Characterization and expression of plasma and tonoplast membrane aquaporins in primed seed of *Brassica napus* during germination under stress conditions

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Abstract

Two aquaporin genes were isolated from a cDNA library of canola (*Brassica napus* L.). The first aquaporin, Bn*PIP1* of 1094 bp, encoding a putative polypeptide of 287 amino acids with a predicted molecular mass of 30.4 kDa and a pI of 7.8, belongs to the family of plasma membrane intrinsic protein (PIPs) aquaporins. The *B. napus* aquaporin showed 85–94% identity to the *Arabidopsis thaliana* PIPs. ABA priming of seed induced high levels of Bn*PIP1* transcript which remained after subsequent re-drying of the seed. The second aquaporin, Bnγ-*TIP2* of 1020 bp, encoded a putative polypeptide of 253 amino acids with a predicted molecular mass of 25.8 kDa and a pI of 5.8. Bnγ-*TIP2* showed 83–90% identity to γ-*TIP* genes from a variety of plant species. Bnγ-*TIP2* was expressed only when radicle protrusion occurred in either untreated or primed seeds. Seeds primed with PEG or ABA germinated earlier and showed a higher final percentage of germination than unprimed seed, particularly under salt and osmotic stresses at low temperature. Transcripts of both Bn*PIP1* and Bnγ-*TIP2* genes were present earlier during germination of primed seeds than non-primed seed. From these results, we conclude that Bn*PIP1* is related to the water transportation required for enzymatic metabolism of storage nutrients at the early stages of canola seed germination whereas Bnγ-*TIP2* expression is related to cell growth associated with radicle protrusion. Priming induced the expression of Bn*PIP1* but had no effect on Bnγ-*TIP2*.

Introduction

Seed germination involves a sequence of biological events initiated by water imbibition followed by enzymatic metabolism of storage nutrients and, finally, radicle protrusion. Water movement into and out of living cells governs the process of seed germination. Recent studies have suggested that a class of water channel proteins, aquaporins, might finely control intra- and intercellular water flow in plant cells (Maurel *et al.*, 1997). Aquaporins have been isolated from both the tonoplast vacuolar membrane (Johnson

et al., 1991) and the plasma membrane (Kammerloher *et al.*, 1994) of many different plant species. Weig *et al.* (1997) report that the major intrinsic proteins (MIPs) of *Arabidopsis* have three distinct groups with functional aquaporins in each group. Among the *Arabidopsis* tonoplast intrinsic protein (TIPs) orthologues, α-*TIP* is seed-specific and highly conserved in the membrane of protein storage vacuoles (PSVs) (Johnson *et al.*, 1991). It has been postulated that α-*TIP* may play a role in maintaining the integrity of the tonoplast during the dehydration process of seed maturation (Maurel *et al.*, 1997). A second Tip orthologue, γ-*TIP*, is expressed in vegetative organs (Höften *et al.*, 1992). During germination, seed reserves are mobilized and hydrolyzed. Coincident with this process,

α-*TIP* is successively replaced by γ-*TIP* and the latter is expressed in elongating tissues throughout plant development (Ludevid *et al.*, 1992; Culianez-Macia and Martin, 1993; Fray *et al.*, 1994; Maeshima *et al.*, 1994). Unlike TIPs, little is known concerning the role of plasma membrane intrinsic proteins (PIPs) in relation to seed germination.

Priming involves imbibing dry seeds in an osmoticum for a given period of time followed by washing and re-drying the seeds to their original moisture content. Generally, seed germination, particularly under stress conditions, is enhanced by priming (Bradford, 1986). By a broader definition, any treatment that permits almost complete imbibition without the initiation of radicle protrusion could also be classified as priming. For example, exogenous ABA used in priming pea (Sivritepe and Dourado, 1995) and tomato seeds (Finch-Savage and McQuistan, 1991) arrests the progress of germination prior to radicle protrusion. Prehydration and re-drying treatment also resulted in a priming-like effect in canola (Zheng *et al.*, 1998). Canola (*Brassica rapa*, *B. napus*) seeds after priming showed a higher, more rapid and uniform germination under salt and water stresses at low temperature than non-primed seed (Zheng *et al.*, 1994, 1998). Higher yields were obtained from primed seeds, than non-primed seeds, particularly at early seeding dates when the soil temperature was 10 °C or less (Zheng *et al.*, 1996). Compared to a pre-hydration and re-drying treatment or priming with PEG-8000, canola seeds primed with ABA had higher seedling emergence, stronger seedling vigor and higher seed yield under field conditions (Gusta *et al.*, unpublished data). It has been proposed that priming causes 'effective invigoration' of dry seed (Heydecker and Coolbear, 1978). Effective invigoration is the initiation of metabolic events that normally occur during imbibition and that are subsequently 'fixed' by drying (Hanson, 1973). However, there is little published work to support this hypothetical proposal. Rao *et al.* (1978), studying lettuce seeds, reported that priming reduced the frequency of chromosomal aberrations, increased the rate of root growth, and decreased the frequency of morphologically abnormal seedlings. Priming pea seeds with PEG-8000, ABA or distilled water (Sivritepe and Dourado, 1995) increased the final germination and decreased the mean germination time. Sivritepe and Dourado (1995) concluded that priming ameliorated some of the chromosomal damages resulting from aging, and that ABA arrested germination and facilitated repair of age-induced genetic damage in pea

seeds. Liu *et al.* (1997) found priming induced nuclear DNA synthesis in the radicle tip cells of tomato seeds. Similar results were also reported for pepper (Lanteri *et al.*, 1993, 1996; Saracco *et al.*, 1995), maize (Garcia *et al.*, 1995) and leek seeds (Bray *et al.*, 1989; Clark and James, 1991; Ashraf and Bray, 1993). A more recent study found priming induced the expression of β-tubulin, suggesting activation of cell cycle-related processes (de Castro *et al.*, 1995).

This water uptake and its subsequent cell-to-cell movement during germination may be finely controlled by aquaporins (Chrispeels and Maurel, 1994). It is therefore reasonable to assume that priming may induce the expression of aquaporins, resulting in enhanced germination of seeds under stressful conditions. In western Canada, saline conditions induced by fertilization, and drought are the two main stresses that occur during the germination of canola in the spring when the soil temperature is often below 10 °C.

In the present study, we isolated and characterized PIP (Bn*PIP1*) and TIP (Bnγ-*TIP2*) aquaporins from the seedling of *B. napus*. The expression of these genes was studied in *B. napus* seeds that were primed with either an osmoticum (PEG 8000), plant growth regulator (ABA) or distilled water and germinated under salt or osmotic stresses at low temperatures.

Materials and methods

Priming

Seeds of *Brassica napus* L. cv. Quest (1996 crop) were obtained from the Research Center, Agriculture & Agri-Food Canada, Saskatoon, SK, Canada. All priming treatments were conducted at 23 °C, as described by Zheng *et al.* (1994). Seeds (10 g) were soaked in 100 ml of distilled water, or 20% PEG (polyethylene glycol 8000) solution or 100 μM ABA (*S*-abscisic acid, Toray Chemical, Japan) solution in an open plastic container (25 cm long, 10 cm wide, 10 cm high) on a rotax (100 rpm) for 12 h (priming with water) or 40 h (priming with ABA and PEG), after which the seeds were briefly blotted on paper towels and dried in a forced-air dryer set at 23 °C for 24 h. The primed and untreated (control) dry seeds were stored at 4 °C prior to analysis. The concentrations of ABA and PEG and the priming time that resulted in optimum germination performance (assessed by percent germination and the time to reach the maximum germination) and minimal radicle protrusion were determined from preliminary experiments.

Germination test

Fifty seeds, either primed or control, were placed in a 9 cm Petri dish fitted with 2 layers of Whatman No. 1 filter paper moistened with 3 ml of either distilled water, or 15% PEG, or 100 mM NaCl solutions to create either osmotic or salt stresses. The dishes were sealed with Parafilm and placed at 8 °C in the dark. The number of seeds exhibiting radicle emergence was recorded every day until germination was complete. Germination was calculated as a percentage of total seeds sown in each dish. The results were expressed as the means and standard errors calculated using Minitab statistical analysis program (Minitab, PA) from 4 replications per treatment. For RNA extraction, 2 g seeds were collected on a daily basis from each treatment. After rinsing with double-distilled water, the seeds were frozen in liquid nitrogen and stored at −80 °C.

Screening of B. napus seedling cDNA library

The λZAPII cDNA library protocol (Stratagene) was used for the construction of a cDNA library from 7-day old seedlings of *B. napus*. To isolate Bn*PIP1*, the library was screened using a PCR-amplified cDNA from *Arabidopsis thaliana* as a probe. A mixture of 100 ng *A. thaliana* cDNA, 1× RT buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl), 0.25 mM dNTPs, 0.5 mM MgCl$_2$, 0.05 μM upstream primer (5′-CACAAAAGCAAAACACTTAC-3′) and 0.05 μM downstream primer (5′-CACCAAACTTACATACG-3′), both designed from At*RD28* (Weig *et al.*, 1997) was used with 1 unit of *Taq* polymerase (Perkin-Elmer) in a total volume of 50 μl. The PCR amplification was carried out at 94 °C for 3 min (denaturation) followed by 30 cycles of 50 °C for 1 min (annealing) and 72 °C for 2 min (extension). The PCR product was subcloned into the PCR II vector using the TA-cloning kit (Invitrogen, San Diego, CA). The cDNA purified from a single clone was used as the probe for screening PIP genes from the *B. napus* cDNA library.

A cDNA Atγ-*TIP2*, a Atγ-*TIP* gene cloned from *Arabidopsis* (AF057137) was used as to screen the *B. napus* cDNA library for Bn*TIP* genes.

About 5.0×10^4 phage were plated on a 150 mm NYZ agar plate and a total of 8 plates were grown at 37 °C for 6–9 h. After cooling at 4 °C overnight, plaques were lifted onto Hybond-N$^+$ nylon membranes. The filters were autoclaved at 120 °C for 1 min to fix the plaques and then washed with 2× SSC for 20–30 min to remove any top agarose and bacterial debris. The filters were incubated for 3–5 h at 42 °C in prehybridization solution (2× SSC, 1% SDS, 5× Denhardt's solution (polyvinylpyrrolidone (MW 40 000) 1 mg/ml, bovine serum albumin 1 mg/ml, and Ficoll 400 1 mg/ml), 50% deionized formamide and 50 μg/ml of sonicated herring sperm DNA). The cDNA probe labeled with ^{32}P using the Oligolabelling Kit (Pharmiacia Biotech) was added to the prehybridization buffer and incubated at 37 °C overnight, the filters were washed in 2× SSC, 0.1% SDS and 0.2× SSC, 1.0% SDS each for 20 min at 37 °C. The filters were than exposed to X-Omat (Kodak) films at −80 °C overnight and developed with Developer D-19 (Kodak). The remainder of the isolation procedure was conducted according to the λZAPII cDNA library protocol (Stratagene).

DNA sequencing and sequence analysis

One putative PIP cDNA and two putative TIP cDNAs were sequenced completely from plasmid primers and synthetic oligonucleotides on an automated Model 373A DNA Sequencer (Applied Biosystems) at the Plant Biotechnology Institute, National Research Council, Saskatoon, SK, Canada. Overlapping sequence data from both strands for each clone were assembled and analyzed using DNAStar computer software (Madison, WI).

RNA extraction and northern analysis

Seeds (2 g) from each treatment were finely ground in a mortar in liquid nitrogen and then continuously ground in 3 ml of buffer-saturated hot (65 °C) phenol for 2 min. After adding 3 ml of 0.2 M sodium acetate, pH 5.2, and 1 ml of 10% SDS, the sample was centrifuged at 5000 rpm for 5 min. The supernatant was collected and washed twice with chloroform/IAA (24:1) and the RNA was ethanol-precipitated and dissolved in 50 μl of DEPC-treated water. RNA was quantified by measuring the absorbance at 260 nm. Total RNA (10 μg) was fractionated in a denaturing formaldehyde agarose gel and transferred overnight onto Hybond-N (Amersham) nylon membranes using 10× SSC buffer (pH 7.4). Northern blots were prehybridized in 10% dextran sulfate (MW 500 000), 1% SDS, 2× Denhardt's solution, 50% deionized formamide and 50 μg/ml of sonicated herring sperm DNA for 4–6 h at 42 °C. Hybridization was carried out in the same solution at 42 °C with the addition of ^{32}P-labeled probe for 12–16 h. After hybridization,

the membranes were washed with $2\times$ SSPE at room temperature for 15 min, $0.2\times$ SSPE, 0.1% SDS at 42 °C for 20 min followed by $0.2\times$ SSPE, 1% SDS at 42 °C for 10 min. The filters were exposed to X-Omat films (Kodak) at -80 °C overnight and developed with Developer D-19 (Kodak).

Results

Seed germination

Seeds primed with either 100 μM ABA or 20% PEG germinated 2 to 3 days earlier than control seeds at 8 °C. The final percentage of germination hardly differed between primed and control seeds (Figure 1A). Final germination was significantly ($P<0.01$) higher for primed seeds than control seeds under stress conditions, either salt (Figure 1B) or osmotic (Figure 1C). Over 80% of the ABA-primed and 64% of the PEG-primed seeds germinated whereas only 24% of the unprimed control seeds germinated in a 100 mM NaCl solution at 8 °C (Figure 1B). ABA-primed and PEG-primed seeds had 66% and 41% final germination, respectively, versus 5% germination for the unprimed control seeds in a 15% PEG solution at 8 °C (Figure 1C). These results indicated that seeds primed with 100 μM ABA were more tolerant to salt and osmotic stress conditions than seeds primed with PEG. Our previous study (Zheng *et al.*, 1996) also indicated that ABA-primed seeds were more tolerant to salt and water stresses than water-primed seeds. In addition, ABA-primed *B. napus* seeds produced higher yields than either water-primed or PEG-primed seeds, particularly when the seeds were sown in early spring and if the seed bed was relatively dry (Zheng *et al.*, 1996). This study was focused on the elucidation of the effect of priming on seed germination and seedling emergence in canola (*B. napus*) by isolating and studying the expression of aquaporins during priming and germination.

Characterization of BnPIP1 gene

A PCR-amplified At*RD28* (Yamaguchi *et al.*, 1992) cDNA was used to probe a cDNA library constructed from 7-day old *B. napus* seedlings. One positive clone (Bn*PIP1*) was sequenced completely on both strands. Sequence analysis using DNAStar showed Bn*PIP1* to be 1094 bp long containing a complete open reading frame (ORF). A putative polyadenylylation signal
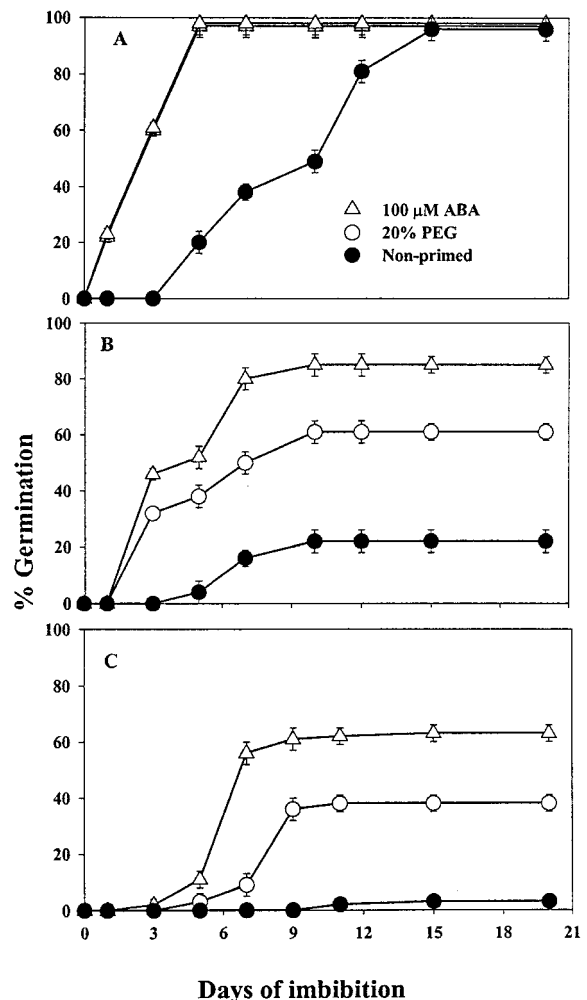

Figure 1. Germination of non-primed control seeds (*Brassica napus* cv. Quest) and seeds primed with either 100 μM ABA or 20% PEG 8000 when imbibed in (A) distilled water, (B) 100 mM NaCl, or (C) 15% PEG 8000 solutions at 8 °C in the dark.

(AATTA) is located at position 942, encoding a putative polypeptide of 287 amino acids with a predicted molecular mass of 30.4 kDa and a pI of 7.8 (Figure 2). The deduced amino acid sequence of Bn*PIP1* shows 94% identity with *PAQ2* from radish (accession number B012045) and 85% identity with At*PIP2a*, At*PIP2b* (Kammerloher *et al.*, 1994) and At*RD28* (Yamaguchi *et al.*, 1992) from *A. thaliana* (Figure 3).

Characterization of Bnγ-TIP gene

The Atγ*TIP2* cDNA (AF057137) was used to probe a cDNA library constructed from 7-day old *B. napus* seedlings. Two of the isolated positive clones were sequenced completely on both strands. Sequence

```
GAAACTAGTTATGGCGAAGGACGTGGAAGCCGTTTCCGGAGAAGGGTTTCAGACAAGA        58
           M  A  K  D  V  E  A  V  S  G  E  G  F  Q  T  R         16

GACTATCAAGATCCACCGCCAGCGCCTCTGTTTGACCCGGCGGAGCTGACCAAGTGGTCT       118
 D  Y  Q  D  P  P  P  A  P  L  F  D  P  A  E  L  T  K  W  S        36

TTCTACAGAGCAGTCATCGCTGAGTTCGTAGCTACTCTCCTCTTCCTGTACATCACCGTT       178
 F  Y  R  A  V  I  A  E  F  V  A  T  L  L  F  L  Y  I  T  V        56

TTGACAGTCATCGGTTACAAGATTCAGACCGATAGTACTGCCGGCGGCGTTGACTGCGGT       238
 L  T  V  I  G  Y  K  I  Q  T  D  S  T  A  G  G  V  D  C  G        76

GGAGTTGGTATCCTAGGTATCGCTTGGGCTTTTGGTGGCATGATCTTCATCCTTGTCTAC       298
 G  V  G  I  L  G  I  A  W  A  F  G  G  M  I  F  I  L  V  Y        96

TGCACCGCCGGTATCTCTGGTGGTCACATTAACCCGGCAGTGACATCGGGCTACTCTTG        358
 C  T  A  G  I  S  G  G  H  I  N  P  A  V  T  F  G  L  L  L       116

GCACGTAAAGTGTCGTTGGTTAGAGCCATATTGTACATGGTAGCTCAGTGTTTGGGTGCG       418
 A  R  K  V  S  L  V  R  A  I  L  Y  M  V  A  Q  C  L  G  A       136

ATATGTGGAGTTGGGTTCGTCAAAGCCTTCCAAAGCTCTTACTACGTCCGTTACGGCGGT       478
 I  C  G  V  G  F  V  K  A  F  Q  S  S  Y  Y  V  R  Y  G  G       156

GGAGCCAACTCTCTAGCCGATGGTTACAGCACAGGAACCGGACTAGCCGCAGAGATCATT       538
 G  A  N  S  L  A  D  G  Y  S  T  G  T  G  L  A  A  E  I  I       176

GGTACTTTCGTTCTTGTCTACACCGTCTTCTCCGCCACTGACCCTAAACGTAGTGCCAGA       598
 G  T  F  V  L  V  Y  T  V  F  S  A  T  D  P  K  R  S  A  R       196

GACTCCCACGTCCCAGTGTTGGCGGCCACTTCCCATTGGATTTGCGGTGTTCATGGTGCAC       658
 D  S  H  V  P  V  L  A  P  L  P  I  G  F  A  V  F  M  V  H       216

TTGGCTACCATTCCCATCACTGGAACTGGAATCAACCCGGCAAGGAGTTTCGGGAGCAGCC       718
 L  A  T  I  P  I  T  G  T  G  I  N  P  A  R  S  F  G  A  A       236

GTTATCTTCAACGAGAGCAAGCCATGGGATGACCACTGGATATTTTGGGTGGGACCATTC       778
 V  I  F  N  E  S  K  P  W  D  D  H  W  I  F  W  V  G  P  F       256

GTTGGAGCTGCGATAGCTGCATTCTACCACCAGTTCGTTTTGAGGGCTTCAGGTTCCAAG       838
 V  G  A  A  I  A  A  F  Y  H  Q  F  V  L  R  A  S  G  S  K       276

TCTCTCGGATCTTTCAGAAGTGCTGCCAACGTTTAAATACTCTCATTTCCACCAATCA        898
 S  L  G  S  F  R  S  A  A  N  V                                287

GATTCTGAAATAATAAAGATGATAATCTTTTACTCTATGTACAAATTATCCAAATCAGTT       958
TCAGTTTGTTATCTTATTTGTGTGTATTCCACTTGCTCTTTAGTGTGTTTTCGTTGAGTG      1018
CAATTGTTCTTATTTTACGCCAATGCCAAGTTTATGAAGTGACTAGAATTGAAAATCAAA      1078
AAAAAAAAAAAAAAA                                                  1093
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Figure 2. Nucleotide sequence and deduced amino acid sequence of Bn*PIP1*. Nucleotide and amino acid numbers are indicated to the right and a potential polyadenylation signal is underlined.

analysis using DNAStar showed both clones to be identical and that they are 759 bp in length with a poly(A) tail. Another 261 bp of sequence of Bnγ-*TIP2* was obtained by 5′-RACE using the upstream primer 5′-TCTCCGATCATGCCGA-3′ designed from *VM23* (D84669) and Atγ*TIP2* (AF057137) and downstream primer 5′-CCAATAGAGGATACCAG-3′ designed from the previously identified Bnγ-*TIP2* sequence. The ORF of Bnγ-*TIP2* encodes a putative polypeptide of 253 amino acids with a predicted molecular mass of 25.8 kDa and a pI of 5.8 (Figure 4). Bnγ-*TIP2* showed 90% identity to a radish γ-*TIP* (*VM23*) (Higuchi *et al.*, 1998), 82–85% identity to Atγ-*TIP2* (AF057137) and At*TIP* (X63552) isolated from *A. thaliana*. The deduced amino acid sequence of Bnγ-*TIP2* showed 87–92% identity to the deduced amino acid sequence of Atγ-*TIP2* and *VM23* (Figure 5).

```
BnPIP1    MAKDVEAVSGEGFQTRDYQDPPPAPLFDPAELTKWSFYRAVIAEFVATLL        50
PAQ2      **************************E**********************
AtPIP2a   *********************FI*g***K*****************
AtPIP2b   ******g-p********e****t*F**aD******L************
AtRD-28   ******g--pd******e****t*F**aE*****L*************

BnPIP1    FLYITVLTVIGYKIQTDSTAGGVDCGGVGILGIAWAFGGMIFILVYCTAG       100
PAQ2      **************e*********************************
AtPIP2a   ***************s*tD****************************
AtPIP2b   ***************s*tK****************************
AtRD-28   ***v***********s*tK****************************

BnPIP1    ISGGHINPAVTFGLLLARKVSLVRAILYMVAQCLGAICGVGFVKAFQSSY       150
PAQ2      ***********************************************A
AtPIP2a   **************F*******P**l**ii******************
AtPIP2b   ***************F******i**v*****************RQs*****
AtRD-28   ***************F******i**v*****************H

BnPIP1    YVRYGGGANSLADGYSTGTGLAAEIIGTFVLVYTVFSATDPKRSARDSHV       200
PAQ2      ***********************************************
AtPIP2a   *T*********************************************
AtPIP2b   *D*******************************************n******
AtRD-28   **N******F***********************N******

BnPIP1    PVLAPLPIGFAVFMVHLATIPITGTGINPARSFGAAVIFNESKPWDDHWI       250
PAQ2      ***********************************************
AtPIP2a   ***********************************v*K*********
AtPIP2b   **********************************s**S*K*********
AtRD-28   *********************************K*********

BnPIP1    FWVGPFVGAAIAAFYHQFVLRASGSKSLGSFRSAANV                287
PAQ2      ******i***********************
AtPIP2a   ******i***********************
AtPIP2b   ******i***********************
AtRD-28   ******i**t*********************
```

Figure 3. Alignment of the deduced amino acid sequence of the Bn*PIP1* (AF118382) from *Brassica napus* with three *Arabidopsis thaliana* PIPs, At*PIP2a*, At*PIP2b* and At*RD28* (X75883, X85774 and D132440) and one radish PIP, *PAQ2* (AB012045) encoding sequences. Amino acid numbers are shown to the right. Amino acid identity is indicated by an asterisk, upper-case letters indicate non-conserved changes and lower-case letters indicate conserved changes.

```
TCTCCGATCATGCCGACCAGAAACATCGCCATCGGCGGAGTCCAAGAAGAAGTGACTCAC        60
         M  P  T  R  N  I  A  I  G  G  V  Q  E  E  V  T  H         17

CCCAGCGCACTTAGGGCGGCCCTCGCTGAGTTTATCTCGACTTTGATCTTCGTCTTCGCC       120
 P  S  A  L  R  A  A  L  A  E  F  I  S  T  L  I  F  V  F  A        37

GGCTCAGGCTCAGGAATCGCTTTCAACAAGCTCACTGACAATGGAGCCACCACTCCTTCA       180
 G  S  G  S  G  I  A  F  N  K  L  T  D  N  G  A  T  T  P  S        57

GGCCTTGTCGCCGCTGCCTTAGCTCATGCTTTCGGTCTCTTCGTCGCTGTTTCTGTCGGT       240
 G  L  V  A  A  A  L  A  H  A  F  G  L  F  V  A  V  S  V  G        77

GCCAACATCTCCGGTGGACACGTTAACCCAGCCGTTACCTTCGGTGCTTTCGTCGGTGGA       300
 A  N  I  S  G  G  H  V  N  P  A  V  T  F  G  A  F  V  G  G        97

AACATCACTCTCCTCCGTGGTATCCTCTATTGGATTGCTCAGCTTCTTGGCTCAGTCGTC       360
 N  I  T  L  L  R  G  I  L  Y  W  I  A  Q  L  L  G  S  V  V       117

GCTTGTCTCCTCCTTAAATTCGCCACCGGTGGCTTGGCAGTTCCAGCTTTTGGTCTCTCT       420
 A  C  L  L  K  F  A  T  G  G  L  A  V  P  A  F  G  L  S       137

GCTGGAGTTGAATCCTTAAACGGTTTCGTCTTCGAGATCGTGATGACCTTCGGGCTCGTC       480
 A  G  V  E  S  L  N  G  F  V  F  E  I  V  M  T  F  G  L  V       157

TACACCGTCTACGGCCACAGCCGTTGACCCCAAGAACGGTAGTCTCGGAACAATCGCCACCA       540
 Y  T  V  Y  A  T  A  V  D  P  K  N  G  S  L  G  T  I  A  P       177

ATCGCCATAGGTTTCATCGTTGGAGCTAACATCCTCGCCGGTGGAGCTTTCAGCGGAGCC       600
 I  A  I  G  F  I  V  G  A  N  I  L  A  G  G  A  F  S  G  A       197

TCCATGAACCCAGCCGTGGCTTTTGGACCAGCCGTCGTGAGCTGGACGTGGACCAACCAC       660
 S  M  N  P  A  V  A  F  G  P  A  V  V  S  W  T  W  T  N  H       217

TGGATCTACTGGGCTGGTCCTCTTGTCGGTGGTGGACTCGCTGGACTCATCTACGAGTTT       720
 W  I  Y  W  A  G  P  L  V  G  G  G  L  A  G  L  I  Y  E  F       237

GTCTTCATCAACCAAAACGGCCACGAGCAATTGCCCACCACTGATTACTGAAGACTGCCA       780
 V  F  I  N  Q  N  G  H  E  Q  L  P  T  T  D  Y               253

AAACTCAATATGATTTGATTTGTTTTTATTTCGCATCGTAATGGGGTCGTTGGATCTTTT       840
TTAGATGAATCGTTGTATGTTGATTTTATTGTGTATTTGGGTTTGTCGTTGGAGTTTTAG       900
TTCCCTGGAATGCTTTTGTGCAATTGTATTGTACGGTACGGTACGGTACTGATGACTATG       960
AATGATAAAACCAGGGAATGTGAATTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA      1020
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Figure 4. Nucleotide sequence and deduced amino acid sequence of Bnγ-*TIP2*. Nucleotide and amino acid numbers are indicated to the right.

```
Bnγ-TIP2   MPTRNIAIGGVQEEVTHPSALRAALAEFISTLIFVFAGSGSGIAFNKITD      50
Atγ-TIP2   *********N*******************************
Atγ-TIP    *********-RPd*A***D**k*************V*****m******e
VM23       ****************************************
```

```
Bnγ-TIP2   NGATTPSGLVAAALAHAFGLFVAVSVGANISGGHVNPAVTFGAFVGGNIT     100
Atγ-TIP2   *************s***************** ********Li*****
Atγ-TIP    ******************************************i*****
VM23       ****************************************
```

```
Bnγ-TIP2   LLRGILYWIAQLLGSVVACLLLKFATGGLAVPAFGLSAGVESLNGFVFEI     150
Atγ-TIP2   ***************A**F**S****Epi************aL****
Atγ-TIP    *************************i******************v********
VM23       ****************m*******F***********pAS******
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```
Bnγ-TIP2   VMTFGLVYTVYATAVDPKNGSLGTIAPIAIGFIVGANILAGGAFSGASMN     200
Atγ-TIP2   ********************a***************************
Atγ-TIP    ****************************************
VM23       ****************************************
```

```
Bnγ-TIP2   PAVAFGPAVVSWTWTNHWIYWAGPLVGGGLAGLIYEFVFINQNGHEQLPT     250
Atγ-TIP2   ******************v******i*********d****de*a******
Atγ-TIP    ***************s**************-*****TT******
VM23       ****************************************
```

```
Bnγ-TIP2   TDY     253
Atγ-TIP2   ***
Atγ-TIP    ***
VM23       ***
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Figure 5. Alignment of the deduced amino acid sequence of the BnTIP2 (AF118381) from *Brassica napus* with *Arabidopsis thaliana* Atγ TIP2, and *γ-TIP* (AF057137 and X63552) and one radish *γ-TIP, VM23* (D84669). Amino acid numbers are shown to the right. Amino acid identity is indicated by an asterisk, up-per-case letters indicate non-conserved changes and lower-case letters indicate conserved changes.
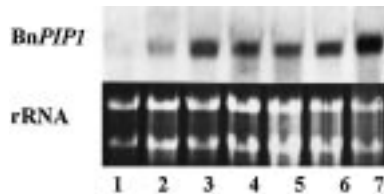

Figure 6. Differential expression of the Bn*PIP1* gene in *B. na-pus* cv. Quest after imbibition and re-drying at 23 °C. Total RNA (10 μg) from dry seed (lane 1), seeds imbibed in distilled water for 12 h before re-drying (lane 2) and after being re-dried (lane 3), seeds imbibed in a 20% PEG 8000 solution for 40 h before re-drying (lane 4) and after being re-dried (lane 5), seeds imbibed in 100 μM ABA solution for 40 h before re-drying (lane 6) and after being re-dried (lane 7). rRNA was visualized by UV illumination as a control for RNA-loading amount.

Differential expression of the BnPIP1 gene during priming and germination under stress conditions

As shown in Figure 6, Bn*PIP1* mRNA was not present in dry control seeds, however, it was induced in seeds imbibed in water for 12 h at 23 °C, or in 100 μM ABA or in 20% PEG 8000 solutions for 40 h. Fur-thermore, the transcript level remained high after the imbibed seeds were re-dried to their original moisture level (Figure 6).

During germination in water at 8 °C, the unprimed control seeds showed no Bn*PIP1* mRNA accumula-tion until day 5 after which Bn*PIP1* mRNA increased gradually (Figure 7A). In contrast, re-dried seeds

(day 0) primed either with 100 μM ABA or 20% PEG, showed a high level of Bn*PIP1* mRNA (Figure 7A). Bn*PIP1* mRNA increased rapidly in the primed seeds after one day imbibition in water at 8 °C. Under stress conditions, the expression of Bn*PIP1* was delayed, with the delay being more pronounced for the un-primed control seeds than for the primed seeds. For example, when imbibed in 100 mM NaCl, Bn*PIP1* mRNA was not produced in control seeds until 9 days whereas ABA primed seeds contained high levels of Bn*PIP1* mRNA from day 0 (Figure 7B). When im-bibed in a 15% PEG solution at 8 °C for 11 days, unprimed control seeds did not germinate (Figure 1C) and had no detectable level of Bn*PIP1* mRNA whereas primed seeds showed detectable levels of Bn*PIP1* mRNA by day 0 (Figure 7C) and had initiated germi-nation by day 5 (Figure 1C). There was no significant difference in the expression of Bn*PIP1* between PEG-primed and ABA-primed seeds imbibed in water at 8 °C (Figure 7A); however, ABA-primed seeds exhib-ited a higher transcript level than PEG-primed seeds when they were imbibed in 15% PEG or 100 mM NaCl solutions (Figure 7B and C). In all cases, seeds primed with ABA had higher levels of Bn*PIP1* mRNA on day 0 than seeds primed with PEG.

Differential expression of the Bnγ-TIP2 gene during priming and germination under stress conditions

In contrast to Bn*PIP1*, which was expressed in primed dry seeds (Figures 6 and 7), northern blot analysis showed no Bnγ-*TIP2* mRNA transcripts in either dry unprimed control or PEG or ABA primed seeds (Fig-ure 8, days 0). Primed with water for 12 h, 100 μM ABA solution, or 20% PEG solution for 40 h at 23 °C did not induce Bnγ-*TIP2* mRNA expression in either freshly primed seeds or the seeds subsequently dried after priming in northern blot analysis (Figure 8, day 0).

Figure 8 shows the Bnγ-*TIP2* mRNA transcripts of control and primed seeds during germination in water (A), 100 mM NaCl (B), or 15% PEG (C) solution at 8 °C in the dark. In control seeds, Bnγ-*TIP* mRNA ap-peared after 7 days of imbibition in water (Figure 8A). Bnγ-*TIP2* transcripts were not observed in unprimed control seeds imbibed in either 100 mM NaCl (Fig-ure 8B) or a 15% PEG solution (Figure 8C). In seeds primed with 100 μM ABA or 20% PEG, a high level of Bnγ-*TIP2* transcripts was observed 3 days after the seed were imbibed in water (Figure 8A), and 7–9 days after imbibition in 100 mM NaCl or 15% PEG solu-
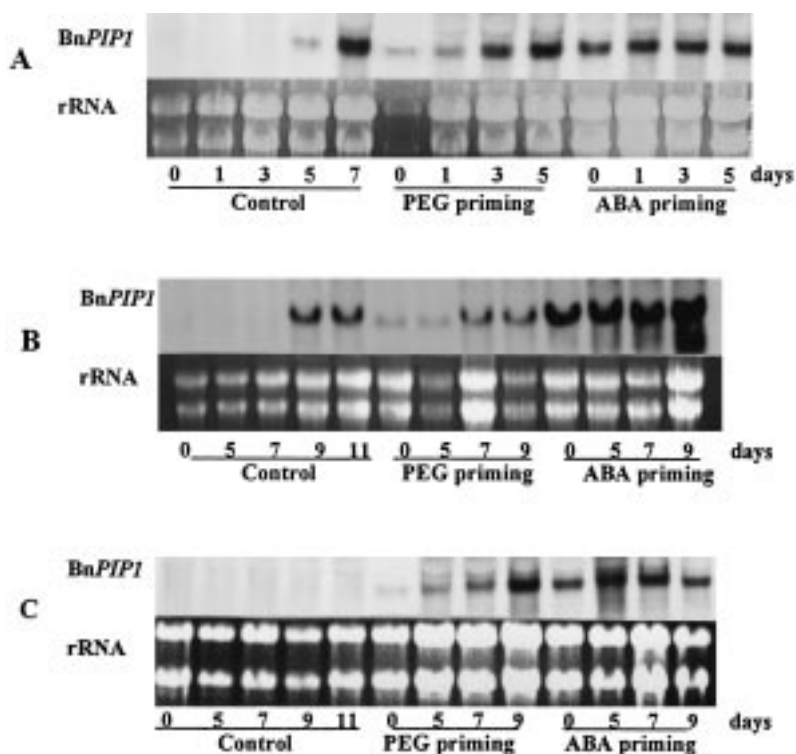
Figure 7. Differential expression of the Bn*PIP1* gene during germination in primed *B. napus* cv. Quest seed at 8 °C in the dark. A. Total RNA from control seeds after 0 (dry seed), 1, 3, 5 and 7 days of imbibition in distilled water or from seeds primed either with 100 μM ABA or 20% PEG after 0 (re-dried seeds), 1, 3 and 5 days. B, C. Total RNA from unprimed seed after 0, 5, 7, 9 and 11 days of imbibition in 100 mM NaCl (B) or 15% PEG (C) and from seeds primed with 100 μM ABA or 20% PEG 8000 after 0, 1, 3 and 5 days of imbibition in 100 mM NaCl (B) or 15% PEG 8000 (C). rRNA was vizualized by UV illumination as a control for the RNA-loading amount.

tions (Figure 8B, C). Compared to seeds primed with PEG, ABA-primed seeds showed an earlier accumulation of Bnγ-*TIP2* mRNA as well as a higher level of Bnγ-*TIP2* mRNA under salt and osmotic stress conditions (Figure 8B, C).

Discussion

Canola is a small-seeded oil crop, which has to be sown at a depth of 2 cm or less to ensure emergence of seedlings. Therefore the seed germinates in a portion of soil which is subjected to water and temperature stresses. Studies have shown that both seed germination and seedling emergence of canola is reduced and delayed by low soil temperature, low soil water potentials and salinity (often caused by fertilization) stresses (Zheng *et al.*, 1998). Our previous studies have demonstrated that priming, particularly with ABA, significantly improved seed germination, seedling emergence and seed yields (Zheng *et al.*, 1996). The present study demonstrated priming with

ABA was superior to PEG when the seeds of *B. napus* cv. Quest were subjected to osmotic and salt stresses at low temperature (8 °C). The effect of priming may be explained by the expression of aquaporins.

Aquaporins are encoded by a relatively large gene family and are widely distributed in plant cells (Maurel, 1997; Maurel *et al.*, 1997; Weig *et al.*, 1997). Though genes encoding aquaporins have been identified from a large number plant species (Schaffner, 1998), these genes have not previously been reported in *B. napus*. In the present study, we report the isolation and characterization of a γ-*TIP* and a *PIP* gene expressed during priming and germination of *B. napus* seed. From amino acid sequence similarity, we conclude that both genes encode aquaporins (Daniels *et al.*, 1994, 1996). It has previously been reported in *A. thaliana* seed that the initially highly abundant tonoplast intrinsic protein, α-*TIP*, is progressively substituted during germination by its γ-*TIP* homologue (Maurel *et al.*, 1997). In recent reviews (Maurel, 1997; Maurel *et al.*, 1997) it was proposed that the
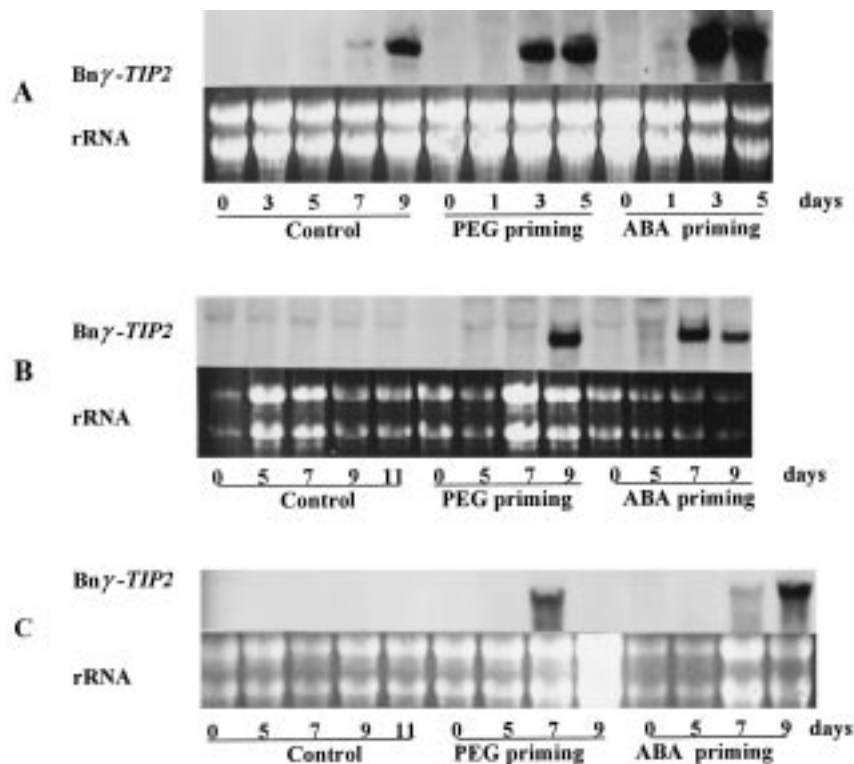
Figure 8. Differential expression of the Bnγ-*TIP2* gene during germination in primed *B. napus* cv. Quest seed at 8 °C in the dark. A. Total RNA from control seeds after 0 (dry seed), 3, 5, 7 and 9 days of imbibition in distilled water or from seeds primed either with 100 μM ABA or 20% PEG 8000 after 0 (re-dried seeds), 1, 3 and 5 days. B, C. Total RNA from unprimed seed after 0, 5, 7, 9 and 11 days of imbibition in 100 mM NaCl (B) or 15% PEG 8000 (C) and from seeds primed with 100 μM ABA or 20% PEG 8000 after 0, 5, 7 and 9 days of imbibition in 100 mM NaCl (B) or 15% PEG 8000 (C). rRNA was vizualized by UV illumination as a control for RNA-loading amount.

function of α-*TIP* might be restricted to regulating the rehydration of the protein storage vacuoles during early stages of water imbibition whereas changes in lipid membrane composition and fluidity of vacuolar membranes in the later phases of germination is regulated by γ-*TIP*. In the present study, Bnγ-*TIP2* was not expressed either during priming (imbibition prior to radicle protrusion), or in the early stages of seed germination. The expression of Bnγ-*TIP2* was coincident with radicle protrusion irrespective of seed treatment or germination medium (Figures 1 and 8). These results suggest that Bnγ-*TIP2* functions during the late phase of germination, probably during cell enlargement and/or proliferation of the root tip. By controlling water and/or solute transportation across the tonoplast membrane, the turgor pressure during the growth of vegetative vacuoles would be maintained (Maurel, 1997; Maurel *et al.*, 1997). From these results we also conclude that priming had no effect on the expression of Bnγ-*TIP2* although the effect on α-*TIP* was not determined.

Maurel's model (Maurel, 1997; Maurel *et al.*, 1997) proposes that plasma membrane proteins regulate water transportation during seed germination, although their exact role has not been identified. In two separate studies, Kammerloher *et al.* (1994) and Daniels *et al.* (1994) speculate that PIPs may regulate water transport through living tissues in plants as they do in animal cells. In the present study, we isolated one *PIP* clone from a cDNA library of germinating *B. napus* seeds, which showed identity to the plasma membrane aquaporins, *PAQ2* from radish (Maeshima *et al.*, 1994) and At*PIP2a*, At*PIP2b* and At*RD28* reported in *A. thaliana* (Yamaguchi *et al.*, 1992; Kammerloher *et al.*, 1994; Daniels *et al.*, 1994, 1996). In agreement with Daniels *et al.* (1994), the Bn*PIP1* transcript was not found in dry unprimed seeds (Figures 6 and 7). PIP was also not identified in green seed of *A. thaliana* either (Daniels *et al.*, 1994). These results suggest that *PIP* is not seed-specific and may not be involved in the formation of protein storage vacuoles during seed maturation. In contrast to Bnγ-*TIP2*

which was not expressed even after seeds underwent several days of germination once fully imbibed (Figure 8), Bn*PIP1* was expressed shortly after water imbibition. Interestingly, the Bn*PIP1* induced during imbibition remained after the seeds were redried (Figures 6 and 7). This was clearly indicative of a role of Bn*PIP1* in the early development of seed germination and priming treatment. Traditional physiological studies have shown that seed germination initiates with water imbibition, followed by enzymatic metabolism of large reserve molecules to smaller molecules for use in cell proliferation and growth. Water transportation across plasma membranes is essential for the initial enzymatic metabolic events to occur. It has previously been postulated that intracellular water transport is mediated by aquaporins (Schaffner, 1998). This is supported by the appearance of Bn*PIP1* transcript in the early stages of germination in this study. The presence of Bn*PIP1* in dry primed seeds further suggests that priming induced the expression of plasma membrane aquaporins essential for water transport in the early stages of germination. The primed synthesis of Bn*PIP1* transcript improved subsequent seed germination, particularly under stress conditions where moisture was limited (salt and osmotic stresses) or the movement of water was suppressed (low temperature).

The level of Bn*PIP1* transcript was quite different after each priming treatment; priming with ABA induced a much higher transcript level of Bn*PIP1* than water priming (Figure 6). The expression of Bn*PIP1* from seeds primed with either ABA or PEG was coincident with the germination curve (Figures 1 and 7). These results suggest that the expression of Bn*PIP1* in *B. napus* was enhanced by the presence of ABA in the priming medium which in turn contributed to higher germination percentage under osmotic and salt stresses. From this study, we conclude that the effect of priming on seed germination of *B. napus* can be partially explained by the induction of Bn*PIP1* although other factors such as the repair of damaged DNA (Rao *et al.*, 1978; Sivritepe and Dourado, 1995; Schaffner, 1998) or enhanced DNA replication (Bray *et al.*, 1989; Clark and James, 1991; Lanteri *et al.*, 1993, 1996; de Castro *et al.*, 1995; Liu *et al.*, 1997) may also contribute to the effect of the priming treatment.

Acknowledgements

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