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Molecular characterization of fruit-specific class III peroxidase genes in tomato (*Solanum lycopersicum*)

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ABSTRACT

In this study, expression of four peroxidase genes, *LePrx09*, *LePrx17*, *LePrx35* and *LePrxA*, was identified in immature tomato fruits, and the function in the regulation of fruit growth was characterized. Analysis of amino acid sequences revealed that these genes code for class III peroxidases, containing B, D and F conserved domains, which bind heme groups, and a buried salt bridge motif. *LePrx35* and *LePrxA* were identified as novel peroxidase genes in *Solanum lycopersicum* (L.). The temporal expression patterns at various fruit growth stages revealed that *LePrx35* and *LePrxA* were expressed only in immature green (IMG) fruits, whereas *LePrx17* and *LePrx09* were expressed in both immature and mature green fruits. Tissue-specific expression profiles indicated that only *LePrx09* was expressed in the mesocarp but not the inner tissue of immature fruits. The effects of hormone treatments and stresses on the four genes were examined; only the expression levels of *LePrx17* and *LePrx09* were altered. Transcription of *LePrx17* was up-regulated by jasmonic acid (JA) and pathogen infection and expression of *LePrx09* was induced by ethephon, salicylic acid (SA) and JA, in particular, as well as wounding, pathogen infection and H_2O_2 stress. Tomato plants over-expressing *LePrx09* displayed enhanced resistance to H_2O_2 stress, suggesting that *LePrx09* may participate in the H_2O_2 signaling pathway to regulate fruit growth and disease resistance in tomato fruits.

Introduction

Peroxidases (EC 1.11.1.X) are present in almost all living organism and reduce hydrogen peroxide to water by catalyzing the oxidation of various substrates. Peroxidases comprise a superfamily, which is divided into three classes: intracellular class I (EC 1.11.1.5/.6/.11); class II of fungal origin (EC 1.11.1.13/.14); and the secretory class III plant peroxidases (EC 1.11.1.7) (Welinder, 1992). Class III peroxidases comprise a single polypeptide chain of approximately 300 amino acid residues, Fe (III) protoporphyrin IX (usually referred to as a heme group) and two calcium atoms. Also, they share a very similar three-dimensional structure with four disulphide linkages, based on the conserved cysteine residue pairs, and a buried salt bridge motif containing invariant Asp and Arg residues (Welinder, 1992; Edwards et al., 1993; Veitch, 2004; Smulevich et al., 2006). Except for the conserved residues, Class III peroxidases display low identity of primary amino acid sequences and have distinct functions and reaction mechanisms.

Class III peroxidases form a large multigene family in all land plants (Duroux and Welinder, 2003; Passardi et al., 2004). Previous studies have suggested that Class III peroxidases may have played a critical role during land colonization of plants, either by enabling the formation of rigid plant structures or by adapting organisms to a more oxygenated environment (Duroux and Welinder, 2003; Passardi et al., 2004). Plant peroxidases are generally secreted into cell walls and vacuoles or into the surrounding medium (Welinder et al., 2002; Matsui et al., 2003). Diverse functions of peroxidases have been reported, including lignification, suberization, stress protection and defense response (as reviewed in Passardi et al., 2005; Cosio and Dunand, 2009).

Tomato (*Solanum lycopersicum* (L.)) is one of the major crops in the world due to the diverse uses and high nutritional value of the fruit. Numerous parameters have been reported to influence the growth and development of tomato fruits. One of the major

Abbreviations: IMG, immature green; MG, mature green; JA, jasmonic acid; SA, salicylic acid.

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factors is the activity of peroxidases, which has been suggested to be involved in the maintenance of mechanical properties of fruit pericarp (Andrews et al., 2002a). Previous studies have reported that the activities of several peroxidase isoforms were detected in the exocarp of tomato fruits at the stage of growth cessation, suggesting that peroxidases may be involved in the regulation of fruit growth (Andrews et al., 2000, 2002b). Further studies demonstrated that peroxidases may act as a catalyst to modify cell walls and a regulator to modulate reactive oxygen intermediates via hydroxylic or peroxidative cycles in the fruit skin, thus promoting the termination of fruit growth (Andrews et al., 2002b; Dumville and Fry, 2003; Passardi et al., 2004). Previously, only two peroxidase genes had been identified in tomato fruits, which is *LePrx75* (*TAP2*) and *LePrx76* (*TAP1*) (Roberts and Kolattukudy, 1989; Mohan and Kolattukudy, 1990; Sherf and Kolattukudy, 1993). In addition to fruit growth, peroxidases were also reported to regulate the growth of various plant organs, including hypocotyls of mung bean (Goldberg et al., 1987) and zucchini (Cosio and Dunand, 2009), coleoptiles of maize (Hohl et al., 1995), and the elongation of *Arabidopsis* roots (Passardi et al., 2006).

The aim of the present study was to identify and characterize peroxidase genes expressed in tomato fruits, and to elucidate the functions of peroxidases during fruit growth. Four peroxidase genes were identified in tomato fruits, with specific expression patterns in immature green (IMG) but not in ripening fruits, suggesting that the peroxidases may be involved in the regulation of fruit growth. Among these, the expression of *LePrx09* was specifically detected in the mesocarp of tomato fruits, and was particularly induced by phytohormone, wounding stress, pathogen infection and H_2O_2 treatments. Moreover, tomato over-expressing *LePrx09* displayed high resistance to H_2O_2 stress. The results suggested that *LePrx09* expressed in mesocarp enhances the anti-oxidative capacity. Therefore, *LePrx09* may be involved in the defense mechanism against environmental stress during the active fruit growth period.

Materials and methods

Plant materials

Hualien ADRVC #5, a tomato (*Solanum lycopersicum* (L.)) cultivar, grown in a net house with 16 h photoperiod at 25 °C, was used in the present study. Tomato fruits at various growth stages, plus leaves and flowers, were collected to verify the expression patterns of identified peroxidase genes. According to size, immature green (IMG) fruits were classified into five stages: IMG1 (fruit diameter 0.5 to 1.0 cm); IMG2 (fruit diameter 1.0 to 1.5 cm); IMG3 (fruit diameter 2.0 to 2.5 cm); IMG4 (fruit diameter 3.5 to 4.0 cm); and IMG5 (fruit diameter 6.0 to 7.0 cm). In addition, mature fruits were divided into three stages: mature green fruits (MG), breaker and ripe, based on the definition described by Lashbrook et al. (1994).

Peroxidase gene identification in tomato fruits

Total RNA was extracted from tomato fruits by the PineTree method (Chang et al., 1993) and used as the template for reverse transcription-polymerase chain reaction (RT-PCR). First-stand cDNA was reverse transcribed from 4 μg total RNA with SuperScript III Reverse Transcriptase and oligo-$(dT)_{12–18}$ (Invitrogen) was used as the primer according to the manufacturer's protocol. A degenerate primer (5′-CAYTTYCAYGAYTGYTTYGT-3′) was designed based on the conserved amino acid sequence of Class III peroxidase genes of *Solanaceae* crop species in the NCBI database, i.e. HFHDCFV. The degenerate primer and an oligo$(dT)_{18}$V primer were used for the amplification of putative peroxidase genes in tomato fruits. The amplified fragments, approximately 900 bp in length, were ligated into a TA vector (pGEMTeasy, Clontech) for sequence verification by an automated DNA sequence analyzer (model 310, Applied Biosystems). To obtain the full-length nucleotide sequence, 5′ rapid amplification of cDNA ends (5′-RACE) was performed with a commercially available kit (GeneRacer, Invitrogen) according to the manufacturer's instructions. A gene-specific primer for each cloned cDNA were designed on the basis of the identified nucleotide sequences (*LePrx09*: GTTGACTCCGTCCTTTCTTCCTAAGGGTACATCGTAGTTGG; *LePrx17*: GAGGAATGTGTGGCCCTCCTAGAGCAACAATACCATCTCTA; *LePrx35*: CCCTAATAACACATTCCATGAAGGACCACCTGCCAG; *LePrxA*: CCTCTTCTACCTGTCTCTACTTCATAAGATTCTCCGCCAGCCA). Full-length cDNA of peroxidase genes were then amplified by RT-PCR with gene-specific primers according to the sequence of fragments amplified by 5′-RACE.

Sequence alignment and phylogenetic analysis

The homologous sequences of peroxidases were searched with BLAST in the PeroxiBase database (http://peroxibase.toulouse.inra.fr/) (Passardi et al., 2007). Nomenclature was used following the PeroxiBase database, with 'LePrx' for class III peroxidases of tomato, followed by a number or letter. Amino acid sequences of the four identified peroxidases were aligned with peroxidases in Solanaceae crop species by use of default alignment parameters in Clustal X (Thompson et al., 1997) and subsequently adjusted manually according to their conserved ion binding sites. The molecular mass and isoelectric point (pI) of mature protein was estimated by the Compute pI/MW tool in Swiss-Prot/TrEMBL (http://www.expasy.org/tools/protparam.html).

A phylogenetic tree of the four identified peroxidases together with 20 *Solanaceae* peroxidases obtained from the PeroxiBase database was constructed using *Arabidopsis thaliana* peroxidase AtPrx01 as an outgroup. Distance and maximum likelihood analyses were performed with the PHYLIP suite (Felsenstein, 1993). Distances between proteins were computed with the PRODIST program using the maximum likelihood estimated on Dayhoff PAM matrix; 1000 bootstrap replicates were carried out with SEQBOOT option. The KFITCH program estimated the phylogenies from the distances in the matrix data using the Fitch–Margoliash algorithm (Fitch and Margoliash, 1967); no rough analysis, no global rearrangements, and multiple jumbles (10 times) options were selected. Phylogenetic trees were drawn by the TREEVIEW program and Adobe illustrator software according to output data of the PHYLIP program. Bootstrap values were shown as support percentage on the branches from 1000 replicates. The bootstrap support values of branches smaller than 50% were collapsed.

Hormone treatments and stress challenge

For hormone treatments, detached IMG3 fruits were placed in solutions of IAA, GA, zeatin, ethephon, ABA, salicylic acid (SA) and jasmonic acid (JA), at concentration 20 μM, for 6 h. The fruits were placed in H_2O as a control for IAA, GA, zeatin, ethephon, and ABA or 1 μM ethanol as the control for SA and JA treatments.

For wounding treatment, fruits at stage IMG3 on tomato plants were cut on the surface using a knife. Fruits collected from plants without damage were used as control.

For pathogen inoculation, conidia of *Alternaria solani* were harvested from diseased leaves of tomato and grown on agar culture medium. To collect the conidia, 2 mL of sterile deionized H_2O was applied to the medium and the conidia were gently scraped with a glass microscope slide. The conidial suspension was filtered through a 0.5-mm^2-pore strainer to remove mycelial debris. The resulting spore suspension was diluted to 1×10^4 conidia per mL and stored at 4 °C. Detached IMG3 fruits were