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Short Communication

Identification and Characterization of Two Paralogous Plastid Terminal Oxidase Genes in Soybean

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Abstract

Plastid terminal oxidase (PTOX) is a plastoquinol oxidase, which plays several important roles in plants. Previous studies suggested that PTOX is encoded by a single gene with only one copy in higher plants. Here we report the identification of two possible paralogous *PTOX* genes on different chromosomes of soybean, both of which are highly homologous to known *PTOX* genes in other species. These two paralogs have quite different introns and nearly the same exons and were predicted to encode membrane protein with chloroplast transit peptide. The deduced PTOX protein encoded by both paralogs were proposed to be functional, since the existence of highly conserved amino acid sites necessary for a typical PTOX, including six iron-binding sites and Exon 8 domain. Moreover, soybean PTOX also exhibit clear sequence similarity to alternative oxidase (AOX). Organ-specific expression analysis showed high transcript levels of soybean *PTOX* in stems, leaves and flowers, while the levels in pods and roots were relatively low. In addition, a light-inducible character was also suggested for soybean *PTOX* in the present study.

Keywords: Alternative oxidase (AOX); IMMUTANS; PTOX; Phytozome; Soybean

Introduction

Plastid terminal oxidase (PTOX), a thylakoid membrane-located quinol oxidase, exists widely in photosynthetic species including higher plants and algae (McDonald *et al*., 2011). It transfers electrons from plastoquinol to O_2 with formation of H_2O and acts as the terminal oxidase of chlororespiration, which represents a respiratory electron transport chain in thylakoid membrane. Moreover, PTOX was also regarded as an important co-factor of carotenoid biosynthesis by transferring the electrons derived from precursors to O_2 via plastoquinol (McDonald *et al*., 2011).

Function of plant PTOX was indicated to be important for chloroplast biogenesis and beneficial for stressed-plants (Aluru *et al*., 2006; Sun and Wen, 2011). But the exact physiological role of PTOX is still unclear, since the limited data are available from only a few model species. More information from extensive species, especially important crops, is necessary for understanding the properties of plant PTOX.

In the present study, we identified the *PTOX* gene in soybean, an important crop worldwide and analyzed the characters of soybean *PTOX* gene as well as the deduced protein. In addition, we also detected the expression of soybean *PTOX* in different organs and compared the transcript levels under light with that in the dark.

Materials and Methods

Seeds of soybean (*Glycine max* L. Merr. cv. Gongxuan 1, supplied by the Key Laboratory of Crop Eco-physiology and Farming System in Southwest China, Ministry of Agriculture) were germinated and grown in regular growing season. For light and dark treatments, soybean plants were divided into two groups and grown under continuous light or in complete darkness for 12 h, respectively, in growth chambers. Different organs, including roots, stems, leaves, flowers and pods were collected to extract total RNA as described by Lei *et al*. (2010).

To obtain cDNAs, reverse transcription (RT) was carried out with total RNA using M-MLV reverse transcriptase (TaKaRa Biotech. Co. Ltd., Dalian, P.R. China) and universal Oligo $(dT)_{16}$ primer. Then, degenerate primers designed by Amirsadeghi *et al*. (2006) were used for PCR. The product was subsequently ligated into pMD19-T vector (TaKaRa Biotech. Co. Ltd., Dalian, P.R. China) and sequenced. Resulting sequence was applied to pick out candidate soybean *PTOX* through BLAST using the soybean database in Phytozome (Goodstein *et al*., 2012). The identity of soybean *PTOX* was confirmed by alignment of the candidate sequences with known *PTOX* genes. Based

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upon the sequence of soybean *PTOX*, gene-specific primers were used for RT-PCR with total RNA to detect *PTOX* transcripts in different organs.

Sequence data of PTOX and alternative oxidase (AOX) used in the present study was obtained from Phytozome and GenBank database. Transit peptide was predicted by Target P 1.1 (Emanuelsson *et al*., 2007) and transmembrane domains were predicted by TMHMM 2.0 (Krogh *et al*., 2001). Sequence alignment was performed by Clustal X 2 (Larkin *et al.*, 2007). *Cis*-acting regulatory elements were searched for soybean *PTOX* 1.0 kb promoter at PLACE database (Higo *et al*., 1999).

Results

A product of 432 bp was obtained by PCR amplification with degenerate primers and then sequenced. After BLAST, two candidate loci with names of Glyma09g01130 and Glyma15g11950 were picked out. Transcript sequences of these two candidate loci display high homologies with known *PTOX* genes, *e.g.* about 80% sequence identity with Arabidopsis *PTOX* gene (also known as *IMMUTANS*, GenBank accession number: AF098072), confirming the identity of soybean *PTOX*. The existence of two *PTOX* sequences in soybean genome suggested that there are two copies of *PTOX* gene appearing as paralogs on different chromosomes (9 and 15, respectively).

Glyma09g01130 and Glyma15g11950 both have eight introns and nine exons (Fig. 1). Their introns display clearly sequence differences, especially for the 4th and 6th introns. Glyma15g1195 has much longer 4th and 6th introns, with insert fragments more than 2400 bp and 300 bp, respectively, compared with Glyma09g01130. However, their nine exons have nearly the same sequences, indicating almost the same amino acid-encoding.

Transcript sequences of Glyma09g01130 and Glyma15g11950 both have an open reading frame of 999 bp, which encodes a protein of 332 amino acids with a 36-amino acid chloroplast transit peptide (Fig. 2). The mature protein of 296 amino acids has a calculated molecular mass of about 34.3 kDa. Two transmembrane domains were predicted in the deduced polypeptide (Fig. 2), indicating the membrane protein property. Six iron-binding sites, including four glutamate and two histidine residues (E116, E155, H158, E207, E276, H279), were also proposed (Fig. 2). These iron-binding sits are conserved in all PTOX proteins examined to date and showed no change (Fu *et al.*, 2005; McDonald *et al.*, 2011). Moreover, E155 and H158, E276 and H279 also display as the EXXH motifs, which have strong iron-binding property. Exon 8 domain is crucial for activity and stability of PTOX proteins (McDonald *et al.*, 2011). This domain has not been found in other proteins (Fu *et al.*, 2005), further confirming the identity of soybean PTOX. Besides, another five vital sites (L115, H131, Y192, Y214, D274), which have functional importance for PTOX activity such as substrate binding (Fig. 2).

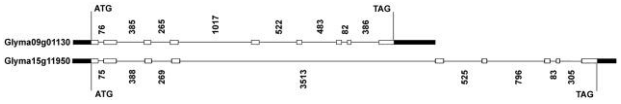

Fig. 1: Genomic organization of soybean *PTOX* paralogs. Exons are indicated by *empty boxes* and introns by *lines*. *Filled boxes* indicate the untranslated regions. Numbers indicate the length of introns. Translation initiation site (ATG) and stop site (TGA) are presented

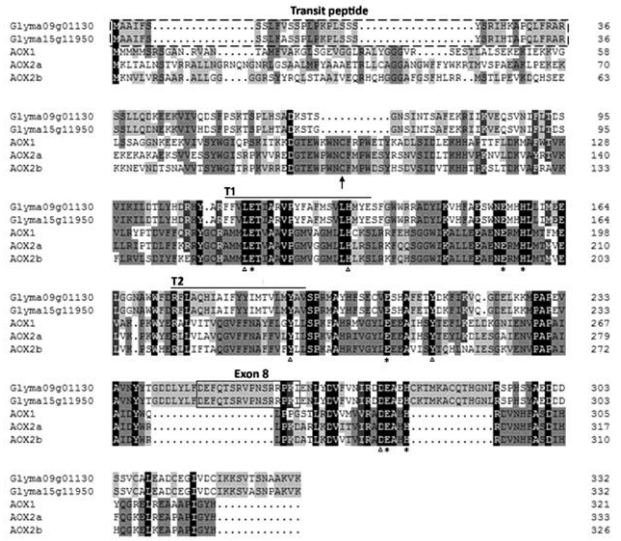

Fig. 2: Sequence alignment of soybean PTOX with AOX1 (Phytozome ID: Glyma04g14800), AOX2a (Phytozome ID: Glyma08g07700), and AOX2b (Phytozome ID: Glyma08g07690). Transit peptide is indicated by *dashed box* and the Exon 8 domain by *solid box*. T1 and T2 represent the two putative transmembrane domains (*solid lines*). *Asterisks* denote the six iron-binding sites, and *triangles* denote the other five conserved sites. *Arrow* indicates the conserved cysteine in AOX, which is lacking in PTOX

Sequence alignment revealed that the deduced amino acid sequences of Glyma09g01130 and Glyma15g11950 share 19–22% identity with the three members of soybean AOX (Fig. 2), which act as the terminal oxidase of alternative pathway in mitochondria. Many residues are conserved between PTOX and AOX, including six iron-binding sites and five conserved activity sites (Fig. 2). However, PTOX and AOX also have their own unique domains. For example, the Exon 8 domain in PTOX is missing in all the members of soybean AOX (Fig. 2). On the other hand, a conserved cysteine in all of the AOX members, which participates in disulfide bond formation between adjacent monomers and gives rise to a dimer, is not found in PTOX (Fig. 2), implying that PTOX cannot dimerize and exists only as a monomer.

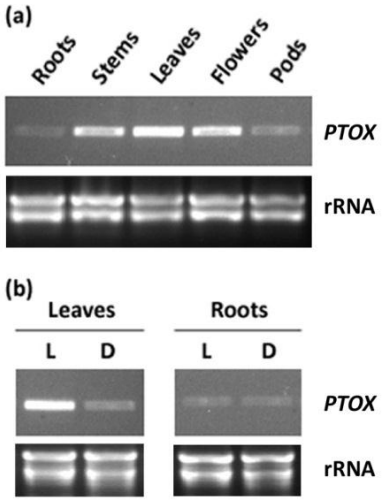

Fig. 3: Expression analysis of soybean *PTOX* in different organs. (a) Detection of *PTOX* transcripts in roots, stems, leaves, flowers and pods. (b) Comparison of *PTOX* transcript level in the light with that in the dark. L: light; D: dark. rRNA is shown as a loading control and indicator of RNA intactness

Data further detected *PTOX* transcripts in different soybean organs (*i.e.* roots, stems, leaves, flowers and pods). Primers used for RT-PCR were designed based on the same coding sequences from both paralogs, in order to detect total transcripts. Results showed high transcript levels in stems, leaves and flowers, while the levels in pods and roots were relatively low (Fig. 3a). *Cis*-acting regulatory elements analysis indicated a widely distribution of light-responsive motifs (*e.g.* GATA-box, GT1-motif, REalpha) in the promoters of both Glyma09g01130 and Glyma15g11950 (data not shown) and suggested a light-inducible character for soybean *PTOX*. Subsequently, we detected the transcript levels of *PTOX* under light and dark conditions, respectively. Results showed a higher level in leaves under light compared with that in the dark (Fig. 3b), further confirmed the light-inducible character of soybean *PTOX*. But transcripts remained at the same level in roots under both light and dark conditions (Fig. 3b), suggesting a different expression pattern other than light-induction in roots.

Discussion

Soybean is a diploidized ancient tetraploid, whose genes are often present as multiple copies since the chromosome duplication events in evolution process (Schmutz *et al*., 2010). So, existence of two paralogous *PTOX* genes in soybean can be considered as a result of this duplication events. However, Glyma09g01130 and Glyma15g11950 are both described as *AOX*, which encodes mitochondria-located protein, in Phytozome database. Many studies indicated that PTOX and AOX are homologs, which have the same origin (McDonald and Vanlerberghe, 2006). But they have distinct subcellular localizations and unique conserved domains. Since the existence of chloroplast transit peptide and Exon 8 domain in deduced amino acid sequences, we hold the opinion that these two loci should be described as *PTOX*.

Previous studies suggested that *PTOX* always appears as a single gene with only one copy in the genomes of higher plants (Wu *et al*., 1999; Kong *et al*., 2003), while a recent study showed a second gene with homology to *PTOX* in rice genome (Tamiru *et al*., 2014). But this gene was predicted to encode a polypeptide which lacks many of the conserved residues necessary for PTOX, such as iron-binding sites and the Exon 8 domain and is therefore unlikely to execute function (Tamiru *et al*., 2014). In the present study, deduced amino acid sequences of the two soybean *PTOX* paralogs have all of the conserved sites, involving the six iron-binding sites and Exon 8 domain. Therefore, both of these paralogs can be proposed to encode functional PTOX protein.

Our present study showed that soybean *PTOX* has an organ-dependent expression pattern. The expression of *PTOX* gene was also indicated to be organ-dependent in Arabidopsis and rice (Aluru *et al*., 2001; Tamiru *et al*., 2014), implying that PTOX has distinct roles in different organs. In addition, transcript detection and *cis*-acting regulatory elements analysis suggested a light-inducible character for soybean *PTOX*. But this character is not proposed for underground organ, the roots.

Conclusion

This study identified two possible paralogous *PTOX* genes in a higher plant for the first time. Both of these paralogs in soybean were suggested to encode functional PTOX protein, which possess all of the conserved amino acid sites necessary for PTOX. The expression of soybean *PTOX* was suggested to be organ-dependent and light-inducible.

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