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A soybean gene encoding a proline-rich protein is regulated by salicylic acid, an endogenous circadian rhythm and by various stresses

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Abstract A cDNA clone of a soybean gene encoding a proline-rich protein (PRP) was characterized and designated *SbPRP* (*So*y*b*ean *P*roline-*r*ich *P*rotein). The SbPRP protein is a putative bimodular protein of 126 amino acids with a proline-rich domain and a hydrophobic cysteine-rich domain plus a signal peptide at the N terminal. Southern analysis indicates the presence of a single copy of the *SbPRP* gene in the soybean genome. The *SbPRP* gene expression was investigated and the results demonstrate that it accumulates in leaves and epicotyls of soybean seedlings, but not in cotyledons, hypocotyls and roots. The *SbPRP* mRNA was also expressed in response to salicylic acid and virus infection. In addition, the *SbPRP* gene transcription was regulated by circadian rhythm, salt stress, drought stress and plant hormones. These results indicate that the *SbPRP* gene might play a role in plant responses to multiple internal and external factors.

Keywords Soybean [*Glycine max* (L.) Merr.] · Proline-rich protein · Salicylic acid · Circadian rhythm · Stress

Introduction

Biotic and abiotic stresses, including pathogen attack, drought stress and salt stress, have a serious influence on plant growth, development and crop yield. To adapt to unfavorable environmental conditions, plants develop many responses and many genes are induced. These induced genes fall into two groups. One group contains a regulatory gene that is generally involved in the signal transduction process (Zhang and Klessig 1997; Komjanc et al. 1999; Sakamato et al. 1999). Another group contains functional genes that possibly play roles in stress tolerance or defense reaction (Metraux et al. 1988; Merkouropoulos et al. 1999; Zhang et al. 1999; Li and Chen 2000; He et al. 2001a). Although some of the upstream and downstream genes have been well-studied and their association established, more genes need to be characterized to facilitate the full-understanding of plant responses to changes in their living conditions.

Soybean is one of the important crops in China. Each year, its yield is reduced due to unfavorable field conditions. To avoid such a situation, a transgenic approach is being adopted to improve the ability of soybean to survive extreme conditions. For this purpose, a series of genes have been cloned in our laboratory and their introduction into the plants resulted in an improved stress tolerance (Guo et al. 1997; Liu et al. 1997; He et al. 1999; Li and Chen 2000; Shen et al. 2001). Recently, a full-length gene from soybean has been cloned and showed homology to the tobacco mosaic virus resistance gene *N*. This gene has been mapped to the L linkage group using a genetic map established from a RIL population (He et al., unpublished results; Wu et al., unpublished results) and two of its homologs have been mapped to the J and F linkage groups (He et al. 2001b). A mitochondrial *atp6* gene was also characterized and it may play a role in the defense response (He et al. 2001a). Although some of the genes related to defense or abiotic stress have been isolated, genes that may function in both defense and abiotic stress responses were less investigated.

In the present study, a gene encoding a proline-rich protein was isolated from soybean seedlings and designated *SbPRP*. Its genomic organization and expression pattern in response to different factors were examined. Its possible roles in defense and stress response are also discussed.

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Materials and methods

Plant materials and treatments

Seeds of soybean [*Glycine max* (L.) Merr.] cultivar Kefeng1 were grown in the greenhouse or in pots filled with vermiculite. Leaves

were harvested for DNA extraction. Two-week-old seedlings were used in the following treatments. Samples from all the treatments were harvested at the indicated times and stored at –70 °C for RNA isolation.

For salicylic acid (SA) treatment, seedlings were either sprayed with 2.0 mM of SA until running off, or immersed with their roots in different SA solutions for 24 h. For virus inoculation, each leaf of the soybean seedling was gently rubbed with carborundum and inoculated with a suspension of the soybean mosaic virus (SMV) strain Sa by using a soft brush. For Circadian study, seedlings were grown under natural conditions of May (16 h light/8 h dark). Leaves were harvested at 4-h intervals beginning at 6:00 pm, and the experiment lasted for 3 days. Plants were transferred to continuous light (100–120 µE s^{-1} m^{-2}) with a constant temperature of 25 °C, or continuous light (20 µE s^{-1} m^{-2}) with a constant temperature of 4 °C, for the study of the regulation of the Circadian rhythm. For salt-stress treatments, seedlings were watered with different concentrations of NaCl and leaves were collected at 9:00 am, 12 h after the initiation of the treatment. For drought treatments, water was withheld from seedlings 3 days before the first harvest at 9:00 am. The drought condition was maintained and the leaves were harvested at the same time each day for the following 4 days. For plant-hormone treatments, seedlings were carefully pulled out of the soil and their roots were immersed in the following solutions: indoleacetic acid (IAA, 100 µM), naphthaleneacetic acid (NAA, 100 µM), gibberellin (GA_3, 60 µM), kinetin (90 µM), abscisic acid (ABA, 0.2 mM), ethephon (2.0 mM) and methyl jasmonate (MeJA, 0.2 mM) for 18 h.

Genomic Southern-blot analysis

DNA isolation and genomic Southern-blot analysis were carried out as described previously (Chen et al. 1991). Hybridization was performed for 16 h at 65 °C with α-^{32}P-dCTP-labeled full-length *SbPRP* cDNA as a probe. The filters were washed with 2 × SSC, 0.1% SDS; 1 × SSC, 0.1% SDS and 0.5 × SSC, 0.1% SDS for 15 min at 68 °C, respectively.

RNA gel-blot analysis

Total RNA isolation and RNA gel-blot analyses were performed following the description by Zhang et al. (1995). Northern hybridization was performed overnight at 65 °C by using α-^{32}P-dCTP-labeled *SbPRP* cDNA as a probe. Filters were washed with 2 × SSC, 0.1% SDS and 1 × SSC, 0.1% SDS for 15 min at 52 °C, respectively, and then washed with 0.5 × SSC, 0.1% SDS for 10 min at 68 °C. After stripping the probes, the same blots were re-probed with a 18S rDNA gene. The mRNA levels were quantified using the Imaging DensitoMeter (Model GS-670, Bio-Rad). The resulting values were normalized with those obtained from the 18S rDNA hybridization. The experiments were repeated 2–3 times and the results were the same. Only typical ones are presented.

Sequencing analysis

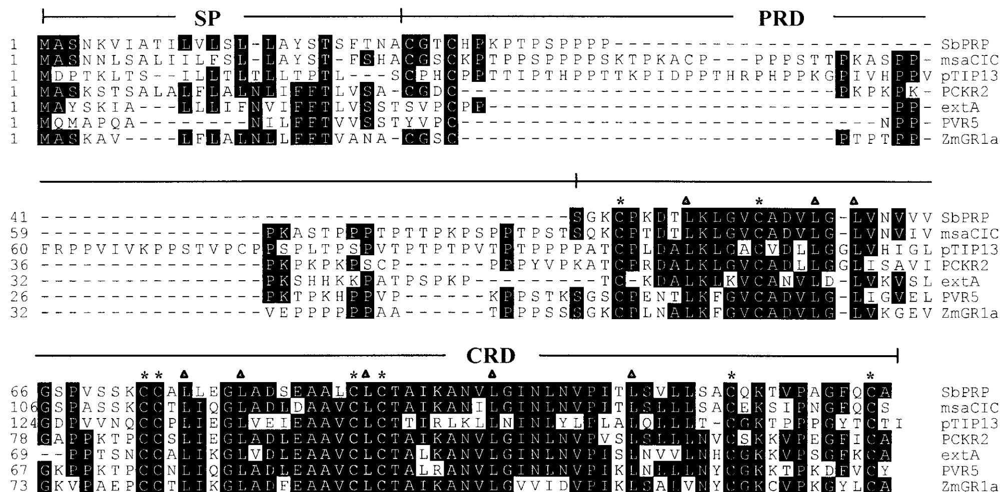
The *SbPRP* cDNA sequences were determined using the *Taq* Dye Primer Cycle Sequencing Kit (Amersham) and ABI 373A automatic sequencer. The nucleotide and amino-acid sequences were compared with those released in GenBank databases by using the BLAST analysis program. The domain structure of SbPRP was analyzed in the EMBL Database (http://smart.embl-heidelberg.de). The nucleotide sequence of *SbPRP* has been deposited in GenBank database under the accession number AF248055.

Results

Sequence analysis of the *SbPRP* gene

A cDNA was obtained from salicylic acid (SA)-treated soybean seedlings in an attempt to clone defense-related genes (He et al. 2001b). Sequence analysis revealed that it encoded a protein homologous to a series of proline-rich proteins (PRP). The gene corresponding to this cDNA was designated *SbPRP* (*S*oy*b*ean *Proline*-*r*ich *P*rotein). The *SbPRP* gene consisting of 760 bp, containing a 5′-untranslated region of 51 bp, a coding region of 381 bp and a long 3′-untranslated region of 328 bp (data not shown). The encoded SbPRP protein was composed of 126 residues. The first 25 residues represented a putative signal peptide (Fig. 1). After this, a typical bimodular arrangement of PRP proteins was clearly identified. The first do-

Fig. 1 Comparison of the deduced amino-acid sequence of the *SbPRP* gene with other PRP proteins. msaCIC (L22305) was from *Medicago sativa*, pTIP13 (X82413) was from *Asparagus officinalis*, PCKR2 (X85206) was from *Catharanthus roseus*, extA (X67421) was from *Arabidopsis thaliana*, PVR5 (U34333) was from *Phaseolus vulgaris*, and ZmGR1a (AB018587) was from *Zea mays*. The *numbers* on the left indicate the position of residue. The *asterisks* indicate the conserved cysteine residue and the *triangles* indicate the conserved Leucine residue in the cysteine-rich domain. *Dashes* were introduced to maximize the alignment. Residues shaded in *black* indicate identity. SP: signal peptide; PRD: proline-rich domain; CRD: cysteine-rich domain



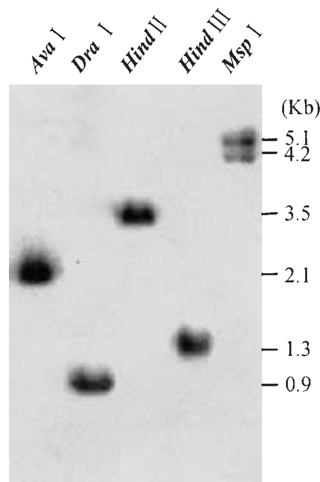


Fig. 2 Southern analysis of the soybean genomic DNA using the *SbPRP* cDNA as a probe. Ten micrograms of genomic DNA were digested with *Ava*II, *Dra*I, *Hind*II, *Hind*III and *Msp*I. After electrophoresis on a 0.8% agarose gel, fragments were transferred onto a Hybond N+ nylon membrane and hybridized with a labeled *SbPRP* probe. *Numbers* on the right indicate the size of the corresponding DNA fragment

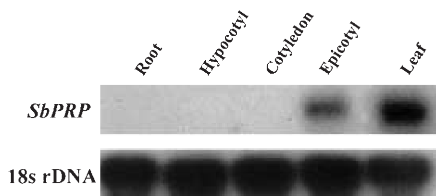


Fig. 3 Expression of the *SbPRP* gene in different soybean organs. The samples were harvested at 9.00 am and total RNA was isolated. Thirty micrograms of total RNA were run on a 1.0% agarose gel containing formaldehyde, transferred onto the Hybond N+ nylon membrane and then hybridized with the labeled *SbPRP* probe. The membranes were stripped and hybridized with the 18S rDNA probe to normalize the RNA loading

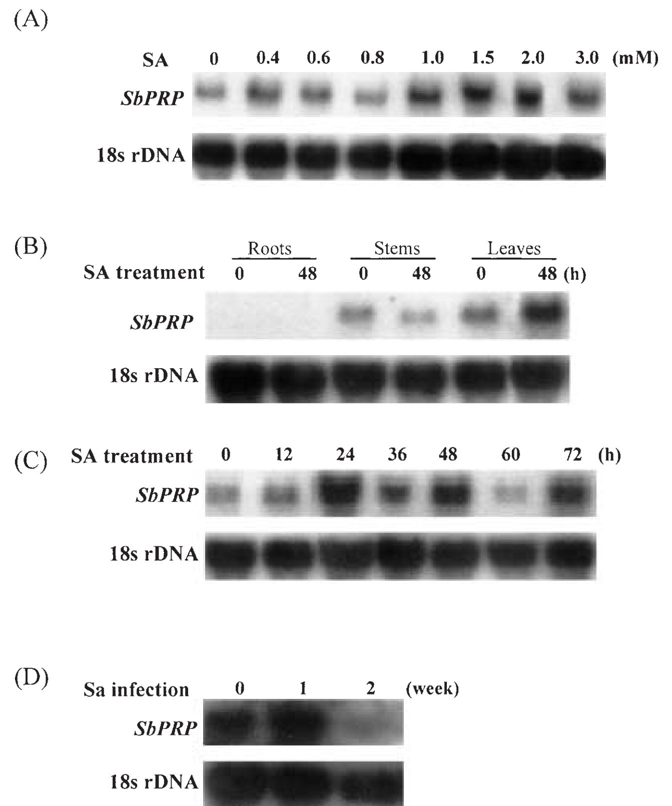


Fig. 4A–D Differential mRNA accumulation of the *SbPRP* gene in soybean seedlings in response to salicylic acid (*SA*) treatment and virus infection. The samples were harvested at 9.00 am and total RNA was isolated from leaves, unless stated. The method of hybridization is the same as in Fig. 3. **(A)** Expression of the *SbPRP* gene after seedlings were immersed with their roots in different SA solutions for 24 h. **(B)** SA induction of *SbPRP* gene expression in different organs of soybean. SA treatment was performed by both spraying and immersion with the roots in 2.0 mM of SA solution. **(C)** Time-course of *SbPRP* gene induction in soybean seedlings after spraying with 2.0 mM of SA solution. Please note the diurnal variation of the*SbPRP* gene expression. **(D)** *SbPRP* expression upon infection by soybean mosaic virus Sa for different times. The samples were harvested at 1.00 pm

main in this arrangement was a proline-rich domain located between residue 26 and 42, and a higher percentage of the proline residue was observed. The length of this domain varied among the proteins compared.

The second domain was a long cysteine-rich hydrophobic domain located between residue 43 and 126. Eight cysteines were present in this domain and were conserved among the different PRP proteins compared (Fig. 1). The spacing among the eight cysteines may be necessary for the formation of disulfide bridges within the protein or with other proteins (Deutch and Winicov 1995; Terras et al. 1995). The conserved spacing of residue L was also observed in these PRP proteins (Fig. 1) and possibly associated with the formation of a leucine zipper motif, as suggested in the case of HyPRP (Subramaniam et al. 1994). The present SbPRP shared 23.4–68.3% identity when compared with other PRPs and was more-related to a cold-induced PRP protein (MsaCIC) from alfalfa (Castonguay et al. 1994), which may play a role in the cold response. Other PRP proteins may function in the stress response and the developmental processes regulated by various factors or plant hormones.

Genomic organization of the *SbPRP* gene

To investigate the genomic organization of *SbPRP*, Southern-blot analysis was performed using the *SbPRP* cDNA as a probe. Because the recognition sites of *Ava*II, *Hind*II, *Hind*III, *Msp*I and *Dra*I were not detected in the *SbPRP* cDNA, genomic DNA of Kefeng1 was digested with these restriction enzymes and the hybridization results (Fig. 2) indicated the possible presence of a single-copy *SbPRP* gene in the soybean genome. A pattern of more than one band observed in the *Msp*I digestion was possibly caused by the introns in the genomic DNA.

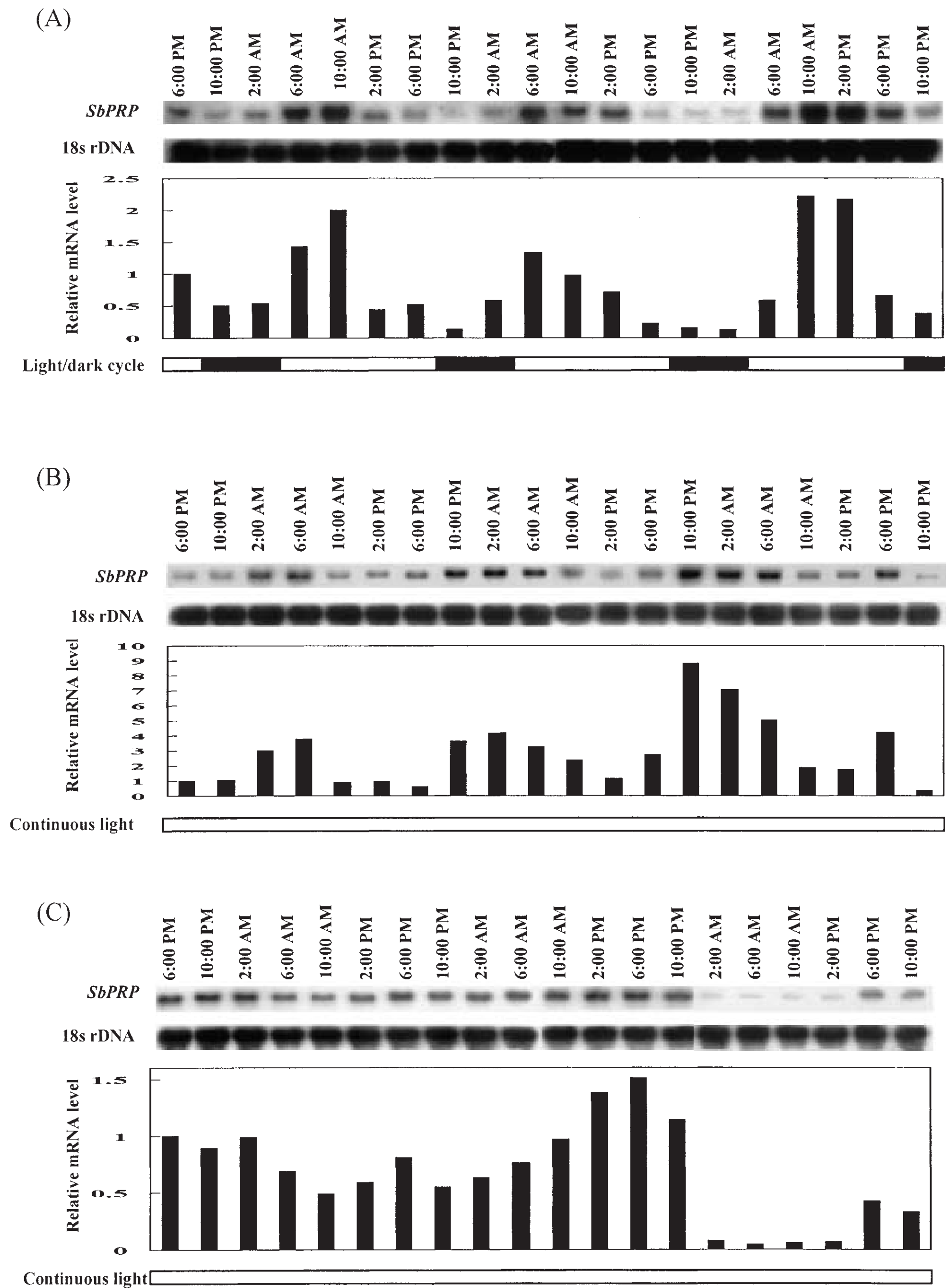


Fig. 5A–C Circadian regulation of *SbPRP* gene expression. Leaves were harvested at the indicated times. Total RNA isolation and hybridization were performed as in Fig. 3. The hybridization signals were quantified and the relative mRNA levels were determined by the ratio of the *SbPRP* density to the 18S rDNA density. The value in the first point was arbitrarily set to 1 and all other values were compared with it. **(A)** Changes of the *SbPRP* gene expression under natural conditions (16-h light/8-h dark, 10°–30 °C). **(B)** Changes of the *SbPRP* gene expression under continuous light (100–120 µE s^{-1} m^{-2}) at 25 °C. **(C)** Changes of the *SbPRP* gene expression under continuous light (20 µE s^{-1} m^{-2}) at 4 °C.

Organ-specific expression of the *SbPRP* gene

To elucidate the expression pattern of the *SbPRP* gene, total RNA was extracted from different organs of soybean seedlings and RNA gel-blot analysis was carried out. The results, in Fig. 3 show that the *SbPRP* mRNA accumulated in an organ-specific manner. It was expressed at a higher level in leaves and a lower level in epicotyls. No expression was detected in cotyledons, hypocotyls and roots.

Expression of the *SbPRP* gene in response to salicylic acid and virus infection

Since the *SbPRP* gene was isolated from SA-treated soybean seedlings, RNA gel-blot analysis was performed to investigate whether the *SbPRP* mRNA was accumulated in response to SA. The results are presented in Fig. 4. It can be seen that *SbPRP* mRNA accumulation tended to increase upon SA treatment and reached a maximum at 1.5–2.0 mM of SA (Fig. 4A). However, the expression of the *SbPRP* gene was only induced in leaves, and not in stems and roots (Fig. 4B). The *SbPRP* gene expres-

sion over the time course of the SA treatment was also investigated in leaves (Fig. 4C). It can be seen that the *SbPRP* transcript levels fluctuated depending on the time of day when the leaves were harvested. Leaves harvested at 9:00 am, and subsequently at 24, 48 or 72 h after the initiation of treatment, accumulated apparently more *SbPRP* mRNA than the leaves harvested at 9:00 pm, and subsequently at 12, 36 or 60 h after the initiation of treatment. These observations indicate the possible involvement of an endogenous circadian rhythm in the regulation of the *SbPRP* gene. Roots were also harvested at a 12-h interval and no expression of the *SbPRP* gene was detected (data not shown).

Based on the established relationship of SA with plant disease resistance (Delaney et al. 1994; Ryals et al. 1996; Durner et al. 1997), the SA-responsive nature of the *SbPRP* gene may implicate its involvement in the soybean defense response to pathogen attack. Therefore, the mRNA level of the *SbPRP* gene was examined in response to soybean mosaic virus (strain Sa) infection. The results in Fig. 4D show that mRNA accumulation of the *SbPRP* gene increased 1 week after inoculation and decreased 2 weeks after inoculation when compared with the 0-week control. This expression pattern indicates that the *SbPRP* gene might play a role in the soybean disease response.

Accumulation of the *SbPRP* transcript follows an endogenous circadian rhythm

To further examine the pattern of the *SbPRP* mRNA accumulation over time, soybean seedlings were grown under natural conditions for 2 weeks and leaves were harvested at 4-h intervals beginning at 6:00 pm and lasting for 3 more days. The results presented in Fig. 5 show that diurnal variation of the *SbPRP* transcript level occurred with minimal levels at 10:00 pm and maximal levels at 6:00–10:00 am (Fig. 5A). The periodic increase and decrease with a 24-h cycle suggests the involvement of a Circadian rhythm. To determine whether the rhythmic variations of the *SbPRP* mRNA accumulation were affected by light, seedlings grown under a natural photoperiod for 12 days were transferred to conditions of continuous light at a constant temperature of 25 °C for 2 days' adaptation, then the leaves were collected at indicated times for RNA gel-analysis. The results in Fig. 5B showed that the seedlings shifted to continuous light accumulated the *SbPRP* mRNA with a rhythmicity similar to seedlings grown under natural conditions. However, the peaks occurred 4–8 h earlier than those under natural conditions.

An effect of temperature on the Circadian rhythm of the SbPRP mRNA levels was also investigated. Seedlings grown under a natural photoperiod for 12 days were shifted to conditions of continuous light at a constant temperature of 4 °C for more than 3 days. The results of RNA gel-analysis indicate that low temperature interrupted the circadian rhythm and plants lost subtle control over the rhythm, although fluctuations were still observed (Fig. 5C). High temperature showed no effects on the rhythm (data not shown).

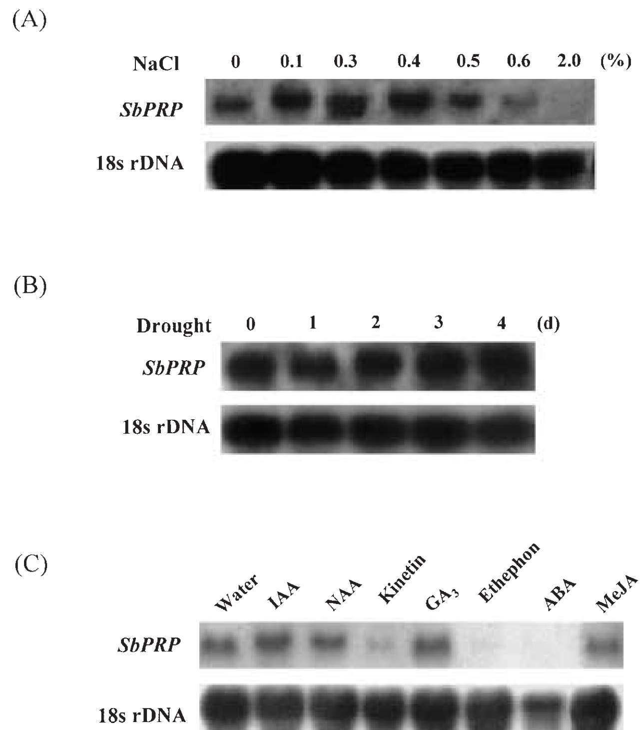


Fig. 6A–C Expression of the *SbPRP* gene in response to NaCl, drought and plant hormones. Total RNA extraction and hybridization were performed as in Fig. 3. **(A)** Soybean seedlings were treated with different concentrations of NaCl for 12 h. **(B)** Time-course of *SbPRP* expression in response to drought stress. **(C)** Soybean seedlings were treated for 12 h with the following plant hormones: 100 μM of IAA, 100 μM of NAA, 60 μM of kinetin, 90 μM of GA_3, 2.0 mM of ethephon, 0.2 mM of ABA and 2.0 mM of MeJA

Differential accumulation of the *SbPRP* transcript in response to NaCl, drought and plant growth regulators

Salinity and drought-responsive genes were well studied in our laboratory (Zhang et al. 1995, 1999; Li and Chen 2000). RNA gel-analysis was performed to elucidate the effect of salt and drought stress on *SbPRP* expression. The results in Fig. 6A show that *SbPRP* mRNA accumulation increased gradually and reached the highest level at 0.3% to 0.4% NaCl and then decreased. No transcript was detected in 2.0% NaCl treatment. In roots, the *SbPRP* mRNA was not detected after the same treatment (data not shown). In response to drought stress, an increasing tendency of the *SbPRP* mRNA-accumulation was observed in soybean seedlings, as presented in Fig. 6B.

Plant growth regulators play an important role in growth and development. The results in Fig. 6C indicated that IAA, NAA and GA_3 showed no significant effects on the level of the *SbPRP* transcript, but kinetin, ethephon, ABA and MeJA inhibited the transcription.

Discussion

In the present study, we described the characterization of a soybean proline-rich protein gene *SbPRP*. *SbPRP* encoded a putative bimodular protein (Fig. 1) which contains a proline-rich domain as well as a cysteine-rich domain. Homology analysis revealed that SbPRP shared similarity with many PRP proteins. Southern and Northern analysis indicated that the *SbPRP* gene was unique in the soybean genome and had an organ-specific expression pattern (Figs. 2 and 3). Its expression was observed in leaves and epicotyls but not in other organs. Organ-specific expression was also reported for another *PRP* gene (*PVR5*) from bean and it was specifically expressed in the roots of bean seedlings (Choi et al. 1996).

Accumulating evidence suggests that SA plays an important role in both systemic acquired resistance signalling and disease resistance (Durner et al. 1997; Zhang and Klessig 1997; Martinez et al. 2000). Upon infection, the endogenous SA level is increased and leads to a cascade of resistance signal transduction, and ultimately develops an enhanced resistance to further pathogen attack not only in the area of primary infection but also in distal uninfected areas (Ryals et al. 1996; Durner et al. 1997; Martinez et al. 2000). Moreover, the application of exogenous SA to plants would influence the expression of many genes and lead to a plant-resistance response (Ryals et al. 1996; Zhang and Klessig 1997; Komjanc et al. 1999; Merkouropoulos et al. 1999; Sakamato et al. 1999). In the present study, the *SbPRP* mRNA accumulation was significantly up-regulated in leaves when soybean seedlings were subjected to SA treatment (Fig. 4). An increase of the *SbPRP* mRNA level was also detected in the 1st week infection during with the soybean mosaic virus (Sa strain). These observations indicated that the SbPRP protein may function at a relatively early stage of Sa invasion, and induction by both SA and the mosaic virus demonstrated the involvement of the SbPRP protein in the soybean defense response, possibly as one component in the related cascade.

Genes regulated by the circadian rhythm are prevalent in plants and animals, and even in prokaryotes (Piechulla 1993; Dunlap 1999; Xu et al. 2000). Two classes of Circadian-regulated genes have been reported in plants. One class involves photosynthesis-related genes. Another involves photosynthesis-unrelated genes. Photosynthesis-related genes, like *rbcS* and *Cab*, exhibited a usual Circadian rhythm that peaks at the end of the dark period and the beginning of the light phase (Guilno et al. 1988; Millar and Kay 1991). Whereas other genes, such as the *Arabidopsis* genes *Ccr1* and *Ccr2* (encoding glycine-rich proteins), the *Pharbitis PNZIP* gene (encoding a protein with a leucine zipper motif) and the *Arabidopsis* and maize catalase genes *CAT3* and *cat3*, exhibited an unusual Circadian rhythm pattern that peaks at the end of the light period and the beginning of the dark phase (Redinbaugh et al. 1990; Carpenter et al. 1994; Zhong and McClung 1996; Zheng et al. 1998). The present *SbPRP* gene exhibited a 24-h rhythm under natural conditions (Fig. 5A) and was more similar to those of photosynthetic genes. Under continuous light at 25 °C, the rhythm was not significantly changed although the peak came earlier (Fig. 5B). At low temperature (4 °C) with continuous light, however, the rhythm was completely disturbed (Fig. 5C). The disruption of the Circadian rhythm by low temperature was also reported for the *Cab* gene (Martino-Catt and Ort 1992). These observations indicated that temperature was one of the important factors for the regulation of the rhythm. Regulation by the Circadian rhythm has not been reported for other PRP genes. The physiological relevance of the *SbPRP* mRNA oscillation with respect to its functions remains to be investigated.

Plant *PRP*s are expressed in response to many external factors. *Wcor518* from *Triticum aestrivum* L. (L173214), *PRP* from *Brassica napus* L. (X94976) and *MsaCIC* from alfalfa (L22305) were cold-regulated (Castonguay et al. 1994). *MsPRP2* from *Medicago sativa* L. (L37017) was salt-inducible (Deutch and Winicov 1995). A *PRP* from *Lycopersicon chilense*, however, was negatively regulated by water stress (Yu et al. 1996). Additionally, many *PRP*s were regulated by plant growth regulators, and some were down-regulated while others were up-regulated (Datta et al. 1993; Subramaniam et al. 1994; Neuteboom et al. 1999; Ogawa et al. 1999). In the present study, the *SbPRP* gene was induced by salt stress, drought stress, SA treatment and virus infection, but inhibited by kinetin, ethephan and ABA treatment. These results indicated that the *SbPRP* gene was regulated by multiple internal and external factors and may play a role in plant responses to changes in living conditions. The differential accumulations of the *SbPRP* mRNA in response to environmental or endogenous factors are probably due to the turnover of mRNA transcription or mRNA stability. Although the expression patterns of the *SbPRP* gene in response to various factors provided clues to understand its roles, further precise subcellular location and transgenic study should give a good elucidation of its biological functions.

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