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Constitutive expression of the pea ABA-responsive 17 (ABR17) cDNA confers multiple stress tolerance in *Arabidopsis thaliana*

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Summary

The constitutive expression of the pea *ABR17* (abscisic acid-responsive 17) cDNA, which is a member of the group 10 family of pathogenesis-related proteins (PR 10), in *Arabidopsis thaliana* is reported. The presence of *ABR17* transcripts and the protein in the three transgenic lines is demonstrated by reverse transcriptase-polymerase chain reaction (RT-PCR) and two-dimensional electrophoresis followed by tandem mass spectrometry, respectively. Three independently derived transgenic lines containing *ABR17* germinated better in the presence of salt, cold temperature or both. Furthermore, the transgenic plants also exhibited enhanced tolerance to freezing temperature, suggesting the potential utility of the *ABR17* gene to engineer multiple stress tolerance. In order to obtain insights into the mechanism underlying *ABR17*-mediated stress tolerance, we have compared the proteome of a transgenic line with that of its wild-type counterpart. Several proteins were observed to be significantly altered in the transgenic line, including some with a role(s) in photosynthesis, stress tolerance and the regulation of gene expression. Our findings are discussed within the context of available genes to engineer multiple stress tolerance as well as the biological activities of the ABR17 protein.

Introduction

Plants encounter a wide range of environmental stresses, including drought, salinity, temperature extremes, flooding and ultraviolet radiation, which severely limit crop productivity worldwide (Boyer, 1982). Abiotic stresses can occur simultaneously and can affect multiple stages of plant growth and development (Chinnusamy *et al*., 2004), leading to serious morphological, physiological, biochemical and molecular changes. This can cause a concomitant decrease in the average yield of most major crops by more than 50% (Boyer, 1982; Bray *et al*., 2000; Wang *et al*., 2003). Soil salinity alone can lead to a significant decrease in the yield, affecting as much as 7% of the world's arable land (Hasegawa *et al*., 2000; Zhu, 2003). Furthermore, increased salinization is expected to reduce agricultural land by an estimated 30% by the year 2025 and by up to 50% by the middle of the 21st century (Wang *et al*., 2003). Therefore, it is extremely important to characterize plant responses to abiotic stresses and to identify genes that may be useful for improving tolerance to such stresses.

The availability of high-throughput genomics and proteomics technologies has enabled us to conduct a comprehensive study of the genome, the transcriptome and the proteome under different abiotic and biotic stress conditions (Ramonell and Somerville, 2002; Agrawal *et al*., 2005). Previously, through an analysis of salinity-induced changes in pea root proteome, we reported an increase in the levels of several members of a pathogenesis-related (PR) protein family (PR 10; Kav *et al*., 2004), including PR 10.1 and the abscisic acid (ABA)-responsive protein ABR17 (PR 10.4). The PR proteins are part of a multicomponent defence response in many plants that respond to various abiotic and biotic stresses (Walter *et al*., 1990). They are grouped into 14 different families on

the basis of their serological relations, homology at the nucleotide/amino acid sequence level and similarities in biological functions (Van Loon *et al*., 1994; Van Loon and Van Strien, 1999).

Proteins belonging to the PR 10 family have been detected in a variety of angiosperms, monocots and dicots (Biesiadka *et al*., 2002). PR 10 proteins are small (15–18 kDa), acidic and intracellular, unlike other PR proteins that are extracellular (Walter *et al*., 1990; Van Loon *et al*., 1994). These intracellular PR (IPR) proteins were first described in cultured parsley cells on elicitor treatment (Somssich *et al*., 1988) and, as described earlier, have subsequently been detected in many species. *PR 10* genes are known to be induced by pathogens (Fristensky *et al*., 1985; McGee *et al*., 2001; Borsics and Lados, 2002) as well as other stresses, including salinity, drought, wounding and darkness (Osmark *et al*., 1998; Hashimoto *et al*., 2004; Kav *et al*., 2004). In addition to the induction by stimuli, PR 10 proteins occur in high concentrations in roots, flowers and pollen (Biesiadka *et al*., 2002; and references therein). These observations suggest that, in addition to their role(s) in plant stress response, they could play an important role during the normal growth and development of plants (Wu *et al*., 2003).

The pea ABA-responsive protein ABR17 is similar to pea disease resistance response proteins; it is produced late in seed development, and is induced by the exogenous application of ABA (Iturriaga *et al*., 1994; Colditz *et al*., 2004). Many proteins with significant homology to the pea ABA-responsive protein include an IPR protein from bean (Walter *et al*., 1990), garden pea (Fristensky *et al*., 1988), parsley (Somssich *et al*., 1988), major birch pollen allergen Betv1 (Breiteneder *et al*., 1989), potato (Constabel and Brisson, 1992) and SAM22 from soybean (Crowell *et al*., 1992). Several gene products homologous to ABR17, proteins also known as dehydrins and late embryogenesis abundant (LEA)-related proteins, have been identified in different plant systems (Skriver and Mundy, 1990; Close *et al*., 1993; Goday *et al*., 1994).

Despite the identification of proteins with significant similarities to ABR17 (and other PR 10) in many species, a direct role for these proteins in mediating plant responses to abiotic stresses has not been convincingly demonstrated. We have reported previously (Srivastava *et al*., 2004) that the constitutive expression of a *PR 10* (*PR 10.1*) gene from pea in *Brassica napus* ameliorates the effects of salinity stress during germination and early seedling growth. In this article, we demonstrate that *ABR17* (classified as a member of the *PR 10* family in pea) is able to enhance germination of *Arabidopsis thaliana* under multiple abiotic stresses, and also enhances tolerance to these stresses. Furthermore, we have compared the proteomes of *ABR17* transgenic and wild-type seedlings in order to probe the mechanisms underlying the enhanced stress tolerance. Our findings are discussed within the context of the known biological activities of *PR 10*, as well as its potential utility in engineering multiple stress tolerance in plants.

Results

Generation of transgenic *Arabidopsis* plants and confirmation of gene expression

Transgenic *A. thaliana* plants were generated using the floral dip method (Clough and Bent, 1998). Seeds from T0 plants (T1 seeds) of 33 independent transgenic lines were selected and screened for their ability to germinate in the presence of 75 and 150 mM NaCl. The number of copies of ABR17 cDNA in these lines was estimated on the basis of their segregation on kanamycin plates. Three transgenic lines (6.9, 14.9 and 25.20) with single insertions of the ABR17 cDNA, which showed best germination in preliminary experiments under saline conditions, were selected for the production of homozygous lines and further characterization.

The expression of the transgene was verified using *ABR17*-specific primers together with 18s rRNA primers as an internal control. The presence of an *ABR17*-specific transcript was confirmed by this reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, which produced the expected 319-bp amplification product, which was absent in the wild-type as well as the negative control (Figure 1a). The quality of mRNA, cDNA and the amplification reactions was confirmed by the successful amplification of the expected 18s rRNA product in all samples (Figure 1a). The presence of the ABR17 protein in all three transgenic lines was confirmed by Western blot analysis with PR 10.4-specific polyclonal antibodies. Protein extracts prepared from the transgenic plants revealed the presence of a strongly reacting band with a molecular weight corresponding to that of ABR17, whereas extracts from the wild-type plants did not show the presence of this band (Figure 1b).

Effects of salinity and cold on germination

The ability of the three transgenic lines to germinate in the presence of NaCl was evaluated at room temperature and at 10 °C, and the results are shown in Figure 2. At room temperature, in the absence of NaCl, we observed 100% germination in the wild-type as well as the three transgenic lines. At the lower temperature (10 °C), although germination in

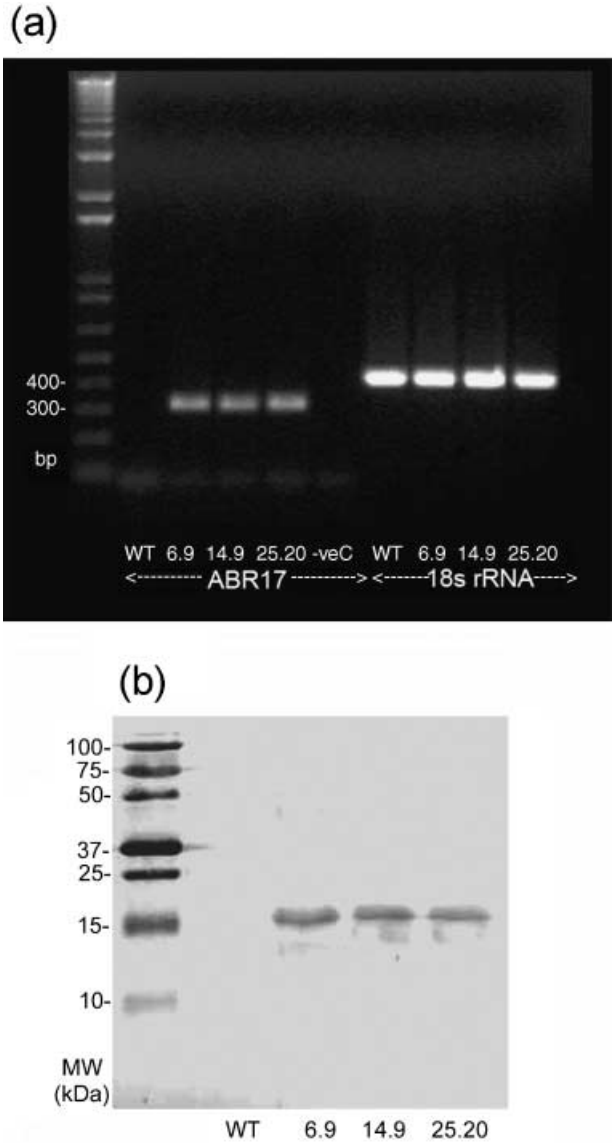

Figure 1 Expression of ABR17 cDNA in transgenic *Arabidopsis thaliana*. (a) Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of *ABR17* and *18s rRNA* expression, demonstrating the presence of *ABR17* transcript in the three transgenic lines (6.9, 14.9 and 25.20) and its absence in the wild-type (WT), as well as the presence of 18s transcript throughout. (b) Western blot analysis of protein extracts (from 2-week-old seedlings), demonstrating the presence of a unique band corresponding to the molecular weight of ABR17 in the three transgenic lines.

all lines was not 100%, there were no significant differences in the numbers of germinated seeds on any day (Figure 2c). However, the transgenic seedlings germinated earlier in the presence of 75 and 150 mM NaCl at both temperatures tested (Figure 2). For instance, the transgenic lines 6.9 and 14.9 initiated germination by day 1 in the presence of 75 mM NaCl at room temperature, whereas no wild-type or 25.20 transgenic seeds had germinated at this point (Figure 2a). Similarly, in the presence of 150 mM NaCl at room temperature, all three transgenic lines had initiated germination by day 3, whereas no significant germination in the wild-type had occurred at this time (Figure 2b). The maximum germination observed at room temperature in the presence of 75 mM NaCl at the conclusion of the experiment (on day 14) in the wild-type was ~74%, compared with 99%, 99% and 90% for the transgenic lines 6.9, 14.9 and 25.20, respectively (Figure 2a). In the case of 150 mM NaCl at room temperature, the maximum germination in the wild-type was 47%, compared with 85%, 89% and 60% in the transgenic lines 6.9, 14.9 and 25.20, respectively (Figure 2b).

When the germination of the seeds was assessed at 10 °C, in the presence of either 75 or 150 mM NaCl, the differences in the germination rates of the transgenic seeds were obvious, with the transgenic lines clearly performing better than the wild-type (Figures 2d,e). At 10 °C, on day 5, in the presence of 75 mM NaCl, all three transgenic lines showed a significantly higher germination rate than the wild-type, with the transgenic line 6.9 appearing to be the best of the transgenics (Figure 2d). The trend remained the same throughout the entire period of the experiment (14 days) for 75 mM NaCl treatments. At the end of the 14-day experiment, in the presence of 75 mM NaCl at 10 °C, 70% of the wild-type seeds had germinated, whereas the germination rates were 94%, 94% and 86% in the three transgenic lines 6.9, 14.9 and 25.20, respectively (Figure 2d). In the presence of 150 mM NaCl, the difference between the wild-type and the three transgenics was apparent on day 6 and was very clear on day 8 (Figure 2e). At this salt concentration, only 17% of the wild-type seeds had germinated by day 14, whereas the germination rates were 83%, 67% and 50% in the three transgenic lines 6.9, 14.9 and 25.20, respectively. Once again, the transgenic line 6.9 appeared to be better than the other two lines (Figure 2e). All the observed differences between the germination rates of the transgenic and wild-type seeds were statistically significant ($P < 0.05$).

Appearance of seedlings germinated and grown in the presence of NaCl

The appearance of the wild-type and transgenic seedlings grown on Murashige–Skoog (MS) plates containing 0, 75 or 150 mM NaCl at room temperature after 1 and 2 weeks is shown in Figures 3(a) and 3(b), respectively. It is clear that there are no obvious differences between the wild-type and the three transgenic lines in the absence of salt after 1 week of growth (Figure 3a). However, after 2 weeks, in the absence

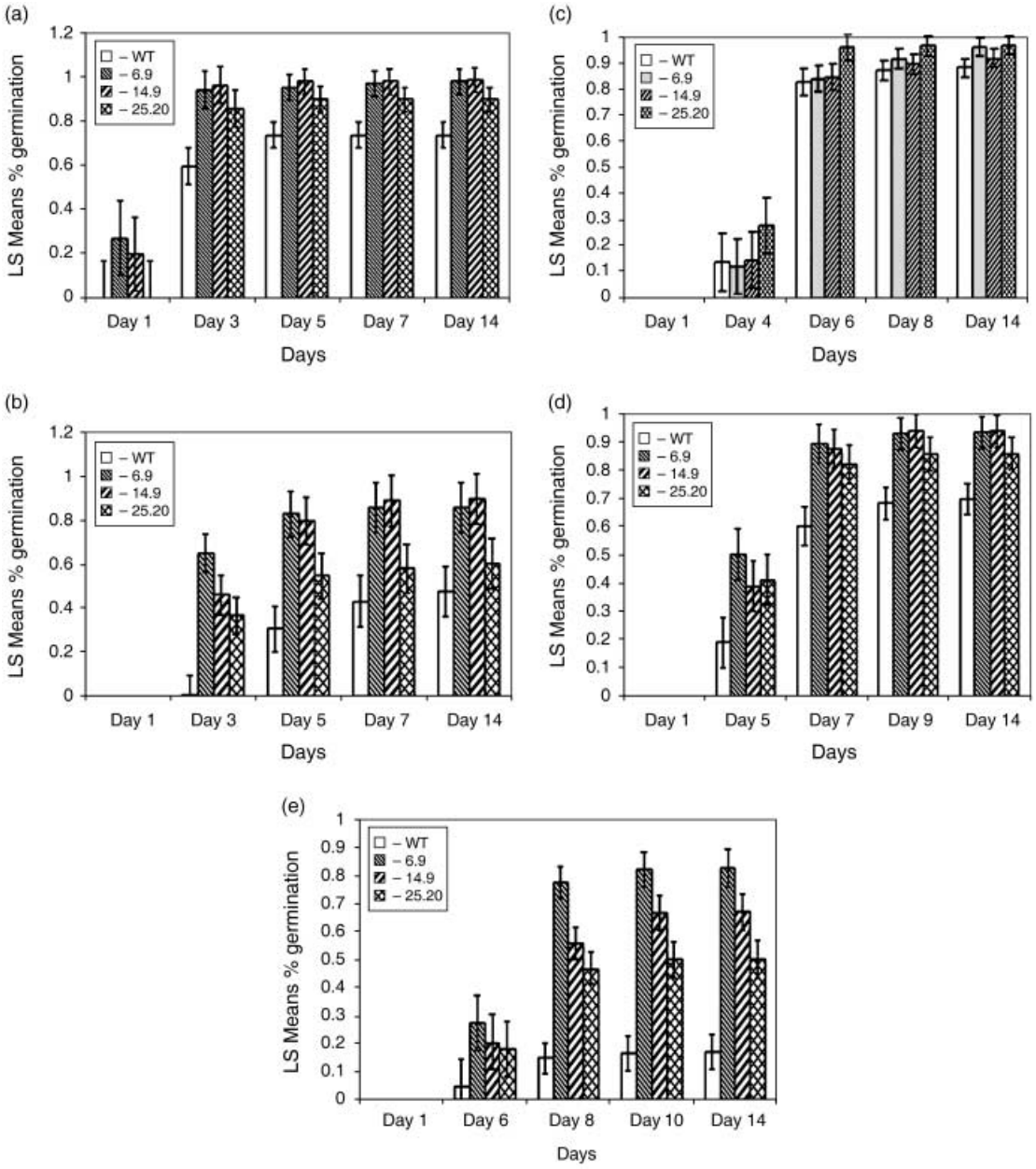

Figure 2 Effects of NaCl and low temperature on the germination of wild-type (WT) and *ABR17* transgenic seedlings. Effects of 75 mM NaCl (a) and 150 mM NaCl (b) at room temperature. Effects of 0 mM NaCl (c), 75 mM NaCl (d) and 150 mM NaCl (e) at 10 °C. Experiments were repeated three times (n = 42) and the bars indicate the standard errors.

of salt, it appears as though both the roots and shoots of the transgenic seedlings are better developed than those of the wild-type (Figure 3b). As described previously for the germination experiments, the differences between the wild-type and transgenic seedlings were more apparent in the presence of NaCl (Figure 3), with the deleterious effects on root and shoot development being more pronounced at the higher (150 mM) NaCl concentration. It is clear from the appearance of the seedlings (Figure 3) that the deleterious effects of 150 mM NaCl at both 1 and 2 weeks are considerably less in the transgenic lines than in the wild-type seedlings. These observations were confirmed by evaluating the effect of the salinity treatments on the chlorophyll and carotenoid levels, which were measured in 2-week-old control and 75 mM NaCl-treated tissues. The chlorophyll levels were significantly ($P < 0.05$) decreased in the wild-type seedlings, whereas the levels in the transgenics grown in 75 mM NaCl were not significantly different from those of the untreated

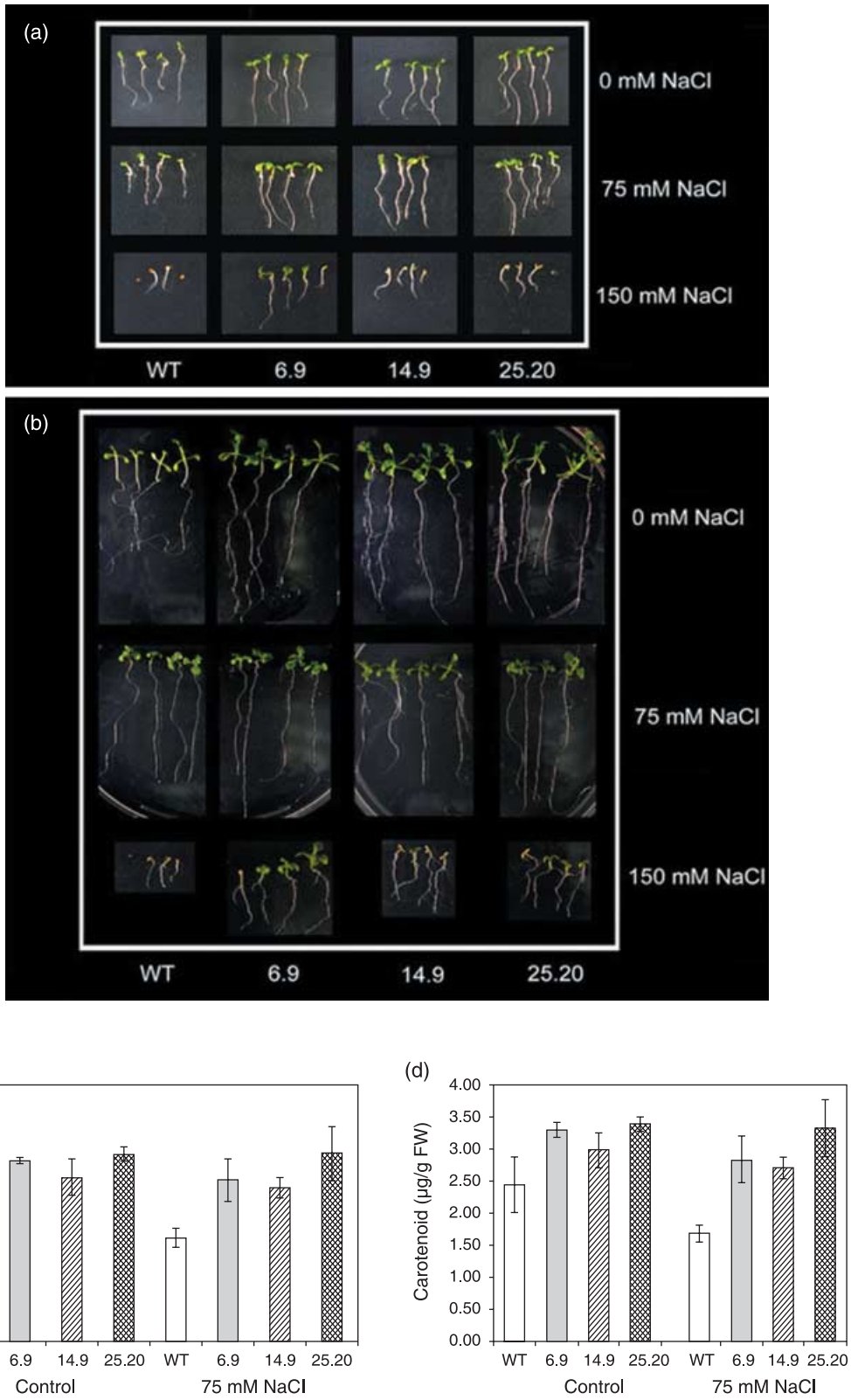

Figure 3 Effects of salinity on the seedlings germinated and grown at room temperature. Morphology of seedlings germinated after 1 week (a) and 2 weeks (b) of growth at 0, 75 and 150 mM NaCl. Total chlorophyll (c) and carotenoid (d) levels following 2 weeks of growth at 0 and 75 mM NaCl. WT, wild-type.

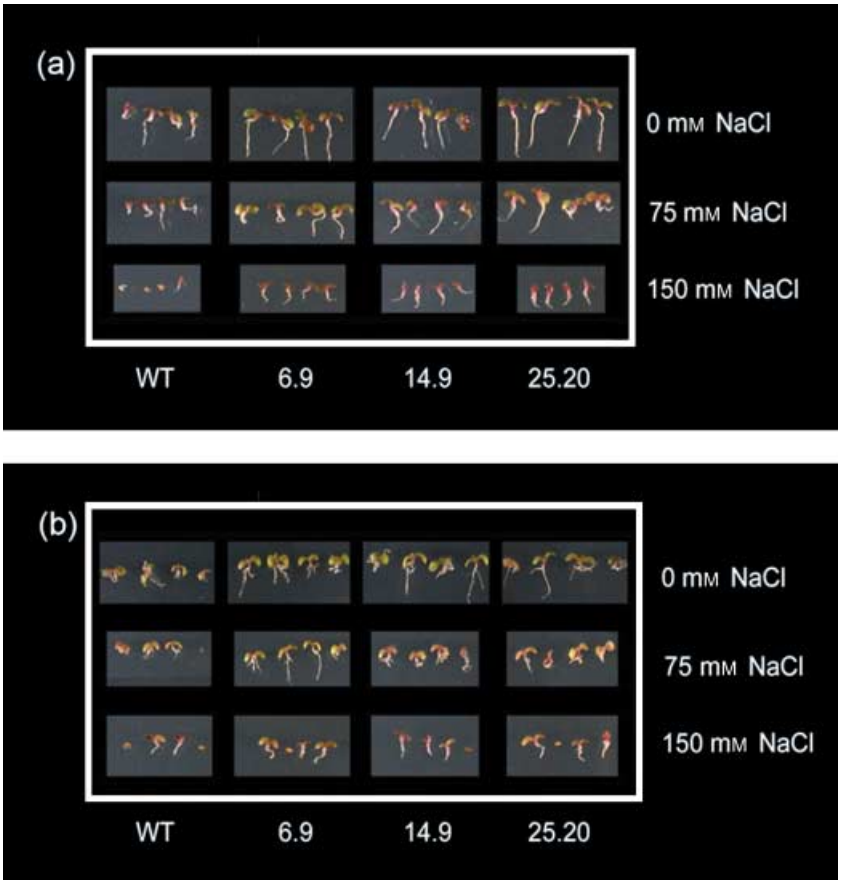

Figure 4 Effects of salinity on the seedlings germinated and grown at 10 °C. Morphology of seedlings after 2 weeks (a) and 3 weeks (b) of growth at 0, 75 and 150 mM NaCl. WT, wild-type.

samples (Figure 3c). The effect of 75 mM NaCl was more pronounced on the carotenoid levels of the wild-type seedlings, with the salt treatment reducing the carotenoid levels significantly ($P < 0.05$) compared with those of the transgenics (Figure 3d). It is also apparent that the carotenoid levels in the untreated transgenic seedlings were higher than those of untreated wild-type seedlings (Figure 3d). These elevated levels of carotenoids may contribute to the better tolerance to NaCl exhibited by the transgenic lines.

The appearance of seedlings after 2 and 3 weeks at 10 °C, in the presence or absence of NaCl, is shown in Figure 4. It is evident that, at this lower temperature, in the presence or absence of NaCl, the transgenic seedlings appear to be healthier than the wild-type after 2 (Figure 4a) and 3 weeks (Figure 4b) of growth, suggesting that they are able to better tolerate the lower temperature stress. Chlorophyll and carotenoid levels could not be measured in the seedlings subjected to NaCl treatment at this lower temperature because of the lack of sufficient tissue to perform these experiments. Therefore, the appearance of the seedlings also supports the data obtained from the experiments that assessed the abilities of the transgenic lines to germinate under these stress conditions.

Assessment of tolerance to freezing temperature

In addition to the ability to germinate and grow under stress conditions, it is also important to enhance the ability of plants to tolerate subzero temperatures. We were therefore interested in evaluating whether these transgenic lines were capable of tolerating exposure to freezing temperature (–5 °C). The appearance of plants grown on MS plates 24 h after a 4-h treatment at –5 °C is shown in Figure 5. From the appearance of the plants 24 h after returning to room temperature, it is apparent that almost all the wild-type plants had collapsed, whereas the transgenic lines appeared healthier, indicating that these lines were more tolerant to the imposed stress (Figure 5). In order to confirm that this was indeed the case, we determined the percentage ion leakage in the wild-type and transgenic seedlings after 4 h of stress and 4 h of incubation at 4 °C, as well as after the 24-h recovery at room temperature (Table 1). The percentage ion leakage, which is indicative of membrane damage, after the stress and 4 °C incubation, was significantly less in all three transgenic lines than in the wild-type; line 6.9 seemed to suffer the least amount of membrane damage when compared with the other two transgenic lines (Table 1). These observations were

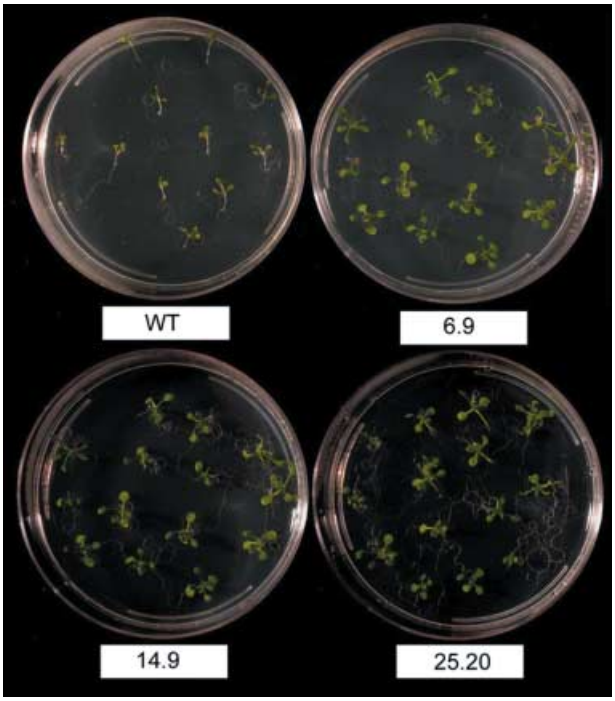

Figure 5 Freezing tolerance of *ABR17* transgenic plants. Appearance of seedlings following 1 day of recovery after exposure to cold stress (–5 °C) for 4 h. Experiments were repeated three times (n = 28). WT, wild-type.

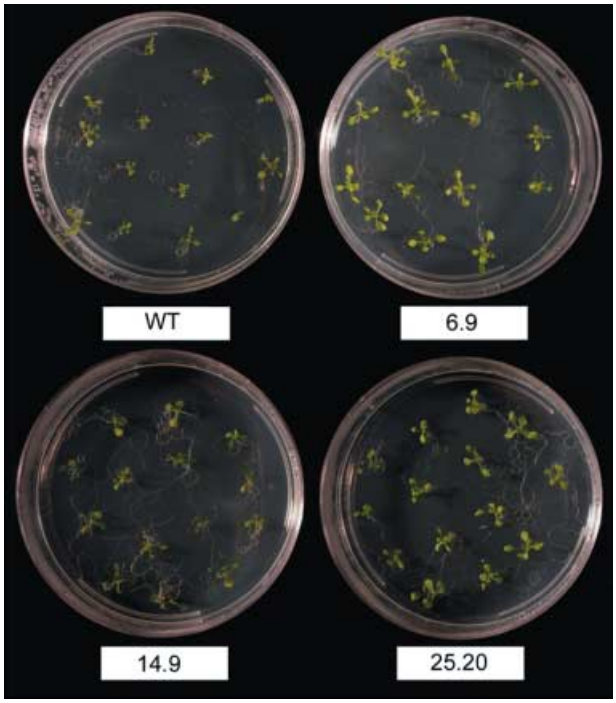

Figure 6 Heat tolerance of *ABR17* transgenic plants. Appearance of seedlings following 1 day of recovery after exposure to heat stress (48 °C) for 2 h. Experiments were repeated three times (n = 28). WT, wild-type.

Table 1 Effects of cold stress on ion leakage and chlorophyll and carotenoid content

Percentage ion leakage (cold stress –5 °C for 4 h and 4-h incubation at 4 °C)	
Wild-type	61.69 ± 3.32
6.9	37.17 ± 3.66
14.9	47.22 ± 1.49
25.20	44.84 ± 1.17
Percentage ion leakage (1 day recovery after cold stress –5 °C for 4 h)	
WT	51.36 ± 4.17
6.9	38.26 ± 4.41
14.9	44.57 ± 6.94
25.20	38.31 ± 7.40
Chlorophyll (μg/g fresh weight) (1 day recovery after cold stress –5 °C for 4 h)	
WT	72.45 ± 1.02
6.9	98.47 ± 18.93
14.9	81.83 ± 4.29
25.20	74.41 ± 15.64
Carotenoid (μg/g fresh weight) (1 day recovery after cold stress –5 °C for 4 h)	
WT	2.50 ± 0.05
6.9	2.95 ± 0.56
14.9	2.68 ± 0.16
25.20	2.37 ± 0.56

also supported by the ion leakage observed after the 24-h recovery period where, once again, it was observed that line 6.9 showed the least amount of membrane damage (Table 1). Although the appearance and ion leakage data indicated that the transgenic plants were significantly more tolerant to cold stress, there were no significant differences in the chlorophyll or carotenoid content when measured after the stress, as well as after the recovery period (Table 1).

The enhanced germination of the transgenic lines in the presence of NaCl and the increased tolerance of these lines to cold temperature stress prompted us to test whether these *ABR17* transgenic plants would exhibit enhanced tolerance to heat stress. Wild-type and transgenic plants (2-week-old) were exposed to 48 °C for 2 h and allowed to recover for 1 day at room temperature. The appearance of these plants after the 1-day recovery period is shown in Figure 6. It is evident that all three transgenic lines appeared healthier than the wild-type after 1 day of recovery, suggesting that *ABR17* expression could confer multiple stress tolerance. However, we did not investigate the heat tolerance of these transgenic lines in greater detail as it was beyond the scope of this study.

Characterization of proteome-level differences between wild-type and transgenic *A. thaliana*

In order to probe the physiological basis for the observed enhancement of stress tolerance in transgenic *A. thaliana* seedlings expressing the pea *ABR17* gene, we performed two-dimensional electrophoresis to compare proteome-level

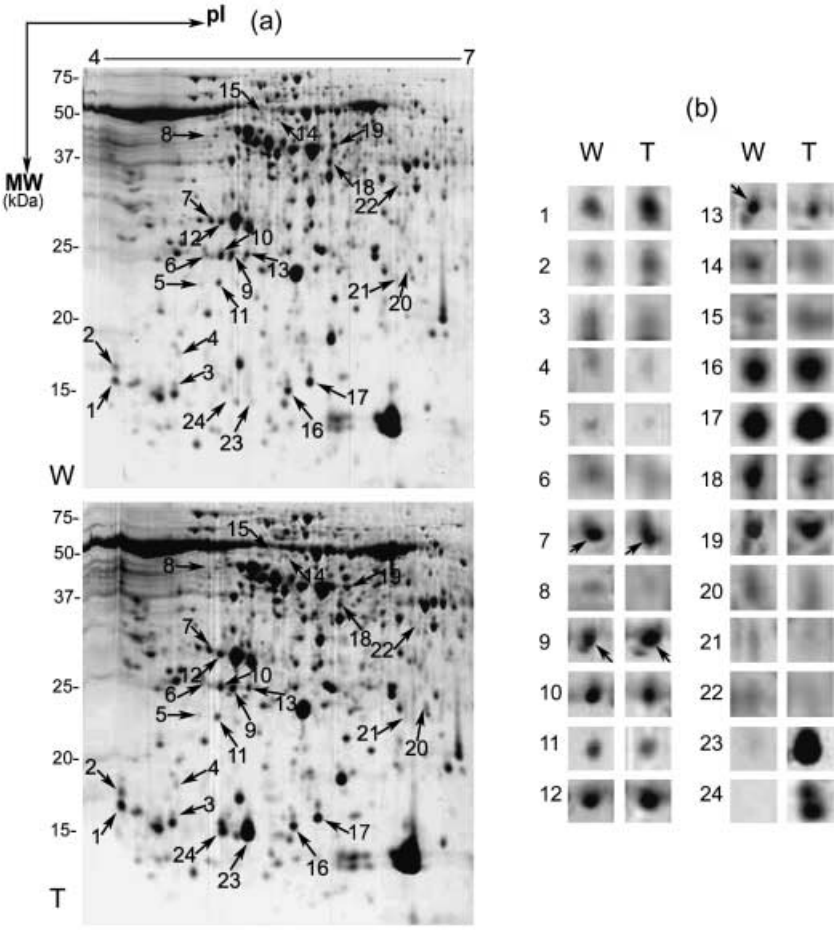

Figure 7 Images of two-dimensional gels of protein extracted from wild-type (W) and transgenic (T) plants. (a) Representative images of whole gels and (b) a closer view of changes in the intensities of the spots selected for identification.

changes brought about by the transgene expression. We selected the transgenic line 6.9 for these comparisons because of the fact that it exhibited the highest degree of stress tolerance. Representative images of two-dimensional gels obtained from wild-type and 6.9 protein extracts are shown in Figure 7. The intensities of 24 protein spots were observed to be significantly ($P < 0.01$) and reproducibly altered in the transgenic line compared with the wild-type (Figure 7). Of these 24 protein spots, the intensities of seven were greater, 15 were lower and two spots were unique in the transgenic line (Figure 7). The identities of these 24 spots were established using tandem mass spectrometry and are presented in Table 2. In all cases, Mascot searches of the National Center for Biotechnology Information (NCBI) non-redundant database generated significant hits with scores above the threshold value, and, in most cases, these were a result of multiple peptide matches (Table 2). These scores are based on individual ion scores, where the ion score is –10 log *P* and *P* is the probability that the observed match is a random event. A score above the threshold value indicates sequence identity or extensive homology ($P < 0.05$).

Of the proteins that were identified in this study, two were unique and the identities of both were established as pea ABR17 (spots 23 and 24; Figure 7, Table 2). Although only one protein corresponding to ABR17 was expected, the fact that there are two suggests the possibility that the additional spot may be the result of post-translational modification. Most of the proteins identified in this study were associated with photosynthesis and primary metabolic pathways, including putative photosystem I (PSI) subunit PSI-E protein (spot 2; Figure 7, Table 2), chlorophyll-binding proteins (spots 6, 10 and 13; Figure 7, Table 2), Cp29 (spot 7; Figure 7, Table 2), PSBO-2/PSBO2, oxygen-evolving protein (spot 12; Figure 7, Table 2), ribose-5-phosphate isomerase (spot 9; Figure 7, Table 2), phosphopyruvate hydratase (spot 14; Figure 7, Table 2) and Ribulose bisphosphate carboxylase/oxygenase (RUBISCO) activase (RCA) (spot 19; Figure 7; Table 2). Other interesting proteins identified in this study were DRT112 (spot 1; Figure 7, Table 2), which is thought to be involved in DNA damage repair, and glycine-rich proteins (spots 16 and 17; Figure 7, Table 2), which are involved in post-transcriptional gene regulation.

Table 2 Details of proteins identified by electrospray ionization quadrupole time-of-flight tandem mass spectrometry (ESI-Q-TOF MS/MS)

Spot	Protein identity	ESI-Q-TOF MS/MS			Access. No.‡	M_r/pI	Fold change
		PM (%)*	Score†	Sequence			
1	DRT112 [*Arabidopsis thaliana*]	14	88 (> 48)	NNAGYPHNVVFDEDEIPSGVDVAK	gil166696	17089/5.06	3.49 ± 0.57
2	Putative photosystem I subunit PSI-E protein [*Arabidopsis thaliana*]	23	153 (> 49)	AAEDPAPASSSSK RESYWFK NVGSVVAVDQDPK	gil24030202	14756/9.92	18.17 ± 10.23
3	GDCH [*Arabidopsis thaliana*]	15	147 (> 49)	FCEEEDAAH VKPSSPAELESLMGPK	gil15226973	18050/5.24	0.14 ± 0.13
4	Unknown protein [*Arabidopsis thaliana*]	7	85 (> 42)	VEVTEAEVELGFK	gil18422918	17603/4.80	0.68 ± 0.07
5	Bas1 protein [*Hordeum vulgare*]	27	140 (> 48)	YVILFFYPLDFTFVCPTEITAFSDR SGGLGDLKYPLVSDVTK EGVIQHSTINNLGIGR	gil861010	23398/5.48	0.47 ± 0.02
6	LHB1B2; chlorophyll binding [*Arabidopsis thaliana*]	59	483 (> 49)	GPSGSPWYGSDR YLGPFSGEPPSYLTGEFPGDYGW DTAGLSADPETFAR WAMLGALGCVFPELLAR FGEAVWFK LAMFSMFGFFVQAIVTGK GPLENLADHLADPVNNNAWAFATNFVPGK YLGPFSGEPPSYLTGEFPGDYGWDTAGLSADPETFAR	gil18403546	28093/5.28	0.42 ± 0.11
	LHB1B1; chlorophyll binding [*Arabidopsis thaliana*]	60	445 (> 49)	ASKPTGPSGSPWYGSDR YLGPFSGEPPSYLTGEFPGDYGWDTAGLSADPETFAR WAMLGALGCVFPELLAR FGEAVWFK VAGDGPLGEAEDLLYPGGSFDPLGLATDPEAFAELK LAMFSMFGFFVQAIVTGK GPLENLADHLADPVNNNAWAFATNFVPGK	gil18403549	28209/5.15	
7	Cp29 [*Arabidopsis thaliana*]	27	499 (> 49)	VNAGPPPPK SSYGSGSGSGSGSGSGNR LYVGNLSWGVDDMALENLFNEQGK GFGFVTLSSSQEVQK AINSLNGADLDGR VSEAEARPPR	gil681904	34602/5.23	0.86 ± 0.05

Table 2 *Continued*

Spot	Protein identity	ESI-Q-TOF MS/MS			Access. No.‡	M_r/pI	Fold change
		PM (%)*	Score†	Sequence			
	Inorganic diphosphatase/magnesium ion binding/pyrophosphatase [*Arabidopsis thaliana*]	22	326 (> 49)	VQEEGPAESLDYR VFFLDGSGK VSPWHDIPLTLGDGVFNFIVEIPK IVAISLDDPK HFPGTLTAIR IPDGKPANR	gil15242465	33644/5.71	
8	Putative aspartyl protease [*Arabidopsis thaliana*]	9	177 (> 49)	GDLASESILLGDTK SSVSLVSQTLK VIYDTTQER LGIVGENCR	gil12324588	47745/6.06	0.47 ± 0.10
	Ribosomal protein S1 [*Spinacia oleracea*]	8	151 (> 49)	VSDIATVLQPGDTLK AEEMAQTFR IAQAEAMAR	gil18060	45044/5.41	
9	Ribose-5-phosphate isomerase [*Arabidopsis thaliana*]	53	727 (> 49)	AVEAIKPGMVLGLGTGSTAAFAVDQIGK LLSSGELYDIVGIPTSK SLGIPLVGLDTHPR IDLAIDGADEVDPNLDLVK EKMVEAVADK MVEAVADK FIVVADDTK VDGDGKPYVTDNSNYIIDLYFK FQGVVEHGLFLGMATSVIIAGK NGVEVMTK	gil15229349	29401/5.72	1.46 ± 0.09
10	Chlorophyll *a/b*-binding protein (LHCP AB 180) [*Arabidopsis thaliana*]	67	516 (> 49)	GPSGSPWYGSDR YLGPFSGESPSYLTGEFPGDYGWDTAGLSADPETFAR WAMLGALGCVFPELLAR FGEAVWFK VAGNGPLGEAEDLLYPGGSFDPLGLATDPEAFAELK LAMFSMFGFFVQAIVTGK GPIENLADHLADPVNNNAWAFATNFVPGK	gil16374	25036/5.12	0.46 ± 0.02
	LHB1B2; chlorophyll binding [*Arabidopsis thaliana*]	31	376 (> 49)	GPSGSPWYGSDR WAMLGALGCVFPELLAR FGEAVWFK LAMFSMFGFFVQAIVTGK GPLENLADHLADPVNNNAWAFATNFVPGK	gil18403546	28093/5.28	

Table 2 *Continued*

Spot	Protein identity	ESI-Q-TOF MS/MS			Access. No.‡	M_r/pI	Fold change
		PM (%)*	Score†	Sequence			
11	2-cys peroxiredoxin-like protein [*Arabidopsis thaliana*]	31	334 (> 41)	AQADDLPLVGNK APDFEAEAVFDQEFIK LNTEVLGVSVDSVFSHLAWVQTDR SGGLGDLNYPLVSDITK EGVIQHSTINNLGIGR	gil9758409	29714/5.55	0.8 ± 0.04
12	PSBO-2/PSBO2; oxygen evolving [*Arabidopsis thaliana*]	31	451 (> 42)	RLTYDEIQSK GTGTANQCPTIDGGSETFSFK FCFEPTSFTVK VPFLFTVK GGSTGYDNAVALPAGGR NTAASVGEITLK SKPETGEVIGVFESLQPSDTDLGAK	gil15230324	35226/5.92	2.92 ± 0.30
	33-kDa oxygen-evolving protein [*Arabidopsis thaliana*]	31	430 (> 42)	RLTYDEIQSK FCFEPTSFTVK NAPPEFQNTK VPFLFTVK GGSTGYDNAVALPAGGR GDEEELVKENVK NTAASVGEITLK SKPETGEVIGVFESLQPSDTDLGAK	gil22571	35285/5.68	
13	Chlorophyll *a*/*b*-binding protein (LHCP AB 180) [*Arabidopsis thaliana*]	59	393 (> 41)	GPSGSPWYGSDR YLGPFSGESPSYLTGEFPGDYGWDT AGLSADPETFAR WAMLGALGCVFPELLAR FGEAVWFK VAGNGPLGEAEDLLYPGGSF DPLGLATDPEAFAELK GPIENLADHLADPVNNNAWAFATNFVPGK	gil16374	25036/5.12	0.29 ± 0.03
	LHB1B2; chlorophyll binding [*Arabidopsis thaliana*]	24	259 (> 41)	GPSGSPWYGSDR WAMLGALGCVFPELLAR FGEAVWFK GPLENLADHLADPVNNNAWAFATNFVPGK	gil18403546	28093/5.28	
14	Phosphopyruvate hydratase [*Arabidopsis thaliana*]	20	416 (> 42)	SAVPSGASTGIYEALELR NQADVDALMLELDGTPNK IGMDVAASEFFMK AAGWGVMVSHR SGETEDNFIADLSVGLASGQIK IEEELGNVR YAGEAFR	gil15221107	51841/5.79	0.41 ± 0.13

Table 2 *Continued*

Spot	Protein identity	ESI-Q-TOF MS/MS			Access. No.‡	*M*r/pI	Fold change
		PM (%)*	Score†	Sequence			
15	TUA3 [*Arabidopsis thaliana*]	16	363 (> 41)	QLFHPEQLISGK EDAANNFAR SLDIERPTYTNLNR LISQIISSLTTSLR IHFMLSSYAPVISAAK DVNAAVGTIK	gil15241168	50250/4.95	0.66 ± 0.06
16	ATGRP8 (GLYCINE-RICH PROTEIN 8); RNA binding/nucleic acid binding [*Arabidopsis thaliana*]	44	515 (> 41)	CFVGGLAWATNDEDLQR TFSQFGDVIDSK GFGFVTFK GFGFVTFKDEK VITVNEAQSR SGGGGGYSGGGGGGYSGGGGGGYER	gil15235002	16626/5.58	2.23 ± 0.02
17	Glycine-rich RNA-binding protein 7 [*Arabidopsis thaliana*]	53	581 (> 42)	CFVGGLAWATDDR ALETAFAQYGDVIDSK GFGFVTFK SGGGGGYSGGGGSYGGGGGR EGGGGYGGGEGGGYGGSGGGGGW	gil21553354	16934/5.85	1.73 ± 0.15
18	AT4g38970/F19H22–70 [*Arabidopsis thaliana*]	36	650 (> 41)	LDSIGLENTEANR TLLVSAPGLGQYVSGA ILFEETLYQSTTEGKK MVDVLVEQNIVPGIK TAAYYQQGAR TVVSIPNGPSALAVK YAAISQDSGLVPIVEPEILLDGEHDIDR ATPEQVAAYTLK ALQNTCLK YTGEGESEEAK	gil16226653	43029/6.79	0.65 ± 0.02
19	RCA (RUBISCO ACTIVASE) [*Arabidopsis thaliana*]	22	620 (> 42)	LVVHITK VPLILGIWGGK SFQCELVMAK SFQCELVMAK MCCLFINDLDAGAGR IKDEDIVTLVDQFPGQSIDFFGALR LMEYGNMLVMEQENVK VQLAETYLSQAALGDANADAIGR	gil18405145	52347/5.87	2.22 ± 0.37

Table 2 *Continued*

Spot	Protein identity	ESI-Q-TOF MS/MS			Access. No.‡	M_r/pI	Fold change
		PM (%)*	Score†	Sequence			
20	Glutathione *S*-transferase (GST6) [*Arabidopsis thaliana*]	24	288 (> 41)	QEAHLALNPFGQIPALEDGDLTLFESR GMFGMTTDPAAVQELEGK VLFDSRPK	gil20197312	24119/6.09	0.55 ± 0.02
21	Unknown protein [*Arabidopsis thaliana*]	10	169 (> 41)	LSVIVAPVLR FADNLGDDVK IENIGQPAK	gil2829916	29986/6.40	0.44 ± 0.06
22	Unknown protein [*Arabidopsis thaliana*]	13	257 (> 41)	SAVADNDNGESQVSDVR GKDPIVSGIEDK LSTWTFLPK	gil6437556	31235/5.91	0.67 ± 0.03
23	ABA-responsive protein [*Pisum sativum*]	48	487 (> 41)	GVFVFDDEYVSTVAPPK LDAVDEANFGYNYSLVGGPGLHESLEK VAFETIILAGSDGGSIVK GDAALSDAVR GDAALSDAVRDETK	gil20631	16619/5.07	Unique
24	ABA-responsive protein [*Pisum sativum*]	39	250 (> 41)	DADEIVPK EAQGVEIIEGNGGPGTIK LSILEDGK VAFETIILAGSDGGSIVK GDAALSDAVR	gil20631	16619/5.07	Unique

*Percentage identity between the amino acids present in the MS/MS tag and the sequences in the databases.

†Ion score is –10 log *P*, where *P* is the probability that the observed match is a random event. Individual ion scores: > value indicates identity or extensive homology ($P < 0.05$).

‡Accession number is Mascot search result using National Center for Biotechnology Information (NCBI) and other databases.

Discussion

Abiotic stresses, such as salinity, drought, cold and heat, negatively affect all stages of plant growth and development. Enhancing plant tolerance to abiotic stresses by introducing genes is a desirable method for many crop plants, but the nature of the genetically complex mechanisms of abiotic stress tolerance, and the potential detrimental side-effects of manipulating gene expression, make the task of generating useful transgenic plants very difficult. However, current research efforts to improve plant stress tolerance have had significant success. For example, the over-expression of the vacuolar Na^+/H^+ antiporter *AtNHX1* promoted growth and development in saline conditions (up to 200 mM NaCl) in *Arabidopsis* (Apse *et al*., 1999) and *B. napus* (Zhang *et al*. 2001). With respect to tolerance to cold temperatures, transgenic plants over-expressing various transcription factors have been demonstrated to have a higher tolerance. For example, in *Arabidopsis*, significant improvement of freezing stress tolerance was demonstrated by the over-expression of the transcription factor *CBF1* (Jaglo-Ottosen *et al*., 1998), enhancement of drought and freezing tolerance by *CBF4* (Haake *et al*., 2002), and increased tolerance to freezing, water and salinity stress by over-expression of the *DREB1A* gene (Kasuga *et al*., 1999), whereas transgenic rice over-expressing *CBF3* demonstrated elevated tolerance to drought and salinity, but very low freezing tolerance (Oh *et al*., 2005). It appears, therefore, that these transcription factors are able to enhance tolerance to a variety of stresses; however, such enhancement may be species specific (Oh *et al*., 2005). Therefore, it is extremely important to identify other genes/gene products capable of enhancing multiple stress tolerance so as to augment existing tools to engineer stress tolerance. In the extreme cold conditions of the Canadian prairies, as well as in other parts of the world, successful growth of many crops is based on their ability to germinate and survive better in cold temperatures (Coursolle *et al*., 1998). As a result of the risk of early spring or autumn frosts, the identification of novel stress-tolerant genes and the generation of transgenic plants with enhanced germination and early maturity may have very useful application for farmers. The results described here suggest that: (i) the constitutive expression of the pea *ABR17* gene in *Arabidopsis* enhances germination and early seedling growth in the presence of salt or cold, or when both stresses are combined; and (ii) 2-week-old transgenic *Arabidopsis* plants exhibit increased tolerance to multiple environmental stresses.

The phytohormone ABA plays a very crucial role in response to environmental stresses such as desiccation, salinity and cold, and is also involved in regulating events such as dormancy and maturation during late seed development (McCarty, 1995; Leung and Giraudat, 1998). Many ABA-inducible genes share *cis*-regulatory elements (i.e. ABA-responsive elements, ABREs; Guiltinan *et al*., 1990) and have been demonstrated to be involved in the regulation of plant stress responses. Constitutive expression of *ABF3* in *Arabidopsis* (Kang *et al*., 2002) and rice (Oh *et al*., 2005) enhanced tolerance to drought stress, as well as enhancing tolerance to multiple stresses in *Arabidopsis* (Kim *et al*., 2004). The pea ABA-responsive *ABR17* gene is known to be induced during late embryogenesis as well as by the exogenous application of ABA (Iturriaga *et al*., 1994), and the level of the ABR17 protein has been demonstrated to increase in response to salinity stress (Kav *et al*., 2004).

Several gene products homologous to ABA-responsive proteins (also known as dehydrins or LEA proteins) have been identified in different plant systems (Skriver and Mundy, 1990; Close *et al*., 1993). It has been suggested that dehydrins (members of LEA proteins) and small heat shock proteins (sHSPs) may protect cells from the deleterious effects of dehydration (Pnueli *et al*., 2002). Expression of the *HVA1* gene of barley, which encodes an LEA protein, conferred tolerance to salinity and water-deficit stresses (Xu *et al*., 1996), a tomato *Le25* gene in yeast increased freezing and salinity tolerance (Imai *et al*., 1996) and a wheat chloroplast LEA-like protein (WCS19) in *Arabidopsis* (Ndong *et al*., 2002) resulted in increased freezing tolerance. It is possible that ABR17 proteins may have a similar function to dehydrins (or LEA); this possibility is based on the fact that the ABR17 protein contains a larger amount of polar amino acids (about 30%), as do dehydrins (50%; Pnueli *et al*., 2002). However, the biological function of ABR17 is currently unknown, and therefore it is also possible that the mechanism by which ABR17 is able to protect plants is entirely different from that of the dehydrins and must be investigated further.

Despite the identification and characterization of the ABA-responsive protein ABR17, which is similar to the PR 10 proteins from various plant species, its biological function(s) remains unclear. Sequence similarities (60%–70% sequence identity) have been found between an isolated ribonuclease (RNase) from a callus cell culture of *Panax ginseng* and two IPRs from parsley, suggesting that IPR proteins may be RNases (Moiseyev *et al*., 1994). Other studies have also demonstrated RNase activity of some PR 10 proteins (Bufe *et al*., 1996; Bantignies *et al*., 2000; Biesiadka *et al*., 2002; Wu *et al*., 2003; Park *et al*., 2004). Recently, we have demonstrated that the pea PR 10.1 protein is an RNase (Srivastava *et al*., 2006) and, because of the similarities between pea PR 10.1 and ABR17, it is possible that pea ABR17 may also be a RNase. We also demonstrated recently that the constitutive expression

of pea PR 10 cDNA in *B. napus* enhanced germination and early seedling growth (Srivastava *et al*., 2004).

In order to understand the biological mechanisms underlying the ABR17-mediated multiple stress tolerance, we have compared the proteome of a transgenic *A. thaliana* line expressing the pea *ABR17* gene with its wild-type counterpart. As described previously, most of the proteins whose levels were significantly ($P < 0.01$) affected in transgenic line 6.9 are involved in photosynthesis or primary metabolic pathways (Table 2). Only those with reported roles in enhancing plant growth and development and/or mediating tolerance to abiotic stresses are discussed further. For example, we observed a significant increase in the levels of PSI-E (spot 2; Table 2), a component of PSI. PSI, a multiprotein complex, consists of core proteins and a peripheral antenna containing four different light-harvesting chlorophyll *a/b* protein complexes (LHCs) that mediate the light-driven transfer of electrons from plastocyanin to the ferredoxin-NADP complex (Jensen *et al*., 2003). PSI-E is crucial for the growth and development of *A. thaliana*, as demonstrated by a 50% decrease in growth as a result of disruption of the *psaE1* gene (Varotto *et al*., 2000). Therefore, it is possible that an increase in PSI-E protein in our *ABR17* transgenic lines may contribute to the observed increase in stress tolerance.

Another protein which showed a significant up-regulation of approximately threefold in the transgenic line is an oxygen-evolving protein, PSBO-2/PSBO2 (spot 12; Table 2). The light reaction in oxygenic photosynthesis is catalysed by the oxygen-evolving PSII and PSI (Steppuhn *et al*., 1988). Oxygen-evolving proteins, such as PSBO-2/PSBO2, also known as oxygen-evolving enhancer (OEE) proteins, and two other molecules form the oxygen-evolving complex, which functions in water splitting and the maintenance of an environment conducive for oxygen evolution (James *et al*., 1989). Levels of OEE have been reported to be affected by various abiotic stresses, including salinity (Abbasi and Komatsu, 2004) and cytokinin treatment (Kasten *et al*., 1997). Furthermore, it has been demonstrated that OEE from green algae possesses thioredoxin activity (Heide *et al*., 2004); thioredoxins are well-characterized proteins involved in diverse physiological processes, including the amelioration of oxidative stress (Balmer *et al*., 2004). Therefore, it is possible that OEE2 may enhance the tolerance of *ABR17* transgenic *A. thaliana* by reducing the oxidative damage that accompanies abiotic stresses.

In addition to the aforementioned proteins involved in the light reactions of photosynthesis, we also observed an increase of approximately twofold in the level of RCA (spot 19; Figure 7, Table 2), which is involved in the activation of RUBISCO (Parry *et al*., 2003). RCA, a member of the AAA^+ family, a class of chaperone-like ATPases, activates RUBISCO by removing sugar phosphates from its active site (Wang and Portis, 1992; Neuwald *et al*., 1999; Portis, 2003). RCA levels have been shown to be affected by abiotic stresses, including salinity (Parker *et al*., 2006) and drought (Salekdeh *et al*., 2002). It has been suggested that this increase in RCA levels may be responsible for mediating the tolerance to abiotic stresses by increasing RUBISCO activity under conditions of low stomatal conductance and CO_2 levels (Parker *et al*., 2006). It is possible that the transgenic line is able to grow and develop better under stress as a result of increased photosynthetic efficiency mediated by increased levels of specific photosystem components and enhanced RCA levels. However, the precise role(s) of these proteins in mediating the observed tolerance must be investigated further, and such studies are currently underway in our laboratory.

Another enzyme involved in primary metabolism, phosphopyruvate hydratase (spot 14; Table 2), also known as enolase (2-phospho-D-glycerate hydrolase), was observed to be decreased in the transgenic line. The role of enolase in mediating plant stress responses has been reported previously (Yan *et al*., 2005; and references therein), and it has been demonstrated that a mutation in *los2*, which encodes an enolase, enhances the tolerance of *Arabidopsis* to freezing temperature stress (Lee *et al*., 2002). Therefore, the observed decrease in enolase levels in the *ABR17* transgenic *A. thaliana* line may be important for its tolerance to cold temperature stress, and must be investigated further.

Two proteins involved in post-transcriptional regulation, glycine-rich RNA-binding proteins (GR-RBPs; spots 16 and 17; Table 2), were also observed to be increased in the transgenic *A. thaliana* line used in this study. GR-RBPs contain RNA-recognition motifs at the N-terminus and a glycine-rich region at the C-terminus that are important for their function (Sachetto-Martins *et al*., 2000; Kim *et al*., 2005). These proteins have been identified in various species, and their involvement in plant development, hormone signalling and stress responses has been suggested (Sachetto-Martins *et al*., 2000). Although the role of GR-RBPs *in planta* is still not very clear, Kim *et al*. (2005) demonstrated that the over-expression of *AtRZ-1a* (a cold-inducible zinc finger-containing GR-RBP) contributed to the enhancement of freezing tolerance in *Arabidopsis*. Our finding that two GR-RBPs are enhanced in the transgenic line provides further evidence that this group of proteins may be involved in mediating the tolerance to various abiotic stresses.

The results described in this article demonstrate, for the first time, that pea ABR17 is capable of protecting plants from multiple abiotic stresses, in particular cold and salinity.

This protection was evident during the germination of the transgenic seeds in the presence of salt or a combination of salt and cold, and also when 2-week-old plants were subjected to freezing stresses. The over-expression of pea ABR17 protein also resulted in several interesting protein changes, including photosynthesis-related proteins, enolase and GR-RBPs. Many of these proteins have previously been shown to be associated with enhanced stress tolerance; however, the precise roles of these proteins in mediating the observed tolerance in our transgenic lines remain speculative at this stage. Furthermore, the exact nature of the connections between ABR17 and the proteins that are altered in the transgenic lines need to be investigated further, which may shed additional light on the mechanism(s) underlying ABR17-mediated multiple stress tolerance.

Experimental procedures

Plant expression vectors

The pea ABR17 cDNA clone was kindly provided by Dr Trevor Wang, Department of Metabolic Biology, John Innes Centre, Norwich, UK. The cDNA was amplified using the forward (5′-GTGGTCG*AAGCTT*ATGGGTGTCTTTGTTTTTGATGATGAATAC-3′) and reverse (5′-TATATAG*CTCGAG*TTAGTAACCAGGATTT-GCCAAAACGTAACC-3′) primers, respectively. The restriction enzyme sites *Hin*dIII and *Xho*I are highlighted on the forward and reverse primers, respectively. The amplified cDNA fragment was ligated between the cauliflower mosaic virus (CaMV) 35S promoter and the rbcS3′ terminator in the binary vector pKYLX71 (Schardl *et al*., 1987), and the resulting gene construct was sequenced to ensure ligation of the cDNA in the correct orientation and the absence of any mutations/rearrangement.

The gene construct was subsequently introduced into the disarmed *Agrobacterium tumefaciens* strain GV3101 through a triparental mating technique using *Escherichia coli* strain HB101 carrying the helper plasmid pRK2013. Transconjugant *Agrobacterium tumefaciens* was selected on solid medium containing rifampicin, gentamicin and tetracycline. Plasmid DNA was extracted from *Agrobacterium tumefaciens* and analysed by restriction digestion to ensure that no rearrangements had taken place during the introduction into this bacterium.

Transformation of *A. thaliana*

A. thaliana ecotype WS was transformed using the floral dip method (Clough and Bent, 1998). Briefly, plants were grown in growth chambers at 25 °C until flowering with a 16-h light/8-h dark photoperiod. Primary racemes were clipped to encourage proliferation of secondary bolts. *Agrobacterium tumefaciens* cells at the mid-log stage of growth were centrifuged and resuspended to a density of 0.8 (Abs_{600}) in 5% sucrose solution containing 0.05% silwet L-77. Above-ground parts of *A. thaliana* plants were dipped in this *Agrobacterium* solution with gentle agitation. Dipped plants were covered with plastic wrap (for 16–24 h) to maintain high humidity and were returned to growth chambers. Harvested, dry seeds (T0) were surface sterilized and placed on half-strength MS plates containing 50 mg/L kanamycin. The seeds were cold treated at 4 °C for at least 2 days, and grown at 25 °C with a 16-h light/8-h dark cycle (100 μE light) for 7–10 days. Plants that survived on the kanamycin plates were transferred to soil and maintained in growth cabinets. A number of independent transgenic lines were tested for their abilities to germinate on MS plates containing 75 or 150 mM NaCl, and those with enhanced germination were selected for the production of homozygous plants. Seeds from the transgenic plants (T1 seeds) were screened on kanamycin plates to determine segregation ratios, and those with a monogenic (3 : 1) segregation ratio and exhibiting enhanced germination in the presence of NaCl were used for homozygous T2 seed production. Bulked homozygous seeds from T2 plants were used in all subsequent experiments.

RT-PCR analysis

Total RNA, isolated from wild-type and transgenic seedlings, was reverse transcribed and subsequently amplified to detect *ABR17* transcripts. RNA was isolated using the QIAGEN RNeasy Plant Mini Kit (Qiagen, Mississauga, ON, Canada) from pooled 2-week-old seedling tissue. The isolated RNA was treated with RNase-free DNase (Qiagen) to ensure the complete removal of DNA prior to RT-PCR. Reverse transcription and first-strand cDNA synthesis of total RNA (50 ng) were performed using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). PCRs were carried out using the newly synthesized cDNA (2 μL) as the template and primers (forward, 5′-GGTGATCAAGGAAGCACAAGG-3′; reverse, 5′-TTTGGCC-TTTGTTTCATCACG-3′) specific to the pea *ABR17* coding sequence, employing a PCR Master Mix (Promega, Madison, WI, USA). The *ABR17* transcript was amplified using the following thermocycling parameters: 94 °C for 2 min; 35 cycles at 94 °C for 1 min, 62 °C for 1 min and 72 °C for 1 min; and a final extension of 72 °C for 10 min. Plant *18s rRNA* primers (forward, 5′-CCAGGTCCAGACATAGTAAG-3′; reverse, 5′-GTACAAAGGGCAGGGACGTA-3′; Duval *et al*.,

2002) were used as an internal control. PCR products were separated by electrophoresis on a 1.2% agarose gel and visualized under ultraviolet light after staining with ethidium bromide.

Western blot analysis

Two-week-old pooled *Arabidopsis* seedlings were crushed in liquid N_2, and 500 μL of extraction buffer [0.5 M Tris-HCl, pH 6.8, containing 10% glycerol, 10% sodium dodecylsulphate (SDS) and 60 mM dithiothreitol (DTT)] was added to 200 mg of ground tissue. The tubes were vortexed and a small amount of protamine sulphate on the tip of a spatula was added and incubated at room temperature for 15 min prior to centrifugation at 12 500 ***g*** for 15 min. Ice-chilled acetone containing 0.07% DTT (five equal volumes) was added to the supernatants, which were centrifuged once more as described above. The pellets were dried for 15 min under vacuum and resuspended in 100 μL of 50 mM Tris, pH 6.8, containing 0.5% SDS. The concentration of protein in the resuspended samples was determined using a modified Bradford assay, and the samples were stored at –20 °C before being subjected to Western blot analysis.

Protein (30 μg) was applied to polyacrylamide gels (15%) and subjected to electrophoresis according to Laemmli (1970) using a Mini PROTEAN 3 vertical slab system (Bio-Rad) at a constant value of 160 V until the dye front reached the bottom of the gel. After electrophoresis, the gel was equilibrated in transfer buffer (48 mM Tris, pH 9.2, containing 39 mM glycine, 20% methanol and 1.3 mM SDS) for 15 min and transferred to polyvinylidene fluoride (PVDF) membrane for 25 min at 15 V using a Trans Blot SD, semidry transfer apparatus (Bio-Rad). Following transfer, the membrane was blocked with TBS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 5% non-fat skimmed milk powder for 2 h and rinsed with TTBS (TBS containing 0.05% Tween 20) for 10 min. The primary antibody solution (rabbit anti-PR 10.4 in TTBS; 1 : 20 000) was added to the membrane and, after 1 h of incubation, the membrane was washed three times for 5 min each with TTBS. The membrane was then incubated with secondary antibody solution [goat anti-rabbit immunoglobulin G conjugated with horseradish peroxidase (Abcam, Cambridge, MA, USA) in TTBS; 1 : 10 000] for 1 h, after which the membrane was washed three times for 5 min each with TTBS, followed by a 5-min wash with TBS. Bands on the blot were visualized by staining the membrane with a TMB Peroxidase Substrate Kit (Vector Laboratories Inc., Burlingame, CA, USA) according to the manufacturer's instructions.

Plant growth and germination experiments

Wild-type and transgenic (lines 6.9, 14.9, 25.20) *A. thaliana* ecotype WS seeds were grown on half-strength MS medium containing 1.5% sucrose, 0.8% agar, pH 5.7 and various salt concentrations (0, 75 and 150 mM NaCl) in 25 × 100-mm Petri dishes. Seeds were surface sterilized by immersing in 70% ethanol for 1 min, followed by rinsing twice with sterile deionized water. The seeds were then placed in a solution of 20% bleach for 15 min with occasional mixing, after which they were rinsed four times (5 min each) with sterile deionized water. At least five plates per treatment and 14 seeds per plate were used in these experiments, and the plates were incubated under continuous light at either 22 or 10 °C. The germination of seeds was monitored every day and the numbers of germinated seeds were recorded daily for 2 weeks. Those seeds in which the radicles had emerged were considered to have germinated. Each experiment was repeated at least three times.

Imposition of stresses

Wild-type and transgenic *Arabidopsis* lines (6.9, 14.9 and 25.20) were evaluated for tolerance to cold and high temperature stresses as described by Kim *et al*. (2004). For the imposition of cold stress, open MS plates containing the plants were placed at –5 °C for 4 h, after which the plates were returned to room temperature and photographed after 24 h of recovery. In the case of heat stress, 2-week-old plants in closed MS plates were placed in an incubator set at 48 °C for 2 h, after which they were returned to room temperature and photographed after 1 and 3 days of recovery.

Measurement of chlorophyll, carotenoid and ion leakage

The chlorophyll and carotenoid contents, and ion leakage, were used to assess plant damage as a result of stresses. Total chlorophyll and carotenoid were extracted from the pooled tissue of 2-week-old plants grown on MS plates, using a procedure modified from Kirk and Allen (1965). Pooled leaf tissue (0.05 g) was homogenized in 5 mL of ice-cold 80% acetone and incubated at room temperature for 10 min in the dark. Samples were then centrifuged at 2500 ***g*** for 15 min. The supernatant was decanted and the absorbance was measured at 663, 645 and 480 nm. Total chlorophyll ($a + b$) was estimated using a nomogram (Kirk, 1968), and the carotenoid levels were calculated using the formula (Kirk and Allen, 1965):

$$\Delta ACAR_{480} = \Delta A_{480} + 0.114\Delta A_{663} - 0.638\Delta A_{645}$$

where *A* is the absorbance and CAR is the carotenoid content.

Membrane damage was assessed by measuring ion leakage from cold-stressed leaves (Vettakkorumakankav *et al*., 1999). Leaves from control and stressed plants were incubated in 20 mL of distilled water and agitated at room temperature for 1 h, following which the conductivity of the solution (initial value) was determined using a conductivity meter (HI 8733, Hanna Instrument, Woonsocket, RI, USA). The solutions were incubated at 4 °C overnight, subsequently autoclaved to release total ions and the final conductivity values were determined. Ion leakage is expressed as a percentage of the initial to final values.

Two-dimensional electrophoresis

Protein extracts for two-dimensional electrophoresis were prepared according to the method described by Subramanian *et al*. (2005) with some modifications. Wild-type and transgenic (6.9) seedlings (2-week-old) from MS plates were homogenized to a fine powder in liquid nitrogen. Homogenized tissue (0.3 g) was further homogenized in acetone containing 10% (w/v) trichloroacetic acid (TCA) and 0.07% DTT, transferred to Eppendorf tubes and the volume was adjusted to 1.5 mL with acetone containing 10% (w/v) TCA and 0.07% DTT. Samples were incubated at –17 °C for 1 h, centrifuged at 13 000 ***g*** for 15 min and the supernatants were discarded. The pellets were washed by resuspension in ice-cold acetone containing 0.07% DTT and centrifuged as described above. This step was repeated four additional times, the pellets were dried at room temperature in a speed vacuum for 30 min and resuspended in 400 μL of rehydration/sample buffer (Bio-Rad, Mississauga, ON, Canada) containing 8 M urea, 2% w/v 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS), 40 mM DTT, 0.2% Bio-lyte 3-10 and 3 μL of 200 mM tributylphosphine (TBP). The samples were mixed vigorously, incubated overnight at 4 °C and centrifuged at 4 °C for 15 min at 13 000 ***g***. The supernatants were placed in fresh tubes and the protein concentrations were determined using a modified Bradford assay (Bio-Rad) using bovine serum albumin (BSA) as standard.

Two-dimensional electrophoresis of protein extracts was performed as described previously (Subramanian *et al*., 2005). Briefly, immobilized pH gradient (IPG) strips (17 cm, Bio-Rad) were passively rehydrated overnight with 300 μg of protein in 300 μL of rehydration buffer (8 M urea, 2% CHAPS, 40 mM DTT, 0.2% Bio-Lyte and 2 mM TBP). Isoelectric focusing (IEF) was performed using a Bio-Rad PROTEAN IEF unit to provide an optimum maximum field strength of 600 V/cm and a 50 μA limit/IPG strip at 10 000 V for 60 000 Volt hours (Vh). Prior to second dimension separation, proteins in the rehydrated strips were reduced by incubation twice in a solution (5 mL/strip) containing 6 M urea, 2% SDS, 0.375 M Tris-HCl, pH 8.8, 20% glycerol and 130 mM DTT for 10 min each. The strips were then incubated in the above solution containing 135 mM iodoacetamide instead of DTT twice for 10 min each in order to alkylate the reduced proteins. SDS-PAGE separation of the proteins was performed on 13% polyacrylamide gels (20 × 20 cm, 1 mm thickness) using a PROTEAN II XI system (Bio-Rad) at constant voltage (90 V) until the dye front reached the bottom of the gel. Protein spots were visualized using a Colloidal Blue Staining Kit (Invitrogen, Carlsbad, CA, USA) according to the instructions provided. Two-dimensional electrophoresis was performed at least three times with the extracted protein samples.

Image analysis

Images of the two-dimensional gels were acquired using a GS-800 calibrated densitometer (Bio-Rad) and analysed using PDQuest software (Bio-Rad). Three gels each of wild-type and transgenic (6.9) samples were used to generate the match sets, and individual spots were matched using the automated detection and matching feature of the software, followed by manual refinements in order to eliminate artefacts and include spots that were missed by the automated detection process. In order to identify the protein spots whose levels were significantly different between the transgenic and wild-type seedlings, the match sets from three replicate gels of wild-type and 6.9 samples were analysed using the Student's *t*-test feature of PDQuest software as described by the manufacturer. Those spots which were reproducibly altered in all three replicates and exhibited a significant ($P < 0.01$) difference were excised from the gels using sterile scalpels, and were subjected to electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-Q-TOF-MS/MS) analysis.

ESI-Q-TOF MS/MS analysis

Tandem mass spectrometry was performed at the Institute for Biomolecular Design, University of Alberta, on protein extracted from isolated gel spots, as described previously (Subramanian *et al*., 2005). Briefly, gel pieces were de-stained, reduced with 10 mM DTT, alkylated with 55 mM iodoacetamide and digested with 6 ng/μL trypsin (Promega Sequencing Grade Modified) in 50 mM ammonium bicarbonate (25 μL) for 5 h at 37 °C in a fully automated fashion on a Mass Prep

Station (Micromass, Manchester, UK). The tryptic peptides were subjected to liquid chromatography tandem mass spectrometry (LC/MS/MS) analysis on a Micromass Q-ToF-2 mass spectrometer (Micromass) coupled with a Waters CapLC capillary HPLC (Waters Corp., Milford, MA, USA). Peptides were separated on a PicoFrit capillary reversed-phase column (5 μm BioBasic C18; pore size, 300 Å; 75 μm ID × 10 cm; 15 μm tip; New Objectives, Woborn, MA, USA), using a linear water–acetonitrile gradient (0.2% formic acid), after desalting on a 300 μm × 5 mm PepMap C18 column (LC Packings, Sunnyvale, CA, USA). Eluent was introduced directly to the mass spectrometer by electrospray ionization at the tip of the capillary column, and data-dependent MS/MS acquisition was performed for peptides with a charge state of two or three. Proteins were identified from the MS/MS data by searching the NCBI non-redundant database with Mascot Daemon (Matrix Science, London, UK), including carbamidomethylation of cysteine, possible oxidation of methionine and one missed cleavage per peptide as search parameters.

Statistical analysis

All analyses were performed using the mixed model procedure of SAS version 8e (Statistical Analysis System, 1985, SAS Institute, Cary, NC, USA). Mixed model methodology was employed to perform analysis of variance and to estimate least-square means and standard errors of the genotype.

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