Identification and expression analysis of two Arabidopsis LRR-protein encoding genes responsive to some abiotic stresses

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Abstract
Two Arabidopsis thaliana genes, psr9.2 and psr9.4 appeared to be highly similar to a phosphate-starved induced gene, psr9, isolated from Brassica nigra suspension cells. Sequence analysis classified the encoded polypeptides as members of leucine-rich repeat (LRR) proteins superfamily. The sequence of psr9 proteins comprise a unique N-terminal region encompassing a coiled-coil structure proceeding eleven LRRs along the C-terminal. Expression pattern analysis showed the responsiveness of these genes to various environmental conditions. Although both psr9 genes, psr9.2 and psr9.4, are expressed throughout the plant, the expression of psr9.2 was higher in the root whereas psr9.4 expression was prominent in the shoot. The expression levels were increased proportional to the duration of phosphate deprivation treatment. Plants exposed to cold temperature expressed both genes at high levels in both roots and shoots. In contrast, heat shock increased the expression levels of both genes in the shoots while reducing it in roots. High-salt treatment upregulated the expression of psr9 genes only in the roots. These data may suggest distinct roles for psr9 genes during plant response to various environmental conditions.
Keywords: *psr9; Cold stress; High salt stress; Phosphate starvation*

INTORDUCTION

Plants have evolved adaptive mechanisms to deal with harsh environmental conditions that are launched upon signal perception and transduction. Characterization of relevant components have elucidated plant signal transduction mechanisms to some extent. Nevertheless, the plant signaling mechanisms for nutrient deficiencies, including phosphate starvation, and other abiotic environmental conditions are still rather vague.

Since 1985, when Leucine-rich repeats (LRRs) was reported for the first time, numerous LRR-containing proteins have been identified in all prokaryotic and eukaryotic organisms (for a review see Kobe and Kajava, 2001). The three-dimensional structures of several LRR proteins have been determined. LRRs are generally 20-29 residue long encompassing a conserved 11-residue segment with consensus sequence of LXXLXLXX[NC]XL (X, any amino acid; L, leucine, valine, isoleucine and phenylalanine). Each structural unit of LRRs consists of a β-sheet and an α-helix connected by loops. The structural units are arrayed around a common axis resulting in a non-globular horseshoe-like molecule so that parallel elements are placed on the concave side (Kajava, 1998; Kobe and Kajava, 2001). In this model, substantial variations within the LRR region as well as the number of repeats are responsible for the specific activities.

A survey of LRR functions suggests that LRRs provides an ideal platform for protein-protein interactions involved in many biologically important processes such as hormone-receptor interaction, enzyme inhibition, cell adhesion, cellular trafficking and plant resistance to pathogens (for reviews see Ashfield *et al.*, 2004; Dievart and Clark, 2004). However, there are quite a few reports about the identities and possible roles of LRR proteins responsive to environmental

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stresses. A carrot LRR protein with antifreeze activity is believed to be evolved from a polygalacturonase inhibitor protein, which is involved in defense against fungi (Meyer *et al*., 1999). A functional subgroup of LRR receptor kinases are involved in brassinosteroid signal transduction as shown in dwarf mutants of *Arabidopsis* (Li and Chory, 1997). Besides, regions of ten LRRs were found in a novel type of cell wall proteins that were designated as LRR-extesins (Baumberger *et al*., 2003). Recently, Forshoefel and colleagues (2005) reported a novel class of plant interacellular LRR (PIRL) proteins composed of 9 members in Arabidopsis. They have shown that all of PIRL encoding genes, except for PIRL7, are actively transcribed in root and shoot.

Here, we have reported the identification of two plant LRR proteins responsive to a range of abiotic stresses. The corresponding cDNA clone, named *psr9*, was initially isolated from phosphate-starved *Brassica nigra* cells (Malboobi and Lefebvre, 1995). Sequence analysis showed that there are two *psr9* ortologs in *A. thaliana* genome. Expression patterns of the *psr9* genes were investigated to find out whether they are responsive to specific environmental conditions and regulated differently.

MATERIALS AND METHODS

Plant culture: *Arabidopsis thaliana* (var. Columbia) seeds were surface-sterilized and grown on MS medium (Murashige and Skoog, 1962) containing 2% sucrose and 0.8% agar for 12 days prior to transfer into liquid culture as described by Malboobi and Lefebvre (1997). The seedlings were incubated in 20 ml of half-strength MS medium with 1% sucrose for two days prior to treatments. Then, all plant cultures were continued for 14 days under sterile hydroponics conditions refreshing the medium every three days. For different phosphate ion (P_i) concentrations, filter-sterilized KH_2PO_4 adjusted to pH 5.8 with KOH was added to the medium. For the minus P_i treatments, 5 mM filter-sterilized KCl replaced the KH_2PO_4. For all investigated stresses other than P_i starvation, the initial concentration of P_i was 5 mM. For the high-salt treatment, sterilized NaCl solution was added to a final concentration of 100 mM. For the minus nitrogen treatment, KNO_3 and NH_4NO_3 were replaced by an equal molarity of KCl. For medium lacking sulfur, $MgSO_4$ was replaced by an equal molarity of $MgCl_2$. All plants were harvested after 14 days of such treatments, except for plants grown without nitrogen that were harvested after 9 days because of the onset of severe deprivation symptoms. Heat shock was exerted by incubating 28 day-old plants at 39°C for 2h. For cold treatments, 28 or 27 day-old plants were incubated at 4°C for 2h or 24h, respectively. For elicitor treatment, 28 days old plants were incubated in the 20 ml fresh medium containing 1 μg/ml chitin for 4h. Plants were also grown in pots by sowing seeds in the mixture of soil perlit in regular green house conditions.

Identification and sequence analysis of *psr9* genes: A cDNA clone for *psr9* gene was isolated by differential screening from *B. nigra* suspension cells treated with or without P_i (Malboobi and Lefebvre, 1995). Nucleotide sequence of *B. nigra psr9* is available at GenBank Accession No. AY090611. Sequence comparisons and structural features predictions were performed by the use of on-line databases and related softwares including BLASTN, X and P (Gish and State, 1993), Pfam (Bateman *et al*., 2000), Prints (Attwood *et al*., 2002), Blocks (Pierrokovski *et al*., 1996), SMART (Letunic *et al*., 2002) and PSORT (Nakai and Horton 1999).

RNA extraction and cDNA synthesis: Direct isolation of mRNA from plant tissue was carried out using oligo(dT)-cellulose as described (Noppen *et al*., 1996). The mRNA extracted from roots and shoots of *A. thaliana* seedlings subjected to various stresses were used for cDNA synthesis. For the reverse transcription reactions 100 ng mRNA was mixed with 40 pmole oligo-$(dT)_{15}$ primers and heated at 65°C for 10 min. After cooling on ice, typical 20 μl reactions of 10 mM DTT, 1 mM dNTP, 20 units RNase inhibitor and 50 units of Expand™ reverse transcriptase enzyme (Roche Molecular Biochemicals) were prepared. Samples were incubated at 42°C for 1h and placed on ice to stop the reaction.

Comparative-Quantitative RT-PCR: A pair of *psr9*-specific primers (5´-AGAGCTTCTAATTGCATTGCTTCTCC-3´ and 5´-CTCGTTGAAACTCACATCCAGCTC-3´) that share similarity with two *A. thaliana psr9* genes were used along with α-tubulin primers for the same PCR reactions. PCR reactions were performed in a reaction consisting of 50 mM KCl, 15 mM Tris-HCl (pH 8.4), 2 mM $MgCl_2$, 0.25 mM dNTP mix, 0.25-0.5 μM of each primer, 0.1-1 μl of cDNA synthesis products and 1unit *Taq* DNA polymerase (CinnaGen, Tehran, Iran). The PCR reactions were performed on a thermocycler (Perkin-Elmer, GeneAmp 9600) with the following program: 4 min incubation at 94°C and then 30 cycles of 1 min, 94°C; 1 min, 60°C; and 1 min, 72°C; before final extension

at 72°C for 5 min. To distinguish between bands representing two genes, the PCR products were digested with *Bgl*II at 37°C for 2h before loading onto 1% agarose gels. These experiments were repeated at least three times for each sample. Ethidium bromide-stained gel photos were scanned to obtain numeric data representing the band intensities. All calculations and chart drawings were performed on Photo-Capture and Microsoft Excel softwares. Relative band intensities were calculated through dividing the intensity of each *psr9* band by that of the α–tubulin band and standardized with respect to the length of DNA fragments.

Nuclear run-off experiments: Five microgram of *psr9* and α-tubulin cDNA inserts were alkaline denatured and applied on Nytran-plus membrane (Schleicher and Schuell, Guelph, Canada) at per dot using Bio-Dot Microfilteration apparatus (Bio-Rad). In order to produce probes from newly synthesized mRNA, transcriptionally active nuclei were isolated from 5-6 g root and shoot tissues of 14-days old P_i -fed and starved plants according to Willimitzer and

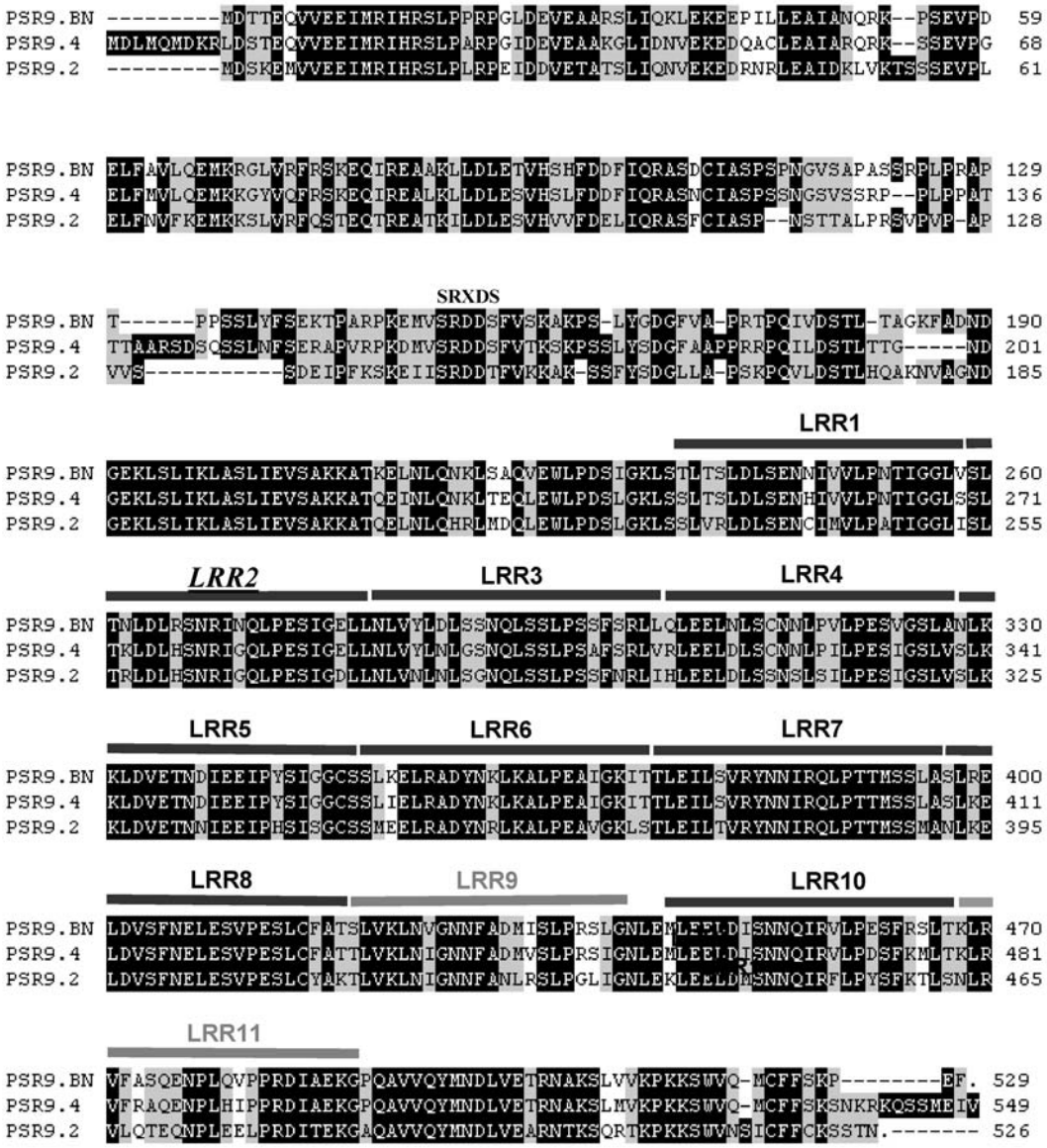


Figure 1. Comparison of predicted amino acid sequences of *B. nigra* PSR9 with *A. thaliana* PSR9.2 and PSR9.4. Residue identities and equivalent substitutions are highlighted in black and gray blocks, respectively. Gaps introduced to optimize the alignments are marked by dashes. The positions of LRRs are indicated with black or gray bars depending on the level of similarities to the consensus sequence. Sequence with similarity to catalytic site of mammalian adenylate cyclase is shown.

Wanger (1981) method. Transcription was allowed to continue within the isolated nuclei in the presence of ^{32}P-UTP for 60 min at 30°C as described by Chappel and Hahlbrock (1984). The labeled RNA was purified using Somassich and colleagues (1989) protocol. The RNA probes were hybridized with the dot blots as described before (Malboobi and Lefebvre 1995). After the last wash at high stringency conditions, blots were treated with a solution of 20 µg/ml ribonuclease A in 2X SSC for 30 min at room temperature. The blots were then washed twice with 2X SSC, 0.5% SDS and twice with 0.2X SSC before exposing to X-ray films and autoradiography. The 1X SSC solution consisted of 0.15 M NaCl and 15 mM trisodium citrate.

RESULTS

Sequence analysis of *psr9* genes: As reported earlier, *psr9* clone was isolated by differential screening of a *B. nigra* cDNA library with cDNA probes derived from P_i-fed or P_i-starved suspension cells (Malboobi and Lefebvre, 1995). For annotation purpose, the nucleotide and deduced amino-acid sequences were subjected to various database searches and computational analysis. The first two putative protein sequences identified by BLAST P algorithm were highly similar to *psr9* (Fig. 1). These two, named *psr9.2* and *psr9.4*, encoded by At2g17440 and AT4g35470 loci on chromosome 2 and 4.

Psr9.2 and *psr9.4* genes are predicted to encode polypeptides with 58.7 and 61.0 kD masses with 81% and 86% similarity to *Brassica nigra* PSR9 protein**,** respectively. In spite of the existence of some short hydrophobic regions as predicted by the Kyte-Doolittle method (Kyte and Doolittle, 1982), no likely transmembrane domain was found. A nuclear targeting motif (KPKK) was found at the carboxyl end of the

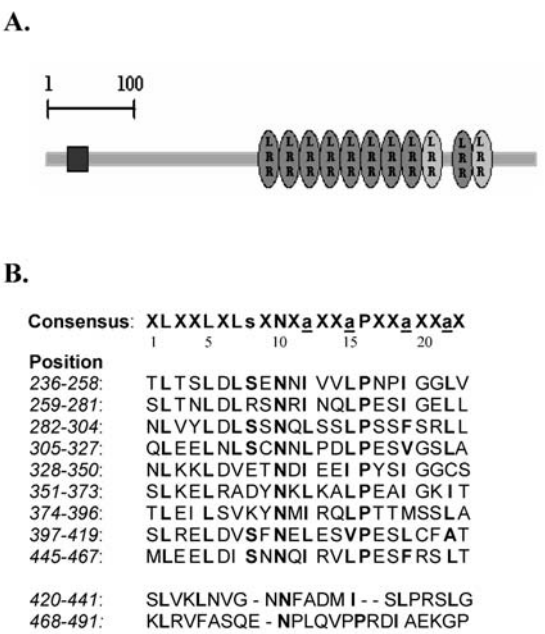


Figure 2. Predicted domains within the amino acid sequence of PSR9. A) A region with coiled coil structure (dark box) at N-terminal and eleven conserved LRRs (dark and gray oval shapes) are shown. B) Alignment of LRRs predicted within PSR9 sequence with the 24-amino acid LRR consensus that is reported for fungal adenylate cyclases (Buchanan and Gay, 1996). Nine LRR sharing more identities to the consensus LRR sequence are taken apart from the other two with little similarity. Abbreviations are: L, Leucine; S, serine; N, Asparagine; P, Proline; a, aliphatic residue; X, any amino acid.

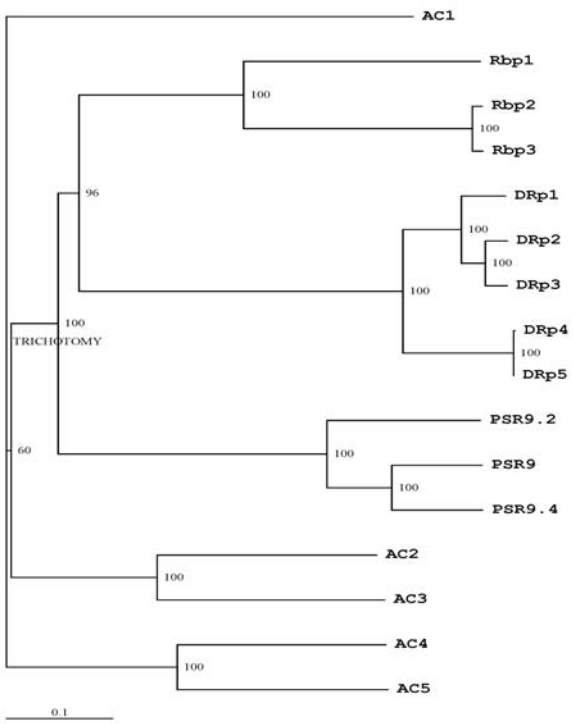


Figure 3. Phylogenetic relationship between PSR9 and representatives of three LRR protein families. Tree was drawn based on multiple alignments of amino acid sequences by Clustal X method. Bootstrap percentage values for 1000 replicates are indicated at branches. The names of gene and species and accession numbers are as below: PSR9, *B. nigra* AY090611; PSR9.2, *A. thaliana* AC002329; PSR9.4, *A. thaliana* AL117188; Rbp1, *Caenorhabditis elegans* AF068919; Rbp2, *Homo sapiens* AF068920; Rbp3, *Mus musculus* AF068921; DRp1, *Lycopersicon esculentum* AAC78593; DRp2, *Lycopersicon pimpinellifolium* AAC15780; DRp3, *Lycopersicon esculentum* AF053993; DRp4, *Lycopersicon pimpinellifolium* AAC15779; DRp5, *Lycopersicon pimpinellifolium* AAC78594; AC1, *Schizosaccharomyces pombe* P14605; AC2, *Saccharomyces kluyveri* P23466; AC3, *Saccharomyces cerevisiae* P08678; AC4, *Podospora anserina* Q01513; AC5, Q01631 *Neurospora crassa*.

Table 1. The effects of nutrient deficiencies and high salt concentration on the growth rate of hydroponically cultures *A. thaliana* seedlings. The averaged total, root and shoot fresh weights and the root/shoot ratios are given for fifty seedlings grown in the medium with or without nutrients as described in Materials and Methods. Seedlings were grown in half-strength MS medium containing 1.2 mM P_i for 14 days prior to each treatment. The control plants were grown in half-strength MS medium containing 5 mM P_i (well fed) during the course of experiment.

Days of treatment	Treatments	Fresh weight (g)			Root/Shoot ratio
		Total	Root	Shoot	
0	None	0.58	0.05	0.53	0.094
8	Control	3.85	0.57	3.28	0.174
	No Pi	1.53	0.26	1.27	0.205
	No nitrogen	1.82	0.49	1.33	0.370
14	Control	7.88	1.08	6.80	0.158
		2.89	0.67	2.22	0.302
		5.73	1.26	4.47	0.282
		6.32	1.65	4.67	0.353

polypeptide, by PSORT analysis. Interestingly, sequence analysis by SMART program revealed a coiled-coil structure at N-terminus and a region of eleven leucine-rich repeats (LRR) at the C-terminal half of all predicted amino acid sequences, (Fig. 2). The conservation of proline 16, aspargine 10, and serine 8 in addition to leucine residues within the LRRs resemble the consensus sequence of LRRs in fungal adenylate cyclases (Buchanan and Gay, 1996; Fig. 2B). No nucleotide binding site (NBS) was detected in PSR9 sequences.

Similarity searches showed that PSR9 aligns with various members of other LRR-proteins families, particularly Ras-binding proteins, such as yeast and fungal adenylate cyclases and also disease resistance proteins. To indicate the relationship among these proteins, a phylogenic tree was drawn based on multiple alignments with Clustal X method (Fig. 3). Despite very little simliarities, conservation of the catalytic site of mammalian adenylate cyclases (SRXDS/T; Zhang *et al.*, 1997) at position 154-158 was noticeable (Fig. 1).

Hydroponic plant cultures and nutrient manipulations: A hydroponic plant-culture system was developed that allows manipulations of available nutrients with the least effect on other environmental parameters. To show the appropriateness of the methodology, collected data for the effect of some nutritional treatments on shoot and root growth rates of fifty seedlings are reported in Table 1. In compare to P_i-fed control plants (5 mM P_i), lower fresh weight of both root and shoot tissues were observed for the P_i -starved seedlings during the course of experiments. Despite considerable loss in the total biomass (Approx. 2.7 times), about two folds differences could be seen when comparing the root/shoot ratios after two weeks of the treatments. This is due to the increased root growth as a result of P_i starvation. Similarly, the elimination of nitrogen caused decline in growth while the root/shoot ratio was doubled by the day eight. The incubation of these plants was ceased earlier than others as the leaves turned yellow, severely. The growth rates of plants cultured in media with 100 mM NaCl or without sulfur were slightly lower than the control plants, though the root/shoot ratios were almost as high as P_i-starved ones at day fourteen.

Expression analysis of *psr9* genes: We have previously reported that the expression levels of two messages with approximate molecular size of 1.7 and 2.9 Kb increased in *B. nigra* cells as the P_i concentration is decreased from 10 mM to 1.2 mM and to no P_i (Malboobi and Lefebvre, 1995). Since the expression level of this gene is very low, we decided to use comparative-quantitative RT-PCR to investigate the expression level of *psr9* homologous genes in hydroponically grown *A. thaliana* seedlings. The identity of the RT-PCR products were confirmed by using several nested-PCR reactions in which six different pairs of primers were applied (data not shown).

In general, the *psr9* expression was low as compared to another P_i–starvation induced gene, *psr3* (data not shown) and *α-tubulin* (Fig. 4-6). Since the transcripts of *psr9* genes generate two PCR bands with indistinguishable sizes (Fig. 5, lane1), the RT-PCR products were digested with *Bgl*II restriction enzyme. As shown in Figure 4 to 6, this endonuclease cut amplification products into two fragments with molecular sizes of 0.4 and 0.5 kb for *psr9.2* and into two 0.1 and 0.8 kb fragments for *psr9.4*. Since *psr9* gene was originally isolated in relation to low P_i conditions, its

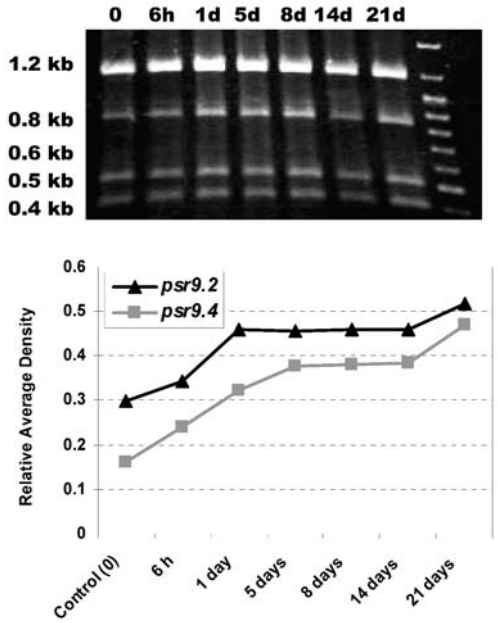


Figure 4. The expression levels of *psr9* genes in *A. thaliana* roots during the time course of growth in no P_i medium. Top, a typical gel electrophoresis photograph of *Bgl* II-digested RT-PCR products of *psr9.2* (0.5 and 0.4 kb), *psr9.4* (0.8 Kb) and a-*tubulin* gene (1.2 kb). Bottom, a graph of standardized data of comparative-quantitative RT-PCR products for samples collected after 6h, 1, 5, 8, 14 and 21 days of growth in no P_i medium. The 0.1 Kb fragment of *psr9.4* is not shown.

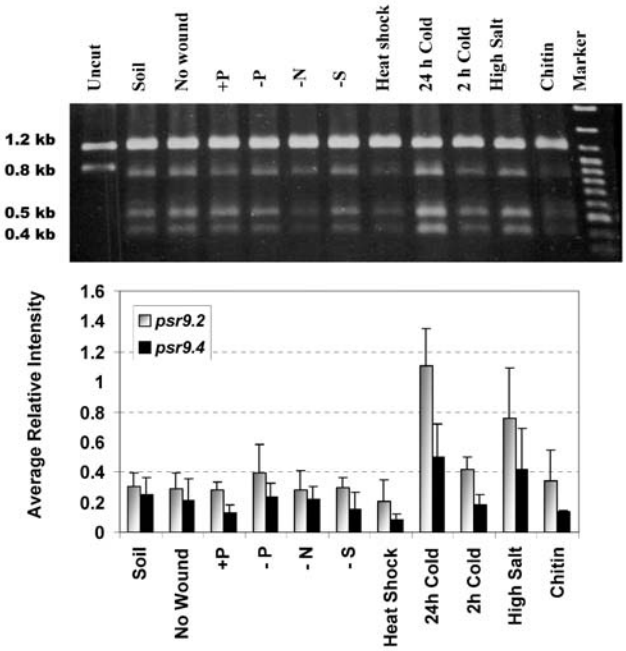


Figure 5. The expression levels of *psr9* genes in *A. thaliana* plant roots cultured under various environmental conditions. Top, a typical gel electrophoresis photograph of *Bgl* II-digested RT-PCR products. Fragments are as described in figure 4. Bottom, histograms of quantitative data for RT-PCR products representing the *psr9* mRNA levels accumulated in root of plants grown under various environmental stresses. The abbreviations are as follow: uncut, undigested RT-PCR products; soil, soil-grown plants; No wound, no cutting at the time of harvest; $+P_i$, phosphate-fed; $-P_i$, phosphate-starved; -N, nitrogen-starved; -S, sulfur-starved; heat shock, grown at 39°C for 2 h; 24h or 2h cold, incubating at 4°C for 24h or 2h; high-salt, grown in the presence of 100 mM NaCl; and chitin, grown in medium containing 1μg/ml chitin for 4h.

expression level was investigated during the course of P_i-starvation treatment. Figure 4 shows that the expression levels of both *psr9* genes increased gradually in the root tissues as the P_i-starvation treatment was prolonged for 21 days. Besides, the appearance of a 0.65-Kb RT-PCR product was consistently noted in P_i-starved shoot samples. Since no other homologous genomic sequence other than the above loci has been reported in *A. thaliana* genome project, the expression of an alternative-splicing product in the shoot could be investigated**.**

Different patterns of expression were observed when *Arabidopsis* seedlings were subjected to the other environmental conditions. There was only slight difference between the expression of *psr9* genes in the soil-grown plant and hydroponically cultured plants (Fig. 5). This shows that possibly there is or little effect of culturing method on *psr9* gene expression. Furthermore, we made certain if wounding during the plant harvest influence the levels of expression by having no wound control plants (Fig. 5). Comparing Figure 5 and 6, higher expressions of *psr9.2* was detected in the roots, while the expression of *psr9.4* was generally higher in the shoots.

Lack of nitrogen and sulfur, or exposure to chitin, as fungal elicitor, did not produce considerable changes in the transcript levels. However, as shown in Figure 5 and 6, remarkable variations in the gene expression levels were observed in the roots and shoots when plants were subjected to heat shock (39°C), cold temperature (4°C), or high-salt concentration (100 mM NaCl) treatments. Compared with the well-fed plants grown at room temperature, 3-fold increase in the expression for both *psr9* genes occurred in heat-shocked shoots, while the repression of these genes in the roots was evident. Also, induction of both genes in the roots and shoots of plants exposed to cold temperature was more pronounced if it was prolonged to 24h, particularly for *psr9.2* expression in the root (ca. 5 folds; Fig. 5). High salt concentration in the medium increased the expression of *psr9.2* and *psr9.4* to 2.7 and 3.3 times, respectively, in the root tissues. No apparent change in the expression of these genes was detected in the shoots of salt-treated plants.

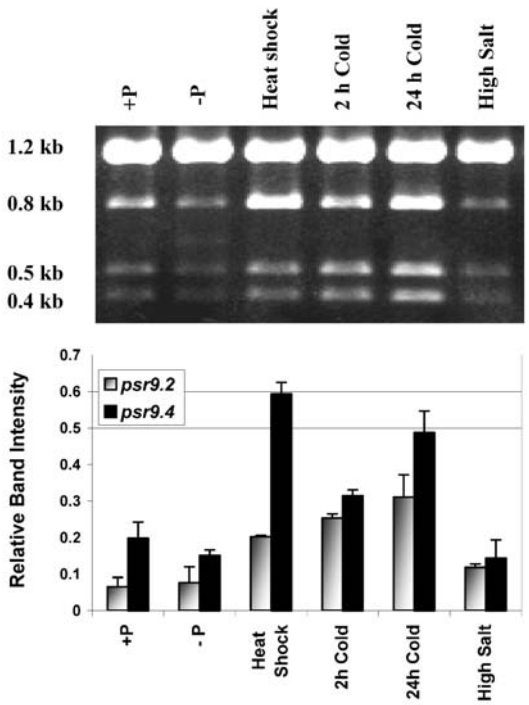
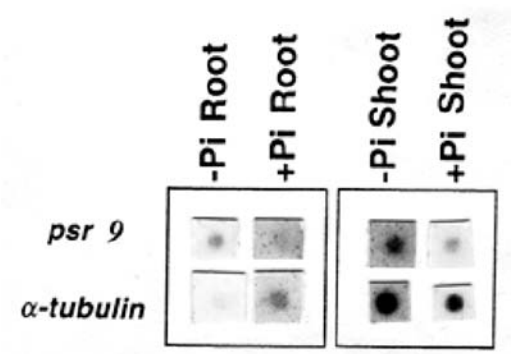


Figure 6. The expression levels of *psr9* genes in *A. thaliana* plant shoots grown under various stresses.Top, a typical gel electrophoresis photograph of *Bgl* II-digested RT-PCR products. Fragments are described in figure 4. An additional 0.65 Kb band is visible in -P_i shoot. Bottom, histograms of quantitative data for RT-PCR products representing the *psr9* mRNA levels accumulated in plant shoots grown under various environmental stresses as described in figure 5 legend.



Relative intensity	2.04	0.27	0.54	0.38

Figure 7. Detection of the level of newly synthesized *psr9* mRNA species. Transcription was allowed in the isolated nuclei from of P_i-fed (+Pi) and P_i –starved (-Pi) roots and shoots of *A. thaliana* in the presence of labeled UTP. The labeled products were hybridized with *psr9* and *α-tubulin* DNA dot-blotted on a nylon membrane. The relative intensities shown in the table below indicate the ratio of expression levels in P_i-fed (+Pi) roots to that of P_i –starved roots and shoots.

In order to investigate whether *psr9* genes are regulated at transcriptional initiation level, nuclei isolated from P_i-starved and P_i-fed plant roots and shoots were allowed to continue RNA synthesis in the presence of ^{32}P-UTP. Hybridization of newly synthesized RNA with *B. nigra psr9* and *α-tubulin* DNA dotted on membrane and washing at high stringency conditions allowed the comparison of *psr9* and *α-tubulin* expressions at transcription initiation levels. Similar to quantitative RT-PCR results, higher transcription rates of *psr9* genes were detected for the P_i-starved root samples (Fig. 7).

DISCUSSION

Since late 1980's, it is known that LRR-proteins constitute a main group of plant disease resistance (R) genes. In recent years, the occurrence of many more LRR proteins have been pointed out in plant genomes (Mondragon-Palomino *et al*., 2002; Meyers *et al*., 2003). The major trend in the relevant publications is to bring about possible signaling mechanisms in plant-pathogen interactions. Diversified length, polymorphic sequences and frequent occurrence of LRRs in the plant genomes has led to the proposed role of this gene family as the innate surveillance system for recognizing the large battery of pathogens in the nature (Dangl and Jones, 2001; Meyers *et al*., 2003). However, the available data in other organisms reflect various roles for LRR proteins in several cell processes, particularly wherever protein-protein interactions necessitate specific events. For instance, a receptor kinase carrying 25 tandem LRR is engaged in signaling of brassinsteroid as shown by mutation analysis (Li and Chory, 1997). Induction of *psr9* genes by P_i starvation prompted us to show if members of this protein family are associated with plant response to abiotic stresses and to what extent.

As shown in Figure 1 to 3, all PSR9 amino acid sequences encompass a coiled-coil region at N-terminal and a number of conserved LRRs at C-terminal. PSR9 LRRs strongly resemble the consensus sequence suggested for fungal Ras-binding proteins, particularly adenylate cyclases (Fig. 2). These proteins are known to be involved in transmitting nutritional signals and regulating cell growth for a long time (Young *et al*., 1991; Buchanan and Gay, 1996). Introduction of insertion or point mutation into any LRR unit abrogated Ras-dependent activation of these adenlyate cyclases and led to sensitivity to heat shock and nutritional starvation and also failure to sporulation (Kido *et al*., 2002). A known amino acid sequence in the catalytic

site of mammalian adenylate cyclase type 1 was also conserved in PSR9 proteins (Fig. 1). These amino acid residues form ventral cavity of ATP-binding site (Zhang *et al*., 1997). In addition, a repeated DNA sequence within the promoter region of *psr3.2* gene encoding a P_i -starvation inducible β-glucosidase (Malboobi and Lefebvre 1997), is highly similar to an element within the promoter of an inducible cAMP early repressor (ICER) gene (Malboobi and Lefebvre, unpublished data). This may provides another support for the postulated role of cAMP signaling pathway in plant response to P_i starvation stress.

The involvement of cAMP and adenylate cyclases in the phosphate-starvation response has already been reported for bacteria (Wanner *et al*., 1988) and yeast (Matsumoto *et al*., 1984). Whether cAMP is an authentic second messenger in higher plants has been controversial for a long time. In year 2001, Moutinho and colleagues detected a resting concentration of 50-100 nM in living maize pollen tubes by using a very sensitive imaging technique for cAMP distribution. These researchers isolated an adenylate cyclase-encoding gene, *PsiP*, from maize pollen and confirmed its functionality by complementation of the *cyaA* mutant of *E. coli* (Moutinho *et al*., 2001). However, we found no significant similarity between primary sequence of PSR9 and PsiP except for the LRR motifs (data not shown). Therefore, it is quite possible that significant divergence at the N-termini and even within the LRR motifs has hindered the identification of plant adenylate cyclases by sequence similarity predictions. For the same reasons, we doubt if yeast complementation assays could help with the function of *psr9* genes. Besides, organ specific response to P_i starvation should not be neglected. Therefore, the functional analysis await for assays in transgenic plants in which each one of the *psr9* gene is overexpressed or silenced. As recently shown both *psr*9.2 and *psr*9.4 are expressed in leave, root, flower and seeds of Arabidopsis (Forshoefel *et al*., 2005).

Different levels of expression for *psr9.2* and *psr9.4* genes in response to environmental stress were monitored by quantitative RT-PCR. Although the *psr9* clone was isolated from *B. nigra* cells subjected to long term P_i starvation treatment, the expression of both genes were elevated after one day and remained almost constant for the duration of experiment. They were found to be even more inducible by other environmental factors. In all experimental conditions, *psr9.2* expression levels were high in roots as compared with *psr9.4* for which high expression levels were detected in shoots. Both genes were induced in roots and shoots of plants exposed to cold temperature and prolonged exposure led to higher gene expression levels. Conversely, heat shock induced both genes in shoots while repressing them in roots. In comparison, the presence of high salt in the medium induced *psr9* genes in roots, but not in shoots. The appearance of a 0.65 Kb band in the starved shoots is an additional evidence for differential expression of *psr9* genes. It is possible that *psr9* genes work together to activate proper signaling pathways in order to derive appropriate responses toward complex environmental conditions in certain plant tissues.

Curiously, the genome of other plants was inspected for the presence of *psr9* ortologs by gene-specific primer pairs as STS markers. Two fragments with the size of 1.3 and 1.1 Kb in length were amplified for *A. thaliana* that are of expected sized predicted from the genome project data. The other chosen genomes were belong to three dicotes subclasses: Dialypetales (*B. nigra* and *B. napus*), Apetales (patellaris and sugar beet), and Gamopetales (tobacco), as well as monocots (*Aeluropus lagopodes*, a salt tolerant plant from gramineae family). One or more loci were detectable in all examined plants indicating well representation of this gene in all examined plants (data not shown).

* The nucleotide sequences reported in this paper are available at GenBank under the accession numbers: *B. nigra psr9* AY090611; *psr9.2,* NM127299; and *psr9.4*, NM119712.

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