Plant Physiology and Biochemistry 47 (2009) 73–79

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Molecular characterization of a novel soybean gene encoding a neutral PR-5 protein induced by high-salt stress

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ARTICLE INFO

Article history:
Received 11 September 2008
Accepted 24 September 2008
Available online 11 October 2008

Keywords:
Glycine max
High-salt stress
Homology modeling
Methyl jasmonate
Pathogenesis-related protein
Salicylic acid
Thaumatin-like protein

ABSTRACT

In this study, we characterized a novel soybean gene encoding a neutral PR-5 protein and compared it to two acidic isoforms of soybean PR-5 protein. This gene, designated as *Glycine max* osmotin-like protein, b isoform (*GmOLPb*, accession no. AB370233), encoded a putative protein having the greatest similarity to chickpea PR-5b (89% identity). Unlike the two acidic PR-5, GmOLPa and P21, the protein had a C-terminal elongation responsible for possible vacuolar targeting and after maturation showed a calculated molecular mass of 21.9 kDa with p*I* 6.0. The 3D models, predicted by the homology modeling, contained four α-helixes and 16 β-strands and formed three characteristic domains. The two acidic PR-5 proteins also showed a 3D structure very similar to GmOLPb, although the electrostatic potential on molecular surface of each PR-5 was significantly different. In the study of the gene expression under conditions of high-salt stress, *GmOLPb* was highly induced in the leaves of the soybean, particularly in the lower part of a leaf. The expression started at 2 h after initiation of the stress and was highly induced between 18–72 h. Gene expression of *P21e* (protein homologous to P21) was transiently induced by high-salt stress, but took place earlier than the gene expressions of *GmOLPa* and *GmOLPb*. Such differential expression was observed also under investigation with methyl jasmonate and salicylic acid. These results suggested that each soybean PR-5 might play a distinctive role in the defensive system protecting the soybean plant against high-salt stress, particularly in the leaves of the soybean.

1. Introduction

Pathogenesis-related protein (PR protein) is known to function in higher plants as a protein-based defensive system against abiotic and biotic stress, especially against pathogen infection. PR protein constitutes a large family of proteins and is generally classified into 16 subfamilies that display some differences in structural features, serological relationships and biological activities [23,31,32]. One of the PR proteins, pathogenesis-related protein family 5 (PR-5), shows high sequence similarity to thaumatin, a sweet-tasting protein from the West African shrub *Thaumatococcus danielli*, and is often referred to as thaumatin-like protein (TLP). Many genes encoding PR-5 proteins, including several homologous proteins, have been identified from a variety of plants, indicating that PR-5 is broadly distributed throughout higher plants. PR-5 proteins appear to be mainly involved in plant defensive systems that act against infection by pathogens, for example, inhibiting fungal spore germination or mycelial growth [1,24,29,34,35]. Consistent with this view, transgenic plants overexpressing PR-5 showed increased resistance to fungal pathogens [6,15]. In addition, the involvement of PR-5 proteins in protection against abiotic stresses, such as osmotic imbalance [13] and freezing stress [5], has been suggested. However, the molecular basis of PR-5 action in these defensive mechanisms against abiotic stresses remains uncertain.

Structural features of PR-5 proteins have been well-characterized by three dimensional (3D) modeling. Modeling studies using several PR-5 protein homologues, such as maize zeamatin [4], tobacco PR-5d [11], and thaumatin [20], suggest that the 3D model of PR-5 is a small protein consisting of three characteristic domains held in a compact fold by disulfide-bridges. Due to the high sequence similarities among known PR-5 homologues, all PR-5 proteins would share these common structural features and some differences between individual 3D structures of PR-5 might reflect their distinctive functions. Several differences among PR-5 proteins have also been found in isoelectronic values (p*I* value), subcellular localizations and regulations of gene expression. PR-5 proteins have p*I* values covering a wide range of 3.4–12.0 [33], which could have an influence on the electrostatic potential of the molecular surface.

Abbreviations: GmOLP, *glycine max* osmotin-like protein; MeJA, methyl jasmonate; P21e, P21-like protein from cv. ENREI; PR, pathogenesis-related; SA, salicylic acid; TLP, thaumatin-like protein.

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doi:10.1016/j.plaphy.2008.09.012

PR-5 proteins are localized mainly in two subcellular compartments, apoplastic space or vacuole, because an N-terminal signal peptide directs these PR-5 proteins to the secretory pathway and, in certain case, a C-terminal polypeptide directs them to the vacuolar compartment [16,18,26]. Expression of many PR-5 proteins is induced by some form of abiotic/biotic stress, whereas, in contrast, other PR-5 proteins are induced at developmental stages [33]. These results suggest that each PR-5 protein may play a distinctive biological or physiological role in the stress response and/or growth process, which is in part a consequence of their physical properties, subcellular distribution and expression patterns. Therefore, to better understand the roles and functions of PR-5 protein-based defensive systems in plants of interest, we need to characterize in detail the expression and structure of many isoforms of PR-5.

We have previously characterized a novel soybean gene encoding an acidic homologue of PR-5 protein, designated as *Glycine max* osmotin-like protein, acidic isoform (GmOLPa) [21]. *GmOLPa* gene, originally identified by cDNA-amplified fragment length polymorphism (cDNA-AFLP) [2], was induced by high-salt stress and therefore was defined as one of salt-inducible genes of soybean. In addition to our study of soybean PR-5, P21 protein (an acidic PR-5) was purified from leaves of healthy soybean [7], however the other isoforms of soybean PR-5 have not been characterized. High-salt stress is known to enhance gene expression and protein accumulation of several PR-5 proteins, including for example, osmotin from tobacco [27] and NP24 from tomato [10]. It was suggested that some of the PR-5 proteins could be involved in a plant defensive system, such as molecular response and/or adaptation against high-salt stress.

In this study, to clarify the roles and functions of soybean PR-5 in the defense against high-salt stress, we identified a novel salt-inducible gene encoding a PR-5 protein, which is different from the known acidic isoforms, GmOLPa and P21. The gene, which was designated as *GmOLP*, b isoform (*GmOLPb*), was characterized by comparison of sequences, physical properties, subcellular localization and transcriptional regulation with GmOLPa and P21. Finally, we discussed the distinctive roles of these PR-5 isoforms in soybean defensive systems against stress induced by high-salt levels.

2. Methods

2.1. Plant material and stress-treatments

We used a cultivar of soybean, *Glycine max* L. Merr. cv. ENREI, as the plant material in this study. A seed was germinated and grown on wetted vermiculite rocks under conditions of 12 h of light followed by 12 h of darkness, temperature 25 ± 1 °C, and humidity 70 ± 1%. A seedling, which has two nodes with expanded leaves (around 14 days after germination), was transplanted onto standard Hoagland's solution (0.8 mM $Ca(NO_3)_2$, 1.2 mM KNO_3, 0.4 mM $MgSO_4 \cdot 5H_2O$, 0.3 mM $(NH_4)_3PO_4$) and then was placed for 3 days of acclimation. After that, the seedling was transplanted onto Hoagland's solution containing the chemical agent, NaCl (final concentration of 100 mM), methyl jasmonate (MeJA, 100 μM) or salicylic acid (SA, 5 mM), and subsequently maintained for an appropriate period of time. MeJA or SA treatment was performed by dipping of the root in the solution for 2 min, and subsequently the seedlings were transplanted again into standard Hoagland's solution.

2.2. cDNA cloning of GmOLPb and P21-like protein

To identify a novel salt-inducible PR-5 protein from soybean, two ESTs, AI437712 and AW348587, which show greater similarities to basic PR-5 proteins than to the acidic ones, were found. On the basis of the EST information, we designed a primer set, GmOLPb-RT5′ (5′-tgc act ctt aac atc agc caa atg g-3′) and GmOLPb-RT3′v (5′-gac att tat tta ctg gtg ggc-3′). For cDNA synthesis, total RNA template was extracted from soybean seedlings treated for 3 days with 100 mM NaCl (see Section 2.4 for a procedure of RNA extraction). The cDNA was synthesized by PowerScript™ Reverse Transcriptase (Clontech) with Adaptor Oligo$(dT)_{18}$VN primer (5′-*ctg atc tag agg tac cgg atc c*gt ttt ttt ttt ttt ttt ttv n-3′; n means all nucleotides and v for n except t; nucleotides of the adaptor primer are in italic) according to the manufacturer's instructions. RT-PCR was done in 50 μl of 1× *TaKaRa Ex Taq* buffer, 0.2 mM dNTP mixture, 0.4 mM each of the primer set, 2.5 U of *TaKaRa Ex Taq* (TaKaRa) and 3 μl of the cDNA solution. Amplification was performed at 95 °C for 5 min, followed by 30 cycles of each 95 °C for 30 s, 51 °C for 30 s and 72 °C for 30 s. The PCR product was sequenced by Thermo Sequenase™ Cycle Sequencing Kit (Amersham Biotech) with an infrared (IRD800)-labeled primer on an automated sequencer, LICOR LIC-4200S (Aloka). After sequencing, a new primer set, GmOLPb-3RA5′ (5′-ccc aac aca ctt gcg gaa tt-3′) and GmOLPb-3RA2nd5′ (5′-ttc tgt cca ttg gga gaa cc-3′), was designed for rapid amplification of 3′-cDNA End (3′-RACE). The 3′-RACE was performed on the cDNA used in the RT-PCR and done in 50 μl of the reaction of *TaKaRa Ex Taq* with a primer set, GmOLPb-3RA5′ and adaptor (final conc., 0.4 mM each). Amplification was performed at 95 °C for 5 min, followed by 30 cycles of each 95 °C for 30 s, 51 °C for 30 s and 72 °C for 30 s. A nested PCR was performed in 50 μl of the reaction containing the 1st PCR product and a primer set, GmOLPb-3RA2nd5′ and adaptor. The target of 3′-RACE was amplified at 95 °C for 5 min and by 30 cycles of each 95 °C for 30 s, 54 °C for 30 s and 72 °C for 30 s.

For Northern hybridization, a partial fragment of a gene homologous to P21 protein in cv. ENREI, designated as P21-like protein from cv. ENREI (*P21e*), was also cloned by RT-PCR with a primer set, P21-5′ (5′-tcg act gca aag ctt acg g-3′) and P21-3′ (5′-tag ttg gat cat cct tgg g-3′). The primers were designed on the basis of soybean EST, BE820785, which shows highest similarity to the original P21 protein (from var. Williams 82; P25096) [7] using tBlastX search. The RT-PCR was done in 50 μl of 1× *TaKaRa Ex Taq* buffer, 0.2 mM dNTP mixture, 0.4 mM each of the primer set, 2.5 U of *TaKaRa Ex Taq* and 3 μl of the cDNA solution. Amplification was performed at 95 °C for 5 min, followed by 30 cycles of each 95 °C for 30 s, 54 °C for 30 s and 72 °C for 30 s.

Sequences for *GmOLPb* and *P21e* were submitted to the DNA databank DDBJ under accession nos. AB370233 and AB370234, respectively.

2.3. Computational structural analyses

Sequence alignments and some physical properties of the PR-5 proteins, such as molecular mass and isoelectronic point (p*I*), were analyzed using software GENETYX ver. 7.0.3 (Genetyx Corp.). Homologues of PR-5 protein were found using BlastX search [3] from DDBJ/EMBL/GenBank databanks, and phylogenetic tree, including the soybean PR-5 proteins and other homologues, was generated using program ClustalW (http://clustalw.ddbj.nig.ac.jp/top-j.html) [30] and drawn on software TreeView [22]. The 3D model (homology modeling) was predicted using SWISS-MODEL (http://swissmodel.expasy.org//SWISS-MODEL.html) and was visualized using DeepView Swiss-Pdb Viewer program [28]. Modeling of the soybean PR-5 proteins was carried out on the basis of several PDB data, for example, 1aun (tobacco PR-5d) [11], 1du5 (maize zeamatin) [4], 1pcv (tobacco osmotin) [17], 1z3q (banana TLP) [14].

2.4. Northern hybridization

Total RNA from the soybean tissue or the entire soybean plant was extracted by phenol-SDS/LiCl method [2], and the extracted