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Full Length Research Paper

Expression, molecular characterization and detection of lipoxygenase activity of *tomloxD* from tomato

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***TomloxD* is a lipoxygenase from tomato (*Lycopersicon esculentum* Mill.), which encodes a protein of 908 amino acid residues with a calculated molecular mass of 102.31 kDa. The predicted three-dimensional structure of *TomloxD* showed a typical lipoxygenase fold: a β-barrel structure in the N-terminal region and the catalytic site in the C-terminal domain. The semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis suggested that, the *TomloxD* transcript was higher in roots, flowers, younger leaves and fruits of breaker+4 days stage and lower in mature leaves, fruits from the mature green and breaker stages. *TomloxD* expression response to mechanical injury and the change of lipoxygenase activities exhibited is the same as that of *TomloxD* transcript after mechanical injury. The *TomloxD* gene was cloned into pPIC9K vector and was transformed into *Pichia pastoris* expression host strain GS115. A transformant strain was selected and expressed efficiently. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis of the concentrated supernatant showed that exogenous *TomloxD* was expressed in transformed yeast. Lipoxygenase activity of the supernatant showed that, *TomloxD* transgenic yeast had high levels of lipoxygenase activities, which demonstrated that the *TomloxD* has lipoxygenase activity. *TomloxD* may play a role as a component of the octadecanoid defense-signaling pathway.**

Key words: Eukaryotic expression, lipoxygenase (LOX) activity, *Lycopersicon esculentum* Mill., mechanical injury, semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR), *TomloxD*, Western blot.

INTRODUCTION

Lipoxygenases (LOX) (linoleate: oxygen oxidoreductase, EC 1.13.11.12; LOXs) are a family of enzymes found ubiquitously in plants and mammals, but have also been detected in coral, moss algae, fungi, yeast and a number of bacteria (Kühn et al., 2005; Lang and Feussner, 2007; Liavonchanka and Feussner, 2006). LOXs catalyze the oxygenation of polyunsaturated fatty acids (Brash, 1999). Plant LOXs are ubiquitous and encoded by multigene families. LOXs can be found in all organs in plant (Loiseau et al., 2001). LOXs are almost involved in various physiological processes (Kolomiets et al., 2000; Veronesi et al., 1996).

Plant LOXs are a class of non-heme, iron-containing, monomeric proteins of about 95 to 100 kDa that is made of two domains. The amino-terminal domain of about 25 to 30 kDa is a β-barrel domain (domain I) (Andreou and Feussner, 2009; Corbin et al., 2007). Its exact function is yet unknown, but an involvement in membrane or substrate binding has been discussed (Andreou and Feussner, 2009; May et al., 2000; Tatulian et al., 1998). The carboxyl-terminal domain of about 55 to 65 kDa consists primarily of a-helices (domain II) and harbors the catalytic site of the enzyme (Andreou and Feussner,

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Abbreviations: LOX, Lipoxygenases; **JA,** jasmonic acid; **ABA,** abscisic acid; **RT-PCR,** reverse transcriptase polymerase chain reaction; **BMGY,** buffered glycerol complex medium; **MM,** modified medium; **SDS-PAGE,** sodium dodecyl sulfate polyacrylamide gel electrophoresis; **cTP,** chloroplast transit peptides; **SMART,** simple modular architecture research tool; **BLAST,** basic local alignment search tool; **BPH,** brown planthopper; **SSB,** stripped stem bore; **LF,** leaf folder.

2009; Schneider et al., 2007). LOX enzymes contain one iron atom per protein molecule. The iron-containing active site is in the center of domain II. The iron active site metal is a non-heme iron that is octaedrically coordinated by 5 amino acid side chains and a water or hydroxide ligand. In case of plant LOXs, these residues are always three histidines, one asparagine and the carboxyl group of the carboxyl-terminal conserved isoleucine (Andreou and Feussner, 2009). The N-terminal domain makes only a loose contact with the C-terminal domain; this may be dispensable for plant lipoxygenases, because all the amino acid side chains responsible for catalysis are located in the C-terminal domain (Boyington et al., 1993; Minor et al., 1996).

LOX catalyzes the insertion of molecular oxygen into polyunsaturated fatty acids (PUFAs) that contain a (Z,Z)-1,4-pentadiene system to produce an unsaturated fatty acid hydroperoxide. Oxygen can be added to either end of the pentadiene system (regiospecificity). In higher plants, the natural substrates for these enzymes are linolenic (18:2) and linoleic (18:3) acids (Conconi et al., 1996; Siedow, 1991). LOX-catalyzed incorporation of molecular oxygen into these fatty acids can occur either at carbon positions 9 or 13 (Brash, 1999), which leads to two possible products, the 9- and 13-hydroperoxy fatty acids (Siedow, 1991). In *in vitro*, most LOXs prefer free fatty acids, though it has been shown that sterified fatty acids are also substrates for LOX *in vivo* (Feussner et al., 2001) suggesting that, membrane lipids could be substrates for oxylipin biosynthesis (Porta and Rocha-Sosa, 2002).

LOXs are responsible for the dioxygenation of polyenoic fatty acid in the production of fatty acid hydroperoxides, which are further metabolized to various lipid mediators. In plants, LOX products can be further metabolized to yield volatile aldehydes and jasmonates (Feussner and Wasternack, 2002; Mosblech et al., 2009). These compounds have physiological functions in a variety of plant processes such as seed development, germination, vegetative growth, generation of fatty acid-derived flavor compounds, wounding, stress responses, senescence and cell signaling (Chen et al., 2004; Padilla et al., 2009; Porta and Rocha-Sosa, 2002; Siedow, 1991). Oxylipins produced by the 9-Lipoxygenase pathway in *Arabidopsis* regulate lateral root development and defense responses through a specific signaling cascade (Vellosillo et al., 2007).

In plants, multiple LOX isoforms which exist are characterized by their different temporal and spatial distributions during plant development (Tamás et al., 2009). LOX genes isolated from different plant species show differential organ specific expression (Griffiths et al., 1999; Kolomiets et al., 2001). In addition, LOX protein and activity levels is regulated by different effectors such as the source/sink status, jasmonic acid (JA), abscisic acid (ABA) and by different forms of stress, such as wounding, insect attack, water deficiency, pathogen attack, or their elicitors (Bohland et al., 1997; Creelman and Mullet, 1997; Gao et al., 2008; Hwang and Hwang, 2010; Jardim et al., 2010; Melan et al., 1993; Porta et al., 2008; Veronesi et al., 1996; Veronico et al., 2006; Wang et al., 2008; Zhou et al., 2009).

Products of the LOX in plants pathway have several diverse functions. LOX has been associated with some processes in a number of developmental stages (Kolomiets et al., 2001; Siedow, 1991). LOX have implicated some of the physiological processes which include wounding, pathogen attack, seed germination, fruit ripening, plant senescence, cell death and synthesis of ABA and JA (Gao et al., 2009; Hayashi et al., 2008; Hwang and Hwang, 2010; Marmey et al., 2007; Porta and Rocha-sosa, 2002). Seed LOXs may function as storage proteins (Siedow, 1991). LOXs participate in the mobilization of storage lipids during germination (Feussner et al., 2001). During normal vegetative and reproductive growth, LOX is also used as storage for protein, participate in transference of lipoid and response to nutrient stress and source/sink relationships (Fischer et al., 1999).

There are at least five lipoxygenases present in tomato (*Lycopersicon esculentum* Mill.) (Chen et al., 2004). Among the five tomato LOX genes, *TomloxA*, *TomloxB*, and *TomloxE* have high homology and are 72 to 77% identical with each other at the amino acid level, while *TomloxC* and *TomloxD* show 46% identity to each other and 42 and 47% identity to the *TomloxA* protein, respectively. *TomloxB* and *TomloxC* expression is enhanced by the ripening hormone ethylene, whereas *TomloxA* expression declines (Griffiths et al., 1999). *TomloxC* is expressed during ripening that can use both linoleic and linolenic acids as substrates to generate volatile C6 flavor compounds (Chen et al., 2004). The individual LOX isoforms are differentially regulated and may have distinct functions during growth and development of tomato plants.

In this study, the expression characters of *TomloxD* gene was analyzed and three-dimensional structure of *TomloxD* protein was predicted and given its characteristics. To determine the functional activity of the *TomloxD*, yeast cells synthesizing this protein were obtained. The enzyme activity analysis of *TomloxD* protein suggested that, *TomloxD* gene code is a lipoxygenase which participate in response to wounding.

MATERIALS AND METHODS

Growth of plants and treatments

All experiments were performed using a near-isogenic line of diploid tomato (*L. esculentum* Mill. cv Ailsa Craig) plants. Tomato plants were grown in peat pots and maintained for 15 h of light (30 µE m^{-2} s^{-1}) at 26°C and 9 h of dark at 19°C. The leaves were wounded by crushing the leaf with a hemostat, but the main vein of each leaflet cannot be wounded. The wounded leaves were incubated on the wet filter paper in closed can. The wounded leaves of seedlings

Table 1. Nucleotide sequences of primers used for PCR amplification in the present study.

S/N	Primer	Sequence (5' and 3')
1	TomD f	5'-ATTATTATGGCACTTGCTAAAGAAATT-3'
2	TomD r	5'-AATTAGATATCGATACACTATTTGGAACA-3'
3	TomDS f	5'-GACAAGCAATAGCAGGAGTG-3'
4	TomDS r	5'-TAAGTGTGCCAACATCAGAC-3'
5	Actin f	5'-TGAAATGTGACGTGGATATTAGG-3'
6	Actin r	5'-TGAGGGAAGCCAAGATAGAGC-3'
7	TomDJ f	5'-CACCGAATTCCATCACCATCACCATCACGATGATGATGATAAGATGGCACTTGCTAAAGAAATT-3'
8	TomDJ r	5'-GTCGGCGGCCGCTCATATCGATACACTATTTGGAA-3'

were collected after 0, 2, 6, 12 and 24 h treatment, respectively, frozen in liquid nitrogen and then stored at -80°C. The roots, flowers, younger and mature leaves, and fruits at the mature green, breaker and breaker + 4 days (B + 4) stages were collected and stored at -80°C, respectively.

Cloning of *TomloxD* gene

Total RNA was extracted from the seedlings using the Trizol reagent (Gibco-BRL, USA). One microgram of total RNA from each treatment was used for reverse transcriptions using olig(dT)18 as 3' primer. Based on the *TomloxD* cDNA sequence (GenBank accession no. U37840.1), a pair of primers, *TomD* f and *TomD* r, was designed (Table 1). The *TomloxD* gene sequence was amplified from cDNA by reverse transcriptase polymerase chain reaction (RT-PCR) using TaKara LA Taq™ polymerase and 30 cycles of 94°C for 30 s, 58°C for 45 s and 72°C for 170 s. This PCR product was cloned into pMD18-T vector (Invitrogen, USA) and sequenced.

Structure prediction and homology modeling of the OsGSTL2 protein

The nucleotide sequence and the putative amino acid sequence were analyzed using blastP (http://www.ncbi.nlm.nih.gov/), the SMART (http://smart.embl-heidelberg.de/) and ChloroP (http://www.cbs.dtu.dk/services/ChloroP/), respectively. The soybean (*Glycine max*) LOX protein (LOX3) (Protein Data Bank code No.: lno3A) was used as templates for constructing a structure model of *TomloxD.* The three dimensional structure of *TomloxD* protein was modeled using SWISS-MODEL, set automatically to seek appropriate known protein crystal structures as templates based on sequence similarity (http://swissmodel.expasy.org/). The models were selected according to model evaluation score calculated by Procheck and Whatcheck (Arnold et al., 2006; Schwede et al., 2003).

Expression analysis of *TomloxD* gene in tomato

In order to analyze expression of *TomloxD* gene in tomato, *TomDS* f and *TomDS* r were employed to produce a 290 bp PCR product (Table 1). The housekeeping gene, *Actin* gene from tomato was used as a control (GenBank accession no. U60480). The primers Actin f and r were designed to obtain a 201 bp amplification product (Table 1). The gene fragments of *TomloxD* and Actin were amplified using RT-PCR reaction. Amplification was carried out by initial denaturation at 94°C for 2 min followed by 28 cycles of 94°C denaturation for 30 s, 58°C annealing for 30 s and 72°C elongation for 30 s. PCR products from each amplification reaction were separated in a 2% (W/V) agarose gel. The relative expression level of *TomloxD* in different tissues was calibrated against the expression level of the inner control gene *Actin*.

LOX activity assays in wounded tomato leaves

Extraction of tomato lipoxygenases were carried out according to the methods of Ohta et al. (1986) for purifying rice lipoxygenases with several modifications. Tomato leaves (0.2 g) were homogenized with 1 ml of 50 mm sodium phosphate and 1.5% (w/v) Triton X-100 (pH 6.8) using a Polytron homogenizer and centrifuged at 18,000 g for 15 min. The supernatant was used as a crude extract and assayed for LOX activity.

LOX activity was measured spectrophotometrically at 234 nm by the methods of Shi et al. (1996). The reaction mixture (3.0 ml) contained 2.5 mM linoleic acid and 0.1% (w/v) Tween 20 in 0.2 M sodium Borate buffer (pH 9.0). Assays were carried out at 25°C for 15 to 30 s and absorbance at 234 nm derived from the conjugated diene chromophore of fatty acid hydroperoxides was monitored with a spectrophotometer (UV-160A, Shimadzu, Kyoto, Japan). One unit of activity was defined as the quantity of enzyme catalyzing an increase in absorbance of 0.01 at 234 nm/min under assay condition. The specific activities of the enzyme fractions were calculated based on the amount of protein in the fraction. Protein was estimated by the Bradford (1976) method using bovine plasma gamma globulin as a standard.

TomloxD eukaryotic expression

Construction of *TomloxD* eukaryotic expression vector

In order to express the *TomloxD* protein in *Pichia pastoris* expression host strain GS115, the reading frame sequence of the *TomloxD* gene was amplified with the combinations of specific primers *TomDJ* f and *TomDJ* r as detailed in Table 1. The forward primer *TomDJ* f was introduced to an *EcoR* I site and a His-Tag at the 5'-end and the reverse primer *TomDJ* r was introduced to a *Not* I site. The amplification product was cloned into pPIC9K vector (Invitrogen, Carlsbad, USA) by *EcoR* I and *Not* I. The recombinant was transformed into *E. coli* top 10 and was identified by restriction enzyme analysis and sequencing (Invitrogen in Shanghai).

Expression of *TomloxD* in *Pichia pastoris* GS115

The correct expression vector were transformed into *P. pastoris* strain GS115 and identified by PCR. Expression of *TomloxD* in *P.pastoris* GS115 was carried out according to the methods of Hu et al. (2009) with several modifications. Positive transformants were

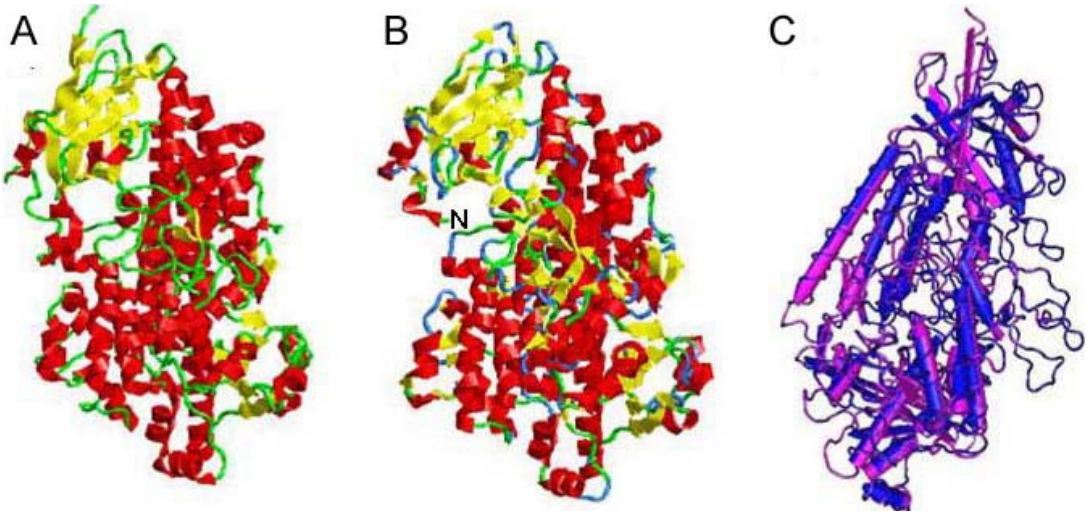

Figure 1. Structure modeling of *TomloxD*: A) The X-ray structure of the soybean LOX3 protein (Protein Data Bank code No.: lno3A); B) Predicted three-dimensional structure of *TomloxD*. N: N-terminus. C) The three dimensional structure alignment of *TomloxD* protein (blue) and soybean LOX3 protein (purple). This three dimensional structural image was generated using the Cn3D program.

grown overnight in 5 ml of buffered glycerol complex medium (BMGY) at 30°C with vigorous shaking. Cell cultures were harvested by centrifugation at 4,000 rpm for 10 min, resuspended in 50 ml of modified medium (MM) and grown for about 3 days at 30°C with vigorous shaking. The expression of *TomloxD* recombinant fusion protein was induced through 1.0% (v/v) methanol. The supernatant was obtained by centrifugation at 4,000 rpm for 10 min at 4°C. Then the supernatant was concentrated by 30-fold ultrafiltration centrifugation. The concentrated supernatant was used to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blot analysis and enzyme assay. Protein concentration in crude extracts was determined with the BioRad Protein Assay Kit (Bradford, 1976).

Western blot analysis

The concentrated supernatant was separated in SDS-PAGE and transferred onto Immobilon membrane (Millipore, USA) with a semi-dry transfer cell (Millipore, USA). The recombinant *TomloxD* polypeptides expressed in *P. pastoris* strain GS115 was detected with an antibody raised against His-tag (Novagen, USA).

LOX activity assay of *TomloxD*

Protein concentration in supernatant was adjusted to 0.5 mg/ml. LOX activity assay of *TomloxD* was measured spectrophotometrically (Shi et al., 1996). One unit of activity was defined as the quantity of enzyme catalyzing an increase in absorbance of 0.01 at 234 nm/min under assay condition.

RESULTS

Structure prediction and homology modeling of the *TomloxD* protein

The ChloroP server predicted that, *TomloxD* has chloroplast transit peptides (cTP), which was a stretch of 77 amino acids in the N-terminal region of protein sequences. Both protein Basic Local Alignment Search Tool (BLAST) and the Simple Modular Architecture Research Tool (SMART) analysis showed that the *TomloxD* belonged to plant lipoxygenase and had two conserved domain. PLAT/LH2 domain spans over 140 residues (in position 74 and 213 of polypeptide), which is predicted to be a β-barrel structure. pfam: lipoxygenase spans over 671 residues (in position 222 and 892 of polypeptide), which is predicted to be the catalytic site in the C-terminal domain (residues 222 and 892).

The three-dimensional structure of *TomloxD* was modeled, based on the X-ray structure of the soybean LOX3 protein (Protein Data Bank code No.: lno3A). The modeled residue range of *TomloxD* was approximately 820 amino acids (positions 88 and 908). Figure 1A shows the crystal structure of soybean LOX3 protein. The structure modeled by a protein structure modeling program modeler is displayed in Figure 1B. This structure was further checked by Procheck and Whatcheck. The three dimensional structure alignments of *TomloxD* protein and soybean LOX3 protein is shown in Figure 1C, which suggested that these two proteins was high overlap. *TomloxD* contained all the functional domains typical of LOXs (Figure 1): a β-barrel structure in N-terminal region (residues 74 to 213) and a catalytic site in the C-terminal domain (residues 222 to 892). The 9 β-strands make the β-barrel structure whose residues are in positions 109 to 116, 129 to 133, 135 to 138, 146 to 152, 164 to 170, 174 to 182, 190 to 193, 196 to 199 and 208 to 211, respectively (Figure 1B). The catalytic site consists of a αβαααββαββββαααααααα structural motif, which contains 14 α-helices and 6 β-strands. The residues of α-helices

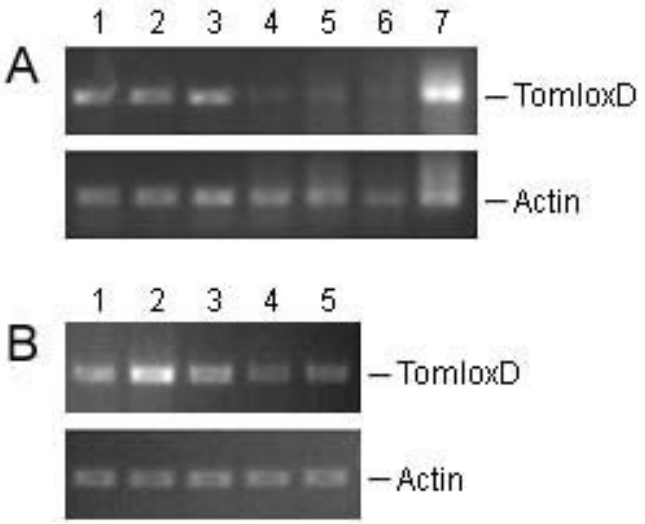

Figure 2. The expression analysis of *TomloxD* by semi-quantitative RT-PCR. A) The expression of *TomloxD* in tomato tissues. Lane 1 to 4, roots, flowers, younger and mature leaves, respectively; Lane 5 to 7, fruits at the mature green, breaker and breaker+4 days stages, respectively. B) The relative expression of *TomloxD* in mechanical injury leaves. Lane 1 to 5 is the treatment time of 0, 2, 6, 12 and 24 h, respectively.

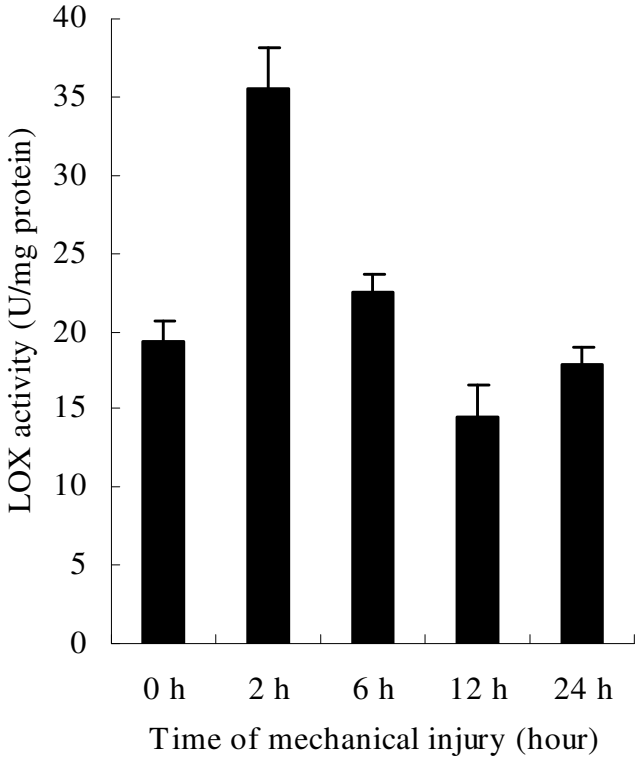

Figure 3. LOX activity in mechanical injury leaves. The average LOX activity and standard deviations were obtained from three independent experiments.

are in position 223 to 238, 320 to 348, 354 to 363, 373 to 379, 414 to 422, 481 to 494, 542 to 581, 591 to 615, 629 to 644, 678 to 702, 705 to 731, 741 to 770, 810 to 824 and 846 to 873, respectively, and the residues of 6 β-strands are in position 252 to 259, 427 to 430, 475 to 480, 501 to 511, 512 to 525 and 530 to 536, respectively (Figure 1B).

Expression characters of *TomloxD* in tomato

Expression of *TomloxD* in tomato tissues

The *TomloxD* mRNA was detected in the roots, flowers, younger and mature leaves and fruits at the mature green, breaker and breaker+4 days stages from tomato plants using semi-quantitative RT-PCR analysis (Figure 2A). The *TomloxD* transcript was higher in roots, flowers, younger leaves and fruits of breaker+4 days stage, whereas lower in mature leaves, fruits from the mature green and breaker stages.

Expression of *TomloxD in* response to mechanical injury

Semi-quantitative RT-PCR analysis demonstrated that, *TomloxD* transcript was up regulated and reached the peak around 2 h, followed by a decrease at 6 h and a further decrease at 12 h after injury (Figure 2B). LOX activity of crude extracts from the tomato leaves was measured with linoleic acid as the substrate. Each point reported is the average of three plants and each treatment was analyzed in three independent experiments. The change of LOX activities exhibited the same as that of *TomloxD* mRNA in wounded leaves. The LOX activity showed a peak level within 2 h, which was about 1.84 times more than that of the untreated leaves, but also had a decline thereafter (Figure 3). The findings showed *TomloxD* expression responses to mechanical injury.

Expression of *TomloxD* transgene in yeast

To investigate the function of *TomloxD* protein, the *TomloxD* gene was cloned into pPIC9K vector (Invitrogen, USA) and was transformed into *P. pastoris* expression host strain, GS115. The supernatant was concentrated by ultrafiltration centrifugation. The concentrated supernatant was analyzed by SDS–PAGE (Figure 4). A novel protein band of about a 100 kDa molecular weight was obtained in *TomloxD* transgenic yeast.

As the target protein contains a 9×His-Tag recognition sequence in its N terminal, we analyzed the recombinant protein by Western blotting with anti-his antibody. A single target protein band was detectable in the expression supernatant after ultrafiltration centrifugation, which showed

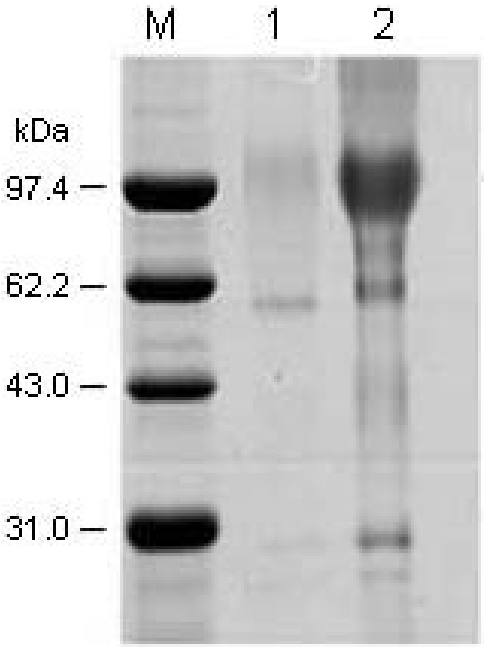

Figure 4. SDS–PAGE analysis of concentrated supernatant from *TomloxD* transgenic yeast. M, Marker; Lane 1, pPIC9K (control); Lane 2, *TomloxD.*

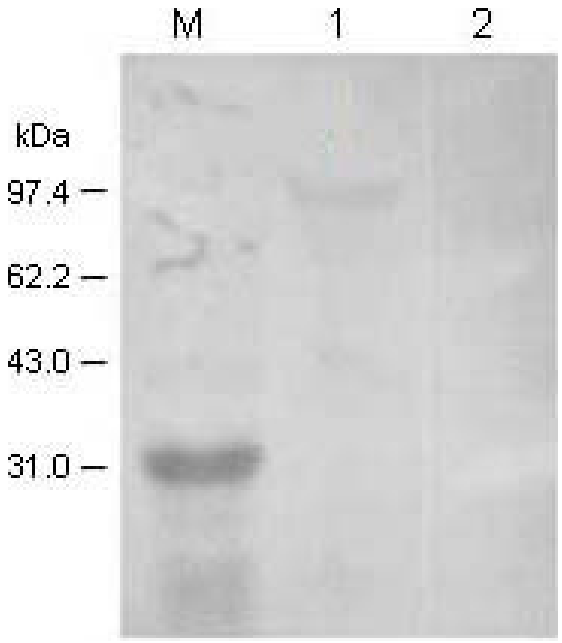

Figure 5. Western-blot analysis of supernatant from *TomloxD* transgenic yeast. M, Marker; Lane 1, *TomloxD*; Lane 2, pPIC9K (control).

that the contained *TomloxD* and His-Tag fusion protein was expressed in *TomloxD* transgenic yeast. But under the same culture conditions, no exogenous *TomloxD* was detected in pPIC9K vector transformed yeast (Figure 5). The target protein band was weaker in Western-blot analysis than SDS–PAGE. That is because *TomloxD* protein is a large molecule with a predicted molecular weighs of 102.31 kilodaltons, it is very difficult to transfer the target protein onto Immobilon membrane.

LOX activity analysis of *TomloxD*

LOX activity of supernatant from the *TomloxD* transgenic yeast was measured with linoleic acid as the substrate. The findings showed that, *TomloxD* transgenic yeast induced with 1.0% (v/v) methanol contains high levels of LOX activities and pPIC9K transformed yeast has hardly LOX activity (Figure 6).

DISCUSSION

TomloxD has a chloroplast transit peptide, which consists of 77 amino acids in the N-terminal region. The three dimensional structure alignments of *TomloxD* protein and soybean LOX3 protein suggested that, *TomloxD* contained all the functional domains typical of LOXs. Western blot analysis showed that *TomloxD* protein was synthesized in transformed yeast cells. The enzyme activity analysis of *TomloxD* protein suggested that *TomloxD* gene code has a lipoxygenase.

Multiple isoforms of LOX have been detected in a wide range of organisms (Chen et al., 2004). Lipogenase activity in plants has been observed in several cell fractions, including chloroplasts, mitochondria, vacuoles, lipid bodies and membranes (Liavonchanka and Feussner 2006; Tamás et al., 2009). According to the position of addition of the -OOH moiety in the primary hydroperoxide product using linoleic or linolenic acids as substrates, LOX enzymes can be grouped into two types: 9-LOX, which specifically forms 9- hydroperoxides (9-HPOs) and 13-LOX, which specifically forms 13- hydroperoxides (13-HPOs). The product of the reaction of linoleic acid with soybean cotyledon lipoxygenase-1 is almost exclusively 13-hydroperoxy linoleic acid (Axelrod et al., 1981). In potato (*Solanum tuberosum*), Lox1 is expressed in tubers and roots and uses linoleic acid to produce predominantly 9-HPOs. The amino acid sequences of *TomloxA, TomloxE,* and *TomloxB* have strong homology to that of potato Lox1 and show 92, 75, and 69% identity, respectively. Taking into account that the majority of the hydroperoxides formed by LOX activity in tomato are the 9-isomers (Galliard and Matthew, 1977; Smith et al., 1997) and that *TomloxB, TomloxE,* and *TomloxA* have high expression levels in fruit, it is most likely that, *TomloxA, TomloxB,* and *TomloxE* may produce 9-HPOs (Chen et al., 2004). *TomloxC* is a chloroplast-targeted lipoxygenase isoform that can use both linoleic and linolenic acids as substrates and produce 13-hydroperoxy products. The 13-HPOs formed in tomato appear to be

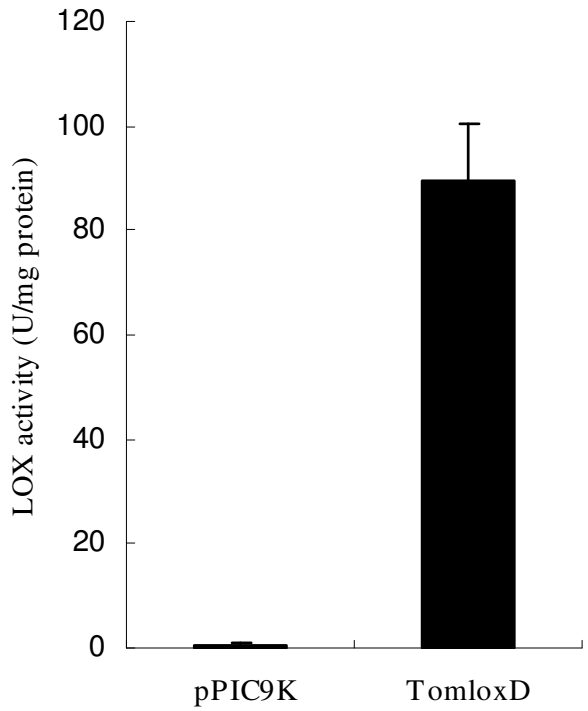

Figure 6. Specific activities of *TomloxD* in protein extracts from the transgenic yeast. The average LOX activity and standard deviations were obtained from three independent experiments.

metabolized further by the action of the 13-hydroperoxide lyases (13-HPLs) to give rise to hexanal and the corresponding alcohols (Chen et al., 2004). The *TomloxD* protein also has a chloroplast target signal. Sequence comparison showed that the *TomloxD* protein sequence has 92% identity at the amino acid level with that of potato Lox3, which is mostly expressed in potato leaves and roots and produces almost exclusively 13-HPOs (Royo et al., 1996). It is most likely that *TomloxD* may produce 13-HPOs.

TomloxD may be capable of generating 13-HPOs, but *TomloxD* does not play a key role in the generation of flavor volatiles in fruit (Chen et al., 2004). 13-HPOs can be used as substrates by several different enzymes (Schaller, 2001). The metabolic fate of 13-hydroperoxides may depend on their colocalization or association with specific downstream enzymes.

LOXs are also involved in JA synthesis, in response to mechanical injury and in defense responses against insect pests and microbial pathogens. For example, in transgenic *Arabidopsis* plants, suppression of the chloroplast targeted LOX2 gene resulted in the absence of wound inducible JA accumulation and reduced expression of the wound and JA-inducible *vsp* gene (Bell et al., 1995). Potato plants with silenced LOX H3, a *TomloxD* homolog, showed greatly reduced proteinase inhibitor expression, indicating a role in defense signaling (Royo et al., 1999). Inactivation of the lipoxygenase ZmLOX3 increases susceptibility of maize to *Aspergillus* spp. (Gao et al., 2009). OsHI-LOX is a chloroplast-localized type 2 13-lipoxygenase gene of rice, whose transcripts were up-regulated in response to feeding by the rice striped stem borer (SSB) *Chilo suppressalis* and the rice brown planthopper (BPH) *Niaparvata lugens*, as well as by mechanical wounding and treatment with JA. Antisense expression of OsHI-LOX (as-lox) reduced stripped stem bore (SSB) or BPH-induced JA and trypsin protease inhibitor (TrypPI) levels, increased the damage caused by SSB and LF (leaf folder) larvae (Zhou et al., 2009). OsLOX1 product is involved in tolerance of the rice plant to wounding and BPH attack (Wang et al., 2008). Semi-quantitative RT-PCR analysis demonstrated that the product of *TomloxD* is expressed principally in roots, flowers, younger leaves and fruits of breaker+4 days stage and at a very low level in mature leaves, fruit at the mature green stage and at onset of ripening (Figure 2A). The *TomloxD* transcript was upregulated by mechanical injury and the change of LOX activities exhibited the same as that of *TomloxD* transcript after mechanical injury (Figure 2B and 3). The findings suggested the *TomloxD* expression was response to mechanical injury. The results of this study are consistent with the previous work of Heitz et al. (1997), which concluded that *TomloxD* is mainly expressed in tomato leaf and is up-regulated in leaves in response to wounding (Heitz et al., 1997). Hence, *TomloxD* may play a role as a component of the octadecanoid defense-signaling pathway, as has been demonstrated for its corresponding homologous genes in potato and *Arabidopsis* plants (Bell et al., 1995; Royo et al., 1999).

In summary, the *TomloxD* protein is a chloroplast-targeted lipoxygenase isoform that contains all the functional domains typical of LOXs and has LOX activity. The *TomloxD* may use both linoleic and linolenic acids as substrates and produce 13-hydroperoxy products. The expression of *TomloxD* gene was induced by mechanical injury. The characteristics of the *TomloxD* gene and its product indicate that, it is a strong candidate and a component of the octadecanoid pathway and may play a role in the defense-signaling pathway in tomato plants. In order to elucidate the precise physiological functions of *TomloxD* gene, further biochemical and molecular experiments are in progress in our laboratory.

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