Phytochemistry 72 (2011) 2275–2287

Contents lists available at SciVerse ScienceDirect

Phytochemistry

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

Eggplant polyphenol oxidase multigene family: Cloning, phylogeny, expression analyses and immunolocalization in response to wounding ☆

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ARTICLE INFO

Article history:
Received 8 April 2011
Received in revised form 14 July 2011
Accepted 31 August 2011
Available online 24 September 2011

Keywords:
Solanum melongena
Solanaceae
Eggplant
Phylogenetic analysis
Polyphenol oxidase gene
PPO
Immunolocalization
Multigene family
Synteny
Wounding

ABSTRACT

Though polyphenol oxidase (PPO) genes from tomato and potato have been extensively studied, information about PPO genes in eggplant (*Solanum melongena*) is lacking. The main objective of this study is to understand the structural and functional aspects of eggplant PPO genes. Six eggplant PPO genes (*SmePPO1–6*) cloned by RACE and genome walking were found to be intronless and correspond to eight eggplant unigenes. Comprehensive sequence analyses indicated that the eggplant PPO genes exhibit considerable variation in the transit peptide regions, copper-binding domains and UTRs, and fall into two distinct structural classes. Further, PPO gene members appear to exist in clusters on eggplant chromosome 8 as seen in the case of tomato and potato PPOs. During normal growth and development, *SmePPO1* and *2* are expressed in roots, whereas the transcript levels of all the eggplant PPO genes vary considerably in leaves, flowers and fruits. *SmePPO1* was expressed in *Escherichia coli* as a GST fusion protein, and immunoblot using rabbit polyclonal antiserum to GST–SmePPO1 detected a major protein band (~70 kDa) and a minor band (~67 kDa) in eggplant fruit extract. Tissue printing indicated the predominant presence of PPO in the exocarp and the areas surrounding the seeds in the mesocarp of eggplant fruits. Immunolocalization of PPOs in eggplant infested with shoot-and-fruit borer revealed localization of the PPO at the site of infection in tender shoots and fruits, and further inside the mature tissues. The upregulation of eggplant PPO gene transcripts following mechanical injury shows that all the genes except *SmePPO2* are induced in the fruit over 6 h. On the contrary, the transcripts of *SmePPO2* and *PPO3* are not detectable in the stem, and expression seems to be prominent over a 2 h period for *SmePPO1* and *SmePPO4–6*. Our results show that eggplant PPO genes are structurally different, and are differentially expressed in various tissues of eggplant indicating their functional diversity.

1. Introduction

In plants, phenol oxidizing enzymes are generically termed as polyphenol oxidases (PPOs) based on their ability to oxidize specific phenolic substrates in the presence of molecular oxygen – cresolases (monophenol oxidase, EC 1.14.18.1), *o*-diphenol oxidases (*o*-diphenol: oxygen oxidoreductase, EC 1.10.3.1) and laccase-like multi-copper oxidases (LMCO, *p*-diphenol: dioxygen oxidoreductase, EC 1.10.3.2) (Aniszewski et al., 2008; Mayer, 2006; Yoruk and Marshall, 2003). Peroxidases (E.C.1.11.1.7, donor: hydrogen-peroxide oxidoreductase) are also capable of oxidizing phenolics but only in the presence of H_2O_2. Ubiquitously distributed in the plant kingdom (Mayer and Harel, 1979), PPOs play key roles in plant defense mechanism against pests and pathogens (Thipyapong et al., 1995; Thipyapong and Steffens, 1997; Wang and Constabel, 2004a), biosynthesis of aureusidine from oxidation of chalcones in *Antirrhinum majus* (Nakayama et al., 2000), oxidation of flavonoids in *Arabidopsis* seeds (Pourcel et al., 2005), and flavonoid biosynthesis (Ono et al., 2006). The phenolic substrates released from vacuoles upon cellular decompartmentation as a result of tissue damage comes in contact with polyphenol oxidases, which catalyze their oxidation to highly reactive quinones, capable of non-enzymatic covalent modification and polymerization of cellular nucleophiles to produce black- or brown-colored pigments (Vámos-Vigyázó, 1981).

Abbreviations: DAP, days after pollination; GST, glutathione-*S*-transferase; MALDI, matrix-assisted laser desorption ionization; PMF, peptide mass fingerprinting; PPO, polyphenol oxidase; RACE, rapid amplification of cDNA ends; SFB, shoot-and-fruit borer; Sme, *Solanum melongena*; UTR, untranslated region; WGS, whole genome shotgun.

☆ A portion of this work was presented at the 6th Solanaceae Genome Workshop (SOL 2009) held during 8–13 November 2009 at New Delhi, India.

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0031-9422/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2011.08.028

Nuclear gene encoded PPOs are synthesized in cytosol as precursor proteins of 67–70 kDa, which subsequently are processed into mature peptides of molecular mass 58–60 kDa during their import into thylakoid lumen (Koussevitzky et al., 1998; Sommer et al., 1994). PPOs are copper metalloproteins with three types of copper ion active sites found together or individually in the native enzymes. *o*-Diphenol oxidases, capable of oxidizing both mono- and ortho-diphenols to corresponding *o*-diquinones, are localized in plastids, and belong to the type 3 copper protein family. Six histidine residues present in two highly conserved copper-binding domains form coordination complexes with two copper ions; the C-terminal domain is homologous with hemocyanin (Klabunde et al., 1998; Marusek et al., 2006).

Of the three classes of PPOs, *o*-diphenol oxidases (catecholases) and their encoding genes have been extensively characterized in many higher plant species. In many dicotyledons like *Vicia faba* (Cary et al., 1992), tomato (Newman et al., 1993; Thipyapong et al., 1997), potato (Thygesen et al., 1995), red clover (Sullivan et al., 2004; Winters et al., 2009) and apple, *o*-diphenol oxidase-encoding genes are organized as a multigene family. In tomato, RFLP mapping has elucidated the location of PPO genes on 165 kb locus of chromosome 8 (Newman et al., 1993). None of the dicotyledonous PPO genes reported so far show the presence of introns. Monocots like banana and wheat, which have at least four and six distinct *o*-diphenol oxidase genes, respectively, show the presence of intervening sequences (Gooding et al., 2001; Massa et al., 2007). Though genes homologous to *o*-diphenol oxidase are absent in *Arabidopsis* genome, laccases with at least 17 genes constituting multigene family are present (McCaig et al., 2005). Characterization of tissue-specific and developmental expression pattern of seven *o*-diphenol oxidase genes in tomato (Thipyapong et al., 1997) and six genes in potato (Thygesen et al., 1995) has been reported. Local and systemic upregulation of PPO gene expression in response to wounding and herbivory of leaves and fruits have been reported in these plants – apple (Boss et al., 1995), Fuji apple (Kim et al., 2001), hybrid poplar (Wang and Constabel, 2004b), pineapple (Zhou et al., 2003), potato (Thipyapong et al., 1995), and tomato (Thipyapong et al., 1997; Bhonwong et al., 2009).

The role of PPO as a defense protein in plants and the fact that post-harvest losses of agricultural produce are caused by enzymatic browning, explain why so many PPO proteins and their encoding genes have been isolated and characterized (Mayer, 2006). Characterization of PPOs from plants in which they have not been previously described will be useful in understanding additional structural and functional features of PPOs considering the enigmatic nature of the role of PPOs in plants. Among the solanaceae family, potato, tomato, eggplant (also known as aubergine or brinjal), and bell pepper are the principal vegetable crops of economic importance (Daunay, 2008); however, whole genome Shotgun (WGS) sequences are available only for potato and tomato (http://www.potatogenome.net; http://solgenomics.net/) (The Potato Genome Sequencing Consortium, 2011). Eggplant is a major vegetable crop in the Indian subcontinent with an annual production surpassing 10.4 million tons in 2009 (http://faostat.fao.org). With the center of eggplant diversity located in India, eggplant makes an important part of the Indian cuisine. Among the proteins described from eggplant, enzymatic activity of PPO due to its role in browning of eggplant fruit (rich in phenolic compounds) has been studied extensively; chlorogenic acid (5-*O*-caffeoylquinic acid and its isomers) typically accounts for 70–95% of total phenolics in eggplant fruit flesh (Whitaker and Stommel, 2003; Singh et al., 2009). Several studies related to the biochemical and enzymatic features of PPO from eggplant have been described (Pérez-Gilabert and García Carmona, 2000), though information on PPO genes is lacking. Nagasawa et al. (2001) have described a partial gene of 742 bp encoding a PPO-like protein from eggplant flowers (3 days following pollination). In view of the biological importance of PPO and very limited information on eggplant PPO genes, it appeared interesting to clone the gene(s) for eggplant PPOs, and to investigate their spatial and temporal expression patterns. Additionally, the study of eggplant PPO genes in relation to disease/pest resistance is much warranted due to their common infestation with the pest, shoot-and-fruit borer (SFB; *Leucinodes orbonalis*).

2. Results and discussion

2.1. Molecular sequence analyses revealed two structurally distinct classes of eggplant PPOs

The conserved sequences from tomato and potato PPOs corresponding to CuA and CuB regions along with eggplant unigenes provided a platform for designing primers required for cloning the eggplant PPOs, constituting a putative multigene family. PCR-based cloning strategy was successfully adapted to isolate six distinct eggplant PPO genes (*SmePPO1–6*) by genome walking and RACE techniques. Both genomic and cDNA clones of eggplant PPOs were obtained, and pair-wise sequence alignment of genomic and corresponding cDNA clones of six eggplant PPO genes clearly showed single open reading frame with no intervening sequences (data not shown). This is in agreement with the absence of introns in all dicotyledonous PPOs reported so far (Cary et al., 1992; Newman et al., 1993; Sullivan et al., 2004). Each of the eggplant PPO genes cloned in this study, with the exception of *SmePPO2* gene, correspond to the sequences of eggplant PPO unigenes available in the SGN database (Table 1). The percent identity among eggplant PPOs at the level of nucleotide sequence is presented in Table 2, and at the level of deduced amino acid sequence in Table 3. It is seen from Tables 2 and 3 that *SmePPO1–3* shares 80% homology at nucleotide level and 70% homology at protein level; similarly, *SmePPO4–6* exhibit 89% nucleotide homology and 80% homology at protein level. Hence, these identity matrices indicate the existence of two structural classes of eggplant PPOs: class A (SmePPO1–3) and class B (SmePPO4–6).

The translated nucleotide sequences of six eggplant PPOs (Fig. 1) were analyzed for delineating the nature of transit peptide and the two copper-binding regions characteristic of all PPOs (Koussevitzky et al., 1998). The N-terminal region of eggplant PPO precursors predicted to harbor positively charged bipartite chloroplast transit peptide consists of stromal and thylakoid luminal targeting domains of total length varying from 80 to 90 amino acids. The stromal targeting pre-sequence (~45 aa) rich in hydroxyamino acids confers import of eggplant PPO from cytosol into chloroplast stroma. The hydrophobic thylakoid targeting domain of 40–45 aa in length is responsible for localization of PPO in thylakoid lumen. All eggplant PPO chloroplast transit peptides (Fig. 1, boxed portion) display conserved cleavage sites for stromal processing peptidase [VSCK/N↓] and thylakoid processing peptidase [L(A/T)A(S/N)A↓] (highlighted in color in Fig. 1) with conspicuous deletions or substitutions in the flanking regions.

The N-terminal regions of mature PPO polypeptides are invariably rich in proline residues. The copper-binding regions A and B show the presence of highly conserved histidine and cysteine residues; the copper-binding region A is relatively more conserved than the copper-binding region B which show a number of amino acid deletions apart from substitutions in eggplant PPO genes 4–6 (Fig. 1) indicating a distinct structural class (class B) in comparison to SmePPO1–3 (class A). Unlike the signal peptide and copper-binding regions, the C-terminal portion of eggplant PPOs reveals highly conserved features having a signature domain (KFDV) that