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RAPID COMMUNICATION

Molecular cloning and characterization of pearl millet polyphenol oxidase and its role in defense against downy mildew

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Abstract

Polyphenol oxidase partial gene *PG-PPO* was cloned and characterized from *Pennisetum glaucum* (pearl millet) which showed 42% identity to a PPO sequence isolated from wheat at the region of Copper B with a score of 40 and e-value of 2.8. Multiple sequence alignment results revealed similarity to polyphenol oxidase (PPO) sequences from wheat, trifolium, lettuce, apricot, tobacco, tomato, pokeweed, apple, grape and poplar especially at the Copper B region of PPO. The 395 bp pearl millet PPO sequence was AT rich (53.3%) and contained the highly conserved amino acids of histidine-rich copper binding sites similar to PPO sequences from other crops. Results also indicated that PPO in pearl millet exists in multi copy. The role of the isolated PPO gene during pearl millet-downy mildew interaction was analyzed and the results showed significantly higher and rapid accumulation of PPO mRNAs in resistant pearl millet seedlings inoculated with *Sclerospora graminicola* in comparison to the susceptible control, demonstrating that the PPO plays a prominent role in pearl millet defense against pathogens, particularly downy mildew pathogen.

Keywords: downy mildew, plant defense, *Pennisetum glaucum*, polyphenol oxidase

Polyphenol oxidases (PPOs; EC 1.14.18.1 or EC 1.10.3.2) are nuclear coded enzymes of almost ubiquitous distribution in plants (Mayer and Harel 1979; Mayer 1986). Due to their conspicuous reaction products and their wound and pathogen inducibilities, it has frequently been suggested that PPOs participate in plant defense against pests and pathogens (Constabel *et al.* 1995; Thipyapong *et al.* 1995; Thipyapong and Steffins 1997). There are many reports showing the involvement of PPO during defense against plant pathogens. Overexpression of PPO in transgenic tomato plants resulted in enhanced resistance to *Pseudomonas syringae* (Li and Steffens 2002). PPO mediated induced resistance against *Fusarium graminearum* in wheat (Mohammadi and Kazemi 2002), against *P. syringae* in tomato (Goel and Paul 2015), and against *Sclerospora graminicola* in pearl millet (Govind *et al.* 2016; Lavanya *et al.* 2017) has been reported. Earlier studies from our laboratory demonstrated the involvement of PPO during pearl millet defense against the downy mildew pathogen (Niranjan Raj *et al.* 2006). In this study we reported on identification and molecular characterization of the gene coding for pearl millet PPO.

In the present study, RNA was extracted from two-day-old pearl millet seedlings (IP18292) following the method of Green (2014). Forward and reverse primers for polyphenol oxidase were designed based on the wheat PPO primers, and the primers were synthesized by Sigma-Genosys, Sigma-Aldrich, Bangalore, India. The primer sequences were as follows:
Forward – PPO F3: 5'-GTGGTGCGAGTAGAAGACG-3'
Reverse – PPO R3: 5'-GACGTCGTCGACTTCAGC-3'

PCR amplification reaction was carried out in a Biometra UNO II thermocycler (Biometra, Germany). The PCR amplified product was run on agarose gel till the bands were clearly separated, and the band of interest was cut out, purified with CleanGenei Kit (gel purification) and reamplified. Ligation of the cDNA fragments was carried out by using pGEM-T Easy Vector Systems Kit (Promega, USA). The PGEM vector had

a 3'-terminal thymidine at both ends. Competent cells (*E. coli* DH 108 strain) were prepared from log phase culture (OD550 = 0.8) and 0.1 ml competent cells were put in 5 ml screw cap tubes in ice, the DNA was added and kept in ice for 40 min. Later, heat shock was given by keeping the tubes in an oven at 42°C for 1.5 min. The heat-shock treated samples were added to 1 ml of Luria-Bertani (LB) medium and incubated at 37°C for 1 h with shaking at 150 rpm. Two hundred µl of the cells were plated by spreading on LB agar plates containing IPTG (isopropyl β-D-thiogalactopyranoside) (0.5 mM), X-gal (5-bromo-4-chloro-3-indolyl β-D-galactoside) (80 µg · ml^{-1}) and ampicillin (100 µg for 1 ml media). After overnight (16 h) incubation at 37°C, selection was carried out by blue/white colony screening. Colony PCR was carried out for the confirmation of the presence of the insert. Respective primers, reaction conditions and concentrations were taken to amplify the insert. A PCR reaction was carried out with the purified DNA as a template using the PPO primers.

The PCR products were PEG precipitated and the purified DNA was sent for sequencing to Microsynth AG, Schützenstrasse 15, 9436 Balgach, Switzerland where a premium run was carried out using PPO F3 and PPO R3 forward and reverse primers. The obtained sequence data was analyzed by BLASTX network services at the National Center for Biotechnology Information. Searches for similarity between the predicted protein product of clones and proteins on the NCBI database were carried out using BLASTX. Multiple sequence alignments of PPO of pearl millet with other known PPO gene sequences was performed using Clustal W program with PAM250 residue weight table.

The previously mentioned primer combinations generated one major band of 400 bp in size and a few faint bands. The 400 bp band from the primer combination was close to the size of the fragment expected for PPO based on the sequences of wheat PPO. Therefore, this amplified band was eluted from the gel and ligated on to pGEM Easy Vector and transformed into *E. coli* DH 10 B cells by heat shock. The presence of inserts in the transformed colonies was confirmed using colony PCR.

DNA sequencing was done by ABI-PRISM, Model 3730 BC1.5.1.0. The complete sequence obtained was as follows:

– A1, Premium RUN, edited 478255 98883 PM1 PPOF CGCCGTTAGCGACTTCAATCATATTAAGG AAACCATCATCATTGACATCCTCTT CAACCGTTGGCTGAGGCAGTGAAG TCGAGGTTTGGCCACCATCTT CAACTGTAATAGTCTTATTCGAA GACGGCCGCAGGTTACCAGACAGAT CCGTAACTATAGTACGTATCGTATT CACCATCATCGAGCGCATCAGGTGG TAGATCGGTTTTAAAGTTGATCACTCTC GGCAAATCATCAATGCTGGTAACTTCA AACTCTCTGGTAATGTTCTGTCCCCAGTGG AAGACTACCAGGTCATTCACGGCAGCGT CACGGTAAGAGGGAATGATAGCTTCCACAC CAAAAGCAATATCGTAGTGATCGAGTT TTGGTCGTCTTCTACTCGCACCACA,

– A1, Premium RUN, edited 478714 988 PM1 PPOR3 GCTGCCGTGAATGACCTGGTAGTCTTC CACTGGGGAGAACATTACCAGAGAGTTT GAAGTTACCAGCATTGATGATTTGCCGA GAGTGATCAACTTTAAAACCGATCTACCAC CTGATGCGCTCGATGATGGTGAATACGA TACGTACTATGTAGTTACGGATCTGTCTGG TAACCTGCGGCCGTCTTCGAATAAGACTAT TACAGTTGAAGATGGTGGCCAAACCTCGACT TCACTGCCTCAGCCAACGGTTGAAGAGGAT GTCAATGATGATGGTTTCCTTAATATGATT GAAGTCGCTAACGGCGTGCATGTAACCG GGACGTATCCATTAATGGCTGAAGTCGAC GACGTCA.

Searches of the EMBL GenBank data base using the BLASTX algorithm revealed that the sequence showed similarity with the other known plant PPO sequences. The probability scores ranged from 42 to 40 with the lowest e-values.

BLASTX results indicated that the PPO clone showed 42% identity to a PPO sequence isolated from wheat (Accession No. AJ613526) at the region of Copper B with a score of 40 and e-value of 2.8. This clone also indicated homology to earlier isolated hypothetical protein rice (Accession No. AP008210). Multiple sequence alignments were performed using Clustal W software with pearl millet PPO and ten most similar PPO sequences from wheat, trifolium, lettuce, apricot, tobacco, tomato, pokeweed, apple, grape and poplar (Fig. 1). The analysis with these known PPO sequences indicated a high similarity, especially at the Copper B region of PPO. The PPO sequence reported in this study was submitted to the gene bank (AY 881993).

The role of the identified PPO gene in pearl millet resistance against downy mildew disease was studied in susceptible (7042S) and resistant (IP 18292) pearl millet seedlings with or without inoculation by the downy mildew pathogen *S. graminicola*. Two-day-old seedlings with or without pathogen inoculation were sampled at different time intervals and the relative quantitation of PPO mRNAs in pearl millet seedlings was done by using PPO F3 and PPO R3 forward and reverse primers. The results showed that constitutive level of PPO transcripts was detected in both resistant and susceptible seedlings with or without pathogen inoculation and the expression level was higher in resistant seedlings than in susceptible controls; and PPO gene expression was higher in inoculated samples than in the uninoculated samples at tested time points (Fig. 2). In the pathogen inoculated samples, PPO gene

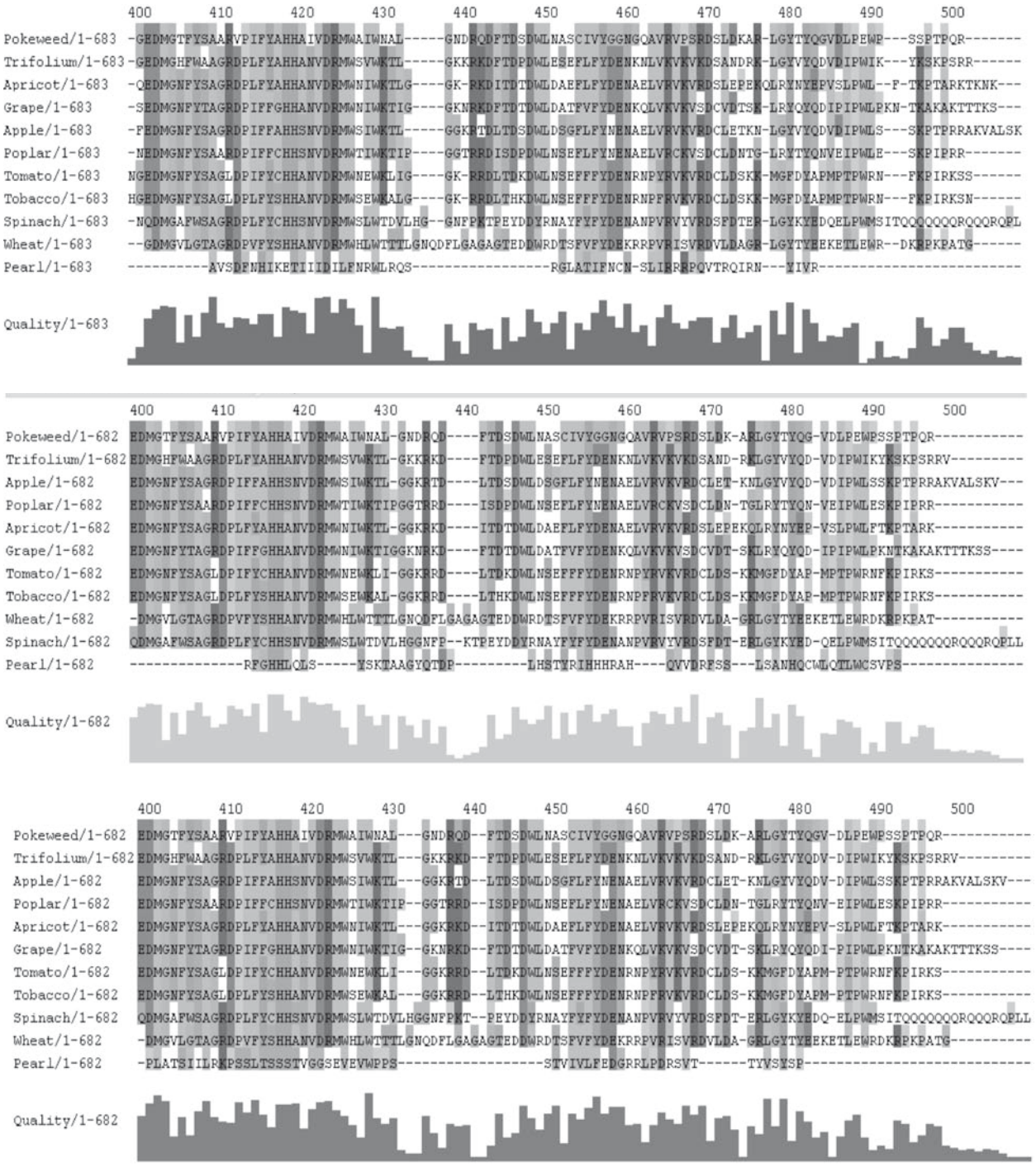

Fig. 1. Alignment of deduced amino acid sequences of the pearl millet polyphenol oxidase (PPO) sequence with that of other PPO sequences of pokeweed, trifolium, apple, poplar, apricot, grape, tomato, tobacco, wheat and spinach

expression gradually increased from 3 hpi (hours post inoculation) and peaked at 24 hpi and decreased thereafter. Among the pathogen inoculated seedlings, at 24 hpi, resistant seedlings recorded the highest PPO gene expression which was 6.08 fold higher than that of the control. In seedlings without pathogen inoculation, the pattern of PPO expression was similar to that of pathogen inoculated seedlings but the level of expression was significantly less. Measurement of PPO mRNA accumulation demonstrated that genes encoding PPO were induced substantially after being challenged with *S. graminicola*, which indicated that the enzyme products of the genes examined here are predicted to be involved in the biosynthesis of defense compounds, so it is not surprising that transcripts accumulated to high levels following pathogen inoculation. High levels of PPO gene expression in IP18292 pearl millet seedlings, in contrast to the low levels of PPO gene expression in 7042S pearl millet seedlings indicated a correlation with the levels of resistance to downy mildew disease. The very rapid and large changes in the resistant seedlings, in contrast to the delayed, smaller changes in the

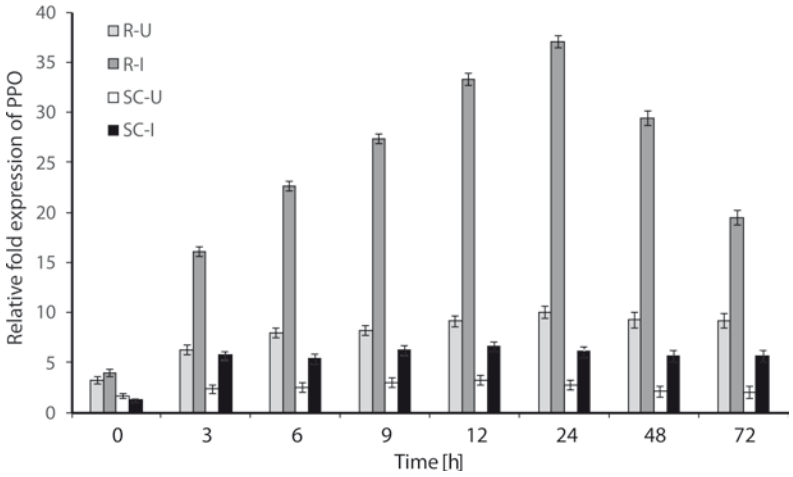

Fig. 2. Real-Time quantitative reverse transcription PCR (qRT-PCR) determined relative expression of PPO genes in two-day-old pearl millet seedlings with (I) or without (U) *Sclerospora graminicola* inoculation harvested 0, 3, 6, 9, 12, 24, 48, and 72 h. R – resistant, SC – susceptible control. Expression levels were measured by quantitative PCR (qPCR) and normalized to the constitutive *PP2A* gene. Values are means of three experiments carried out in triplicate. The bars indicate ± SE and the data were analyzed by one-way ANOVA followed by Tukey's test and *p*-value < or = 0.05

susceptible seedlings suggests that rate and magnitude of PPO gene expression is important for the development of effective resistance against downy mildew.

Although there are some reports which indicate the involvement of PPO in defense against plant pathogens (Li and Steffens 2002; Mohammadi and Kazemi 2002), there is no direct evidence. In this study, we demonstrated the involvement of PPO in pearl millet during defense against the oomycete *S. graminicola* at the molecular level. PCR amplification of pearl millet genomic DNA generated by PCR led to the production of one cDNA fragment of 395 bp, whose amino acid sequence presented a very high homology with that of wheat and rice PPO cDNA. It was deposited in the GenBank database, given the accession AY 881993, and designated as PG-PPO (for *Pennisetum glaucum* polyphenol oxidase). A search of the nonredundant peptide sequence database on the National Center for Biotechnology Information BLAST server using the BLAST program showed high homology of the isolated pearl millet cDNA with PPOs from various sources.

The pearl millet PPO sequence is AT rich (53.3%). Overall, 395 bp of putative pearl millet PPO DNA sequence was obtained. The number of nitrogenous bases was 106, 105, 85 and 98 for A, T, G and C, respectively, which shows the AT rich nature of the pearl millet PPO gene. The 395 bp sequence is not a full-length PPO gene sequence as PPO genes reported so far are about 2,000 bp. This 395 bp pearl millet PPO DNA sequence reported for the first time could be used for antisense technology to study the role of PPO in pearl millet. This sequence may also facilitate the obtaining of the remaining pearl millet PPO DNA sequences. The deduced amino acid sequence of the isolated clone was compared with the sequences of PPOs from other plant species (wheat, trifolium, lettuce, apricot, tobacco, tomato, pokeweed, apple, grape and poplar). A high homology with the PPO of other sequences mentioned above was found particularly at the conserved Copper B region, where there was the highest homology among the aligned sequence. Overall, there is a low level of sequence similarity among PPO genes of different plant species. The highest level of sequence similarity is for the conserved copper-binding regions (Sullivan *et al.* 2004).

This study also confirms the high level of PPO conservation in higher plants. No previously characterized PPO genes possess introns and thus the DNA sequence obtained from amplification of genomic DNA presumably codes for exons only (Demeke and Morris 2002). The pearl millet PPO sequence from the PCR product contained the highly conserved amino acids of histidine-rich copper binding sites similar to other PPO sequences, particularly to that of wheat as described by Demeke and Morris (2002).

Furthermore, we isolated a partial PPO gene from pearl millet for the first time, and indicated that PPO in pearl millet exists in multi copy. We also elucidated the plausible role of this enzyme during pearl millet downy mildew interaction. This partial pearl millet PPO DNA isolated could be used for antisense technology to study the role of PPO in pearl millet. This sequence may also facilitate the obtaining of the remaining pearl millet PPO DNA sequences.

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