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Molecular cloning and expression analysis of duplicated polyphenol oxidase genes reveal their functional differentiations in sorghum

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ABSTRACT

Polyphenol oxidase (*PPO*) is believed to play a role in plant growth, reproduction, and resistance to pathogens and pests. PPO causes browning of grains in cereals. In this study, genetic mapping of sorghum grain for phenol color reaction (PHR) was performed using a recombinant inbred line population. Only one locus was detected between SSR markers SM06072 and Xtxp176 on chromosome 6. Two linked orthologous genes (*Sb06PPO1* and *Sb06PPO2*) within the mapped region were discovered and cloned. Transformation experiments using Nipponbare (a PHR negative rice cultivar) showed that *Sb06PPO1* from LTR108 and two *Sb06PPO2* alleles from both varieties could complement Nipponbare, whereas *Sb06PPO1* from 654 could not. Subsequent quantitative real-time PCR (qPCR) experiments showed that *Sb06PPO1* and *Sb06PPO2* functioned diversely, *Sb06PPO1* was mainly expressed in young panicles before flowering. Sb06PPO2 was strongly expressed in flowering panicles, especially in hulls and branches at filling stage. Moreover, the expression of *Sb06PPO1* was found to be significantly up-regulated by exogenous ABA and salt, whereas *Sb06PPO2* was not changed significantly, further demonstrating functional differentiation between the two genes.

1. Introduction

Polyphenol oxidase (PPO) is the major enzyme that causes browning of fruits, vegetables and cereals grains. It oxidizes a number of phenolic compounds to the corresponding *o*-quinones, which easily undergo secondary reactions with amines, proteins or other phenols to produce dark or brown compounds [1,2].

PPO genes have been isolated and characterized in many plant species, including broad bean [3], tomato [4], potato [5], grape [6], apple [7], sugar cane [8], apricot [9], poplar [10,11], banana [12], wheat [13,14], red clover [15], sweet potato [16], rice [17], barley [18], eggplant [19], and foxtail millet [20]. All of these *PPO* genes have no introns in dicots and have two introns in monocots. PPOs are bi-copper metalloenzymes and have two conserved copper-binding domains, CuA and CuB [21]. Both of the two copper atoms are coordinated by three histidine residues provided by the CuA and CuB sites [13].

Among cereal *PPOs*, sorghum *PPO* is present in the leaves [22,23] and the grain [24–26], while wheat and rice PPOs are expressed only in the grain [17,27]. The exception is in leaf tissue when rice plants were under stress [28]. In barley, two linked *PPO* genes, *HvPPO1* and *HvPPO2*, were found to be differentially expressed in different tissues. The *HvPPO1* gene was expressed in hulls and awns while *HvPPO2* was expressed in caryopses [18]. In addition, PPO may play a key role in plant defense systems by modifying endogenous phenolic compounds, which are toxic to the invading pathogens and pests [10,29–32]. PPO activity in sorghum leaves was found to be in response to fungal invasion [30,33].

Recently, eight *PPO* gene members, *SbPPO1–SbPPO8*, were identified by bioinformatics analysis in sorghum [34]. However, functional analysis of sorghum *PPO* genes has yet to be reported. We actually found that there were nine PPO homologs encoded in sorghum genome.

Abbreviations: PPO, polyphenol oxidase; PHR/Phr, phenol color reaction; RIL, recombinant inbred line; DAP, days after pollination; indel, insertion/deletion; SNP, single nucleotide polymorphism; RT-PCR, reverse transcription PCR; qPCR, quantative real-time PCR; DNA, deoxyribonucleic acids; cDNA, complementary DNA

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In this study, we mapped the gene for the phenol reaction (PHR) of the grain using a sorghum recombinant inbred line (RIL) population. Two linked *PPO* genes were then isolated within the mapped region and their functions were validated through transgenic experiments in rice. Moreover, gene expression profiles revealed the two genes were functionally differentiated.

2. Materials and methods

2.1. Plant materials and phenotyping of PHR

A recombinant inbred line (RIL) population was derived from the cross between two sorghum [*Sorghum bicolor* (L.) Moench] varieties 654 and LTR108. The RIL population consisted of 244 recombinant inbred lines (RILs) [35]. Due to a reddish color of LTR108 seed, many RIL lines showed brown or yellowish-brown in natural seed color through genetic recombination. To unambiguously determine the PHR phenotype, 142 lines with white grains were selected for this study. Each RIL was derived from a single F_2 plant by the single seed descent method until the F_8 generation. PHR phenotype was determined by the method of Oka and Chang [36]. Eight grains per line and parent, and rachis branches with mature spikelets from each parent were collected for identification of PHR. These samples were soaked in 1 mL 1% (v/v) phenol solution using 2.0 mL microcentrifuge tubes, dried after 5 days and then observed by naked eye.

2.2. Determination of PPO activity

The mature grains of two parents were washed several times with tap water, followed by repeated rinsing in deionized water, and then dried and milled to obtain fine flour. PPO was extracted from 50 mg of sorghum flour by continuous stirring with 1.6 mL 0.1 M pyrocatechol solution (prepared using 0.2 M sodium phosphate buffer, pH 6.4) in 2.0 mL microcentrifuge tubes at 37 °C for 5 min. The tubes were then moved to ice for 3 min to terminate the reaction. The solution was centrifuged at 12,000*g*, 4 °C for 5 min and the supernatant monitored using a spectrophotometer (UV-1800; Shimadzu) at 410 nm for 5 min at room temperature. One unit of PPO activity was defined as equal to a change of 0.001 in absorbance per min per gram of flour. The tests were repeated three times.

2.3. Mapping of the SbPhr1

DNA was extracted from 6 to 8-week old seedlings using the CTAB method. Common simple sequence repeat (SSR) markers including Xtxp, Xgap and Xcup [37] and a new set of SSR markers developed by our laboratory according to the most recent versions of the Sbi1 and Sbi1_4 annotations (JGI Phytozome Sorghum bicolor website: http://www.phytozome.net/sorghum) were selected to detect the polymorphism between the two parents, and the polymorphic markers were used to genotype the RIL population. The sequences of the new SSR primers were designed using Primer3 [38] (Supplementary Table 1) and synthesized by Genscript Technology Co., Ltd (Nanjing, China). The linkage map was constructed using MAPMAKER/EXPV3.0 [39]. QTL mapping was conducted with windows QTL Cartographer V2.5 [40].

2.4. Sequence analysis of candidate genomic region

Gene structures were predicted using FGENESH [41], and intron/exon structures were verified by reverse transcription (RT) PCR. The genomic DNA fragments and RT-PCR amplified products of candidate genes from LTR108 and 654 were amplified and sequenced. Sequencing reaction was done by HuaDa Biotech Co., Ltd (Hangzhou, China). Further sequence analysis was performed using Vector NTI from Invitrogen.

2.5. Salt stress and exogenous ABA treatments

For expression analysis of *PPO* genes in response to salt stress and exogenous ABA, seeds of LTR108 were germinated and grown in a growth chamber under normal conditions. Twelve-day-old seedlings were transferred to deionized water containing 100 μM ABA or 300 mM NaCl. Seedlings were harvested after 0, 2, 4, 8, 12 and 24 h ABA treatment and immediately frozen in liquid nitrogen for RNA isolation. Under salt stress, seedling samples were taken after 0, 4, 10 and 24 h after treatment.

2.6. Expression analysis

Root, stem, leaf, sheath and panicle of sorghum plants at the flowering stage and several developing panicles before flowering were harvested from LTR108 and 654. The caryopses, hulls and branches of LTR108 at 1, 5, 10, 20 days after pollination (DAP) were collected. The spikelets, being flowered on the day, were used as samples after removing the previously flowered and un-flowered spikelets. Caryopses were washed with distilled water after removing the endosperm by finger squeezing. Total RNA was extracted from different tissues using RNAiso reagent according to the manufacturer's protocol (Takara, Dalian, China). Young panicles (~2 cm long) were used for semi-quantitative RT-PCR analysis, total RNA (1 μg) was treated with RNase-free DNase and first-strand cDNA was synthesized through reverse transcription by oligo(dT)18 primer (Takara). Subsequently, the first-strand cDNA was used as a template for semi-quantitative PCR analysis after normalized with *SbUbiquitin* gene using the primer SbUbi. The PCR reaction for *Sb06PPO1* and *Sb06PPO2* was performed by using the specific primers 06PPO1-RT1 and 06PPO2-RT1 (Supplementary Table 1), respectively. The PCR procedure was: 94 °C for 4 min, followed by 30 cycles of 94 °C 20 s, 55 °C for 30 s and 72 °C for 30 s and an elongation step at 72 °C for 5 min. The PCR products were analyzed on 1.5% agarose gels. Real-time PCR analysis was performed using a LightCycler (Roche, Switzerland) with the primers 06PPO1-RT2 and 06PPO2-RT2 for *Sb06PPO1* and *Sb06PPO2*, respectively. Amplification reactions were prepared with the Thunderbird SYBR qPCR Mix (TOYOBO, Japan), according to manufacturer's instructions. Each reaction was made in triplicate. The threshold cycle was determined by using the maximum-second-derivative function of the software. Error bars represent the standard error calculated on experiment repetitions.

2.7. Vector construction and plant transformation

For amplification *Sb06PPO1* and *Sb06PPO2* cDNAs, the first-strand cDNAs from 12-day-old seedlings were used as the templates. The following amplifications were carried out using KOD plus DNA polymerase (TOYOBO). PCR products were sequenced by HuaDa Biotechnology. The primer 06PPO1BK was used to amplify the *Sb06PPO1* cDNAs of the two sorghum cultivars, 654 and LTR108. Then, the amplified fragments were inserted into the binary vector pCAMBIA1301-Ubi-NOS, between the *Ubiquitin* promoter and the NOS-3′ terminator, after digesting with *Bam*H I and *Kpn* I. Because the *Sb06PPO1* expression in 654 was low, the second round of PCR was performed by using a small quantity of the first-round reaction mixture as the template. The *Sb06PPO2* cDNAs were amplified using the primer 06PPO2c, and were cloned into pEasy-Blunt vector (TransGen Biotech, China) to generate 06PPO2c-pV654 and 06PPO2c-pV108. Afterwards, the target fragments were obtained by digesting 06PPO2c-pV654 and 06PPO2c-pV108 with *Bam*H I and *Xba* I, respectively, and inserted between the *Ubiquitin* promoter and the NOS-3′ terminator in the binary vector pCAMBIA1390-Ubi-NOS, which was digested with *Bam*H I and *Spe* I. The *Sb03PPO2* gDNAs containing the entire coding regions were amplified using the primer 03PPO2sal from two parents, and were cloned into pEasy-Blunt vector. The target fragments were subsequently obtained by digesting with *Sal* I and *Xba* I, and then inserted into