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GmPOI gene encoding a Pollen_Ole_e_I conserved domain is involved in response of soybean to various stresses

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Abstract

In the previous research, a novel gene *GmPOI* (GenBank acc. No. HM235775) encoding a Pollen_Ole_e_I conserved domain was identified in roots of soybean drought resistant cv. Jindou 23. In the present study, *GmPOI* was cloned and functionally characterized. Real-time quantitative PCR indicated that the expression of *GmPOI* was induced by drought, cold, salt, and abscisic acid in wild-type soybean. The soybean plants overexpressing *GmPOI* showed higher tolerance to drought stress than wild types. We concluded that *GmPOI* is probably a novel gene that is involved in the response to various stresses in soybean.

Additional key words: drought resistance, *Glycine max*, real-time PCR.

Introduction

Soybean growth, productivity, and quality are adversely affected by a wide range of abiotic and biotic stresses (Manavalan *et al*. 2009, Tran and Nguyen 2009, Gutierrez-Gonzalez *et al*. 2010). Among the abiotic stresses, drought is considered to be the most devastating stress in all stages of plant growth (Manavalan *et al*. 2009). Although many genes induced by drought stress have been cloned (Bray 1997, Zhu 2002), their biological functions and mechanisms related to drought stress still remain unclear. Thus, understanding of the genetic and molecular mechanisms controlling drought response has become vital in evolving strategies to enhance drought tolerance in crop plants (Thapa *et al*. 2011). Ole e I was first purified and characterized in *Olea europaea* pollen (Rodríguez *et al*. 2002). Until now, pollen proteins from about 100 different species contain a Pollen_Ole_e_I conserved domain (Bateman *et al*. 2004), which has been defined as "pollen proteins of the Ole e 1 family" within the Pfam protein families database. Ole e I exhibits sequence similarity with the products of genes *Lat52* from tomato, *Zm13* from maize, and lots of extensins from *Arabidopsis thaliana*. The molecular mass of the whole protein family is 8 - 50 kD. These plant pollen proteins consist of around 145 residues in which the conversed domain contains 6 cysteins connected by disulfide bonds. Some members of Ole family are rich in proline (Jiménez-López *et al*. 2011). Some pollen-specific genes encode cytoskeleton proteins, such as Tac25 (Thangavelu *et al*. 1993), TUA1 (Carpenter *et al*. 1992), LeProl (Yu *et al*. 1998), and Mab45a (Kandasamy *et al*. 1999), ascorbic acid oxidase (Weterings *et al*. 1995) or are considered as transcription factors (Zachgo *et al*. 1997). They have been suggested to be involved in pollen formation, protein transformation, and stability maintenance (Stratford *et al*. 2001). However, the existing knowledge of their precise structures and expressions is still limited (Jiang *et al*. 2005) and little is known about their roles in abiotic stress responses. In the present study, we have cloned and characterized *GmPOI*, a novel drought-inducible gene in soybean roots. In addition, we demonstrated that the expression of *GmPOI* can be induced by cold (4 °C), salt, abscisic acid (ABA), and drought treatments. Finally, we showed that over-expression of *GmPOI* in transgenic soybean lines significantly enhanced both superoxide dismutase (SOD) activity and proline content, and improved tolerance to drought.

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Abbreviations: ABA - abscisic acid; EST - expressed sequence tag; ROS - reactive oxygen species; RT-PCR - reverse transcription polymerase chain reaction; SOD - superoxide dismutase; WT - wild type.
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Materials and methods

Soybean [*Glycine max* (L.) Merr.] cv. Jindou 23 was used as a test plant because it reveals the first-degree drought resistance. Seeds were germinated in sandy loam soil irrigated with water. After opening the first trifoliate leaf, the seedling watering was withheld for 6 d. Total RNA was extracted using *Trizol* reagent (*Invitrogen*, Carlsbad, USA). The expressing profile under drought stress was obtained by cDNA microarray chip in roots. The result of hybridization showed that an expressed sequence tag (EST) was up-regulated, therefore we searched in soybean genome database with the corresponding nucleotide sequence of GmaAffx.83637.1.S1 corresponding to the soybean gene chip probe. The open reading frame was predicted by matched sequences with *FGENESH* program. The accuracy of prediction was verified by searching soybean EST data using the predicted sequence as the probe. Specific primers (FP: 5′-ATGAGCAGCTTGGCTTATT-3′, RP: 5′-TTT CTTACGGAAGGTGACC-3′) were synthesized and the full-length cDNA fragment was amplified from the cDNA by reverse transcription - polymerase chain reaction (RT-PCR). The conditions for amplification were 95 °C for 5 min, then 35 cycles at 95 °C for 30 s, 57 °C for 30 s, 72 °C for 40 s, respectively, followed by incubating at 72 °C for 10 min. The PCR product was purified and connected with pMD18-T vector for sequencing.

Multiple sequence alignment was conducted using *Clustal X* software based on the available amino acid sequences which were homologous with our PCR encoding product. The phylogenetic tree was subsequently constructed. The length, molecular mass, isoelectric point, hydrophobicity, and membrane structure were predicted. The conserved domain was analyzed by *InterProScan* of *EBI*. Three-week-old wild type (WT) seedlings were exposed to low temperature (4 °C), high salinity (250 mM NaCl), 100 μM ABA for 1, 3, 6, 12, and 24 h or drought stress (disruption of watering) for 2, 4, and 6 d, respectively. Total RNA of both roots and leaves were extracted and analyzed by real time PCR. *SYBR green* was used to monitor the kinetics of PCR product. As an internal control, the soybean *actin* gene was used to quantify the relative expression of each target gene in each tissue type. Three biological replicates were used.

The pCAMBIA 3301-*GmPOI* was constructed to transform soybean *via Agrobacterium* mediated transformation of cotyledonary node. The *GmPOI* gene was expressed under 35S promoter. Glufosinate-resistant transformants were tested by PCR and RT-PCR analyses. Drought tolerance assays were performed using the control (WT transformed with the empty vector pCAMBIA 3301) and T_2 transgenic plants under identical conditions. Water was withheld from 3-week-old WT and transgenic plants for 0 and 6 d, respectively. Roots were collected for ABA quantification by the ABA immunoassay kit (*R&D Systems*, Minneapolis, USA). Content of proline was measured according to Bates *et al.* (1973). SOD activity was measured by the nitroblue tetrazolium (NBT) method (Beauchamp and Fridovich 1971).

All experiments were independently carried out thrice in WT and transgenic lines L3 and L12. All measurements were performed three times. Data analyses were conducted using the *SPSS v. 16.0* statistical software. Tukey's range test was used at $P \leq 0.05$. Sample variability was given as standard deviation (SD).

Results

GmPOI gene (GenBank acc. No. HM235775) was first isolated from soybean roots *via* the RT-PCR method. The sequence is 612 bp in length with a complete open reading frame of 204 amino acids. The molecular mass of GmPOI protein was predicted to be 22.2 kD with isoelectric point being 8.53. There was a transmembrane segment and a typical conserved domain named Pollen_Ole_e_I. It had neither signal peptides nor introns.

We searched GenBank NR database using the amino acid sequence of GmPOI as the probe. The sequence of GmPOI protein possessed a high sequence similarity with NP_182276 from *Arabidopsis thaliana*, XP_002302729 from *Ricinus communis*, CBI36891 from *Vitis vinifera*, and XP_002523122 from *Populus trichocarpa* (Fig. 1*A*). The phylogenetic tree was constructed (Fig. 1*B*).

GmPOI expressed little in leaves but more in roots treated by low temperature, NaCl, and ABA. Compared to salt or ABA treatment, low temperature induced only slightly the expression of *GmPOI*, without significant increase with the time extension (Fig. 2*A*). The *GmPOI* mRNA accumulated in response to NaCl, reached maximum after 12 h and then gradually decreased (Fig. 2*B*). The expression pattern under ABA treatment was similar to that of salt treatment. The transcripts of *GmPOI* began to accumulate after 1 h and quickly reached maximum after 6 h (Fig. 2C). The *GmPOI* abundance gradually increased and reached the maximum on day 6 of drought stress (Fig. 2*D*).

To examine the *in vivo* function of *GmPOI*, transgenic soybean plants overexpressing *GmPOI* were generated (Fig. 3*A*). Two transgenic lines were selected for functional analysis. Quantitative RT-PCR analysis showed that the *GmPOI* mRNA of 35S-*GmPOI* transgenic lines accumulated to higher levels than that of WT plants in leaves and roots (Fig. 3 *B*,*C*). Three-week-old seedlings of T_2 transgenic plants were exposed to 15-d drought stress in order to evaluate their drought resistance. Plant survival rate before stress treatment was

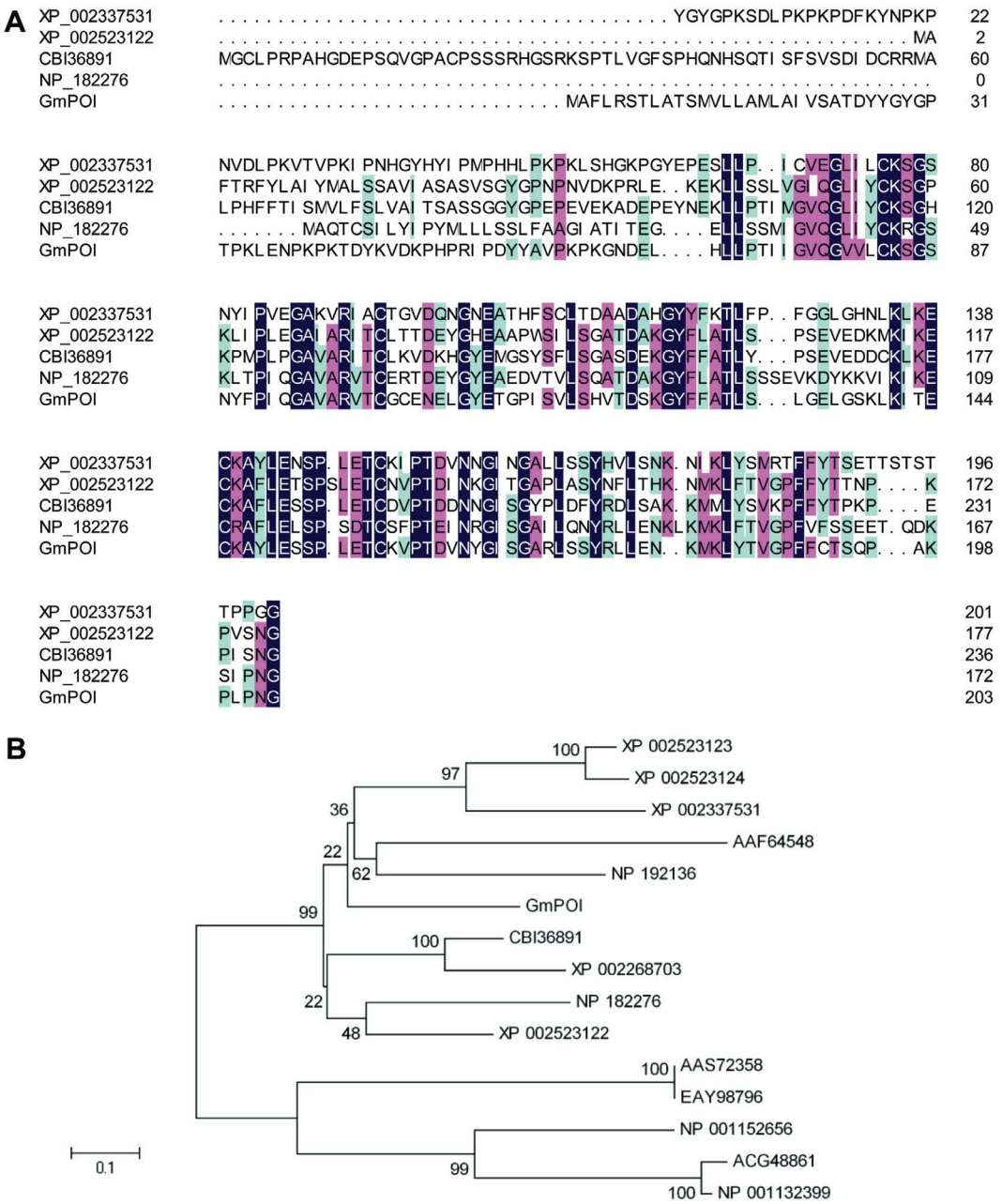

Fig. 1. Characterization of GmPOI. Alignment of the amino acid sequences of the GmPOI protein (*A*) and phylogenic analysis of GmPOI protein by the *DNAMAN* software with the observed divergency distance method (*B*). The appended proteins are as follows: XP_002523123, XP_002523124: *Ricinus communis*; XP_002523122, XP_002337531: *Populus trichocarpa*; CBI36891, XP_002268703: *Vitis vinifera*; NP_182276, NP_192136, AAF64548: *Arabidopsis thaliana*; EAY98796, AAS72358,: *Oryza sativa*; NP_001132399, ACG48861: *Zea mays*.

100 % for WT plants as well as transgenic lines. After re-watering, 61.9 % (L3) and 57.6 % (L12) of transgenic plants recovered whereas only 21.9 % WT did. Taken all the data from three different treatments together, the transgenic plants showed an increased drought tolerance than wild types at the same growth conditions.

The SOD activity in L3 and L12 plants was 12.9 and 9.3 %, respectively, higher than that in WT plants. However, after 6-d drought treatment, the SOD activity in WT plants sharply decreased, while the SOD activity in L3 and L12 plants only slightly decreased, thus being much higher than that in WT plants. The content of proline was higher in transgenic lines than in WT plants both before and after drought treatment. However, there were no significant differences in ABA content both before and after the drought treatment.

Discussion

In the present research, we have cloned and characterized *GmPOI*, a novel drought-inducible gene in soybean roots. Analysis of the deduced amino-acid sequence of the protein revealed that it had a Pollen_Ole_e_I conserved domain. Phylogenetic analysis showed that some Pollen_Ole_e_I proteins from different species had a high similarity whereas some Pollen_Ole_e_I proteins of the same species did not share a high similarity from one another (Fig. 1*B*). Besides, GmPOI was also found similar with some proteins from other species (Fig. 1*B*).

Up to date, proteins containing Pollen_Ole_e_I domain have been studied in regulations of plant growth and development as well as compositions of cell structure. However, few researches on their molecular function and biological process have been carried out. Recently, another novel pollen_Ole_e_I-domain containing protein (NP_568156) was cloned from roots of *Arabidopsis thaliana* and located in endomembrane system. Nevertheless, its functions is still unknown. In this report, *GmPOI* was cloned from soybean roots. It was supposed to be a gene involved in drought resistance since it was induced by drought stress and the transgenic seedlings overexpressing *GmPOI* performed a higher resistance to drought treatment than WT plants. Our observation might resemble that of Rabello *et al* (2008). They analyzed rice roots in both drought tolerant and susceptible cultivars by SSH and found the pollen specific protein C13 precursor only in drought tolerant cultivar. However, the pollen specific protein C13 precursor had not yet been directly related to drought tolerance. Further studies need to be performed to explore its biological function, since this gene might play an important role in crop adaptation to drought stress.

Numerous works reported the expression patterns of pollen genes during anther development in *Arabidopsis thaliana* (Twell *et al*. 1990, 1995, Mitsuda *et al*. 1995) and *Brassica rapa* (Okada *et al*. 1999). However, until now, no information has been available concerning the expression pattern of these genes under abiotic stresses. Our paper showed that the mRNA level of *GmPOI* increased after exposure to low temperature, NaCl, ABA, and drought (Fig. 2), suggesting that *GmPOI* might be involved in plant tolerance to abiotic stresses. *GmPOI* was induced to express little in leaves but more in roots under the above stresses which indicated that the expression of *GmPOI* could probably be regulated in a

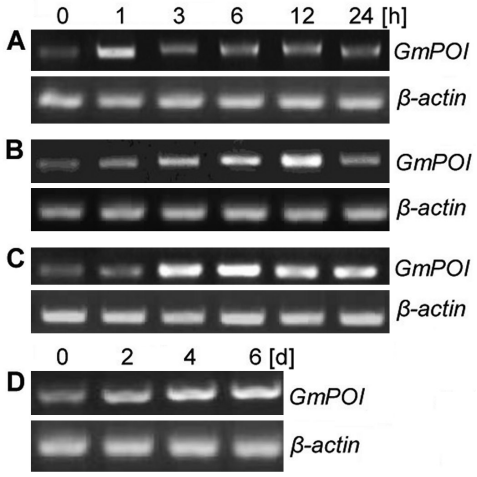

Fig. 2. Expression patterns of *GmPOI* in roots under various treatments: *A* - low temperature (4 °C; *GmPOI* mRNA reached its maximum after 1 h of the cold treatment); *B* - 250 mM NaCl (*GmPOI* mRNA abundance gradually increased to its maximum at 12 h of stress treatment and then quickly decreased); *C* - 100 mM ABA (the transcripts of *GmPOI* began to accumulate after 1 h and quickly reached its maximum after 6 h; *D* - drought treatment (*GmPOI* abundance gradually increased and reached the maximum on day 6).

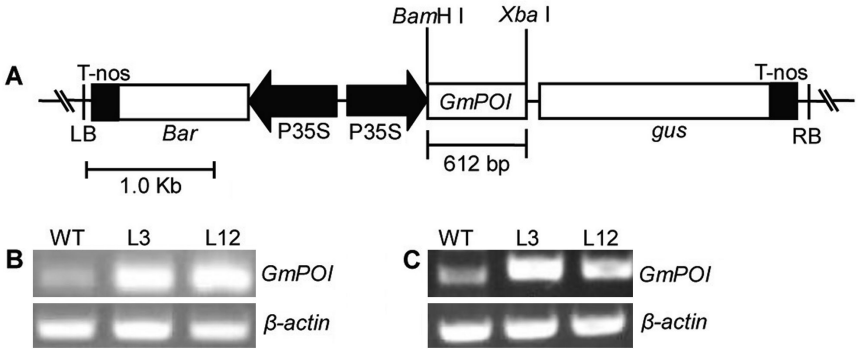

Fig. 3. Overexpression of the *GmPOI* gene in soybean. *A* - T-DNA region of pCAMBIA 3301-*GmPOI* which harbors P35S-*Bar*, P35S-*GmPOI*, and the *gus* gene. The 612 bp *GmPOI* gene (marked with a *full line*), flanked by *Bam*H I and *Xba* I sites on the left and right sides. P35S - *Cauliflower mosaic virus* 35S promoter, LB - left border, RB - right border, *Bar* - biolaphos resistance gene, *gus* - β-glucuronidase gene, T-nos - nopaline synthase terminator. *B, C* - *GmPOI* transcript levels in both leaves (*B*) and roots (*C*) of WT and transgenic lines (L3 and L12) were assessed by quantitative RT-PCR. The soybean *β-actin* gene was used as the internal control. The *GmPOI* abundance in leaves of transgenic lines (L3 and L12) was 12 and 9 times higher than that of WT, respectively; *GmPOI* transcript levels in roots of transgenic lines (L3 and L12) were 10 and 5 times higher than that of WT, respectively.

Table 1. Effect of drought on SOD activity, proline, and ABA content in wild type (WT) and two transgenic lines (L3 and L12) overexpressing *GmPOI*. Means ± SD, n = 3. The means labeled with different letters show significant difference among treatments ($P < 0.05$).

Drought [d]		SOD [U g^{-1}(f.m.)]	Proline [ug g^{-1}(f.m.)]	ABA [ng g^{-1} (f.m.)]
0	WT	1217.14±27.07bc	6.05±0.40c	92.50±3.53b
	L3	1373.88±53.57a	11.84±1.22b	99.00±1.41b
	L12	1330.10±27.80ab	9.89±0.14bc	94.50±3.53b
6	WT	79.66±13.03d	7.71±0.59c	306.00±5.65a
	L3	1200.85±53.76bc	20.18±1.71a	317.00±4.24a
	L12	1140.03±10.58c	18.37±1.20a	310.50±2.12a

tissue-specific manner. The *GmPOI* abundance in roots was detected to increase sharply after 3-h ABA stress. Therefore, the *GmPOI* gene was considered to be an early expressing gene responding quickly to ABA treatment. However, *GmPOI* was upregulated more slowly under drought stress compared with the other stresses. The reason could be that drought stress was induced by the gradual decrease of water content in the soil while the other stresses acted directly and quickly. In addition, *GmPOI* might respond to diverse stresses in different ways.

Although the function of many pollen proteins was studied (Muschietti *et al*. 1994, Qiao *et al*. 2004, Grobei *et al*. 2009), the precise information is still lacking. In addition to their allergenic character, pollen proteins are considered as key proteins for pollen physiology. The role of profilin, another pollen protein, in response to salinity has also been described in some species (Askari *et al*. 2006). Alché *et al.* (2007) speculated Pollen_Ole_e_5 of the olive might play an important role in defence against reactive oxygen species (ROS), as did Pollen Pollen_Ole_e_I of soybean in our experiments. Drought stress led to accumulation of high ROS content (Borsani *et al*. 2001) which may disturb cellular redox homeostasis and result in oxidative injuries. SOD belongs to the enzymatic antioxidants and a higher SOD activity was observed in transgenic plants compared to control plants with or without drought treatment suggesting that *GmPOI* was involved in oxidative stress responses by regulating SOD activity to scavenge ROS.

Proline is one of the important osmoprotectant, which accumulates in plants in response to stresses (Hasegawa *et al*. 2000, Verbruggen and Hermans 2008). In this research, the proline content was much higher in the 35S-GmPOI transgenic plants than that of WT plants under drought stress. These results suggest that *GmPOI* might play an important role in the synthesis of osmolytes to protect plants from water deficit.

Both ABA-dependent and ABA-independent pathways leading to changes in gene expression have been shown (Hirayama and Shinozaki 2007). In this research, *GmPOI* was observed to be induced by drought, low temperature, high salt, as well as ABA, which indicated *GmPOI* could be involved in ABA-dependent signalling pathway in the response to drought stress. However, ABA contents in WT and transgenic plants were not different. It was possible that the expressing levels of *GmPOI* in transgenic lines might not be enough to trigger the expression of ABA synthetizing genes. Further studies need to be performed in order to resolve this issue.

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