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Short Communication

Constitutive Expression of a PR10 Protein Enhances the Germination of *Brassica napus* under Saline Conditions

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Pathogenesis-related (PR) proteins are expressed by virtually all plants in response to pathogen infection and, in many cases, in response to abiotic stresses as well and include the PR10 family. However, the precise roles of the PR10 protein family in abiotic stress responses are not clear. In this paper we report, for the first time, that the constitutive expression of a pea PR10 gene in *Brassica napus* enhances their germination and growth in the presence of NaCl. Our findings are discussed within the context of PR10 protein function and their utility in engineering stress tolerant crops.

Keywords: Abiotic stress — *Brassica napus* — Genetic engineering — Pathogenesis-related (PR) proteins — Salinity.

Abbreviations: MS, mass spectrometry; TCA, trichloroacetic acid; DTT, dithiothreitol; IPG, immobilized pH gradient.

Salinity negatively affects crop production worldwide through numerous deleterious effects on plant cells including membrane disorganization, generation of toxic metabolites and reactive oxygen species as well as the inhibition of photosynthesis (Hasegawa et al. 2000). Because plants respond to salinity by activating a complex set of defense pathways that ultimately culminate in tolerance or susceptibility, the breeding of salinity-tolerant crops has been difficult (Zhu 2002). Transgenic approaches including the enhanced expression of a Na^+/H^+ antiporter have been successful (Apse et al. 1999). With the completion of plant genome projects, rapid development of post-genomic techniques including microarrays and proteomics-based strategies may identify additional targets for engineering salinity tolerance.

We employed a proteomics-based strategy using two-dimensional electrophoresis and mass spectrometry (MS) to characterize proteome-level changes in the roots of pea (*Pisum sativum* L.) plants in response to salinity (Kav et al. 2004). These studies revealed a significant salinity-induced increase in the levels of several members of a pathogenesis-related (PR) protein family (PR10). Similar observations have been made by others with respect to PR10 proteins being expressed in response to pathogen infection as well as abiotic stress such as drought and salinity (Park et al. 2004, Moon et al. 2003, Dubos and Plomion 2001). PR10 proteins are encoded by a gene family and have been characterized from various plant species (Liu et al. 2003). While PR10 genes were originally identified in peas expressing resistance to fungi (Riggleman et al. 1985), PR10 has also been described as responding to stress and abscisic acid, as a pollen allergen and has been shown to be constitutively expressed in roots (see refs. in Tewari et al. 2003a). The precise role of PR10 in these processes has remained elusive although PR10 proteins from ginseng, white lupin and hot pepper have been demonstrated to possess RNase activities (Moiseyev et al. 1994, Bantignies et al. 2000, Park et al. 2004). However, molecular docking experiments have also suggested that a PR10 protein from white lupin may be able to bind cytokinins (Biesiadka et al. 2002) although this remains to be verified experimentally. With respect to roles in disease resistance, while expression of pea PR10.1 in potato conferred resistance to early dying disease (Chang et al. 1993), constitutive expression of this gene in transgenic canola did not result in enhanced resistance to blackleg (Wang et al. 1999).

Our observation that pea PR10 proteins are significantly induced upon salinity stress led us to hypothesize that these proteins may have a significant role in protecting plant cellular components from the deleterious effects of salinity (Kav et al. 2004). Transgenic *Brassica napus* lines constitutively expressing the pea PR10.1 gene were generated to evaluate the utility of this gene to engineer resistance to fungal diseases in *B. napus* (Wang et al. 1999) and the availability of these plants allowed us to quickly test our hypothesis. Furthermore, *B. napus* (canola) is an economically important agricultural commodity and increasing its ability to germinate and survive under water deficit conditions is highly desired. The results presented here indicate that these transgenic plants constitutively expressing pea PR10.1 gene are able to germinate and develop better in the presence of NaCl when compared to untransformed control plants. Our findings therefore support our hypothesis that at least one member of the pea PR10 protein family may have a vital role in protecting plants from abiotic stresses such as salinity.

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B. napus cv. Westar seeds (untransformed and seeds from T_2 plants of a transgenic line GN1-5#22; Wang et al. 1999) were surface sterilized for 1 min in 70% ethanol and 5 min in 5% (v/v) sodium hypochlorite in water. Seeds were rinsed with sterile deionized water 3–4 times and were placed on a sterile filter paper in a Petri dish containing 5 ml of deionized water (control) or 75 mM NaCl solution. The experiment consisted of five seeds per Petri dish and a total of five replicates for both the untransformed control and transgenic seeds at 0 and 75 mM NaCl. The plates were sealed with laboratory film and incubated in the dark at room temperature (20±2°C) for 7 d at which time root and shoot lengths were measured. The data were analyzed using the GLM procedure of SAS (Statistical Analysis System 1985).

Root and shoot tissues from 7-day-old seedlings were pulverized under liquid nitrogen. Total RNA extraction and subsequent DNase treatment was done using RNeasy Plant Mini Kit and RNase-free DNase set, respectively (QIAGEN, Ontario, Canada). Total RNA (50 ng) was reverse transcribed using iScript cDNA synthesis kit (Bio Rad Hercules, CA, U.S.A.). PCR reactions using the newly synthesized cDNA (2 µl) as the template were carried out using primers specific to the pea PR10.1 protein coding sequence (5′-CTAGTTACAGATGCTGATAAC-3′ and 5′-CATCCCCCTTAGCTTTG TCAG-3′) using a PCR Master Mix (Promega, MD, U.S.A.). PCR amplification steps consisted of a preliminary denaturation step at 94°C for 2 min followed by 35 cycles of 1-min steps at 94°C, 62°C and 72°C, performed on Gene Amp PCR System 9700 (Applied Biosystems, CA, U.S.A.). Plant 18S rRNA primers (Duval et al. 2002) were used as an internal control. PCR products were run on 1% Agarose gel and visualized under UV light after staining with ethidium bromide.

Shoot tissue from control and transgenic seeds germinated and grown for 7 d in Petri dishes in the presence or absence of NaCl (75 mM) were ground in liquid nitrogen to a fine powder. The homogenized tissue (0.1 g) was resuspended in 1 ml ice-cold 10% w/v trichloroacetic acid (TCA), 0.07% w/v dithiothreitol (DTT)–acetone solution and incubated at –20°C for 1 h. Samples were centrifuged at 12,500×*g* for 15 min, the supernatants discarded. The pellets were resuspended in 1 ml ice-cold acetone containing 0.07% w/v DTT and incubated at –20°C for 1 h. The samples were centrifuged as before and the wash step was repeated twice. Following the final wash step, the pellets, which contained the precipitated proteins, were dried for 15 min at room temperature and resolubilized with 0.5 ml rehydration buffer containing 5 µl tributylphosphine (TBP, Bio Rad, Mississauga, Canada). Samples were vortexed vigorously and incubated at 4°C overnight after which, they were centrifuged as before. The supernatants were collected and the protein concentrations were determined using a modified Bradford assay (Bio Rad) with bovine serum albumin (BSA; Pierce Biotechnology Inc., Rockford, U.S.A.) as the standard.

Protein extracts were diluted to a concentration of 400 ng μl^{-1} using the rehydration/sample buffer (Bio Rad) and a total of 125 µl (50 µg protein) of this diluted protein solution was used to passively rehydrate linear, immobilized pH gradient (IPG) strips (Bio Rad; 7 cm, pH 4–7) overnight at room temperature. Isoelectric focusing of the proteins was performed on a PROTEAN IEF Cell (Bio Rad) and included a conditioning step (250 V for 15 min). The voltage was increased linearly to 4,000 V over 2 h and focusing continued for an additional 5 h (20,000 V h). Focused IPG strips were equilibrated for 10 min in 0.05 M Tris-HCl, pH 8.8 containing 6 M urea, 2% sodium dodecyl sulfate (SDS), 20% glycerol and 2% (w/v) DTT and then for 10 min in the same buffer containing 2.5% (w/v) iodoacetamide instead of the DTT. Second dimension electrophoresis was performed using a Mini-PROTEAN 3 system (Bio Rad) for 80 min at 150 V. Following electrophoresis, gels were stained using the Silver Stain Plus kit (Bio Rad) according to the manufacturer's instructions.

Silver-stained gels were scanned using the GS-800 Calibrated Densitometer (Bio Rad) and images of the gels were compared using the PDQuest 2D analysis software (Bio Rad). The expected region of the gel where PR10.1 protein normally migrates was carefully analyzed. The unique spot present in the transgenic plant in that region was excised using a sterile scalpel and placed in a microtiter plate containing 40 µl Milli Q water. The excised gel spot was prepared for mass spectrometry using a MassPREP Station (Micromass, Manchester, U.K.). LC/MS/MS analysis of the trypsin-digested protein was performed using a PicoFrit capillary reversed-phase column (New Objectives, MA, U.S.A.) connected to a Quadrupole Time of Flight 2 mass spectrometer (Micromass, Manchester, U.K.) The tandem mass spectrum (MS/MS) data was used to establish the identity of the excised protein.

The transgenic *B. napus* (GN1-5#22) used in this study was one among several lines generated earlier in order to evaluate their resistance to diseases (Wang et al. 1999). This particular transgenic line was chosen for our studies because of the relatively higher levels of PR10.1 mRNA that were detected (Wang et al. 1999) and seeds from T_2 plants were used in all experiments. In order to confirm the expression of the pea PR10.1 gene, we synthesized cDNA from RNA isolated from both roots and shoots of transgenic as well as non-transgenic, wild-type seedlings. PCR analysis of the cDNA using PR10.1-specific primers revealed the presence of an amplified product of the expected size (314 bp) in both the roots (Fig. 1A) as well as the shoots (Fig. 1B) of the transgenic seedlings. However, in the non-transgenic seedlings, a similar amplified product was absent although the presence of the internal control (18S rRNA) was detected in all cases. The fact that an amplification product is not observed by RT-PCR in the non-transgenic *B. napus* germinated in 0 or 75 mM NaCl may be due to the pea PR10.1-specific primers used in this study. Under the conditions used in these RT-PCR experiments, our primers specifically amplify only the pea PR10.1 gene as evidenced by the lack of amplification of the nearly identical PR10.2 gene when a PR10.2 cDNA clone was used as a template (Tewari et al.

2003b). Furthermore, sequence databases searches have revealed no PR10 homologues in *B. napus* or the related *A. thaliana* (data not shown).

The detection of mRNA for the transgene does not provide any evidence for the presence of the protein. Ideally, one can detect the presence of the protein by a Western blot; however, the lack of antibodies to the pea PR10 proteins precluded this. Therefore, to verify the presence of PR10.1 protein in the transgenic seedlings we performed two-dimensional electrophoresis of protein extracted from the shoots of both control and transgenic seedlings. Silver-stained images of the two-dimensional gels of protein extracts from the two seedlings is shown in Fig. 2A and B. The region of the gel where the pea PR10.1 protein is expected to migrate is shown in Fig. 2C and D from which it was clear that an unique protein was present in the transgenic shoot extracts. Again, due to the unavailability of specific antibodies, we decided to identify the unique protein via MS/MS. The MS/MS data was used to deduce the amino acid sequence of the peptides which revealed the identity of the unique protein as pea PR10.1 (Table 1). Our two-dimensional electrophoresis and mass spectrometry thus confirms the expression of the PR10.1 gene and the presence of the protein in transgenic *B. napus*.

In order to assess the relative levels of salinity tolerance, particularly during germination, we germinated both control and transgenic seeds in Petri dishes with or without 75 mM NaCl. Appearance of the transgenic and non-transgenic seedlings after 7 d of continuous germination in Petri dishes in the absence or presence of 75 mM NaCl is shown in Fig. 3. The appearance of five seedlings in a Petri dish which formed a typical experimental setup is shown in Fig. 3A and representative control and transgenic seedlings in the absence or presence of NaCl is shown in Fig. 3B. It is obvious that both the root and shoot lengths of the control Westar seedlings were affected to a greater extent by the NaCl when compared to the transgenic seedlings. In order to fully gauge the extent of tolerance of transgenic seedlings to the imposed stress their root and shoot lengths were measured and compared to the non-transgenic control. From the data presented (Fig. 3C) it is evident that the mean shoot length of 7-day-old transgenic seedlings was significantly (P <0.05) greater than the wild-type controls. Furthermore, the extent of reduction of shoot length as a result of the salinity stress was lower in the case of transgenic seedlings whereas in the wild-type seedlings this reduction was significantly greater (Fig. 3C). In the case of mean root lengths there were no significant differences between the transgenic and wild-type seedlings in the absence of NaCl (Fig. 3D); however, in the presence of NaCl, the extent of reduction in mean root length was once again significantly (P <0.05) greater in the case of the non-transgenic, wild-type control (Fig. 3D). There were no significant differences observed in the shoot masses of 7-day-old transgenic and control seedlings and root masses were too low especially in the case of salinity-stressed control seedlings in order to make valid comparisons (data not shown).

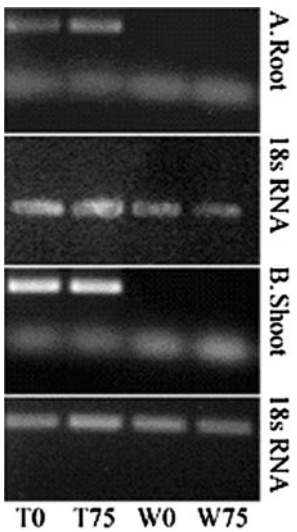

Fig. 1 RT-PCR analysis of the expression of pea PR10.1 and 18S rRNA genes in roots (A) and shoots (B) of *B. napus* seedlings. Seeds from wild-type (W) and transgenic (T) *B. napus* (cv Westar) plants were germinated in 0 or 75 mM NaCl. Shoot and root tissue were excised from 7-day-old seedlings, RNA isolated and used for RT-PCR analysis using pea PR10.1-specific primers.

It is evident from the results presented that the pea PR10.1 gene is expressed and the protein product can be detected in the transgenic *B. napus* line. Furthermore, it is also evident that this transgenic line exhibits enhanced tolerance to salinity specifically during germination and subsequent seedling development, both of which are vital for improved crop productivity in

Table 1 Disease resistance response protein Pi49 (PR10) identified by ESI-Q-TOF

[a] PM/%	MS/MS (ESI-Q-TOF) Sequence	Identity	[b] Access No.	Mr / pI
4/28%	LSAGPNGGSIAK LTFVEDGETK GDAAPSEEQLK ALVTDADNLTPK	Disease resistance response protein Pi49 (PR10)	gi|118933	16794 / 4.94

[a] Number of peptides matched/ sequence percentage coverage.
[b] Accession number is Mascot search result using NCBI and other databases.

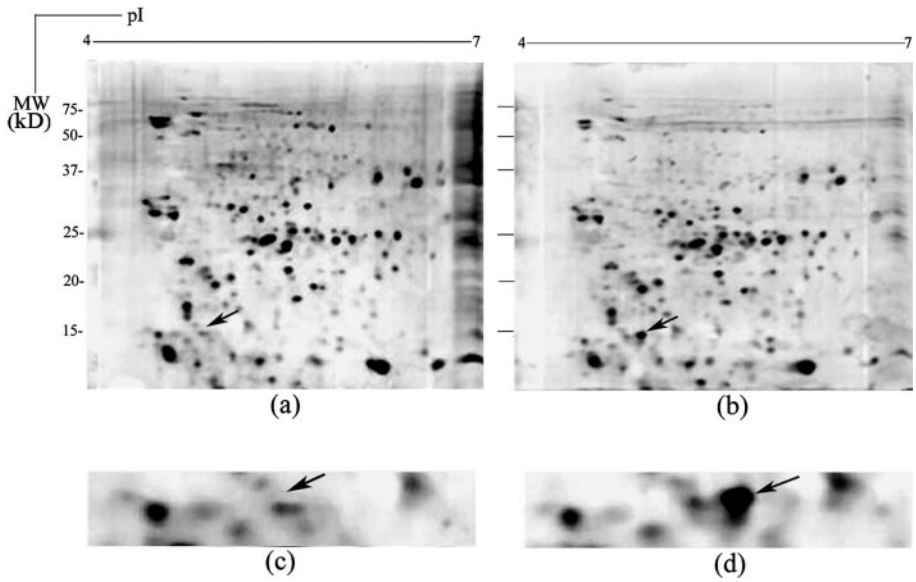

Fig. 2 Two-dimensional electrophoresis of protein extracts prepared from the shoots of wild-type (A) and transgenic (B) *Brassica napus* seedlings. Wild-type and transgenic seeds were germinated for 7 d in water and total protein extracts prepared from isolated shoot tissue. Protein extracts were subjected to two-dimensional electrophoresis and the protein spots visualized by silver staining. Panels (C) and (D) are subregions of the gels where a unique protein with the expected molecular weight and pI of pea PR10.1 protein was detected. The location of this protein on all panels is indicated by the arrow.

marginal environments. Understandably, there is a need to investigate the mechanism by which PR10.1 protein enhances the germination of *B. napus* in the presence of NaCl as well as to test other members of the PR10 protein family for their role(s) in mediating salinity (and perhaps other abiotic) stresses. Based on the preponderance of acidic amino acids in the primary structures of PR10 proteins, Pnueli et al. (2002) have proposed a LEA/dehydrin-like role for PR10 proteins in the protection of cells from water-deficit stress damage. It would also be of interest to determine whether PR10 proteins

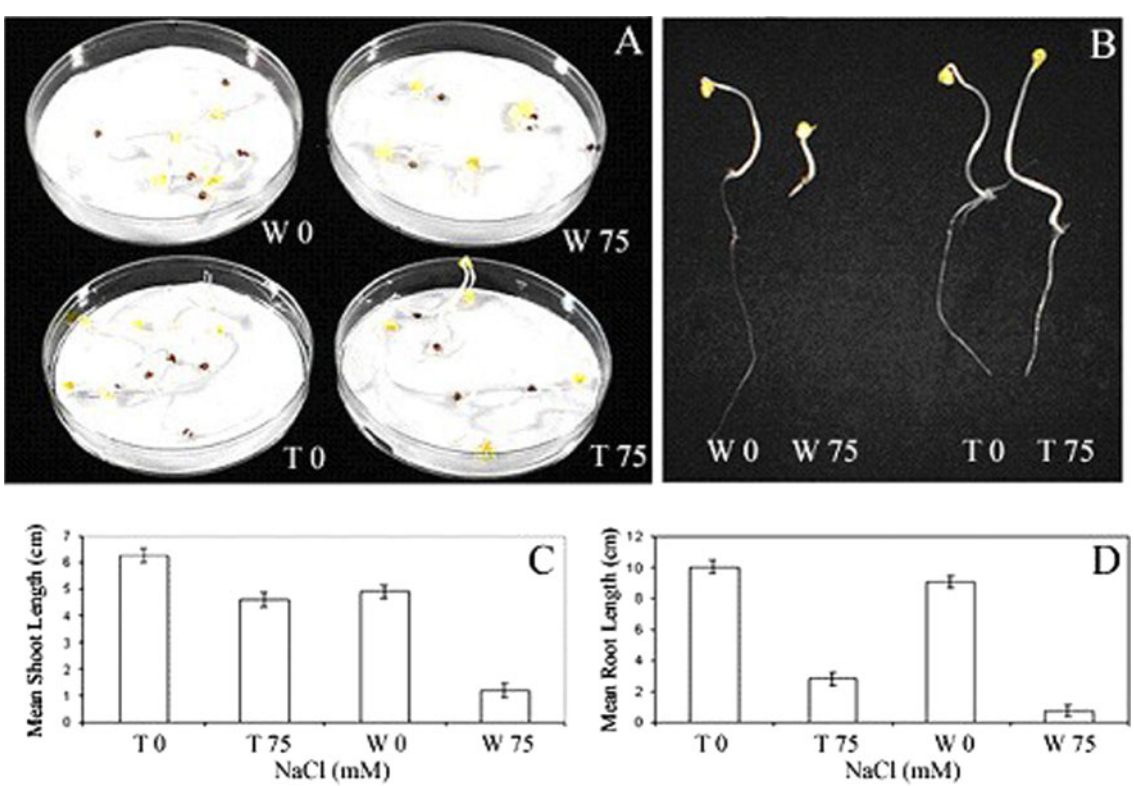

Fig. 3 Effect of NaCl on the germination and subsequent growth of wild-type (W) and transgenic (T) *B. napus* seedlings. (A) Appearance of representative seedlings germinated in Petri dishes, (B) appearance of all seedlings in a typical Petri dish, (C) effect of NaCl on mean shoot length and (D) on the mean root length. Wild-type and transgenic seeds were germinated in water (0) or 75 mM NaCl (75), seedlings were photographed and the root and shoot lengths of 7-day-old seedlings were measured.

can ameliorate other water-deficit stresses such as drought and cold. Indeed, the observation that several members of western white pine PR10 protein family are induced following cold-hardening, suggests that these proteins may afford protection to a diverse range of stresses (Liu et al. 2003).

Our findings, have demonstrated for the first time that a PR10 protein is able to protect seedlings from salinity which may have significance in the genetic engineering of water-deficit stress tolerance in plants. For example, it may be possible to use one or more members of the PR10 family of proteins to further enhance the degree of tolerance to these stresses. Studies are currently underway in our laboratory to determine whether other members of the pea PR10 protein family (specifically PR10.2 and PR10.4) are also able to enhance germination of canola under saline conditions. Moreover, the mechanism underlying PR10-mediated salinity tolerance remains a conjecture at this point and should be investigated. We are in the process of overexpressing recombinant pea PR10 proteins in *E. coli* for detailed structure and function analysis which will provide insights into this aspect of PR10 biology. In addition, we are in the process of characterizing the phytohormone profiles of the transgenic plants in order to explore the relationship between various hormones (especially cytokinins) and PR10. Our studies and those of others will contribute to an enhanced understanding of the biology of PR10 proteins for which a crucial role in plant stress tolerance has now been demonstrated.

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