloned fragment of Dg3 presents 494 bp and encodes a predicted peptide of 102 amino acids (Fig. 1). The longest ORF has a start codon at position $A_{61}TG$ and a stop codon at position $T_{367}AA$ as shown in Fig. 1. According to the alignment of the known plant LTPs with the amino acid sequence of Dg3 (Fig. 2), confirmed that it contains a full length coding region and is similar to the *Arabidopsis* LTP. The deduced amino acid sequence of Dg3 showed greatest homology to two putative lipid transfer proteins (LTP) from *Arabidopsis* (65% identical to *Arabidopsis At5g38195* and 50% identical to *Arabidopsis At5g38160*). The lowest homologies (36%) are observed with the rice LTP. The predicted mature protein has a molecular weight of 9.2 kDa and is acidic, with a predicted isoelectric point (pI) of 6.2. Although the homology detected to lipid transfer protein was not high, the deduced amino acid sequence of Dg3 has all the conserved structural characteristics of plant lipid transfer proteins (Kader, 1997) including a signal peptide at the N terminal, 8 cysteine residues located at conserved positions (shown in Fig. 2) and no tryptophan. Therefore, we concluded that the Dg3 encodes an acidic lipid transfer-like protein in *B. napus*. The PSORT program (Nakai and Horton, 1999) predicts the deduced protein to be localised extracellularly. Using PROSITE program, two protein kinase C phosphorylation sites were predicted in the mature BNPE DG3 protein, at positions 25-28 and 29-32.

Expression analysis of Dg3: Figure 3A shows the results of the tissue culture blot. Dg3 mRNA was detected as abundant in the pre-embryogenic, moderately expressed in mature embryogenic, at a low level in cytokinin-treated tissues and not detectable in non-embryogenic tissue. By using RT-PCR, it was shown that Dg3 transcripts were present in all tested tissues of a mature *B. napus* plant namely leaves, stems, buds, flowers, siliques, roots and carpels. Expression level of Dg3 was very low in all tissues except in silique (Fig. 3B) which the expression level was found to be elevated relative to other tissues, suggesting expression of Dg3 gene is spatially controlled. RT-PCR using *Actin2* primers as internal control showed an amplified PCR product of 300 bp in all lanes except lanes L, B and G (Fig. 3C). In lane G, a PCR product of

```
   1 acttcgataaaaagaccaaaaaatttaaagcaaaaaaaattcctctaggttttcacgac   60

  61 atgaagtttacgactctaatgctcatcacattcgtgataatcgccatgtcgtctcctgtt  120
     M  K  F  T  T  L  M  L  I  T  F  V  I  I  A  M  S  S  P  V

 121 ccaattagagcaaccagggttgaaagtttcggagaagtggcacaatcgtgtgttgtgaca  180
     P  I  R  A  T  R  V  E  S  F  G  E  V  A  Q  S  C  V  V  T

 181 gaactcgccccatgcttaccagcaatgaccttgacaggagacccgactacagaatgttgc  240
     E  L  A  P  C  L  P  A  M  T  L  T  G  D  P  T  T  E  C  C

 241 gacaaactggtagagcagaaaccatgtctttgtggttatattcgaaacccagcctatagt  300
     D  K  L  V  E  Q  K  P  C  L  C  G  Y  I  R  N  P  A  Y  S

 301 atgtatgttacttctccaaacgctcgcaaagtcttagatttttgtaaggtccctttcct  360
     M  Y  V  T  S  P  N  A  R  K  V  L  D  F  C  K  V  P  F  P

 361 agttgttaaatctcccgagacattgctaagaaaaatattattaaaataaaaaaaaaatca  420
     S  C  *

 421 aactagatcttatgtaacaatgactcatcatgttatggttgaagcttatatattgaagtg  480

 481 tttgattttatatt  494
```

Fig. 1: Nucleotide and deduced amino acid sequence of Dg3 (AY570248). The ORF is underlined. The (*) mark indicates the stop codon of the ORF

```
Triticum_aestivum        ------------------------------------ACQASQLAVCAS-A  13
rice                     ------------------------------------AGCNAGQLTVCTG-A  14
maize_seeds              ----------------------------------ANPCNPAQLTPCAGPA  16
Arabidopsis At5g38195    MKFMKLMVITVVIVVMSSPILIKS--------EVSSSCIPTELMPCLP-A  42
DG3                      MKFTTLMLITFVIIAMSSPVPIRATRVESFG-EVAQSCVVTELAPCLP-A  49
Arabidopsis At5g38160    MKFTGVVFILFVLGTMLSPVPVKARVVKGSGEEVNVTCDATQLSSCVT-A  50
                                                           *       *

Triticum_aestivum        ILSGAKPSGECCGNLRAQQGCFCQYAKDPTYGQYIRSPHARDTLTSCGLA  63
rice                     IAGGARPTAACCSSLRAQQGCFCQFAKDPRYGRYVNSPNARKAVSSCGIA  64
maize_seeds              LFGGAVPP-ACCAQLRAQQGCLCGYARSPNYGSYIRSPNAARLFAICNLP  66
Arabidopsis At5g38195    MTTGGQPTKDCCDKLIEQKECLCGYINNPLYSTFVSSPVARKVLEVCNIP  92
DG3                      MTLTGDPTTECCDKLVEQKPCLCGYIRNPAYSMYVTSPNARKVLDFCKVP  99
Arabidopsis At5g38160    VSTGAPPSTDCCGKLKEHETCLCTYIQNPLYSSYVTSPNARKTLAACDVA 100
                                ** *  * *                       *

Triticum_aestivum        VPHC-   67
rice                     LPTCH   69
maize_seeds              MPRCR   71
Arabidopsis At5g38195    YPSC-   95
DG3                      FPSC-  102
Arabidopsis At5g38160    YPTC-  103
                            *
```

Fig. 2: Alignment of the deduced amino acid of Dg3 with the deduced amino acid sequences of other putative lipid transfer proteins. Shaded sequences denote identical amino acids and gaps introduced in the alignment are marked with dashes. The asterisk marks denote 8 cysteine residues present in conserved positions in most plant LTPs. An arrow marks the putative cleavage site of the secretory signal peptide in Dg3. The amino acid sequences were obtained from GenBank: *Triticum_aestivum* (GenBank accession No.P82900), Rice (GenBank accession No. 1L6H_A), Maize (GenBank accession No. P83506); *Arabidopsis At5g38195* (GenBank accession No. NP_568555) and *Arabidopsis At 5g38160* (Gen Bank accession No. NP_198632)

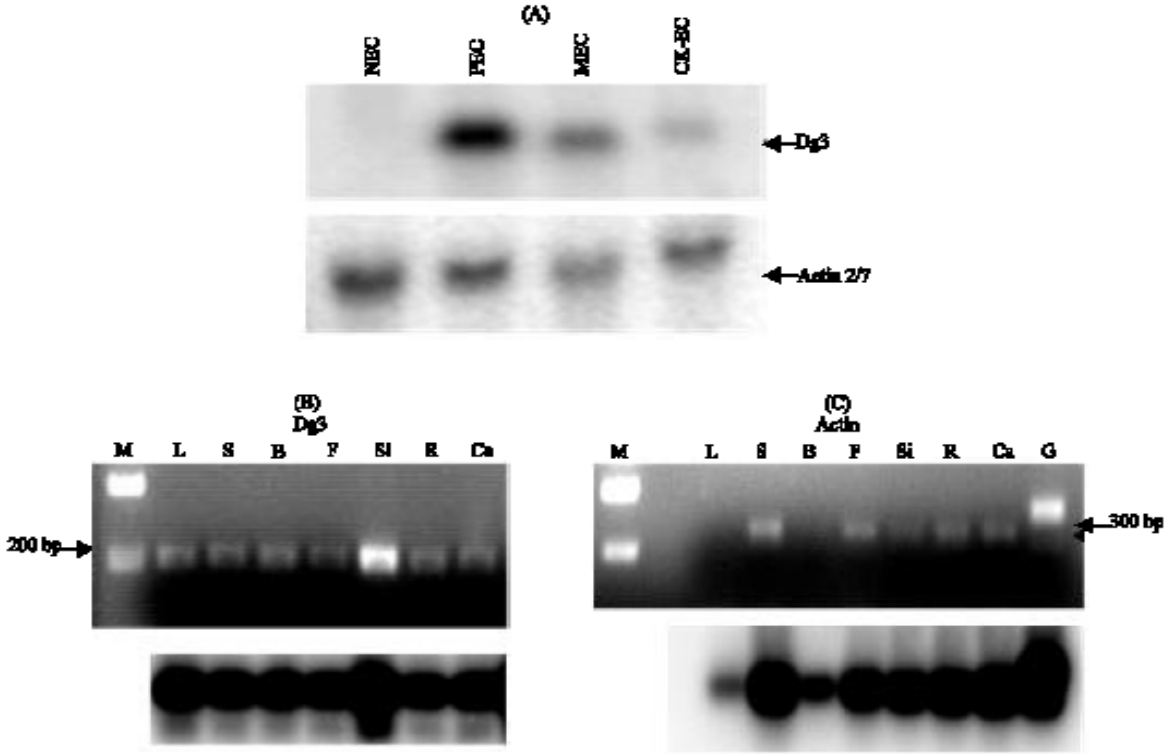

Fig. 3: Expression analysis of Dg3 in *Brassica napus*

A: Tissue culture RNA gel blots containing 10 μg of total RNA per lane were first hybridised to ^{32}P-labelled Dg3 and then to an *Arabidopsis Actin 2/7* cDNA (control). Lanes: NEC, non-embryogenic tissue; PEC pre-embryogenic tissue; MEC, mature embryogenic tissue; CK-EC, cytokinin-treated tissue

B: Top panel show results of RT-PCR analysis of Dg3 gene expression in various tissues of mature *B. napus* C, Top panel shows results of RT-PCR analysis of Actin 2 gene expression. B, bottom panel is southern blot analysis of the RT-PCR gel probed with ^{32}P-labelled Dg3 cDNA

C: Bottom panel is Southern blot analysis of the RT-PCR gel probed with ^{32}P-labelled Actin 2 cDNA. Lanes: M, 1 kb Bioline DNA ladder; L, leaves; S, stems; B, buds; F, flowers; Si, siliques; R, roots; Ca, carpels; G, genomic DNA

380 bp was observed when genomic DNA was used as template. The *Actin2* primers were designed to span different exons so that the amplification product would be of different length from any contaminating genomic DNA which would include introns. The larger band size is due to the intron sequences and also shows that the total RNA did not have any contaminating traces of DNA after the DNAse treatment. However, when a blot of this gel was probed with radiolabelled *Actin2* cDNA in a Southern analysis, signals were detected in both lanes L and B. Thus, the amount of PCR amplification product must have been too low to be visualised on the gel. Although at the start all RT samples had the same amount of RNA and cDNA for PCR, there were variations in signal intensity between lanes for *Actin2*. This may be due to variation of actin abundance in different tissues (McDowell *et al*., 1996).

Southern analysis: In search of sequences in the *B. napus* genome related to the Dg3 gene, genomic DNA blot analysis was performed. The entire ORF of the Dg3 sequence was used to probe the genomic blot, which was then was subjected to low and high stringency washing conditions. Under high stringency condition (Fig. 4), only one fragment hybridised with the probe when genomic DNA was digested with *Bam*HI or *Not*I-there were no restriction sites within the Dg3 sequence for either of those enzymes. The *Dg3* sequence has one restriction site for *Hind*III and none for *Rsa*I. The lane containing *Hind*III genomic digest had two strongly hybridising bands as expected but there were also two additional faint bands in that lane. For *Rsa*I genomic digestion, two smaller bands were observed and that was not expected. Under low stringency washing conditions (data not shown), no additional hybridising bands were detected, only an

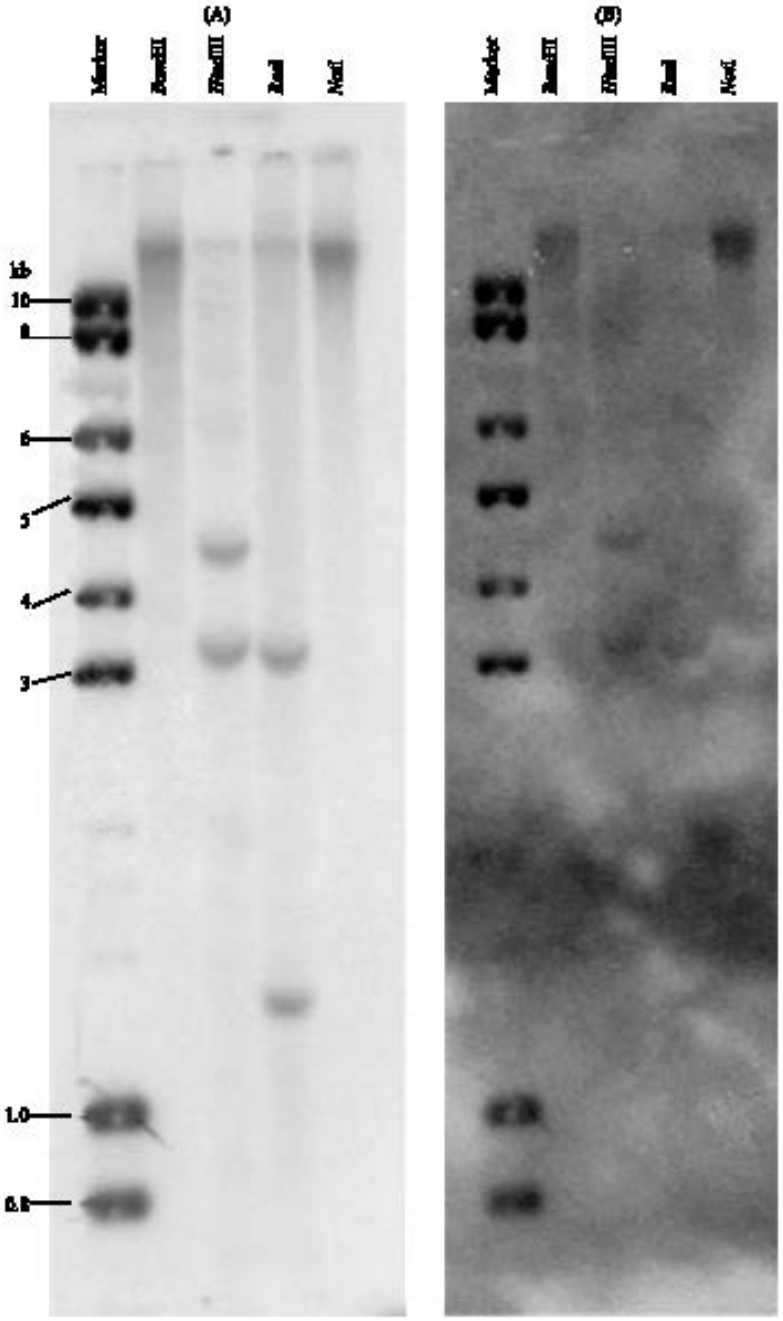

Fig. 4: Genomic Southern blot analysis of Dg3 in *B. napus*, Southern blot of *B. napus* genomic DNA probed with ^{32}P-labelled Dg3, washed under high (A) and low (B) stringency conditions as described in the materials and methods. DNA was digested with BamHI, HindIII, RsaI and NotI. DNA Size markers on the left are in kb.

increase in background. The presence of two additional bands in the *Hind*III and *Rsa*I digestion suggests that the *B. napus* genome may contain another gene with weak similarity to the Dg3 gene.

DISCUSSION

Dg3 encodes a putative acidic lipid transfer protein: The BNPE DG3 clone encodes a protein with all the expected

characteristics of a plant lipid transfer protein. The LTPs are small, soluble basic or acidic proteins ranging in size from 7 to 12 kDa (Kader, 1997; Blanckaert *et al*., 2002). LTPs are synthesised as precursors with a putative signal peptide (Bernhard *et al*., 1991) and are located extracellularly or are associated with the cell wall (Thoma *et al*., 1994; Molina *et al*., 1996). Many genes and cDNAs for plant LTPs have been isolated and characterised (Kader, 1997). The plant LTPs are encoded by a multigene family (Arondel *et al*., 2000) and different isoforms may correspond to different functions (Kader, 1997). They are thought to be involved in various biological roles including cutin biosynthesis (Sterk *et al*., 1991; Meijer *et al*., 1993) or surface wax formation (Pyee *et al*., 1994; Pyee and Katattukudy, 1995), pathogen defence reactions (Molina *et al*., 1993; Garcia-Olmedo *et al*., 1995; Sohal *et al*., 1999a,b), somatic embryo development (Sterk *et al*., 1991; Soufleri *et al*., 1996; Sabala *et al*., 2000) and in adaptations of plants to environmental changes (Dunn *et al*., 1998; Yubero-Serano *et al*., 2003; Jung *et al*., 2003).

The Dg3 gene is a member of a small subfamily: In most plant species, *Ltp* genes are present as a member of a multigene family, as in maize (Tchang *et al*., 1988), tomato (Torres-Schumann *et al*., 1992), broccoli (Pyee and Kollattukudy, 1995), barley (White *et al*., 1994), rice (Vignols *et al*., 1997) and *Arabidopsis* (Arondel *et al*., 2000). However, in *Daucus carota* (Sterk *et al*., 1991) and *Spinacia oleracea* (Bernhard *et al*., 1991), only one *Ltp* gene was detected. Arondel *et al*. (2000) have reported that the *Ltp* genes in *Arabidopsis* exist in small subfamilies of one to three genes which may or may not weakly cross hybridise at low stringency conditions in Southern analysis. Therefore, they suggested that Southern genomic DNA analyses are likely to underestimate the number of *Ltp* genes in a plant genome. Since Dg3 presents an unusual pattern of expression and has relatively low sequence homology with the previously reported *Ltp* genes in *B. napus* (Foster *et al*., 1992; Ostergaard *et al*., 1993), we conclude that the Dg3 gene may belong to a new small *Ltp* gene subfamily comprising one or two genes and distinct from the related genes reported to date.

Expression of Dg3 is developmentally controlled and correlates with embryogenesis: The spatial expression of Dg3 (assuming DG3 is a LTP) is consistent with a function in the secretion and deposition of extracellular lipophilic material such as cutin and wax in the cuticle or epicuticular layer found in aerial parts of the plant as reported in most previous studies (Thoma *et al*., 1994; Pyee and Kollatukuddy, 1995; Canevascini *et al*., 1996; Sohal *et al*., 1999a; Park *et al*., 2000). Sterk *et al*. (1991) suggested that the LTPs may function in transporting a variety of lipid molecules including cutin monomers from their place of synthesis in the endoplasmic reticulum to various extracellular locations such as the outer epidermal cell wall of leaves/stems, developing ovary integuments and later in seed coat. The transported, soluble cutin monomers would be available for polymerisation/cuticle formation which is important for plant defence during pathogen attack and for prevention of water loss (Chugh and Khurana, 2002). Alternatively, LTPs have also been proposed to participate in the deposition of lipophilic material in floral organs such as corolla (Kotilainen *et al*., 1994), nectaries and stigma for adhesion (Thoma *et al*., 1994; Park *et al*., 2000) and anther (including pollen), where sporollenin biosynthesis occurs (Foster *et al*., 1992).

However, neither studies reported by Canevascini *et al*. (1996) nor Sohal *et al*. (1999a) could establish a reason for localisation of *Ltp* gene expression at root tips. In fact, Thoma *et al*. (1994) suggested that the LTP expression in *Arabidopsis* lateral roots could be artefactual based on the premise that roots do not have a cuticular layer equivalent to the shoot. It is not impossible that LTPs are being expressed in the roots because roots do secrete lipophilic material in root mucilage. The surface of the root cap of growing roots is often covered by a thick layer of mucilage (Rougier and Chaboud, 1985). The root exudates play a major role in the interactions between roots, microorganisms and soil such as root penetration, soil aggregate formation, microbial dynamics and nutrient turnover (McCully, 1999). Previous microscopy studies have shown that the root mucilage contained oily droplets in onion root tips (Scott *et al*., 1958) and in radish root hairs (Dawes and Bowler, 1959). More recently, the lipid composition of maize, lupin and wheat root mucilages was analysed by thin-layer chromatography and gas chromatography-mass spectrometry. This study has shown that the lipid content was low and mainly composed of saturated acyl groups (Read *et al*., 2003). The low level of expression of Dg3 in *B. napus* roots may represent a small proportion of root cells expressing Dg3 and low levels of lipid secretion in the root mucilage.

Based on the expression analysis results, Dg3 was expressed at a basal level in all tissues of the mature plant examined but upregulated during somatic and zygotic embryogenesis. This is not surprising because some important genes in embryogenesis have been shown to be expressed in vegetative tissues too (Dodeman *et al*., 1997). Detection of Dg3 transcripts in the pre-embryogenic but not the non-embryogenic tissue

confirms the association of LTPs with somatic embryogenesis reported in carrot (Sterk *et al*., 1991; Meijer *et al*., 1993), grapevine (Coutos-Thevenot *et al*., 1993), barley (Vrinten *et al*., 1999), *Picea abeis* (Sabala *et al*., 2000) and Cichorium (Blanckaert *et al*., 2002). Of the above-mentioned studies, the most relevant was conducted by Blanckaert *et al*. (2002) who reported the presence of 9 kDa acidic and basic LTPs in the culture medium during somatic embryogenesis in Cichorium. They found that the acidic LTP was present during the induction step whereas the basic LTP was only detected during globular embryo formation. Therefore, it was suggested that the acidic LTP might have a role in the initiation of somatic embryogenesis (Blanckaert *et al*., 2002) whereas the basic LTP protein may be involved in the secretion or deposition of extracellular lipophilic molecules on the protoderm layer of the globular embryo (Sterk *et al*., 1991). This is intriguing because the Dg3 mature protein was predicted to encode an acidic LTP and the mRNA transcripts were found to be associated with the pre-embryogenic tissue, a stage at which cells are acquiring embryogenic competence. This is consistent with Blanckaert *et al*. (2002) proposed role for acidic LTP as associated with induction of somatic embryogenesis.

Another interesting observation in regards to gene regulation is that the cytokinin-treated embryogenic tissues showed a low level of Dg3 gene expression. It is intriguing that the transcription of the Dg3 gene appears to be down-regulated in response to cytokinin since there are no previous reports of effects of cytokinin on the regulation of *Ltp* gene expression. Loh *et al*. (1983) reported that if embryoids were treated with cytokinin during the early stages of culture and then transferred to growth regulator-free medium, these embryoids developed directly into plantlets and did not produce secondary embryoids i.e. on cytokinin treatment the tissue lost its embryogenic competence. An important observation made during our study and worth mentioning is that cytokinin-treated embryoids still exhibited the secondary embryogenesis phenotype at a minimal level compared to the untreated embryoid (mature embryogenic culture). Hence, complete loss of embryogenic competence was not observed. As such, low levels of Dg3 transcripts in cytokinin-treated embryogenic tissue may not have been a direct effect of cytokinin treatment but could be related to the low level of secondary embryogenesis in the *in vitro* culture. This could explain why Dg3 transcripts were detectable by Northern analysis in cytokinin-treated tissue but not in the non-embryogenic tissue which does not have embryos at all. If this is true, Dg3 expression correlates strongly with secondary embryogenesis. That raises the interesting question of whether Dg3 expression correlates generally with embryogenesis, in view of the fact that Dg3 expression is elevated in siliques and *in vitro* embryogenic cultures. Future work should involve anti-sense studies to give insights into the contribution of Dg3 to somatic embryogenesis.

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