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番茄果實專一性過氧化酶基因之分子特性

Molecular characterization of fruit-specific class III peroxidase genes in tomato

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本論文係王啟正君（D93B42001）在國立臺灣大學植物科學研究所完成之博士學位論文，於民國 104 年 5 月 26 日承下列考試委員審查通過及口試及格，特此證明

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中文摘要

為了研究番茄果實專一性第三群過氧化酶基因（以下簡稱過氧化酶基因）之特性與功能，本研究從番茄未熟果選殖出來 4 個過氧化酶基因：*LePrx09*、*LePrx17*、*LePrx35* 及 *LePrxA*，其中 *LePrx35* 及 *LePrxA* 為新的番茄過氧化酶基因。蛋白質的二維結構分析顯示此 4 個過氧化酶基因具有第三群過氧化酶的特性，也就是具有 B、D、F 的保守區域，這些區域為與 heme 結構及 2 個鈣離子交互反應的保守氨基酸序列、穩定分子結構的鹽橋及 4 個雙硫鍵，因此確認其為第三群過氧化酶基因無誤。系統分子演化分析 24 個或 118 個茄科作物過氧化酶蛋白序列可分成 3 群，*LePrx09*、*LePrx17*、*LePrx35* 及 *LePrxA* 分別與受傷、病害及木質素合成相關的過氧化酶在同一群中，因此推斷此 4 個基因功能可能與受傷、病害及木質素合成有關。經 RNA 表現部位分析顯示，*LePrx35* 及 *LePrxA* 僅在未熟果中表達，*LePrx17* 及 *LePrx09* 則在未熟果及綠熟果中表達，且 *LePrx09* 則主要在綠色果實中的中果皮表達，其他 3 個基因則主要在果肉組織中表達，利用基因槍傳送 *LePrx09* 啟動子驅動 GUS 基因的啟動子活性分析也支持此一結果。*cis*-element 分析顯示 *LePrx09*、*LePrx17*、*LePrx35* 及 *LePrxA* 之啟動子區域至少含有 1 個負責因糖抑制（sugar- repressive）的調控片段，還有許多與受傷，病原及荷爾蒙反應有關的 *cis*-elements，此外，*LePrx09*，*LePrx17* 及 *LePrxA* 的啟動子有果實專一性的 *cis*-elements：TGTTACA 或 TCCAAAA。使用番茄未熟果進行各種荷爾蒙、受傷處理、病原菌感染及過氧化氫處理， *LePrx09* 之表達量可被 ethephon、茉莉酸、水楊酸、受傷處理、早疫病原菌感染及過氧化氫所誘導而增加，*LePrx17* 表達量可被茉莉酸處理及早疫病原菌感染所誘導增加。利用基因轉殖大量表達 *LePrx09*，可使番茄的轉殖株葉片對過氧化氫具有抗性，大量表達其他 3 個過氧化酶基因之轉殖植株葉片對過氧化氫之抗性則無顯著增加，推論 *LePrx09* 參與過氧化氫的訊息

傳導徑路並調整果實的生長及抗逆境能力，因此，未來若能找到 *LePrx09* 的啟動子區域及其上游基因的序列在抗逆境種原的關聯性，將可作為番茄抗逆境或抗病性育種的分子標誌。

Abstract

To research the characters and function of fruit-specific class III peroxidase gene of tomato, expression of four tomato class III peroxidase (LePrx) genes, *LePrx09*, *LePrx17*, *LePrx35* and *LePrxA*, was isolated and charactered from immature tomato fruits, and the function in the regulation of fruit growth was studied. *LePrx35* and *LePrxA* were identified as novel peroxidase genes in tomato. Analysis of amino acid sequences revealed that these genes are belonged to class III peroxidases group and contain B, D and F conserved domains, which bind heme groups, two calcium atoms, and form a stable molecular structure by a buried salt bridge motif and four disulphide bridges. Phylogenetic analysis for 24 and 118 full-length peroxidase proteins of *Solanaceae* crop species show three groups constructed and some wounding, pathogen and lignin synthesis related peroxidases were grouped with the four LePrxs. The temporal expression patterns at various fruit growth stages revealed that *LePrx35* and *LePrxA* were expressed only in immature green fruits, whereas *LePrx17* and *LePrx09* were expressed in both immature and mature green fruits. *Cis*-element analysis revealed that all the upstream of the four *LePrx*s have at least sugar repressive element and other wounding, pathogen and hormones related *cis*-elements. The promoter of *LePrx09*,

LePrx17 and *LePrxA* has fruit-specific *cis*-elements, TGTTACA or TCCAAAA. Moreover, tissue-specific expression profiles indicated that only *LePrx09* was mainly expressed in the mesocarp but not the inner tissue of immature fruits. Histochemical localization of GUS activity analysis also revealed the mesocarp-expression pattern. 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* displaying 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. Thus, if the sequences of *LePrx09* promoter region and its upstream genes were involved in the stresses resistant tomato entries, it would be as a useful marker for stresses resistance breeding.

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1. Introduction

1.1 The three classes of peroxidases

Peroxidases (EC 1.11.1.X) exist in almost all living organism and reduce hydrogen peroxide to water by catalyzing the oxidation of various substrates. Peroxidases comprise a superfamily which are divided into three classes of peroxidases, the intracellular class I (EC 1.11.1.5/.6/.11), the 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). The intracellular class I peroxidases including three distinct groups: ascorbate peroxidases (APxs; EC 1.11.1.11), cytochrome c peroxidases (CcPs; EC 1.11.1.5) and catalases (CPs; EC 1.11.1.6). Class II peroxidases, encoded exclusively by fungi, are divided into manganese peroxidases (EC 1.11.1.13), lignin peroxidases (EC 1.11.1.14) and versatile peroxidases (EC 1.11.1.16)(Cosio and Dunand, 2009). Class III peroxidases, on which this research is focused, are present as large multigene families in all land plants (Tognolli et al., 2002; Duroux and Welinder, 2003). Previous studies have suggested that class III peroxidases may play a critical role during land colonization of plants, either by allowing the formation of plant rigid structures or by adapting the organism to a more oxygenated environment (Duroux and Welinder, 2003; Passardi et al., 2004).

1.2 The structure of class III peroxidases

All class III peroxidases comprise a single polypeptide chain of approximately 300 amino acid residues, Fe (III) protoporphyrin IX (usually referred to as heme group) and two calcium atoms. Also, they share a very similar three-dimensional structure and are characterized by the presence of highly conserved amino acids, such as two histidine residues interacting with the heme, conserved eight cysteine residues forming disulfide bridges, and a buried salt bridge motif containing invariant Asp and Arg residues (Welinder, 1992; Edwards et al., 1993; Veitch, 2004; Smulevich et al., 2006) (Fig. S1, S2). Except for the conserved residues, the groups of class III peroxidase display low identity of primary amino acid sequences and have distinct functions and reaction mechanisms.

1.2.1 Class III peroxidases contains two different types of metal center

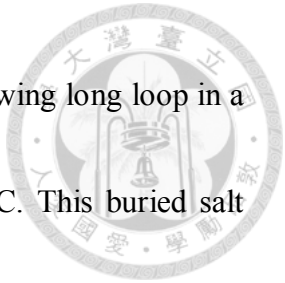
Such class III plant peroxidases contain two different types of metal center, heme group and two calcium atoms. Both are essential for structure and functions of these enzymes. The heme group is attached to peroxidase enzymes at the proximal histidine residue, His170 in horseradish peroxidase isoenzyme C (HRPC), by a coordinate bond between the histidine side-chain atom and the heme iron atom. The distal histidine

reside (His42 in HRPC), together with the distal arginine (Arg38 in HRPC) and heme, catalyzes the heterolytic cleavage of hydrogen peroxide (Welinder, 1992; Veitch, 2004).

The two calcium binding sites are located at positions distal and proximal to the heme plane and are linked to the heme-binding region by a network of hydydrogen bonds. The residues, Thr171, Asp222, Thr225, Ile228 and Asp43, Gly48, Asp50, Ser52, are proximal and distal calcium binding sites (Welinder, 1992; Veitch, 2004). Loss of calcium results in decreases to both enzyme activity and thermal stability (Haschke and Friedhoff, 1978). There is a conserved glutamic acid (Glu64) that also involves the distal calcium and participates in the same hydrogen-bonding network (Cosio and Dunand, 2009).

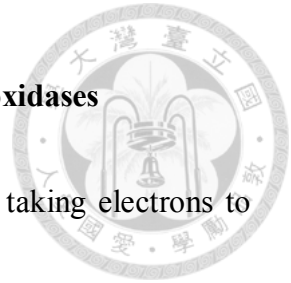
1.2.2 Class III peroxidase proteins have four disulphide bridges, a buried salt bridge and several N-glycosylation sites

The class III peroxidase proteins have eight conserved cysteines forming four disulfide bridges and four amino acids involved in a buried salt bridge. The four disulphide bridges hold the α-helices in a compact globular structure (Schuller at al., 1996). The buried salt bridge motif, Asp99-Arg123 in HRPC, is an invariant part of all three classes of the plant peroxidase superfamily. This motif included the Ser and Asp



residues located at the beginning of helix D and connecting the following long loop in a tight hydrogen bonding network with Gly122 and Arg123 in HRPC. This buried salt bridge is a long loop around half molecular surface of peroxidase proteins to the C-terminal domain and may play an important role in the proper attachment of the N- and C-terminal domains during protein folding (Welinder et al., 1992; Welinder et al., 2002).

The general functions of glycans are protein folding, and stabilizing glycoproteins. Removal of the glycosylation sites of yeast acid phosphate severely affects its folding causing the protein to be retained in the endoplasmic reticulum (Riederer and Hinnen, 1991). The glycan functions about the N-glycosylation in plant peroxidase proteins are still not clear. In HRPC, glycosylation has mild effect in the activities of the enzyme (Tams and Welinder, 1995). However, in the peanut cationic peroxidase (cPrx), site-directed replacement of three glycosylation sites (N60, N144, N185 of prxPNC1) revealed that the three glycans appeared to influence the folding, catalytic ability (Lige et al., 2001). In turnip, removal of oligosaccharide chains from the peroxidase protein caused a decrease in K_m and V_{max} for hydrogen peroxide (Duarte-Vázquez et al., 2003).



1.3 The diverse function and expression patterns of class III peroxidases

Class III plant peroxidases catalyze the reduction of H_2O_2 by taking electrons to various donor molecules such as phenolic compounds, lignin precursors, auxin, or secondary metabolites in the regular peroxidative cycle (Hiraga et al., 2001). Recently, a separate hydroxylic cycle, which leads to the formation of various reactive oxygen species (ROS), has been described. Because of the large number of genes and the two possible catalytic cycles, class III plant peroxidases are involved in a broad range of physiological processes, such as auxin metabolism, lignin and suberin formation, cross-linking of cell wall components, defense against pathogens or cell elongation. (as reviewed in Passardi et al., 2005; Cosio and Dunand, 2009).

1.4 Class III peroxidases are involved in plant growth

The main functions of class III peroxidases involved in plant growth are suberization and lignification (Espelie et al., 1986, Roberts et al., 1988; Müsel et al., 1997; Quiroga et al., 2000), cross-linking of cell wall components (Fry, 1986; Brownleader et al., 1999; Hatfield et al., 1999) and cutin deposition in outer epidermal layers (Ferrer et al., 1991).

Class III peroxidases were reported to regulate the growth of various plant organs, for example, including hypocotyls of mungbean (Goldberg et al., 1987) and zucchini (Cosio and Dunand, 2009), and coleoptiles of maize (Hohl et al., 1995). In *Arabidopsis*, *AtPrx33* and *AtPrx34* could promote cell elongation (Passardi et al., 2006) and *AtPrx37* could reduce growth (Pedreira et al., 2011).

1.4.1 Class III peroxidases mediating plant growth by regulating H_2O_2 and hydroxyl radical

Class III peroxidases can regulate growth by cell elongation through hydroxylic or peroxidative cycle. Those two cycles both can regulate the local concentration of hydrogen peroxide (H_2O_2) (Liszkay et al., 2003)(Fig. S3). The phenoxy radicals can be produced by peroxidative cycle leading to cell wall stiffening. On the other hand, peroxidases can scavenge H_2O_2 and produce the hydroxyl radical ($^{\cdot}OH$) involved in cell wall loosening (Passardi et al., 2004).

The endogenous hydrogen peroxide (H_2O_2) level can be related to the elongation process. For example, the apoplastic H_2O_2 level is lower in the hypocotyl elongation zone of soybean (Schopfer, 1994). In *A. thaliana*, the elongation during root curvature is also regulated by a variation of H_2O_2 concentration (Joo et al., 2001). In onion, the

location of H_2O_2 in the cell wall has been correlated with lignification and elongation during growth (Cordoba- Pedregosa et al., 2003).

Class III peroxidases could control the H_2O_2 level and therefore be directly related to the control of the elongation process. In zucchini, transcripts of the anionic peroxidase gene, ARRX, are accumulated strongly in the elongation zone of the hypocotyl and their accumulations were inversely correlated with lignin level (Dunand et al., 2003). This tissue specific upregulation of peroxidase genes is related with the high elongation rate in the region of hypocotyl of zucchini.

Other compounds such as hydroxyl radical ($^{\cdot}OH$) could be involved in the processes of elongation and expansion. Hydroxyl radicals can be produced at the cell wall level from superoxide radical ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) by class III peroxidase (Schweikert et al., 2000; Schopfer et al., 2002; Liszkay et al., 2003). This highly reactive radical produced by the Fenton reaction (Fry, 1998) or hydroxylic cycle of peroxidase could cleave cell wall polysaccharides such as pectin and xyloglucan. The production of hydroxyl radical near the cell wall could be related to non-enzymatic wall loosening mechanisms (Chen and Schopfer, 1999). Auxin is also involved in this

elongation growth by promoting the release of superoxide radical and production of hydroxyl radical (Schopfer et al., 2002).

1.4.2 Class III peroxidases are involved in the growth of tomato fruit

Tomato (*Solanum lycopersicum*) is one of the major crops in the world due to the diversified usage and high value of nutrition in fruits. The rate and extent of fruit growth are central for crop yield and the fruit size is a major determinant of quality. Numerous parameters have been reported to influence the growth and development of tomato fruits. During the fruit rapid growth stage, all of it occurs by cell expansion rather than division in tomato fruit (Grange, 1995). One of the major factors is the activity of peroxidases, which has been suggested to be involved in the maintenance of fruit mechanical properties (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).

1.4.3 The other two enzymes are involved in mediating tomato fruit growth

Growth and elongation are related to cell extensibility, which occurs with a loosening of the cross-linking of cell wall compounds. In addition to class III

peroxidases, it had been mention that two enzymes, xyloglucan-endotransglycosylase (XET), expansin, involved in cell wall loosening. The function of XET is cleavage and reassembly of xyloglucan and expansin is responsible for the suppression of hydrogen bonds between cellulose and xyloglucan (Cosgrove, 2001). XET activity shows a general correlation with growth rate in tomato fruit (Thompson et al., 1998). Expansins may also be involved in wall loosening (McQueen-Mason et al., 1992) and have been reported from tomato fruit (Rose et al., 1997).

1.4.4 Subcellular localization of class III peroxidases and their effect on the mechanical properties of tomato pericarp

Tomato fruits follow a sigmoidal pattern of growth. An initial short lag phase is followed by a phase of rapid fruit expansion, after which the rate of growth declines approaching maturation (Monselise et al., 1978). Turgor-driven expansion of parenchyma cells within the mesocarp creates tissue pressure, which is expressed on the exocarp layer as tissue tension. Mechanical properties of the exocarp were important in controlling fruit growth (Thompson et al., 1998).

Since the rate and extent of tomato fruit is determined mostly by the pericarp, some cell wall enzymes controlling fruit growth must be concentrated. Class III peroxidases

may be a catalyst to modify cell wall 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). Andrews et al. (2000) found that at least three peroxidase isozymes were increased with increasing fruit age following the phase of rapid fruit expansion. Moreover, studies on the five non-ripening mutants (*nor*, *rin*, *Nr*, *Cnr*, and *Gr*) revealed these isozymes were not involved in ripening. These highly anionic peroxidase isozymes were present in the cell wall of the exocarp in the latter stages of tomato fruit growth (Andrew et al., 2002a). Application of partially-purified peroxidase from mature tomato fruit skin significantly increases the stiffness of fruit skin irrespective of the age of fruit. The results support the hypothesis that the tomato fruit skin plays an important role in the regulation of fruit growth and the mechanical properties may be mediated by peroxidases (Andrew et al., 2002b).

1.5 The current researches of class III peroxidase genes in tomato

Tomato originated in South America where all the wild species related to cultivated tomato grew in the Andean region. Domestication probably started in Peru or Ecuador followed by diversification in Mexico (Blanca et al., 2012). Tomato is one of the most

important vegetable crops in the world. It area for cultivation was covering about 4800,000 ha and the yield was 161 million tones in the worldwide production (http://faostat.fao.org/site/567/ DesktopDefault.aspx?PageID= 567#ancor). Moreover, tomato is a model species for fruit development and ripening, disease resistance and biochemical pathway of important nutrients. The tomato genome has been sequenced and the international tomato genome consortium has released a high-quality reference sequence (The Tomato Genome Consortium, 2012). The available sequence covers 780 Mb of the estimated 900 Mb assembled using a combination of Sanger and next generation technologies from the inbred tomato cultivar 'Heinz 1706'. In addition to the whole tomato genome sequence database, there has been existence of a number of other tomato databases before the release of tomato genome such as, SolEST database (D'Agostino et al., 2009), Tomato functional genomics database (Fei et al., 2011), TOMATOMA (Saito et al., 2011), KaTomicsDB (http://www. kazusa.or.jp/tomato/) and several others (Shirasawa et al., 2013). It is expectative that tomato biotechnique researches will make big progress according to the database.

In tomato, there were 77 putative class III peroxidases in the peroxidase database, PeroxiBase in the past. Since the whole genome of tomato were sequencing, the

numbers of class III peroxidase genes of tomato were decreased to 72 in the database (Table 1). Among them, *LePrx22* and *LePrx27* were still exited in the PeroxiBase database in spite of the two peroxidases did not be found in the genome of the tomato cultivar 'Heinz 1706'.

Among the 72 class III peroxidase genes of tomato (*LePrx*s), only six genes have been study that as follows, *LePrx01* (*TPX1*) and *LePrx02* (*TPX2*) (Botella et al., 1994ab; Medina et al., 1997), *LePrx76* (*TAP1*) and *LePre75* (*TAP2*) (Sherf et al., 1993), *LePrx05* (*CEV1-1*) and *LePrx06*（*EP5C*） (Gadea et al., 1996; Vera et al., 1993; Coego et al., 2005). *LePrx01* is constitutively expressed in the endodermis, exodermis and protoxylem of root and it is transcriptionally activated in this tissue when the plants are exposed to moderate NaCl concentrations (Botella et al., 1994a) and wounding treatment (Botella et al., 1994b). The tomato overexpressed *LePrx01* has more lignin than wild type significant (EI Mansouri, 1999) and has small area in the cross-section of xylem vessel and has more resistance to water flow (Luccna et al., 2003). Talano et al. (2006) demonstrated the physiological role of *LePrx01* in the processing of root cell walls, being part of control mechanisms of ion and water fluxes through the root.

LePrx02 is only weakly expressed in the roots of young tomato seedlings is highly expressed in tomato suspension cells adapted to high external NaCl concentration (Medina et al., 1997). The seeds of transgenic tomato overexpressed *LePrx02* showed lower pore size in the walls. Therefore, the higher capacity of transgenic seeds in retaining water could result in higher germination rates in conditions where the availability of water is restricted (Amaya, 1999).

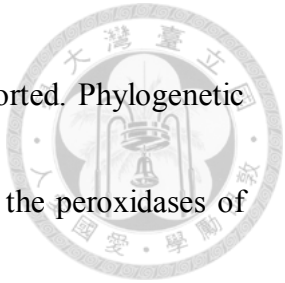
LePrx05 and *LePrx06* are both up-regulated by viroid infection and ethephon treatment in tomato leaves and stems. However, in healthy tomato plants, *LePrx06* display a constitutive expression in roots and *LePrx05* only show weak dateable band in stems (Gadea et al., 1996; Vera et al., 1993). The transcripts of *LePrx06* is rapidly induced by H_2O_2, which is a reactive oxygen intermediate normally generated during the course of a plant-pathogen interaction. The inhibition of LePrx06 protein accumulation in antisense tomato plants established resistance for *Pseudomonas syringae* inoculation and this inhibition represents a novel form of disease resistance based on a loss-of-gene function. (Coego et al., 2005).

Thus far, only two *LePrx*s have been identified in tomato fruits, including *LePrx75* (*TAP2*) and *LePrx76* (*TAP1*) (Roberts and Kolattukudy, 1989; Mohan and Kolattukudy,

1990; Sherf and Kolattukudy, 1993). The *LePrx75* had no reported its function. However, *LePrx76* (*TAP1*) has been shown to be developmental regulated during fruit maturation and its expression levels were significantly enhanced with wounding or fungal attack (Roberts and Kolattukudy, 1989; Mohan et al., 1993). A correlation between sustained anionic peroxidase activity and the ability of the tomato fruit tissue to withstand fungal attack has been previously suggested by Lurie et al. (1997). The fruit-specific E8 promoter droved *LePrx76* transgenic tomato fruit displayed reduced post-harvest decay and increased resistance toward *Alternaria alternata* and *Fusarium solani* (Kesanakurti et al., 2012)

1.6 The aim of this study

Because the class III peroxidases have broad substrate specificity, the various patterns of their temporal and spatial expression and different responses to environmental stimuli, the functions of many peroxidases have been difficult to define. As reported, there are 73 and 138 class III peroxidase genes identified in *Arabidopsis* (Duroux and Welinder, 2003) and rice (Passardi et al., 2004). 44 out of 73 class III peroxidase genes have been reported to putatively involved in a specific mechanism by microarray analysis, proteomics and transgenic approaches (Cosio and Dunand, 2009).



However, little information about the characters of *LePrx*s was reported. Phylogenetic analysis for class III peroxidase of dicotyledon plants revealed that the peroxidases of Solanales (tomato, potato, and sweet potato) were grouped into a special group (T group). Therefore, the class III peroxidase of Solanales may have some different functions compared to the class III peroxidases in *Arabidopsis*. In tomato, the fruit is a special organ. The characters of tomato fruit-specific *LePrx*s are little reported. Only two *LePrx*s have been identified in tomato fruits and other fruit specific class III peroxidase genes have not clear before. The present study aims to identify and characterize *LePrx*s in tomato fruits, and to elucidate the *LePrx*s' functions on fruit growth. In this study, four *LePrx*s that are immature fruits specific had been identified and their structure, phylogenetic analysis, specific expression patterns and function are investigated.

2. Materials and Methods

2.1 Plant materials

Hualien AVRDC #5, a tomato (*Solanum lycopersicum*) cultivar, grown in a net house with 8-10 h photoperiod at 25-28°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 class III 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).

2.2 RNA extraction from various organs of tomato

Total RNA was extracted from tomato roots, stems, leaves, flowers and various stages of fruits and hormones, stress treated fruits by the PineTree method (Chang et al., 1993). 4 g of plant organs in the presence of liquid nitrogen were grind in the presence of liquid nitrogen by using a mortar and pestle. The frozen powder is quickly transfer to the warm extraction buffer (2% (w/v) CTAB, 2% (w/v) PVP (mol wt 40,000), 100

mM Tris-HCl (pH 8.0), 25 mM EDTA, 2 M NaCl, 0.05% spermidine trihydrochloride, 2% ß-mercaptoethanol) at 65°C for 15 min. The solution was extracted twice with an equal volume of chloroform: isoamyl alcohol (24:1), separating phases at room temperature by centrifugation for 10 min at 5,000 x g. 10 M LiCl was added to the supernatant (1:4 v/v), the total RNA was precipitated overnight at 4 °C and harvested by centrifugation at 12,000 x g, 4 °C for 20 min. The RNA pellet was washed with 20 mL of 75 % ethanol, centrifuged at 10,000 x g, 4 °C for 10 min and dissolved in 200 µL of RNase free water. Quality was verified by spectrophotometric analysis (NanoDrop^{TM} Spectrophotometer ND-1000 (Thermo Scientific)) for A260:A280 and A260:A230 ratios.

2.3 Isolation and Identification of the *LePrx*s expressed in tomato fruits

To identify the fruit specific *LePrx*s, a cDNA pool from tomato immature fruits was established. The database searching, a degenerated PCR technique, 5'-RACE, amino acid alignment structure analysis and phylogenetic analysis were performed as follow:

2.3.1 Sequence mining and comparing the class III peroxidase genes of tomato *in Silico*

The amino acid sequences of class III peroxidases of tomato were collected from the PeroxiBase (http://peroxibase.toulouse.inra.fr/) (Passardi et al., 2007) and aligned by use of default alignment parameters in Clustal W/X (Thompson et al., 1997). The conserved regions of class III peroxidase genes were found and the degenerated primers for cloning the *LePrx*s expressed in tomato fruits. All the sequences of *LePrx*s from PeroxiBase were blasted in the sol genomics network website (http://solgenomics.net)(Bombarely et al., 2011) and the position, intron/exon numbers and other information of the LePrxs were figured out.

2.3.2 First-Strand cDNA Synthesis by reverse transcription

Total RNA of IMG3 fruits was used as the template for reverse transcription. First-stand cDNA was reverse transcribed from 4 μg total RNA with SuperScript III Reverse Transcriptase and the protocol is follow:

A mixture contain 4 μg total RNA, 400 ng oligo-$(dT)_{12-18}$ primer and 1 μL 10 mM dNTP in final 13 μL reaction solution was incubated at 65°C for 10 min and quickly chilled on ice for at least 1 min.

After incubation, 7 μL reaction mixture (4 μL 5×Superscript III buffer (Invitrogen), 1 μL 0.1 M DTT, 1 μL RNaseOUT Recombinant RNase inhibitor (40 U/μL; Invitrogen), and 1 μL Superscript III reverse transcriptase (200 U/μL; Invitrogen) were added into the tube and then incubated at 50°C for 1h. The reverse transcriptase was inactivated by incubating the mixture at 70°C for 15 min and the cDNA solution was stored at -20°C.

2.3.3 Polymerase chain reaction with a degenerated primer by annealing temperatures gradient

To amplify partial fragments of the putative *LePrx*s, the cDNA pool from IMG3 tomato fruits by reverse transcription were used as a template for polymerase chain reaction (PCR) and a degenerated primer (5'-CAYTTYCAYGAYTG YTTYGT-3') was designed based on the conserved amino acid sequence of Class III peroxidase genes of *Solanaceae* crop species referring from the NCBI database and the PeroxiBase, i.e. HFHDCFV. The degenerate primer and an oligo$(dT)_{18}$V primer were used for the amplification of putative *LePrx*s in tomato fruits.

The PCR amplification was performed for 40 cycles with a annealing temperature gradient between 50°C and 68°C. The program performed by the thermo cycler (Biometra) is as follow: First denaturation at 94°C, 10 min; denaturation at 94°C, 40 s

and annealing/extension at 68°C, 1 min 30 s for 5 cycles; denaturation at 94°C, 40 s, annealing at 65°C, 40s, extension at 68°C, 1 min 30s for 5 cycles, than the annealing temperatures were changed to 60°C for 5 cycles, 55°C for 25 cycles, and final extension at 68°C for 7min.

2.3.4 Cloning the amplified fragments and Sequencing

The amplified cDNA fragments were separated on a 1% low melt agarose gel and bands between 850 bp and 950 bp were excised. The excised gels were eluted and ligated into a TA vector (pGEMTeasy, Clontech) in a ligation mixture (3 μL Eluted DNA, 5 μL 2X ligation buffer, 1 μL pGEM-T easy vector, 1 μL T_4 DNA ligase). The ligated mixture was incubated at 4°C overnight and the ligated vectors were transformed to *E. coli* (DH5α) competence cell by the heat shock method. The 2-μL ligated vectors and 100-μL competence cell mixtures were heat at 42°C for 90 sec and kept on ice for 2 min and added with 100 μL LB broth to the mixture and incubate at 37°C for 30 min. Spread the mixture with 40 μL 10% X-gal and 7 μL 20% IPTG for Blue White screening on the 100 ppm ampicillin LB medium plate and incubated the plate at 37°C overnight.

Several single colonies were subcultured in a new plate and operated a colony-PCR

to select the colonies transformed successfully. The colony identification was carried out by the Tri-I Biotech Inc. in Taipei, Taiwan for sequencing by an automated DNA sequence analyzer (model 310, Applied Biosystems).

2.3.5 Obtaining the full-length nucleotide sequence of the *LePrx*s

To complete 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. The procedures are as follow:

(1).Dephosphorylation

The dephosphorylation mixtures (5 μL RNA (5 μg), 1 μL 10X CIP Buffer, 1 μL RNaseOut^{TM} (40U μL^{-1}), 1 μL CIP (10U μL^{-1}), 2 μL RNase-free ddH_2O) were incubated in heating plate at 50°C for 1 h and keep in the ice for 2 min. The mixtures were add with 100 μL RNase-free ddH_2O and 100 μL phenol and chloroform and vortexed vigorously for 30 s and delaminated by centrifuging with 13,000 rpm for 5 min at room temperature. The aqueous (top) phase was transferred to a new microcentrifuge tube, add with 2 μL 10 mg mL^{-1} mussel glycogen, 10 μL 3 M sodium acetate (pH5.2) and mix well. The solution was added with 220 μL 95% ethanol, vortexed briefly and centrifugated at 13,000 rpm, 4 °C for 20 min and

the RNA pellet was washed with 20 mL of 75 % ethanol, centrifugated at 13,000 rpm at 4°C for 5 min and dissolved in 7 μL of RNase free water.

(2). Decapping

The decapping mixture (7 μL dephosphorylated RNA, 1 μL 10X TAP Buffer, 1 μL RNaseOUT^{TM}(40 U μL^{-1}), 1 μL TAP(0.5 U μL^{-1})) were incubated in in heating plate at 37°C for 1 h, keep in the ice for 2 min and was added with phenol/ chloroform and ethanol for puring as the same as the procedure of dephosphorylation. The ligated RNA pellet was resuspended in 7 μL RNase-free ddH_2O.

(3). Ligation Reaction

The dephosphorylated and decapped RNA sample (7 μL) was mixed with the pre-aliquoted, lyophilized GeneRacer^{TM} RNA oligo (0.25 μg, 5′-CGACUGGAG CACGAGGACACUGACAUGGACUGAAGGAGUAGAAA-3′) and centrifugated briefly to collect the fluid in the bottom of the tube. The bottom solution was incubated at 65°C for 10 min and ice for least 2 min and centrifugated briefly. The solution was ligated with T_4 RNA ligase (5 U μL^{-1}) and added with phenol/chloroform and ethanol for purification as the same as the procedure of

dephosphorylation. The ligated RNA pellet was resuspended in 10 μL RNase-free ddH_2O.

(4). Reverse transcription

A mixture contain 10 μL of GeneRacer^{TM} RNA oligo ligated RNA, 400 ng oligo-$(dT)_{12-18}$ primer and 1 μL 10 mM dNTP in final 13 μL of solution was incubated at 65°C for 10 min and quickly chilled on ice for least 1 min. The follow reverse transcription steps by Superscript III reverse transcriptase (200 U/μL; Invitrogen) are the same as section 2.3.1.

(5). Touch-down PCR for 5' RACE

According to the sequencing data, a gene-specific primer for each cloned cDNA were designed on the basis of the identified nucleotides sequences :

LePrx09: 5'-GTTGACTCCGTCCTTTCTTCCTAAGGGTACATCGTAGTTGG-3'
LePrx17: 5'-GAGGAATGTGTGGCCCTCCTAGAGCAACAATACCATCTCTA-3'
LePrx35: 5'-CCCTAATAACACATTCCATGAAGGACCACCTGCCAG-3'
LePrxA: 5'-CCTCTTCTACCTGTCTCTACTTCATAAGATTCTCCGCCAGCCA-3'

The PCR mixture for amplified the 5' end fragment of each cDNA clone is containing 2 μL GeneRacerTM 5' Primer and 2 μL Gene-specific primer. The other reagents are the same as section 2.3.2. PCR products were generated from the cDNAs using 35 cycles of touch-down PCR and a gradient of annealing temperatures

between 68 °C and 70°C. The program performed by the thermo cycler (Biometra) is as follow: First denaturation at 94°C, 5 min; denaturation at 94°C, 40 s and annealing/extension at 72°C, 1 min for 5 cycles; denaturation at 94°C, 40 s and annealing/extension at 70°C, 1 min for 5 cycles; denaturation at 94°C, 40 s and annealing/extension at 68°C, 1 min for 25 cycles; final extension at 68°C for 7 min.

The PCR products were check by electrophoresis and eluted, ligated into TA vectors, and sequencing according to the 2.3.3 section.

(6). PCR for full-length cDNA of *LePrx*s

Full-length cDNA of *LePrx*s were then amplified by PCR from cDNA with gene-specific primers according to the sequence of fragments amplified by 5'-RACE. The gene-specific primers are as follows:

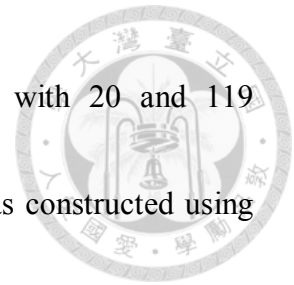
LePrx09 forward primer:	5'-ACAAATTAACTATCTAGCTATAGCCATGGT-3'
LePrx09 reverse primer:	5'-GATCCAAAACACCATGTTCTTACAATA-3'
LePrx17 forward primer:	5'-AAAAGAACAACCTCTACTGACCATTTAG-3'
LePrx17 reverse primer:	5'-AGCAACAAATTAACACTTGTTTCATAACT-3'
LePrx35 forward primer:	5'-ATAGTCTATAGCCTAATAGCTTTCTTGGC-3'
LePrx35 reverse primer:	5'-CATTAAAATTTTAATATATATAAAATTTCAAACACA-3'
LePrxA forward primer:	5'-ATAGCATATTCCTCTCCATTTTATTTTTC-3'
LePrxA reverse primer:	5'-GACGGTCAAGAAAAGGAGTAACTTC-3'

The PCR mixture for amplified the 5' end fragment of each cDNA clone including two specific 2.5 μM primers and Platinum *Taq* DNA Polymerase High Fidelity (5 U

μL^{-1}, Inventrogen) . The other reagents are the same as section 2.3.2. PCR products were generated from the cDNAs with following the program by by the thermo cycler (Biometra): first denaturation at 94°C for 5 min, following by 30 cycles of 94°C for 40 s, 55°C for 40 s and 68°C for 1 min 30 s, and a final extension at 68°C for 7 min.The PCR products were check by electrophoresis and eluted, ligated into TA vectors, and sequencing according to the section 2.3.3.

2.3.6 Sequence alignment and phylogenetic analysis

The homologous sequences of class III peroxidases were searched with BLAST in the PeroxiBase database and the sol genomics network website (http://solgenomics.net). 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 class III peroxidases were aligned with peroxidases in Solanaceae crop species by use of default alignment parameters in Clustal X 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 LePrxs together with 20 and 119 *Solanaceae* peroxidases obtained from the PeroxiBase database was constructed using *Arabidopsis thaliana* class III 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; 1,000 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 1,000 replicates. The bootstrap support values of branches smaller than 50% were collapsed.

2.4 Analysis the promoters of the identified *LePrx*s

To predict the expression pattern and characters of the promoters of the four *LePrx*s, *cis*-elements analyses were done and the promoter of LePrx09 was cloning and analysis

by Transient expression analysis. The methods were as follow:

2.4.1 Putative *cis*-elements analysis of the promoters of the identified *LePrx*s

To analysis the upstream elements of *LePrxs*, the 1.5 kb promoter sequences upstream of the start codon ATG of the four identified *LePrx*s were extracted from sol genomics network (SGN) website database. The putative promoter *cis*- elements were analyzed using PLACE database (http://www.dna.affrc.go.jp/PLACE/).

2.4.2 The phylogenetic analysis for the upstream sequence of the *LePrx*s

A phylogenetic tree of the 2 kb promoter sequences of six *LePrx*s obtained from the SGN database was constructed using the tomato-fruit-specific E8 promoter (AF515784) as an outgroup. Distance and UPGMA analyses were performed with the PHYLIP suite (Felsenstein, 1993). Distances between DNA sequences were computed with the DNADIST program using Kiumura 2-prameter matrix and transition/transversion ratio of 2.0. ; 1,000 bootstrap replicates were carried out with SEQBOOT option. The Neighbor program estimated the phylogenies from the distances in the matrix data using the UPGMA analysis; no rough analysis, no global rearrangements, and multiple jumbles (5 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 1,000 replicates. The bootstrap support values of branches smaller than 50% were collapsed.

2.4.3 Genomic DNA extraction for the *LePrx09* promoter cloning

Total genomic DNA was isolated from young tomato leaves. Leaves samples were ground with mortar and pestle in the presence of liquid nitrogen. The resultant powder was poured into extraction buffer containing 2% (w/v) CTAB, 2% (w/v) polyvinylpyrrolidone, 1 M Tris–HCl (pH8.0), 20 mM EDTA, 1.4 M NaCl and 2% (v/v) β-mercaptoethanol, mixed and incubated for 1 h at 65°C with occasional mixing. The supernatant was mixed with 1 volume of chloroform/isoamyl alcohol (24/1, v/v) and centrifuged for 10 min at 14,000 rpm. The upper phase was recovered and 1 volume of isopropanol was added and incubated for 30 min at －80°C. The suspension was centrifuged for 10 min at 14,000 rpm and the pellet was dried and dissolved in 100 mL of deionized H_2O. Then RNAse was added and incubated at 37°C for 15 min, and equal volume of chloroform/isoamyl alcohol (24/1, v/v) and 100 mL of phenol was mixed with the samples and centrifuged for 10 min at 14,000rpm. The supernatant was precipitated with 2.5 X of ethanol at -20°C for 2 h and centrifuged at 14,000rpm for 20

min. The pellet was air dried and dissolved in 35 mL of deionized H_2O at 65°C. The quality of genomic DNA was assessed by resolving DNA samples on a 0.7% (w/v) agarose gel by electrophoresis, followed by visualization with ethidium bromide staining.

2.4.4 Transient expression analysis for the promoter activity of *LePrx09*

2 Kb length of 5' upstream fragment of *LePrx09* was amplified by PCR from genomic DNA with specific primer pairs. The 1 μg-genomic DNA was used as template and the PCR procedure described in 6th step of section 2.3.4. The specific primers are as follows:

Forwards primer with *Hind*III cutting site:
5'-GCAAGCTTTTATCTCTCTTCTAGTCTTGCTTGTTTCTC-3'
Reverse primer with *Nco*I cutting site:
5'-GTCCATGGCTATAGCTAGATAGTTAATTTGTACAATGATA-3'

The PCR product and the pCAMBIA 1391Z vector (Fig. S1) was digested with *Hind*III and *Nco*I and ligated together so that the digested upstream fragment was ligated into 5' position of GUS gene with an intron in pre-digested pCAMBIA 1391Z vector. (Ligation mixture was 3 μL eluted and digested PCR product, 1 μL digested pCAMBIA 1391Z vector, 5 μL 2X ligation buffer, 1 μL T_4 DNA ligase)

The vector was transformed into *E. coli* DH5α and duplicated, purified. Plasmid

DNA was coated with 0.6 μ golden particle for bombardment. Immature and mature green fruits were cut into thin slices and placed on MS medium within petridishes and the Bio-Rad PDS-1000/He instrument was used.

After 24 h, histochemical staining of GUS assay was performed as described by Jefferson et al. (1987). The tissues were transferred to tubes containing GUS staining solution (50 mM sodium phosphate at pH7.0, 10 mM EDTA, 0.1% Triton X-100, 1 mg/ml X-Gluc, 0.1 mM potassium ferricyanide, 0.1 mM potassium ferrocyanide and 20% methanol) and incubated at 37°C for 16h to 24h. Then the samples were dehydrated by a series of ethanol wash, and finally kept in 70％ ethanol. Tissues were photographed under a stereo light microscope (Leica MZFLIII model)

2.5 Identified the expression patterns of *LePrx*s at various organs and treatments

To identify the expression pattern of *LePrx*s in different organs, developing fruits at various stages and fruits treated by various hormones and H_2O_2 challenge were used for total RNA extraction. Then the RNA samples were applied for RT-PCR and RNA gel blot analysis.

2.5.1 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 placed in 10 mL 1/2X MS liquid medium mixed with equal volume of conidial suspension. To facilitate conidial germination, fruits were incubated in a humidity chamber at 24±1°C.

For H_2O_2 treatment, detached IMG3 fruits were placed in 1/2X MS liquid medium containing 10 μM H_2O_2. Leaf discs from wild-type plants as well as from plants over-expressing *LePrx09* were placed in various concentration of H_2O_2 for 16h.

2.5.2 Reverse transcription-polymerase chain reaction

Total RNA was extracted from fruit samples following the various treatments, and 4 μg of total RNA was used for reverse transcription to produce first-stand cDNA. PCR analysis was performed in a 20-μL reaction mixture with 1 μL cDNA and Platinum *Taq* High Fidelity (Invitrogen) according to the protocol in 6th step of section 2.3.4.

Gene-specific primer pairs for each gene were:

LePrx09 specific primer	5'-CCTCCAATTCTTCAACAACAGTAGC-3'
	5'-CACCCTCTGCCAACACCACA-3'
LePrx17 probe primer	5'-CAGTACATGGAATGTAATGAACATGG-3'
	5'-CTGATCCAATTCCAGACCCAAA-3'
LePrx35 probe primer	5'-CTCTCTTACAATCTAATCTAATTTCTCCATTAG-3'
	5'-CTATTCCTTCTCCTCTTGAAGACATAAATA-3'
LePrxA probe primer	5'-CCAAAGGCTTTAGTGAGAAGGATC-3'
	5'-GAATTACAAACCTTTCTAATCTCTCCTAGTAAT-3'.

Tomato *Actin* gene (U60482.1) was used as an internal control, with primers 5'-GTTCCCATCTATGAAGGTTATG-3' and 5'-CAACCTTGATCTTCATGCTAC-3'. The amplification was carried out with 55°C annealing temperature; the number of

cycles was from 18 to 30 for the test genes. Each experiment consisted of three biological repetitions, and each biological repetition included three replicates.

2.5.3 RNA gel blot analysis

Total RNA (10 μg) was fractionated by formaldehyde-agarose gel electrophoresis and transferred to a positive-charge nylon membrane (Hybond-N, Amersham Pharmacia Biotech). After UV cross-linking, hybridization was performed with DIG Easy Hyb (Roche Diagnostics) containing DIG-labeled DNA peroxidase probes at 50°C overnight. DIG-labeled DNA probes were amplified by PCR, and the specific primers for each *LePrx* were the same with the RT-PCR. The blots were washed twice at 65°C with 1/2X SSPE containing 0.1% (w/v) SDS. Immunological detection was performed to identify the signals.

2.6 Plant transformation and transgenic plant identification

To validate the function of the four *LePrx*s, tomato plants were delivered with the LePrxs by *Agrobacterium*-mediated transformation. The transgenic tomato plants were treated with H_2O_2 stress and the chlorophyll contents were analysis.

2.6.1 Plant transformation

The *CaMV35S* promoter was first inserted into the pCAMBIA2300 binary vector (Fig. S2) at the restriction sites between *XmaI* and *EcoRI*. The identified *LePrx09* and *LePrx17* genes were inserted into the binary vector downstream of the *CaMV35S* promoter at the restriction sites between *Hind*III and *Xba*I. In addition, the identified *LePrx35* and *LePrxA* were inserted into the downstream of *CaMV35S* promoter at the site between *Xba*I and *Bam*HI of the vector. These plasmids were transformed into *Agrobacterium tumefaciens* strain LBA4404 for tomato transformation.

Tomato seeds were surface sterilized with 2% sodium hypochlorite and germinated on 1/2X MS medium with 20 g/L sucrose (pH5.6). Ten days after germination, the cotyledons were used for *Agrobacterium*-mediated transformation according to the procedure described by McCormick et al. (1986). After infection with the *Agrobacterium,* the cotyledon slices were cultured on the coculture medium (1/2X MS medium supplemented with 1.5 μg/L zeatin, 10 g/L sucrose, 10 g/L glucose, 400 μM acetosyringone and 8 g/L agar, pH5.2) at a dark chamber with 24°C for two days. The putative transformants were cultured on 1/2X MS medium supplemented with 1 μg/L zeatin, 20 g/L sucrose and 50 mg/L kanamycin (pH5.6) for selection, followed by

regeneration on rooting medium (1/2 MS medium, 20 g/L sucrose, 0.01 μg/L NAA, 150 mg/L kanamycin, pH5.6). For the T1 generation, seeds were germinated on MS medium supplemented with 150 mg/L kanamycin; the transgenic seedlings were then confirmed by RNA gel blot analysis.

2.6.2 H_2O_2 stress treatment for transgenic tomato leaves

Two explants were chose from each of four *LePrx*-overpressed transgenic events .The 0.5 cm^2 -leaf discs were taken off from the transgenic and wild-type plants and put in the absence and presence of 1% H_2O_2 for 16 h in shaking cultural plates. According the results, three transgenic lines resistant to H_2O_2 stress were chosen and taken their leaf discs assay for treated with various concentrations of H_2O_2 for 16 h.

2.6.3 Measurement of chlorophyll content of transgenic tomato leaves

After treated with H_2O_2, the chlorophylls of the leaf discs were extracted with buffered 80% aqueous acetone according to Porra et al. (1989). The buffered aqueous acetone contains 80% acetone, 20% H_2O and 2.5 mM sodium phosphate buffer pH7.8. After extracted the chlorophylls for 24h, the samples were measured by spectrophotometric analysis (NanoDrop^{TM} Spectrophotometer ND-1000 (Thermo Scientific)). The Chlorophylls *a* + *b* content (μg/ml) = 17.76 $A^{646.6}$ + 7.34 $A^{663.6}$.

3. Results

3.1 Chromosome position and intron/exon numbers of *LePrx*s

Data mining and comparing from the PeroxiBase and the sol genomic network website revealed that 72 whole sequence of *LePrx*s in the PeroxiBase and two *LePrx*s, *LePrx22* and *LePrx27* are not exist in the genomic sequence of inbred tomato cultivar ' Heinz 1706'. There are 72 *LePrx*s located in the 12 chromosomes of tomato, unequally. The 2nd chromosome has 16 *LePrx*s, however, only 2 *LePrx*s in the 9th and 12th chromosomes (Table 1, Fig. S5). 47 *LePrx*s are belonged to classical type class III peroxidases that have 3 introns and 4 exons. Second, 14 *LePrx*s have 2 introns and 3 exons. Specially, the *LePrx55* has 9 introns and 10 exons (Table 1, Fig. S6).

3.2 Characteristics of the four *LePrx*s in tomato fruits

A PCR-based strategy was carried out for the identification of cDNA-encoded class III peroxidases in tomato fruits. Four *LePrx*s, *LePrx09*, *LePrx17*, *LePrx35* and *LePrxA*, were isolated and the sequences were verified and blasted in NCBI, SGN and PeroxiBase databases. The nucleotide sequence, deduced amino acid and molecular characteristics of identified *LePrx*s are listed in Fig.1 and Table 2. The full-length *LePrx* cDNAs were between 1171 bp and 1258 bp, while the predicted molecular weights of

the proteins were between 36.7 kDa and 38.13 kDa. The physical positions of *LePrx09*, *LePrx17*, *LePrx35* and *LePrxA* are in the 4th, 2^{ed}, 10th and 4th chromosome, respectively. According to the PI, LePrx09 and LePrxA were divided into basic peroxidases and LePrx17 and LePrx35 were acidic peroxidases, (Table 2). In addition, *LePrx35* and *LePrxA* were identified as novel class III peroxidase genes in *Solanum lycopersicum*. *LePrx35*, a homologous gene of *NtPrx04a*, also shared 99% identity in the nucleotide sequence with clone TPod3 (Table 2). The other identified novel class III peroxidase gene, *LePrxA*, not only displayed high homology with *StPrx45* but also shared high identity in the amino acid sequence with LePrx77, which was recently registered in PeroxiBase (Table 2). The only difference in the amino acid sequence between LePrxA and LePrx77 was that LePrxA possessed an extra amino acid, lysine, following the conserved region HDCFVQ.

Compared with other class III peroxidases and HRPC, the *LePrx*s identified here contain several highly conserved residues, including distal and proximal histidine residues (His42 and His170 in HRPC) interacting with heme located in the B and F regions. They also possess VSC (A/S) D (L/I), a buried salt bridge motif in the D region.

In addition, all of the identified *LePrx*s have eight cysteine residues to form the disulfide bridges and calcium ion binding sites (Fig. 2).

The phylogenetic trees of 24 and 118 full-length class III peroxidases of *Solanaceae* crop species were constructed using the PHYLIP program (Fig. 3, Fig. S7). All the bootstrap support values were higher than 50% and no branch was collapsed. Based on the genetic distance and bootstrap support values, the *Solanaceae* class III peroxidases family was divided into three groups, A, B and C. Group C was further divided into C1 and C2 subgroups. The results of phylogenetic trees from 24 and 118 class III peroxidase are the same. LePrxA belongs to group A in which several pathogenic- and wounding-related peroxidases, including LePrx06 (EP5C), NtPrx10a (tpoxN1), StPrx28, and CanPrx01, were also present. LePrx09 and LePrx17 belong to group B, along with lignin synthesis-related NtPrx60a (Blee et al., 2003; Kavousi et al., 2010) and pathogen-inducible StPrx14 (Ros et al., 2004). In contrast, LePrx35 was clustered into subgroup C2 in which pathogen-inducible LePrx05 and CanPrx02 were also present (Vera et al., 1993; Choi et al., 2007) (Fig. 3).

3.3 The four *LePrx*s are green-fruit-specific

Expression patterns of the identified *LePrx*s were investigated in various organs and at various developmental stages of fruit. Notably, no expression of the four *LePrx*s was detected in root, stem, leaf and flower (Fig. 4a, Fig. S8a). However, the transcripts of LePrx09 would be detected in the detached roots, stems and leaves when they were not put into liquid nitrogen immediately for extracted RNA (data not show). All of the *LePrx*s were expressed from early developmental to MG stages, but not at the breaker and ripe stages; however, differences in the expression patterns among the *LePrx*s were identified (Fig. 4b, Fig. S8). The expression of *LePrx09* and *LePrx17* was detected in IMG as well as MG fruits; the highest abundance of *LePrx09* occurred in mature green fruits while the peak of *LePrx17* transcripts occurred in IMG2 fruits. As compared with the expression patterns in immature fruits, the expression of *LePrx09* and *LePrx17* decreased markedly in breaker and ripe fruits (Fig. 4b, Fig. S8). These results indicated that *LePrx09* may be involved in the cessation of fruit growth whereas *LePrx17* may be related to the promotion of fruit growth. Similar to the expression profile of *LePrx17*, the transcripts of *LePrx35* and *LePrxA* were also highly abundant in IMG2 fruits. However, the expression of both genes gradually reduced to nearly undetectable levels

in IMG5 fruits, as well as in mature fruits at MG, breaker and ripe stages (Fig. 4b, Fig. S8). These results indicated that both *LePrx35* and *LePrxA* might be involved in the regulation of early fruit growth.

3.4 *LePrx09* has a high mesocarp expression pattern in the immature fruits

The mesocarp and inner tissues, including placenta, funiculus and seeds, of IMG3 fruits were respectively analyzed to examine the expression patterns of the four *LePrx*s. As shown in Fig. 5 and Fig. S9, transcription of *LePrx09* was most abundant in the mesocarp. In contrast, high levels of *LePrx17*, *LePrx35* and *LePrxA* transcripts were detected in the inner tissues (including the immature seeds, funiculus and placenta). The results indicated that all of the *LePrx*s are expressed in tomato fruits, while *LePrx09* was the only one of these *LePrx*s highly expressed in the mesocarp of immature fruits.

3.5 The characters of the promoters of the four *LePrx*s

In tomato, the fruit-specific element TGT(C/T)ACA had been found in the upstream sequence of fruit-specific *E8* gene(Zhao et al., 2009). In this study, the TGT(C/T)ACA motif and the other fruit-specific elements TCCAAAA motif in watermelon (Yin et al., 2009) were search in the promoters of the four *LePrx*s and other *cis*-elements analysis by PLACE database. Putative fruit-specific *cis*-element analysis revealed that the

upstream of *LePrx09* has two TGTTACA elements and one TCCAAAA element, upstream of *LePrx17* only has one TCCAAAA and upstream of *LePrxA* has three TCCAAAA elements and one TGTAACA element. However, upstream of *LePrx35* has not the fruit-specific elements TCCAAAA or TGTAACA (Fig.6). According to *cis*-elements analysis for mining the sugar-repressive element, TTATCC (Tatematsu et al., 2005), TACGTA (Toyofuku et al., 1998) and sucrose responsive element AATAGAAA or AATACTAAT (Grierson et al., 1994), all the upstream of the four *LePrx*s have least one sugar-repressive element. The upstream sequences of *LePrx09* and *LePrx17* reveal one sucrose responsive element in the region (Fig. 6). In addition, there show WRKY, pathogen related and the hormones related elements in the promoter regions of the four *LePrx*s.

The phylogenetic relationship analysis for the 2 Kb upstream sequences of the four *LePrx*s and other two fruit-specific *LePrx75*, *LePrx76* were constructed by using the mature-fruit -specific and ethylene related E8 promoter as outgroup (Fig.7). All the bootstrap support values were higher than 50% and no branch was collapsed. The tree was divided into two groups, A and B group. The upstream sequences of *LePrx09*,

LePrx17 and *LePrxA* were in the A group, whereas, the upstream sequences of *LePrx35* was in the B group which the promoters of *LePrx75* and *LePrx76* were present (Fig. 7).

Because the *LePrx09* is mainly expressed in the mesocarp according to transcripts analysis, the promoter of *LePrx09* drived *GUS* gene for bombardment and in-situ analysis were done for further verification. Histochemical staining manifested that GUS activity was only expressed in the mesocarp rather than other tissues. Noteworthily, the more depth of blue products is present in the mesocarp of fruits at mature green stage than immature green stage (Fig. 8). The results are solid evidence to support the transcripts analysis by RTPCR and RNA blot (Fig 4, 5, Fig. S8, S4).

3.6 The responses of the four *LePrx*s to hormones and stresses

Many hormones are involved in fruit growth and development (Trainotti et al., 2005; 2007; Yin et al., 2008; Hershkovitz et al., 2009). The transcript of fruit-specific *LePrx76* is up-regulated by kinetin (Sherf and Kolattukudy, 1993). To examine expression of the four *LePrx*s in response to hormone treatments of tomato fruit, IMG3 fruits were detached from parent plants and treated with various hormones. Expression of *LePrx09* and *LePrx17* was determined after 6 h of various exogenous hormone treatments (Fig. 9, S10). The expression of both *LePrx35* and *LePrxA* was nearly

undetectable in the detached fruits, not only in the fruits treated with various hormones but also with H_2O or ethanol (Fig. 9, S10). This is similar to results reported for a fruit-specific *LePrx*, *LePrx76*, whose transcripts were not observed in detached tomato fruits (Sherf and Kolattukudy, 1993). Compared to expression in fruits treated with H_2O, the expression of *LePrx09* was reduced by IAA, GA, zeatine and ABA; the greatest reduction was affected by zeatin (Fig. 9, S10). In contrast, ethephon, SA and JA induced the expression of *LePrx09* in IMG3 fruits; the highest level of expression was in fruits treated with JA (Fig. 9, S10). The influences of hormones on the expression of *LePrx17* were different from that of *LePrx09*; that is all of the hormones tested reduced expression of *LePrx17* apart from ABA and JA. The least abundance of *LePrx17* transcripts was in fruits treated with ethephon, where it was only one-tenth of the expression level with H_2O (Fig. 9, S10). The results suggest that the expressions of *LePrx17* and *LePrx09* were mediated by these hormones.

To understand the responses of the four *LePrx*s to wounding stress, the expression levels were determined in IMG3 fruits after mechanical damage to fruits on plants. Only the transcripts of *LePrx09* showed a pronounced increase at 2 h after wounding, and peaked at 8 h post-treatment (Fig. 10a, S11a). The expression levels of *LePrx35* and

LePrxA were also increased at 24 h post-treatment (Fig. 10a, S11a). In contrast, *LePrx17* was the only gene whose transcripts were reduced at all the time points following wounding, indicating that the expression of *LePrx17* might be repressed by wounding (Fig. 10a, S11a). The results revealed that all of the four *LePrx*s responded to wounding treatments but in different manners.

To investigate if the *LePrx*s are involved in defense against pathogen attack, IMG3 fruits were challenged with the phytopathogen *Alternaria solani*. The expression of *LePrx09* was highly induced 2 h post-infection and reduced 4 h and 8 h after inoculation, indicating that *LePrx09* might be an early responding gene to pathogen attack (Fig. 10b, S11b). Another *LePrx* responding to pathogen infection was *LePrx17*, whose expression was induced 4 h post-infection and rapidly reduced 8 h post-infection (Fig. 10b, S11b). In contrast, the expression levels of *LePrx35* and *LePrxA* were extremely low both in the absence and the presence of pathogen infection, suggesting that neither of these is involved in defense mechanism against *Alternaria solani* (Fig. 10b, S11b).

Endogenous H_2O_2 level in tomato fruits was reported to increase at the small green fruit stage and peak at breaker stage, suggesting that H_2O_2 may be involved in the regulation of fruit growth (Jimenez et al., 2002). Our results indicated that all of the

*LePrx*s were highly expressed in immature tomato fruits, before breaker stage (Fig. 10b). We therefore hypothesized that the four *LePrx*s may be involved in the regulation of H_2O_2 molecules in tomato fruits. To verify this hypothesis, detached IMG3 fruits were treated with H_2O_2. As shown in Fig. 6c and Fig. S11c, only the expression of *LePrx09* was elevated 8 h post-treatment, while the expression of the other genes was extremely low in the presence of H_2O_2 (Fig. 10c). These results indicated that among the four *LePrx*s, *LePrx09* may be the only one involved in the regulation of the H_2O_2 signaling pathway.

3.7 Enhanced resistance to H_2O_2 stress in tomato plants over-expressing *LePrx09*

Peroxidases activity is known to be involved in defense mechanisms under oxidative stress. To investigate whether the fruit-specific *LePrx*s play a role in resistance to oxidative stresses in tomato, the four *LePrx*s were each over-expressed in transgenic tomato plants. Expression of the genes in the transgenic lines was confirmed by RNA gel blot analysis (Fig. 11a), and two individual lines with each transgenic cassette were used for H_2O_2 treatments. As shown in Fig. 11b, only the leaf discs from transgenic plants 09-1 and 09-2, with over-expression of *LePrx09*, retained green coloration after exposure to 1% H_2O_2 for 16 h. Leaf discs from the other transgenic

lines and wild-type plants displayed a browning phenotype with H_2O_2 treatments (Fig. 11b). Similar results were observed from analysis of chlorophyll content; only transgenic plants 09-1 and 09-2 displayed significantly higher chlorophyll content compared to wild type (Fig. 11b). There was no significant difference in the chlorophyll content between wild type and transgenic plants over-expressing the other three *LePrx*s (Fig. 11b). The results indicated that among the four *LePrx*s, *LePrx09* appears to be the only one involved in defense mechanism against oxidative stress. To further analyze the anti-oxidation capacity of transgenic plants over-expressing *LePrx09*, leaf discs were treated with various concentrations of H_2O_2 for 16 h (Fig. 11c). As shown in Fig. 7c, leaf discs from transgenic plants over-expressing *LePrx09* (09-1 and 09-2 line) maintained green coloration not only in a low concentration (1%) but also in a high concentration (5%) of H_2O_2; in contrast, the discs from wild-type plants became brownish even when treated with 1% H_2O_2 (Fig. 11c). These results are consistent with the analysis of chlorophyll content; leaves from transgenic plants 09-1 and 09-2 maintained relatively higher chlorophyll content compared to wild type after exposure to the various concentrations of H_2O_2, (Fig. 11c). Although transgenic plant 17-2, with over-expression of *LePrx17*, displayed green color in the leaves and slightly higher

chlorophyll content than wild type with 1% H_2O_2 treatment, no significant difference in leaf color or chlorophyll content was observed between transgenic plant 17-2 and wild type above 1% of H_2O_2 (Fig. 11c). Taken together, the results suggested that among the four *LePrx*s, only *LePrx09* appears to be involved in the mechanism for protection against oxidative stress.

4. Discussion

4.1 Data mining from database supplied some information about the class III peroxidase genes

Arabidopsis has 73 class III peroxidase genes (*AtPrx*s) in the PeroxiBase that have been researched in many literatures (Tognolli et al. 2002; Welinder et al., 2002; Valério et al., 2004). The total numbers of *AtPrx*s are similar to the *LePrx*s(Table 1, Fig. S6). There are 48 *AtPrx*s with 3 introns and 4 exons and they are belonged to classical type class III peroxidases. In addition, 15 *AtPrx*s have 2 introns and 3 exons (Tognollii et al., 2002, Fig. S6). The numbers are also almost same with the *LePrx*s(Table 1, Fig. S6). However, the numbers of intron/exon of *LePrx*s are 4/5, 5/6 even 9/10 (Table 1, Fig. S6) and the patterns are not found in *AtPrx*s. Comparison of the genome of *Arabidopsis*, *Vitis*, *Helianthus*, *Lactuca*, *Mimulus* and *Solanum*, revealed that those crop are separated into three groups at 123-83Myr ago in evolution. The *Solanum* is the farthest specie from *Arabidopsis* in evolution relationship (The Tomato Genome Consortium, 2012). It could be explain that the *LePrx*s have more variation than *AtPrx*s.

4.2 The four *LePrx*s displayed fruit-specific expression and various properties to alleviate environmental stresses

Expression patterns of four *LePrx*s were identified in tomato fruits and their molecular characteristics were analyzed in the present study. These genes all contained several conserved regions, including two heme motifs, a calcium ion binding motif, eight cysteine residues, and a salt bridge motif (Fig. 2). According to the classification of Welinder et al. (2002), the identified peroxidases belong to the Class III peroxidase family. It is notable that the conserved peroxidase distal asparagine residue, Asn70 of horseradish Prx C (HRPC), was replaced by histidine and serine in LePrxA and LePrx17, respectively (Fig. 2). This change from asparagine to serine or histidine has also been reported in AtPrx07, AtPrx21, AtPrx42, AtPrx43 and AtPrx67 (Welinder et al., 2002). Although this property has been observed in several peroxidases, the effect of replacing asparagine with serine or histidine has not yet been studied. A site-directed mutagenesis study found that mutants containing class III peroxidase with the distal Asn70 residue replaced by valine or aspartic acid displayed a significant decrease in the enzyme kinetics in comparison with wild type (Nagano et al., 1996). It is possible that the replacement by serine or histidine also alters the characteristics of peroxidases; a

study of the effects of this replacement may give interesting information on the activity of peroxidases.

In spite of their similar structure, the 24 class III peroxidases of Solanaceae crop species were clustered into three groups, with AtPrx01 as an outgroup (Fig 3), suggesting that class III peroxidase proteins have great diversity in the non-conserved regions. Sequence variation may result in the diverse physiological functions of these proteins (Cosio and Dunand, 2009). From phylogenetic analysis, LePrxA and LePrx06, NtPrx10a, StPrx28, and CanPrx01 were grouped in cluster A (Fig 3). LePrx06, CanPrx01 and StPrx28 are known to be involved in the response to pathogens (Do et al., 2003; Coego et al., 2005; Hamann and Lange, 2006). In contrast, both wounding treatment and pathogen infection induce the expression of *NtPrx10a* (Sasaki et al., 2002). It has therefore been suggested that genes in cluster A may be involved in the response to pathogen and wounding stresses. Our study confirmed that *LePrxA* was expressed in detached tomato fruits in response to wounding (Fig. 10a). However, the response of *LePrxA* to pathogen infection is not yet clear because the expression of *LePrxA* was undetectable under pathogen infection in detached tomato fruits (Fig. 10b).

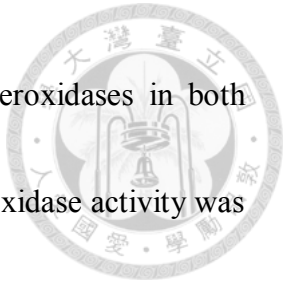
In group B, StPrx14 has been identified as a pathogen-inducible protein (Ros et al., 2004). NtPrx60a plays a role in lignin synthesis (Blee et al., 2003; Kavousi et al., 2010), while StPrx02 was reported to be expressed in response to wounding (Collinge and Boller, 2001). Although as yet the information regarding peroxidases in group B is limited, the expression levels of *LePrx17* and *LePrx09* in detached fruits were analyzed in the presence and absence of wounding and pathogen infection. Different results were obtained on the expression of the two genes after wounding; the expression of *LePrx09* was highly induced by wounding, while the expression of *LePrx17* was not significantly altered (Fig. 10a, S11a). On the other hand, the expression of both *LePrx09* and *LePrx17* was increased after pathogen challenge; a significantly elevated expression of *LePrx09* was observed 2 h post-pathogen infection while an increased expression of *LePrx17* was recorded 4 h post-infection (Fig. 10b, S11b). The expression patterns and phylogenetic analysis of *LePrx09* and *LePrx17* suggested that the two genes may play various roles in protection from wounding and pathogen infection.

Many class III peroxidases in group C have been studied, including LePrx76 (TAP1), LePrx75 (TAP2), StPrx72, LePrx05 (CEV1-1), NtPrx11a (tpoxC1) and CaPrx02 (Fig. 3). Both LePrx75 and LePrx76 are green-fruit-specific class III

peroxidases in tomato. A previous report showed that LePrx76 conferred resistance to pathogen infection in tomato fruits (Kesanakurti et al., 2012). In addition, LePrx35 was classified in subgroup C2 with LePrx05 and CanPrx02, which were reported to be pathogen-related genes (Vera et al., 1993; Choi et al., 2007). However, there was no detectable expression of *LePrx35* in the detached tomato fruits infected with *Alternaria solani* in the present study (Fig. 10b, S11b). The transcripts of *LePrx35* and *LePrxA* were undetectable after green fruits had been detached from the parent vine for 6 h (Fig. 9, Fig. S10), which was similar to the expression pattern of *LePrx76*. Constitutive expression of *LePrx76* became undetectable after fruits were detached from parent vines for 48 h to 72 h (Sherf and Kolattukudy, 1993). This suggests that a particular regulatory factor might be present in green fruits to interact with the fruit-specific peroxidases. Moreover, both *LePrx35* and *LePrx76* were wounding-inducible. Whereas various hormone treatments did not up-regulate *LePrx35,* only kinetin did increase the transcription of *LePrx76* (Fig. 9; Sherf and Kolattukudy, 1993). Due to the different expression patterns between *LePrx35* and *LePrx76*, it may be worthy to study the upstream elements involved in this pathway.

4.3 The four *LePrx*s expression at immature and mature green stages suggests the physiological functions are contributed in fruit growth

Class III peroxidases have been proposed to play a role in the regulation of fruit growth because the peroxidases may be involved in increasing the rigidity of cell walls in the fruit skin (Andrews et al., 2000; 2002a; b). The first classes III peroxidase gene identified in tomato fruits was *TAP1*, also named *LePrx76*, whose expression was detected only in the exocarp of green fruits and not in red fruits (Sherf and Kolattukudy, 1993). In addition, Andrews et al. (2000) reported that four peroxidase isoforms accumulated in the exocarp of fruits from MG to ripe stages; the activity of three isoforms increased in the fruit skin during the IMG stage and decreased from mature green stage to red ripe stages (Andrews et al., 2000). To investigate the possible association of peroxidase activity with fruit growth, Andrews et al. (2002a) partially purified the wall-bound 44, 48, 53 and 58 KDa peroxidases from exocarp tissue of tomato fruits and applied this to exocarp strips excised from fruit at various stages of development. A significant increase in stiffness of exocarp strips was detected after application of the wall-bound peroxidases, suggesting that peroxidase isozymes are associated with the cessation of fruit growth (Andrews et al., 2002a). This result was



further supported by analysis of the sub-cellular localization of peroxidases in both immature and mature fruits (Andrews et al., 2002b). Little or no peroxidase activity was detected in the cell wall of immature fruits; however, peroxidase activity was present in the cell wall of mature green fruits (Andrews et al., 2002b). These studies demonstrated the association of peroxidase activity with the cessation of fruit growth; however, the peroxidases were identified only in the exocarp tissues of fruits. In the present study, all four *LePrx*s showed higher expression associated with fruit growth during IMG and MG stages, and reduced expression at both breaker and ripe stages (Fig. 4b, S8b). The estimated molecular weights of the four peroxidases are between 36.7 to 38.1 KDa (Table 2). The results indicated that the function of the four peroxidases involved in fruit growth is different to the peroixdases reported by Andrews et al. (2002a,b). In addition, the turgor-driven expansion of parenchyma cells within the mesocarp creates tissue pressure, which is expressed on the exocarp layer as tissue tension. Mechanical properties of the exocarp were important in controlling fruit growth (Thompson et al., 1998). The rate and extent of tomato fruit is determined mostly by the pericarp, some cell wall enzymes controlling fruit growth must be concentrated.

The similarity of expression patterns indicated that the four peroxidases might be actively involved in the regulation of fruit growth in tomato. Among the four *LePrx*s, a higher level of transcription of *LePrx09* was found in the mesocarp of green fruits compared with the exocarp according to the result of RTPCR, RNA blot and histochemical localization of GUS activity analysis (Fig. 5, Fig. 8, Fig. S9) suggesting that peroxidases expressed in the mesocarp may also be involved in the cessation of fruit growth. Thus, *LePrx09* is the first peroxidases gene found to have specific expression in the mesocarp tissue of tomato fruits; investigation to elucidate the role of *LePrx09* in the regulation of fruit growth may give interesting results.

4.4 The other peroxidases with similar expression patterns are worthily to be compared

In addition, proteomics study by two-dimensional gel electrophoresis for proteins extracted from pericarp at six tomato fruits growth stages revealed that the glutathione peroxidase GPXle-2 is abundant in the small immature green fruit stage (Faurobert et al., 2007) as same as LePrx09, LePrx17, LePrx35 and LePrxA (Fig. 2), but the function of *GPXle-2* gene was not figured out yet. Moreover, the expression profile of green-fruit-specific *LePrx76* is the same with *LePrx09* that has highest level transcripts

in the mature green stage (Sherf et al., 1993). Those proteins mentioned above are all pericarp or fruit specifics. Specifically, *LePrx09* is the first class III peroxidase gene dominantly expressed in mesocarp. However, only *LePrx67* showed functional with resistance to *Alternaria alternate* and *Fusarium solani* among those fruit-specific *LePrx*s (Kesanakurti et al., 2012). The functional difference between *LePrx09* and those fruit-specific *LePrx*s are necessary to understand.

4.5 Promoter analysis revealed the fruit-specific expression, stress and hormone response

Some fruit-specific *cis*-elements, such as the TGTCACA motif in musk melon (Yamagata et al., 2002) and the TCCAAAA motif in watermelon (Yin et al., 2009), were also studied. The TGTTACA element, found in the upstream of the transcription start site of *LePrx09* and *LePrxA* (Fig. 6) is conserved with the musk TGTCACA motif. The TGT(C/T)ACA element is also found in the promoter of the fruit-specific E8 gene in 16 tomato breeding accessions (Zhao et al., 2009) and is involved in the fruit-specific expression. The watermelon fruit-specific element TCCAAAA, a negative *cis*-regulatory region for inhibiting gene expression in leaves (Yin et al., 2009), is also found in the upstream of the transcription start site of *LePrx09*, *LePrx17* and *LePrxA*

(Fig. 6). Those fruit-specific elements might explain the fruit-specific expression of the *LePrx*s. However, there are not those two fruit-specific elements in the upstream of the *LePrx35* and some elements exist in the sequences responsible for fruit-specific expression might be studied in the future. Phylogenetic analysis revealed the promoter of *LePrx35* were grouped in the A group, unlike the other three promoters in B group (Fig. 7). This results also suggested the characters of upstream sequence of the *LePrx35* were some different with others.

After the mature stage of tomato fruit, it is the time for sugar accumulated and the stage of un-detection for the four *LePrx*s. Therefore, the sugar responded *cis*-elements might exist in the promoter region of the four LePrxs. The sugar-repressive element, TTATCC (Tatematsu et al., 2005), TACGTA (Toyofuku et al., 1998) are all in the promoter regions of the four *LePrx*s suggesting the green-fruit-specific characters in Fig. 4. and Fig. S8. Moreover, there are many WRKY, pathogen related and the hormones related elements in the promoter regions of the four *LePrx*s explaining the hormone, wounding, pathogen and stress responses (Fig. 9, Fig. 10, Fig. S10, Fig. S11)

4.6 Over-expression of *LePrx09* in tomato plants conferred resistance to oxidative stress

Class III peroxidases have been reported to be involved in mechanisms of metabolism related to stress resistance in plants. For example, the involvement of class III peroxidases in the formation of lignin and the cross-linking of cell walls has been reported (Hammerschmidt et al., 1982; Egea et al., 2001). Moreover, class III peroxidases were reported to play a role in the production of reactive oxygen species, which are active in the signal transduction pathway of plant defense mechanism (Laloi et al., 2004). A peroxidase-dependent oxidative burst was proposed to be responsible for resistance to pathogen infection (Bolwell et al., 2002; Bindschedler et al., 2006; Choi et al., 2007). Notably, our present results showed that the expression of *LePrx09* in tomato fruits was induced by wounding treatment, pathogen infection and oxidative stress (Fig. 6), suggesting that *LePrx09* may play a role in stress-related mechanisms. Transgenic plants over-expressing *LePrx09* displayed a high level of resistance to oxidative stress, suggesting that ectopic expression of *LePrx09* conferred protection against oxidative stress (Fig. 11b and 11c). However, further studies are required to determine the function of *LePrx09* in the defense mechanism.

5. Future prospects

In these studies, four tomato green-fruit-specific class III peroxidase genes, *LePrx09*, *LePrx17*, *LePrx35* and *LePrxA*, were cloned and identified. Moreover, their characters, structures, phylogenetic analysis, expression profiles, promoter analysis and functions were verified. Worthily, all the four *LePrx*s are wounding reducible, *LePrx09* and *LePrx17* are upregulated by pathogen inoculation and only *LePrx09* is upregulated by H_2O_2 treatment (Fig. 10, Fig. S11). The *LePrx09*-overexpressed transgenetic tomato had the H_2O_2 resistance (Fig. 11). There are several researches worthily to do as follow:

5.1 The effect of replacing Asn70 with serine or histdine of the LePrxs is worthily to study in the future

The distal asparagine that forms hydrogen bond with the distal histidine can interact with heme for the catalytic ability of class III peroxidase. In LePrxA and LePrx17, the asparagine is replacing to serine or histidine, repectively (Fig. 2). These replacements are also found in AtPrx07, AtPrx21, AtPrx42, AtPrx43 and AtPrx67 (Welinder et al., 2002). The replacement could change the catalytic ability or substrates

but no any report is this asparagine replacement with serine or histidine. Site-directed mutagenesis will be done for assaying the activity of the class III peroxidase enzymes.

5.2 The function of *LePrx09* involved in fruit growth will be cleared by more detail experimental design

The endogenous H_2O_2 level could be related to cell elongation (Schopfer, 1994 ; Joo et al., 2001) and class III peroxidase mediate plant growth by regulating H_2O_2 and hydroxyl radical (Schopfer et al., 2002; Dunand et al., 2003). The growths of transgenetic plants with over-expression of *LePrx09* are slower than wild type plants but not significant (data not show). Because the growth processes are complex results effected by many environment factors and the transgenetic tomato plants were planted in plots for three replications in the nethouse with temperature 24-28°C, the real difference between transgenetic plants and wild types are not discriminated. The difference for growth of tomato could be monitored in a greenhouse with precise temperature, irrigation control and more replications in the future.

5.3 The green-fruit-specific promoters of *LePrx*s are rare and valuable for biotech industry and research

Some fruit-specific promoters, such as the upstream of *T56*, *P119*, *2A11* and *TFM7*, had been used for GM food improvement (Davuluri et al., 2005) and they could be used for health proteins or other metabolism production in fresh fruits. The fruit-specific regulative elements were also studied by deletion analysis. There are four fruit-specific domains in the 5' region of the tomato 2A11 gene (Haaren and Houck, 1993). The TGT(C/T)ACA element, which is similar to the mush melon fruit-specific *cis*-element TGTCACA (Yamagata et al., 2002), is also found in the upstream sequence of the fruit-specific *E8* gene in 16 tomato breeding accessions (Zhao et al., 2009). The fruit-specific and at least one sugar-repressive element exist in the promoter regions of the four *LePrx*s (Fig. 6). The several deletion experiments of upstream sequence of *LePrx*s and the GUS activity analysis will be done and some green-fruit-specific *cis*-elements will be found.

5.4 The DNA sequences in the *LePrx09* or promoter of *LePrx09* and its transcription factors may be used as selection markers in stresses resistant breeding processes.

The tomato genome sequence has been released (The Tomato Genome Consortium, 2012) and many DNA or RNA sequence database have been mentioned in the introduction of this study. The sequence and polymorphism of PCR-based cleaved amplified polymorphic sequence (CAPS) markers, single nucleotide polymorphism (SNP) marker, Microsatellite markers (simple sequence repeats, SSR), InDel (insertion and deletion) and other markers between the tomato breeding accessions and events are found according to those databases (Causse et al., 2013). In this study, *LePrx09* is involved in wounding and pathogen treatments (Fig. 10) and the tomato overexpressed with *LePrx09* has strong resistance to H_2O_2 (Fig. 11). Thus, the tomato has more tolerance to stresses induced H_2O_2 if the transcripts level of *LePrx09* are more than other tomato breeding accession. The tomato breeding accessions with different genes expression and the polymorphism will be found by EST analysis. It is expected that the molecular markers could be used in the breeding processes for tomato *LePrx09* related

stresses resistance if the difference in DNA sequence in the *LePrx09* or upstream of the *LePrx09* or its transcription factors between different tomato accession is found.

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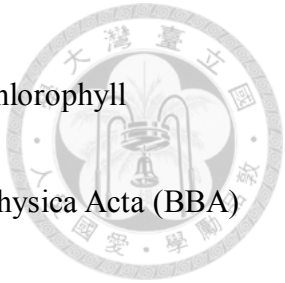

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Table 1 The chromosome position, intron/exon numbers of class III peroxidase genes of tomato.

Gene	SGN accession	ch	position	Intron/exon No.
LePrx01	Solyc07g052510.2.1	7	61020110..61021912	3/4
LePrx02	*Solyc07g047740.2.1*	7	58951739..58953436	4/5
LePrx03	*Solyc02g083490.2.1*	2	46872102..46873459	3/4
LePrx04	*Solyc08g075830.2.1*	8	59920913..59922336	3/4
LePrx05	*Solyc01g006300.2.1*	1	888896..891747	3/4
LePrx06	*Solyc01g105070.2.1*	1	93354312..93356101	2/3
LePrx07	*Solyc04g080760.2.1*	4	64857005..64859239	3/4
LePrx08	*Solyc02g092580.2.1*	2	53627090..53629631	3/4
LePrx09	*Solyc04g071890.2.1*	4	58913334..58915438	2/3
LePrx10	*Solyc10g076240.1.1*	10	59082990..59084119	2/3
LePrx11	*Solyc05g052280.2.1*	5	62559763..62562832	3/4
LePrx12	Solyc05g046010.2.1	5	58047387..58049383	3/4
LePrx13	*Solyc10g076210.1.1*	10	59054478..59055612	2/3
LePrx14	*Solyc02g087190.1.1*	2	49665501..49666487	0/1
LePrx15	*Solyc07g052540.2.1*	7	61035832..61037571	3/4
LePrx16	*Solyc04g071900.2.1*	4	58919910..58922921	2/3
LePrx17	*Solyc02g080530.2.1*	2	44739605..44741297	3/4
LePrx18	*Solyc12g005790.1.1*	12	440801..442920	3/4
LePrx19	*Solyc09g072700.2.1*	9	65312183..65314163	1/2
LePrx20	*Solyc06g076630.2.1*	6	47592055..47594316	3/4
LePrx21	*Solyc03g006700.2.1*	3	1259766..1262506	3/4
LePrx22	*			2/3
LePrx23	*Solyc02g082090.2.1*	2	45813165..45815385	2/3
LePrx24	Solyc02g084790.2.1	2	47880732..47882432	3/4
LePrx25	*Solyc06g050440.2.1*	6	33051778..33054091	3/4
LePrx26	*Solyc11g018800.1.1*	11	9597159..9598442	3/4
LePrx27	*			
LePrx28	*Solyc01g006290.2.1*	1	884779..887621	3/4
LePrx29	*Solyc03g044100.2.1*	3	8096015..8098828	3/4
LePrx30	*Solyc09g007520.2.1*	9	1092950..1099568	5/6
LePrx31	*Solyc01g108320.2.1*	1	95673715..95675247	3/4
LePrx32	*Solyc03g080150.2.1*	3	52050347..52051941	3/4
LePrx33	*Solyc05g046020.2.1*	5	58103304..58104907	3/4
LePrx35	*Solyc10g078890.1.1*	10	58103304..58104907	3/4
LePrx37	*Solyc04g080330.2.1*	4	64553548..64554942	3/4

*:The sequences of the peroxidase genes are not found in SGN website.

Table 1(continue) The chromosome position, intron/exon numbers of class III peroxidase genes of tomato.

Gene	SGN accession	ch	position	Intron/exon No.
LePrx38	*Solyc02g094180.2.1*	2	54788317..54789985	2/3
LePrx39	*Solyc05g055320.2.1*	5	65001185..65002725	1/2
LePrx40	*Solyc01g015080.2.1*	1	16780388..16782933	3/4
LePrx41	*Solyc04g081860.2.1*	4	65729103..65731641	3/4
LePrx42	*Solyc08g007150.1.1*	8	1708656..1709615	0/1
LePrx43	*Solyc01g101050.2.1*	1	90909753-90910895	1/2
LePrx44	*Solyc10g076190.1.1*	10	59045991..59047132	2/3
LePrx45	*Solyc02g090470.2.1*	2	52008574..52010065	3/4
LePrx46	*Solyc05g046000.2.1*	5	58037266..58039451	3/4
LePrx47	*Solyc11g010120.1.1*	11	3243625..3246066	2/3
LePrx48	*Solyc07g017880.2.1*	7	8260969..8263025	3/4
LePrx49	*Solyc01g058520.2.1*	1	66806122..66807766	2/3
LePrx50	*Solyc05g010330.2.1*	5	4476583..4479812	3/4
LePrx51	*Solyc07g052510.2.1*	7	61020180..61021604	3/4
LePrx52	*Solyc08g069040.2.1*	8	58125521..58127215	3/4
LePrx54	*Solyc11g072920.1.1*	11	56077525..56078850	3/4
LePrx55	*Solyc01g007950.2.1*	1	2134714..2143061	9/10
LePrx56	*Solyc12g096530.1.1*	12	65410423..65413887	4/5
LePrx57	*Solyc02g085930.2.1*	2	48699854..48703424	3/4
LePrx58	*Solyc08g013930.2.1*	8	3401624..3406620	3/4
LePrx59	*Solyc01g006310.2.1*	1	899147..902371	3/4
LePrx60	*Solyc07g049240.2.1*	7	59520799..59522115	3/4
LePrx61	*Solyc06g082420.2.1*	6	48242060..48244274	3/4
LePrx63	*Solyc01g104860.2.1*	1	93185250..93187229	3/4
LePrx64	*Solyc01g067860.2.1*	1	76839927..76841525	3/4
LePrx65	*Solyc02g064970.2.1*	2	36095618..36097543	3/4
LePrx66	*Solyc03g033710.2.1*	3	5315137..5318145	3/4
LePrx67	*Solyc02g062510.2.1*	2	34201832..34204123	3/4
LePrx68	*Solyc02g084800.2.1*	2	47888460..47889825	3/4
LePrx69	*Solyc02g090450.2.1*	2	52000059..52001628	3/4
LePrx70	*Solyc02g084780.2.1*	2	47874773..47876593	3/4
LePrx71	*Solyc05g050880.2.1*	5	61090944..61092909	3/4
LePrx72	*Solyc03g025380.2.1*	3	2813769..2816283	2/3
LePrx73	*Solyc04g076770.2.1*	4	61649557..61652559	3/4
LePrx75	Solyc02g079510.2.1	2	44044563..44046955	2/3
LePrx76	*Solyc02g079500.2.1*	2	44040159..44042618	2/3
LePrx77	*Solyc04g064690.2.1*	4	55830684..55834397	4/5

Table 2 Molecular characteristics of four peroxidase genes expressed in the immature tomato fruits.

Clone ID	Gene name	GeneBank accession no.	SGN [a] accession no.	chromosome	Position (bp)	cDNA length (bp)	No. of AA	Molecular weight [b] (KD)	PI [c]	Closest ortholog [d] (identity %)
TPod11	*LePrx09*	AK328918 / AK319424[c]	Solyc04g071890.2.1	4	56506704-56508808	1193	348	38.1	6.50	*Solanum tuberosum* StPrx17 (97)
TPod6	*LePrx17*	AK322518	Solyc02g080530.2.1	2	44739605-44741297	1258	326	37.1	8.80	*Solanum tuberosum* StPrx01 (98)
TPod3	*LePrx35*	AK329186	Solyc10g078890.1.1	10	60552219-60554250	1213	338	37.0	8.78	*Nicotiana tabacum* NtPrx04a (75)
TPod20	*LePrxA*	XM_010321727	Solyc04g064690.2.1	4	54949154-54952867	1171	334	36.7	6.14	*Solanum tuberosum* StPrx45 (96)

[a] The accession number, physical position of the peroxidase genes at SGN (Sol genomic network, http://solgenomics.net/)

[b] Molecular mass and isoelectric point (pI) of the mature protein was estimated by Swiss-Prot/TrEMBL.

[c] Closest orthologous protein in other *Solanaceae* crop species; the percentage identity was determined by BLAST in the PeroxiBase database. (http://peroxibase.toulouse.inra.fr/)

[d] http://www.pgb.kazusa.or.jp/kaftom/

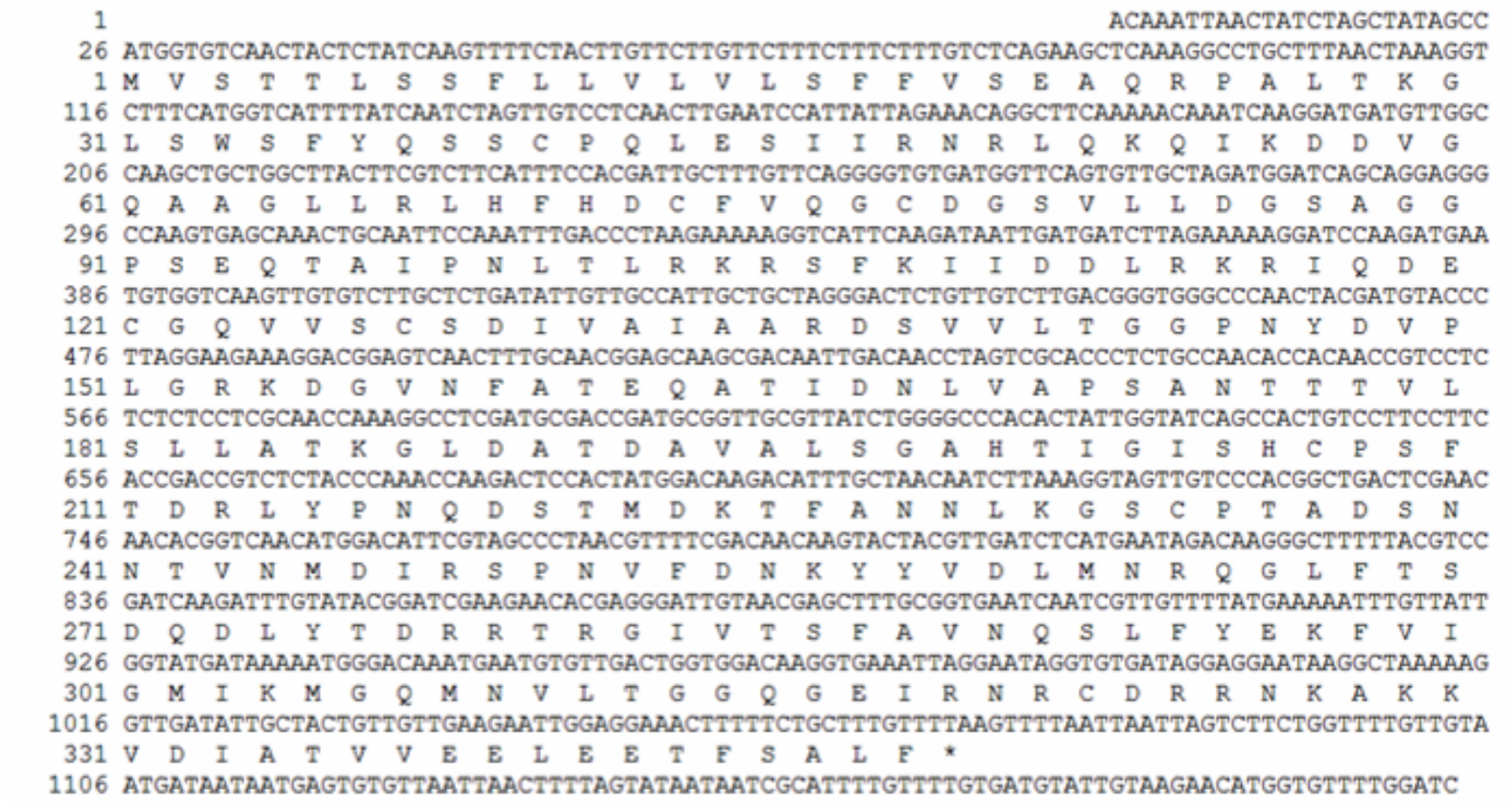


Fig. 1 The nucleotide sequence (above) and deduced amino acid (below) of the *LePrx09*. The nucleotides and amino acids positions are numbered on the left. The predicted amino acid sequence is given in single-letter code. Stop codons are represented with asterisks.

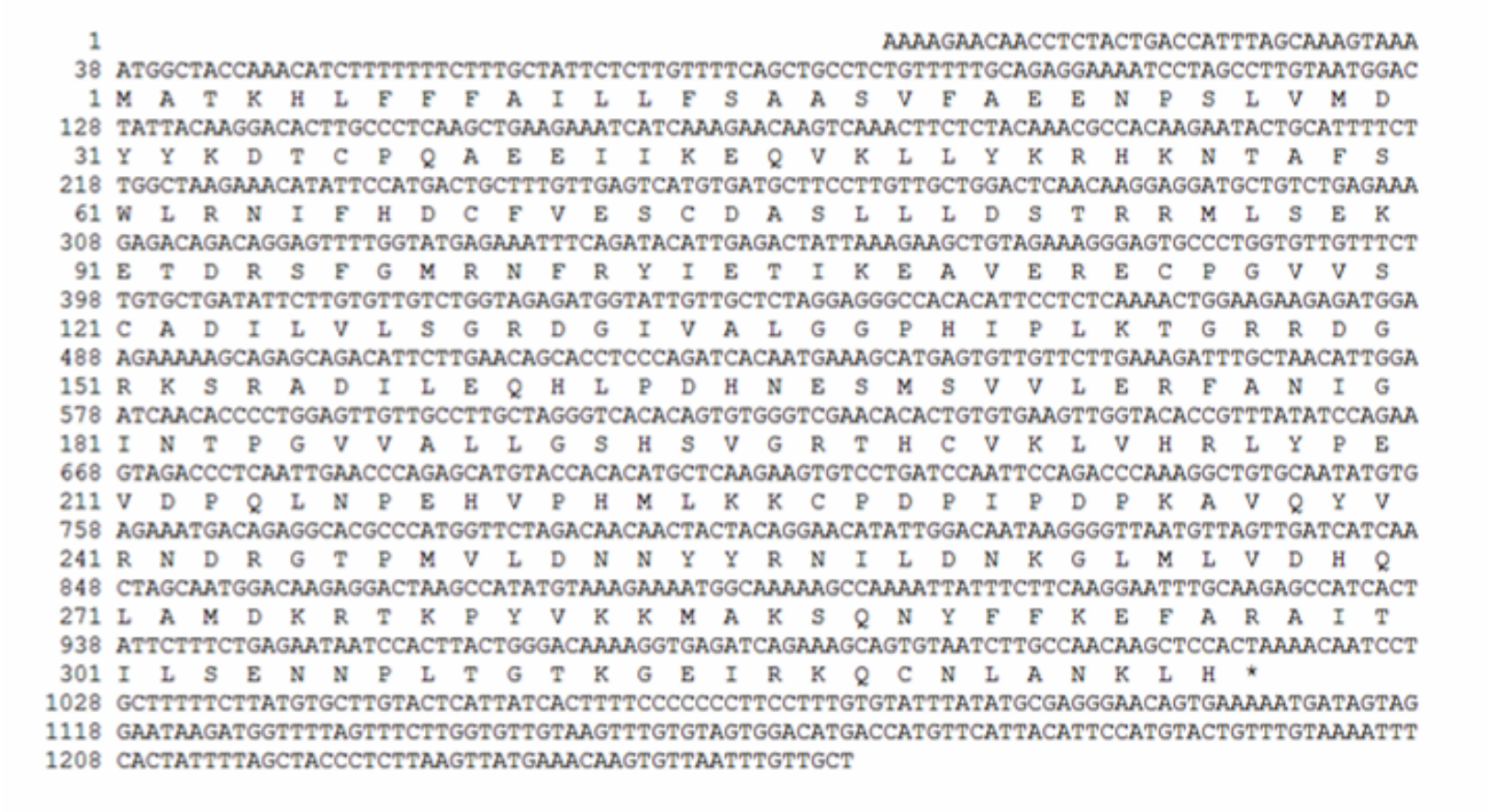


Fig. 1(continue) The nucleotide sequence (above) and deduced amino acid (below) of the *LePrx17*. The nucleotides and amino acids positions are numbered on the left. The predicted amino acid sequence is given in single-letter code. Stop codons are represented with asterisks.

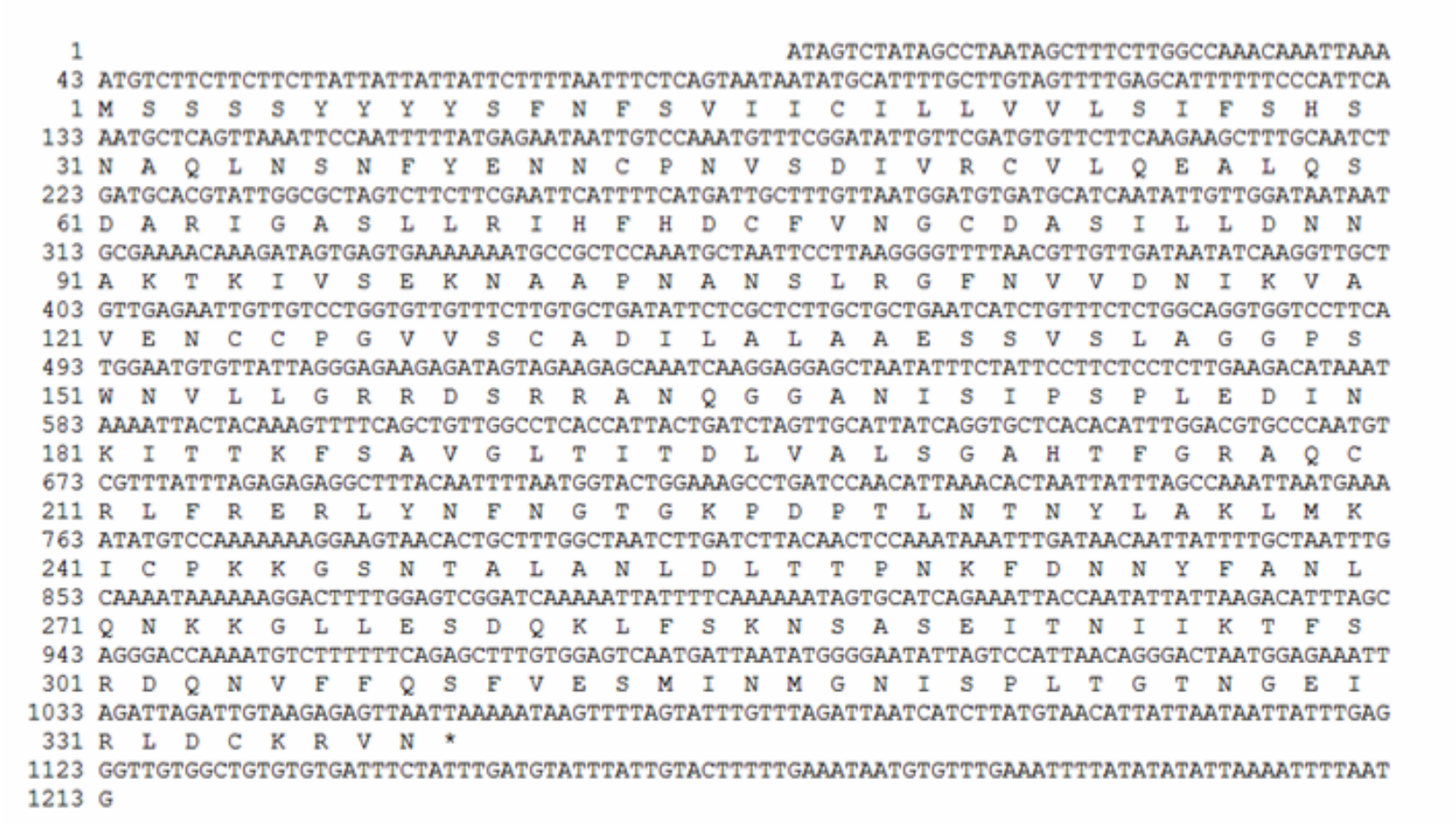
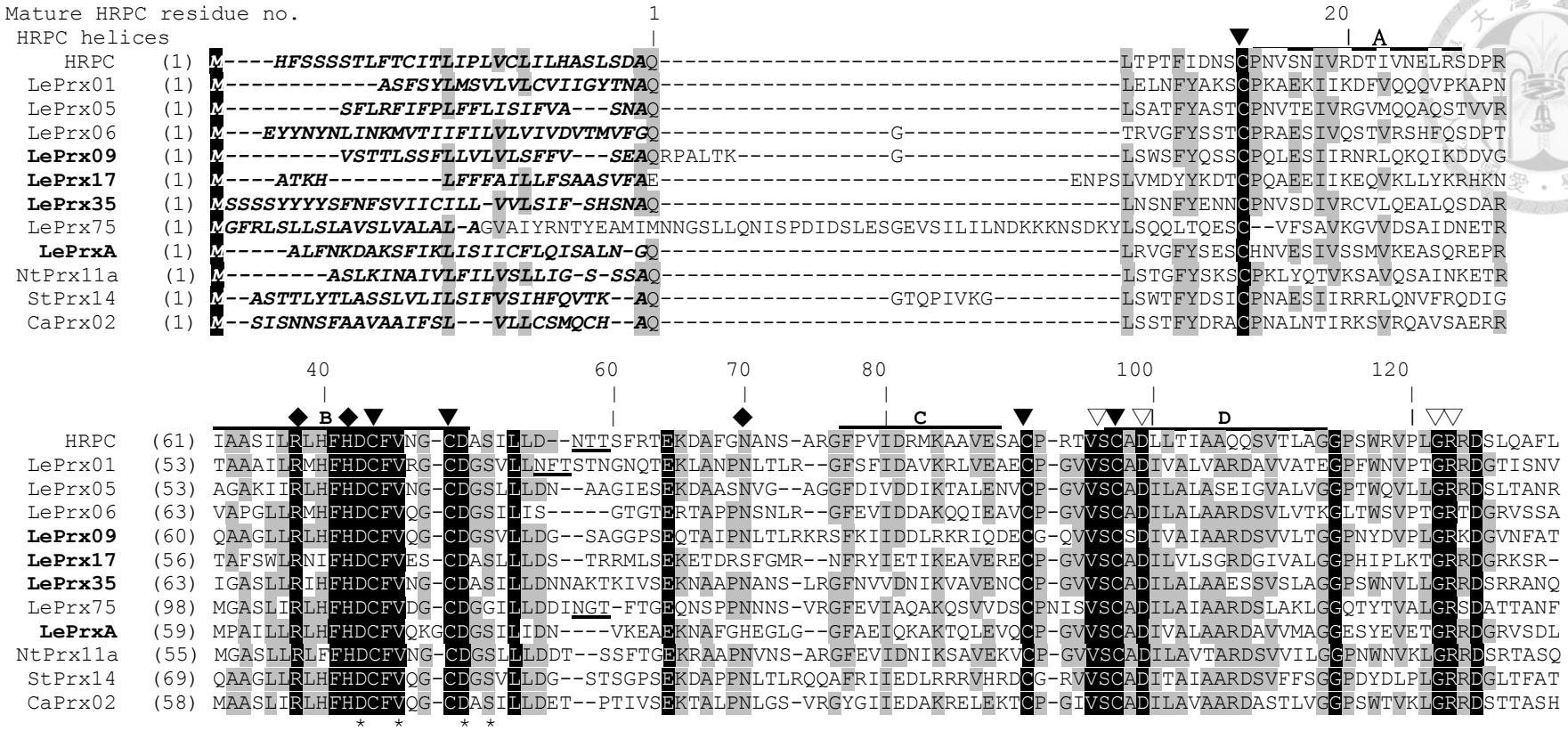


Fig. 1(continue) The nucleotide sequence (above) and deduced amino acid (below) of *LePrx35*. The nucleotides and amino acids positions are numbered on the left. The predicted amino acid sequence is given in single-letter code. Stop codons are represented with asterisks.

Fig. 1(continue) The nucleotide sequence (above) and deduced amino acid (below) of *LePrxA*. The nucleotides and amino acids positions are numbered on the left. The predicted amino acid sequence is given in single-letter code. Stop codons are represented with asterisks.


Mature HRPC residue no.
HRPC helices
HRPC (1)
LePrx01 (1)
LePrx05 (1)
LePrx06 (1)
LePrx09 (1)
LePrx17 (1)
LePrx35 (1)
LePrx75 (1)
LePrxA (1)
NtPrx11a (1)
StPrx14 (1)
CaPrx02 (1)
HRPC (61)
LePrx01 (53)
LePrx05 (53)
LePrx06 (63)
LePrx09 (60)
LePrx17 (56)
LePrx35 (63)
LePrx75 (98)
LePrxA (59)
NtPrx11a (55)
StPrx14 (69)
CaPrx02 (58)

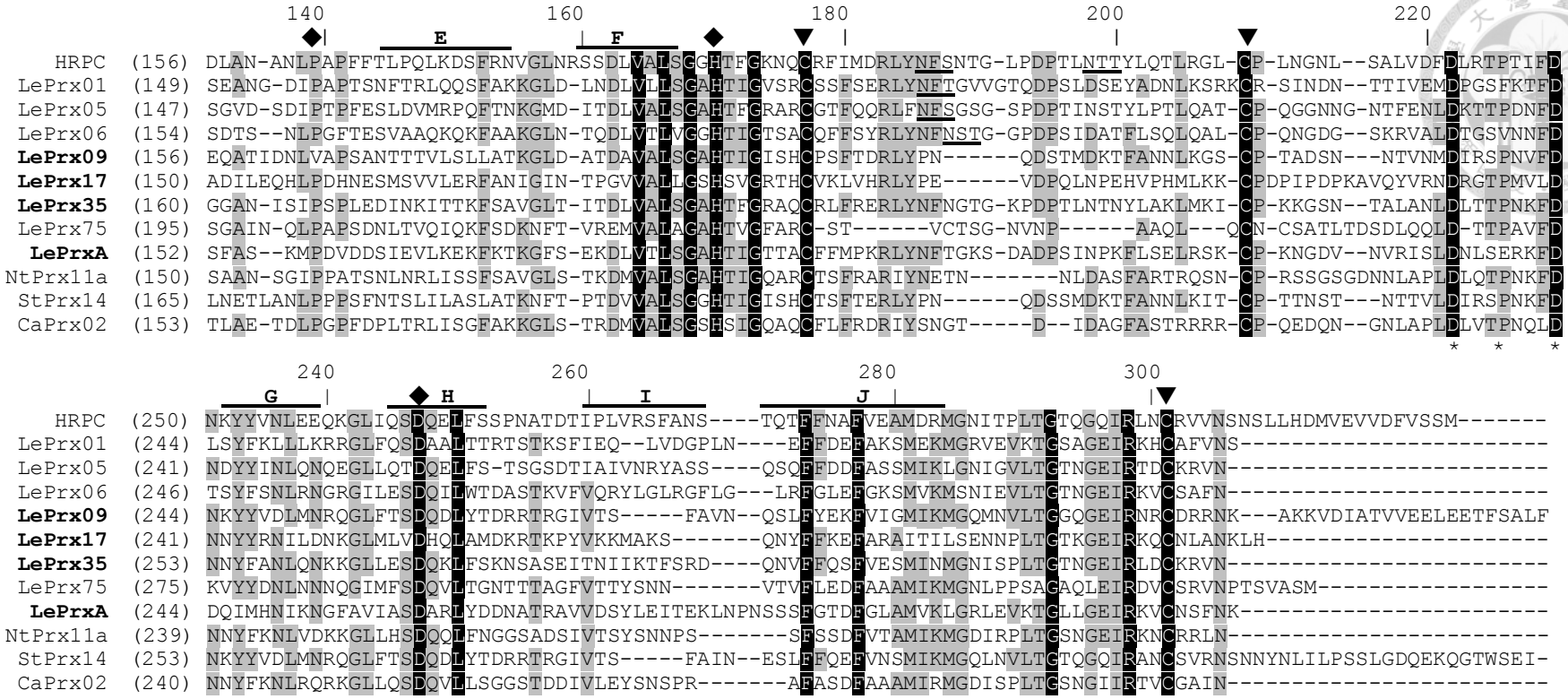


Fig. 2 Alignment of LePrx09, LePrx17, LePrx35 and LePrxA with class III peroxidases in tomato and three *Solanaceae* crops. Numbers along the top represented the residue numbers of mature horseradish peroxidase (HRPC). Putative amino acids identical with HRPC are shaded in black, while similar ones are shaded in grey. Predicted signal peptides are italicized. Helices B, D and F are conserved domains in peroxidases. The heme-binding sites are marked with black diamond symbol (◆) while eight conserved cysteine residues are marked with arrowhead symbol (▼). Star symbol (*) represents the residues involved in calcium binding; residues involved in a salt bridge are marked with invert triangle symbol (▽). Putative *N*-glycosylation sites are underlined.

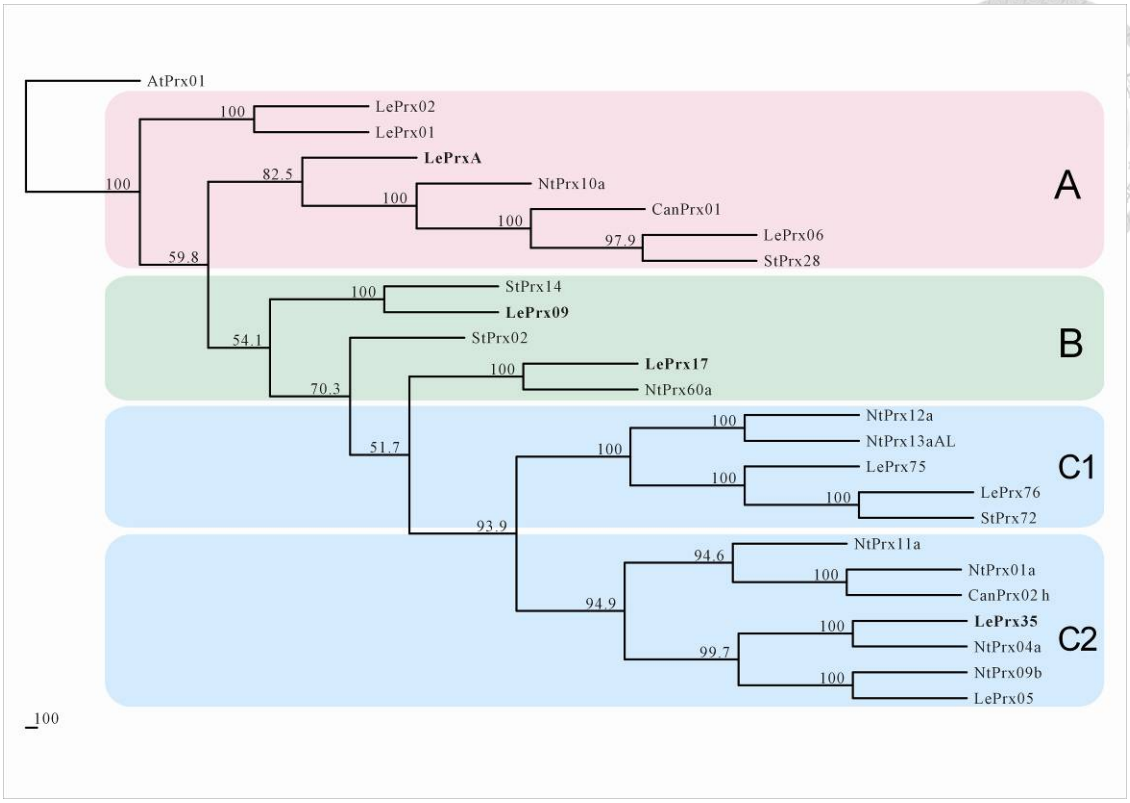


Fig. 3 Phylogenetic tree of LePrx09, LePrx17, LePrx35, LePrxA and class III peroxidases in *Solanaceae* crop species. The un-rooted tree was constructed with PRODIST and KFITCH methods in the PHYLIP package. Random 1000 bootstrap replicates were carried out with SEQBOOT option. Full-length sequences of peroxidases of tomato (Le), tobacco (Nt), potato (St) and pepper (Can) were extracted from the PeroxiBase database. *Arabidopsis thaliana* peroxidase, AtPrx01, was included as an outgroup in the tree. The bootstrap support values shown as percentage are indicated on the branches from 1000 replicates.

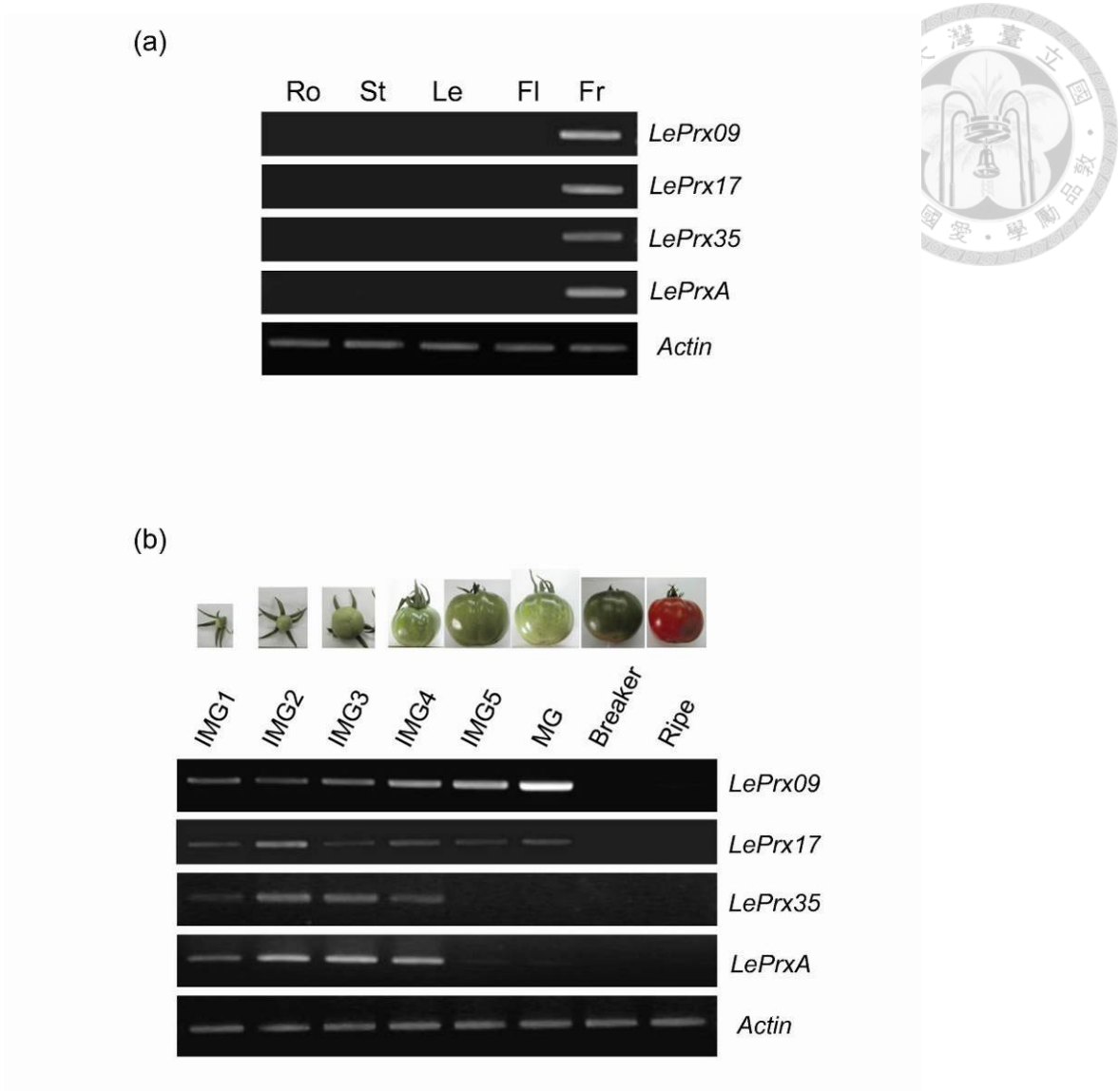


Fig. 4 Organ-specific expression patterns of the four *LePrx*s. (a) Four *LePrxs* displayed fruit-specific expression profiles. Ro: Root; St: Stem; Le: Leaf; Fl: Flower; Fr: Fruit. For RT-PCR analysis, various number of cycles was applied for the amplification of test genes: 25 cycles for *LePrx09* and *LePrx17*; 30 cycles for *LePrx35* and *LePrxA*; and 18 cycles for the internal control, tomato actin (U60482.1). (b) Expression patterns of four *LePrxs* at various growth stages of tomato fruits. The diameters of immature fruits were: IMG1, 0.5 to 1 cm; IMG2, 1.0 to 1.5 cm; IMG3, 2.0 to 2.5 cm; IMG4, 3.5 to 4.0 cm; and IMG5, 6.0 to 7.0 cm. MG: mature green stage. Breaker stage represents tomato fruits with the top turning to red color. At the ripe stage the fruits were completely ripe. The number of cycles for PCR amplification was: 22 cycles for *LePrx09* and *LePrx17*; 28 cycles for *LePrx35* and *LePrxA*; and 18 cycles for the internal control, tomato actin (U60482.1).

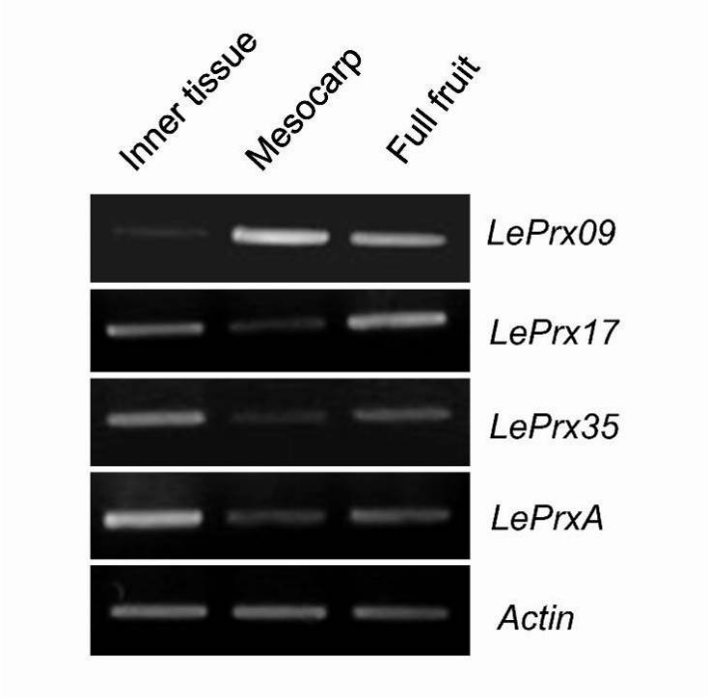


Fig. 5 Expression patterns of four *LePrxs* in the mesocarp and inner tissues of tomato green fruits. For RT-PCR analysis, the number of cycles was: 23 cycles for *LePrx09* and *LePrx17*; 28 cycles for *LePrx35* and *LePrxA*; and 18 cycles for the internal control, tomato actin (U60482.1).

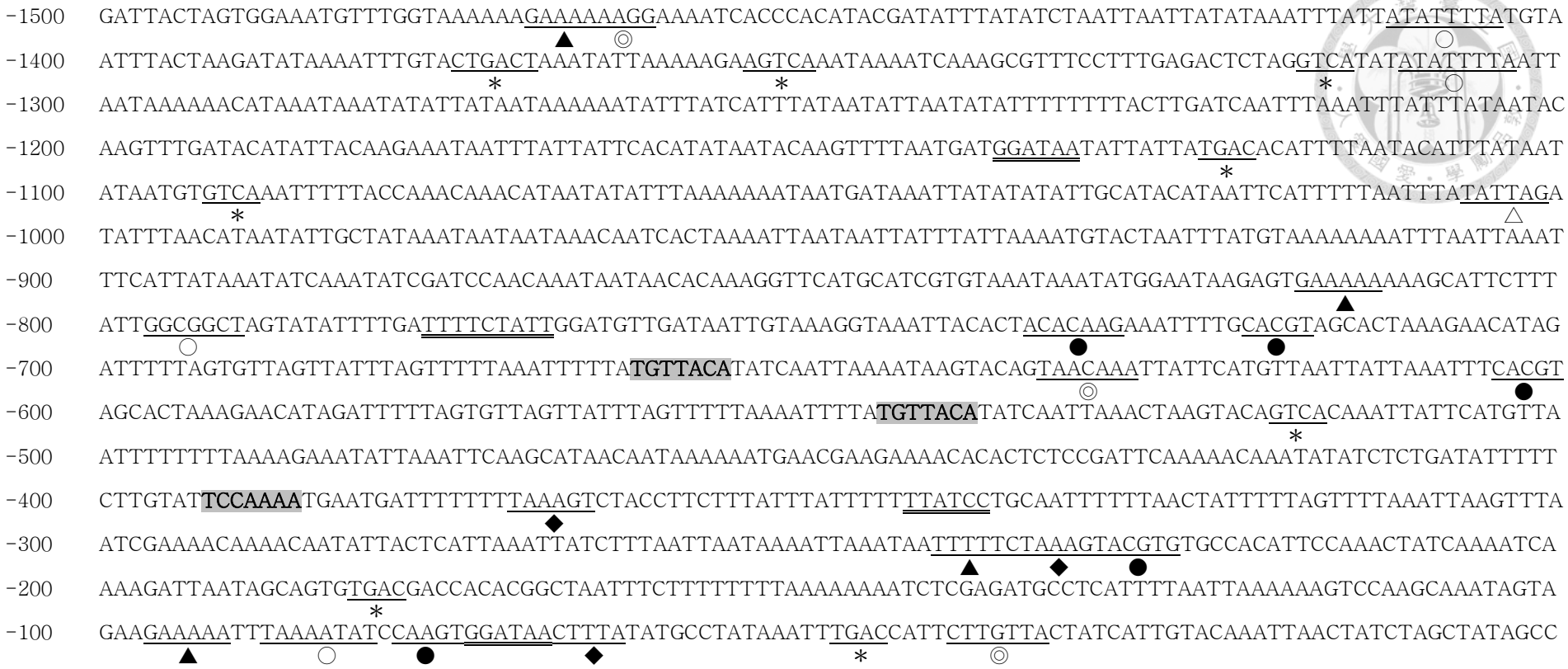


Fig.6 (a) The putative *cis*-element at 5' upstream of *LePrx09*. The fruit-specific elements 'TCCAAAA' and 'TGTTACA' are shaded in grey. The sugar related elements are marked with under double lines. The other *cis*-elements are marked with underlines with signs as follows: ∗ WRKY elements; ▲ pathogen related element; ◆ auxin related element; △ cytokinin related element ; ◎ GA related element ; ○ ethylene related element ; ● ABA related element.

Fig.6 (b) The putative *cis*-element at 5' upstream of *LePrx17*. The fruit-specific element 'TCCAAAA' is shaded in grey. The sugar related elements are marked with under double lines. The other *cis*-elements are marked with underlines with signs as follows: ∗ WRKY elements; ▲ pathogen related element; ◆ auxin related element; △ cytokinin related element ; ◎ GA related element ; ○ ethylene related element ; ● ABA related element.

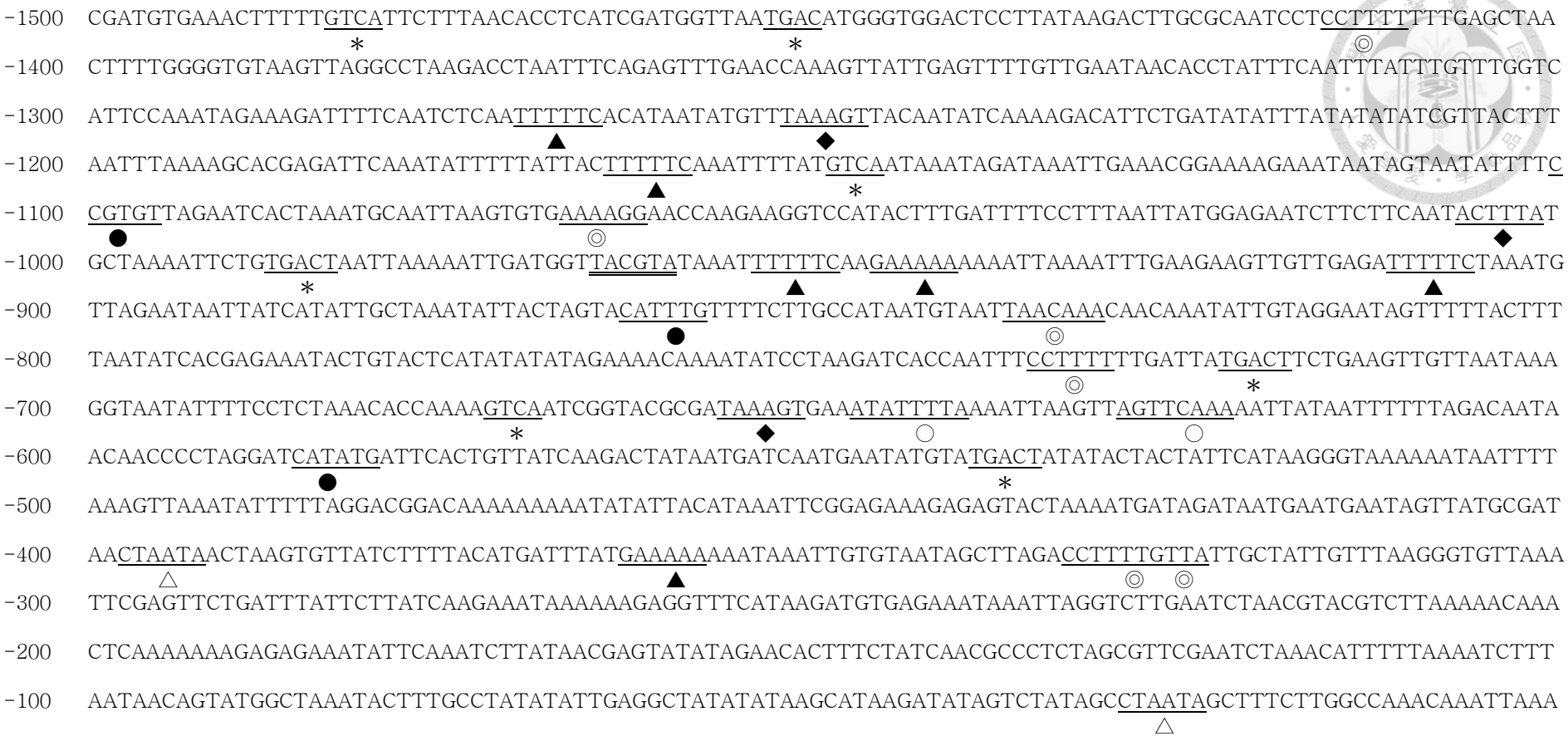


Fig.6 (c) The putative *cis*-element at 5' upstream of *LePrx35*. The fruit-specific elements 'TCCAAAA' and 'TGTTACA' are shaded in grey. The sugar related elements are marked with under double lines. The other *cis*-elements are marked with underlines with signs as follows: ∗ WRKY elements; ▲ pathogen related element; ◆ auxin related element; △ cytokinin related element ; ◎ GA related element ; ○ ethylene related element ; ● ABA related element.

Fig.6 (d) The putative *cis*-element at 5' upstream of *LePrxA*. The fruit-specific elements 'TCCAAAA' and 'TGTTACA' are shaded in grey. The sugar related elements are marked with under double lines. The other *cis*-elements are marked with underlines with signs as follows: ∗ WRKY elements; ▲ pathogen related element; ◆ auxin related element; △ cytokinin related element ; ◎ GA related element ; ○ ethylene related element ; ● ABA related element.

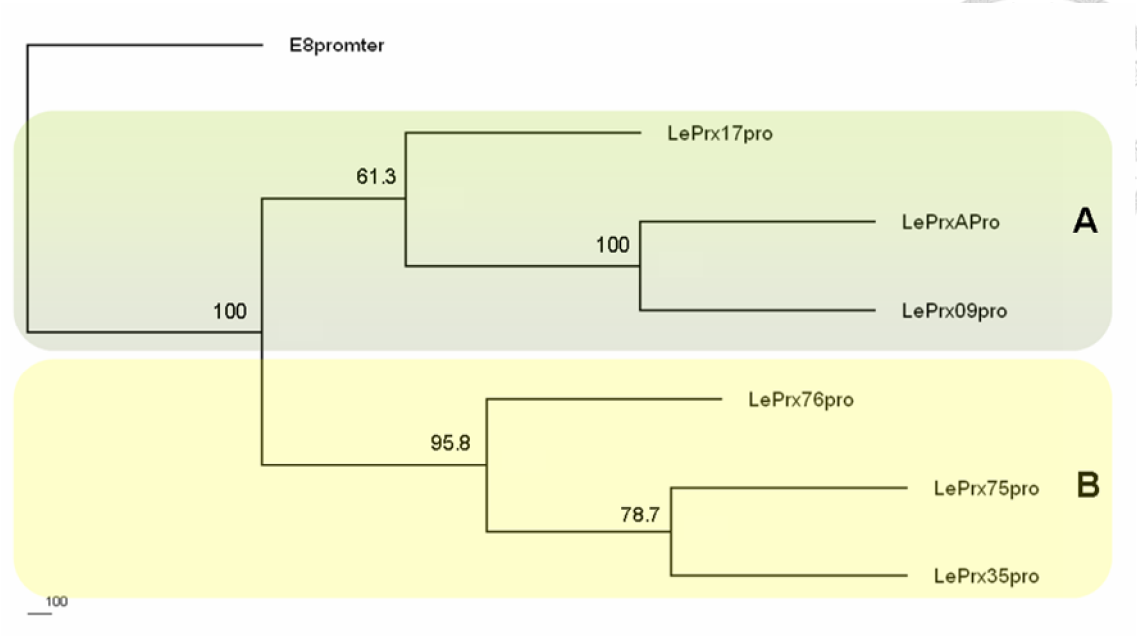


Fig. 7 Phylogenetic tree of the promoter sequences of *LePrx09*, *LePrx17*, *LePrx35*, *LePrxA*, *LePrx75* and *LePrx76*. The un-rooted tree was constructed with DNADIST and NEIGHBOR methods in the PHYLIP package. Full-length sequences of promoter were extracted from the SGN database. The fruit-specific E8 promoter from tomato was included as an outgroup in the tree. The bootstrap support values are indicated on the branches. The LePrx09pro,

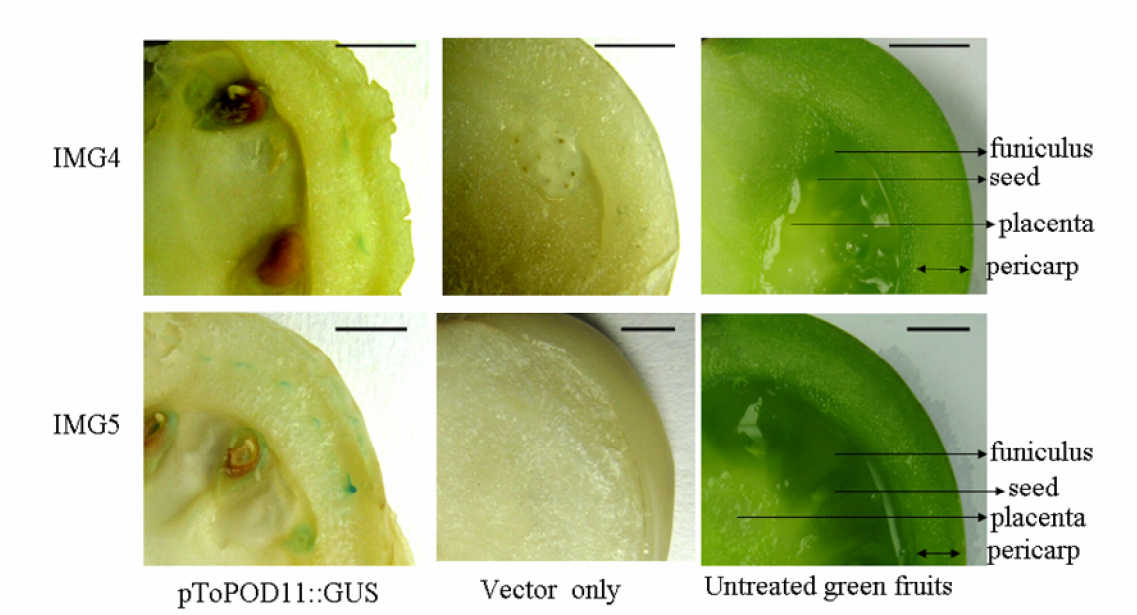


Fig. 8 Histochemical staining of GUS analysis for the slices of tomato fruits after paritcle bombardment with vector harboring pLePrx09:GUS. The length of black bar is 5mm.

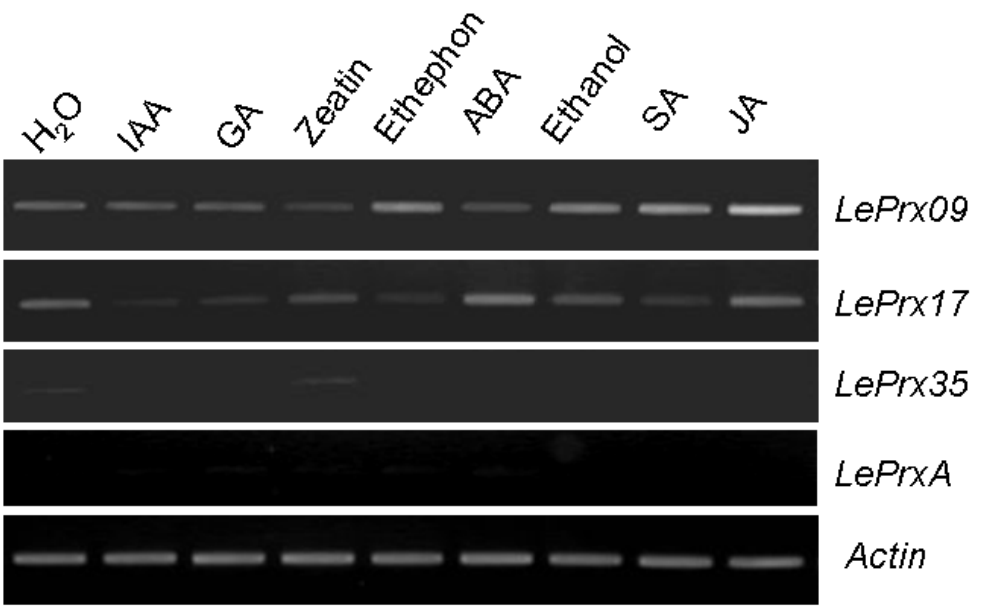


Fig. 9 Expression patterns of four *LePrx*s in immature fruits following hormone treatments. Detached IMG3 fruits were placed in solutions of various hormone (IAA, GA, zeatin, ethephon, ABA, SA and JA), at final concentration 20 μM, for 6 hours. The fruits were placed in H_2O as a control for IAA, GA, zeatin, ethephon, and ABA or 1 μM ethanol as the control for SA and JA treatmentsFor RT-PCR analysis, various number of cycles was applied for the amplification of test genes: 21 cycles for *LePrx09* and *LePrx17*; 28 cycles for *LePrx35* and *LePrxA*; and 18 cycles for the internal control, tomato actin (U60482.1).

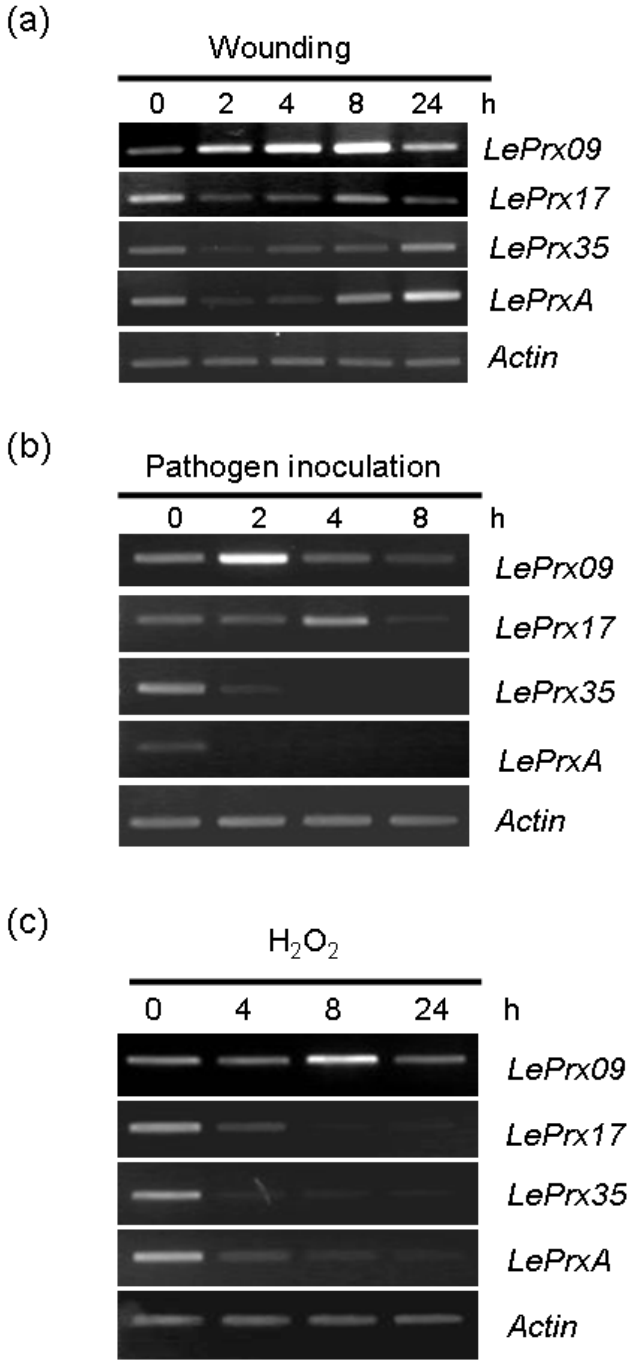


Fig. 10 Response of four *LePrxs* in immature fruits to environmental stresses. (a) The expression of four *LePr*s in tomato fruits 2 to 24 h after wounding. (b) The expression of four *LePrx*s in tomato fruits in the absence and presence of *Alternaria solani* for 2 to 8 h. (c) The expression of four *LePrx*s in tomato fruits treated with 10 μM H_2O_2 for 4 to 24 h. For RT-PCR analysis, the number of cycles was: 21 cycles for *LePrx09*; 23 cycles for *LePrx17*; 28 cycles for *LePrx35* and *LePrxA*; and 18 cycles for the internal control, tomato actin (U60482.1).

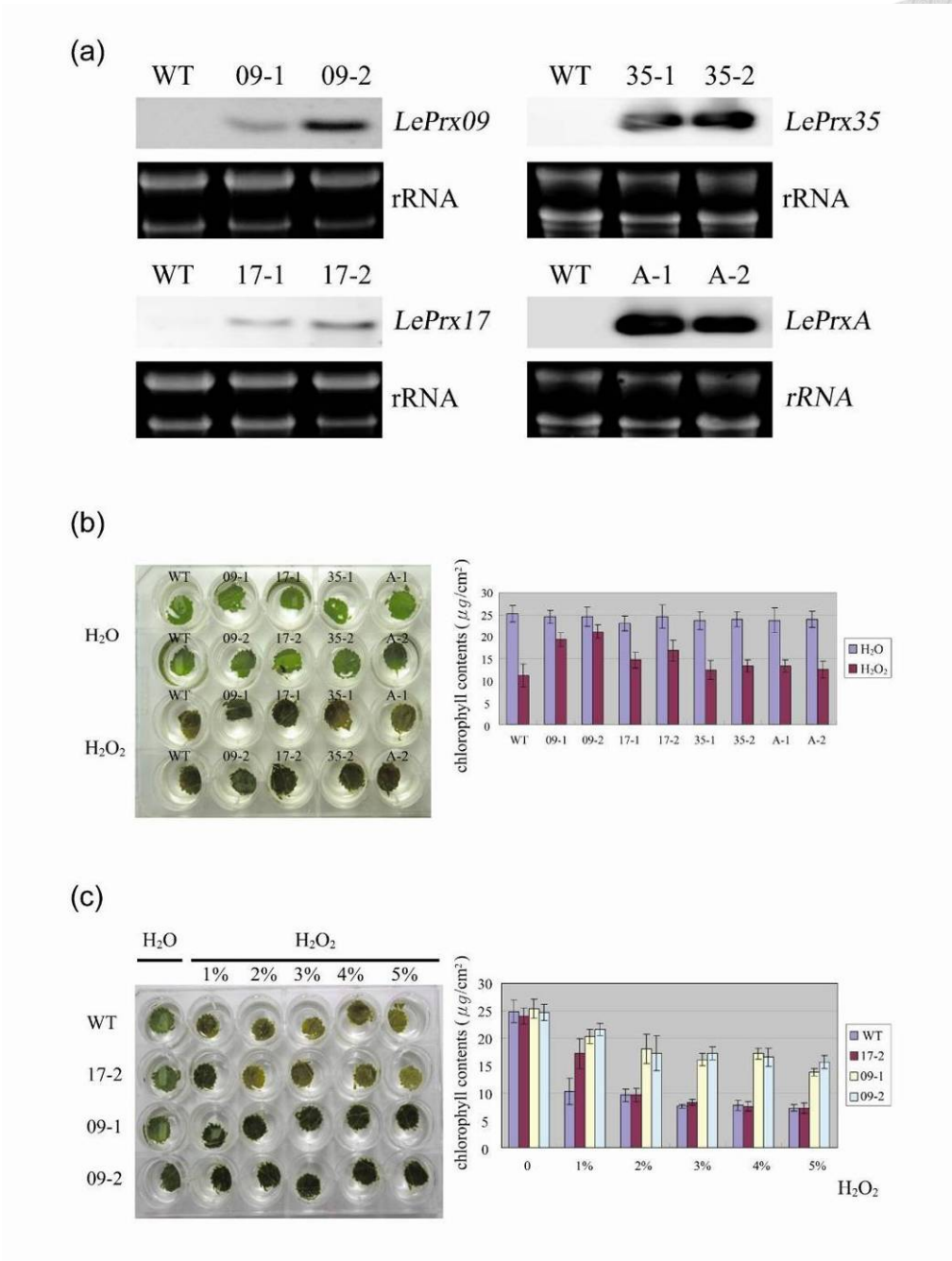


Fig. 11 High resistance of transgenic tomato plants to H_2O_2 treatments. (a) The expression of transgenes in the leaves of transgenic tomato lines. 09-1 and 09-2: *LePrx09* over-expressed lines; 17-1 and 17-2: *LePrx17* over-expressed lines; 35-1 and 35-2: *LePrx35* over-expressed lines; A-1 and A-2: *LePrxA* over-expressed lines. (b) Leaf discs of transgenic tomato lines in the absence and presence of 1 % H_2O_2 for 16 h. The bars in the middle of columns are the standard errors calculated from three replications. (c) Leaf discs of transgenic tomato plants treated with various concentrations of H_2O_2 for 16 h. The data are average of triplicate tests.The bars in the middle of columns are the standard errors calculated from three replications.

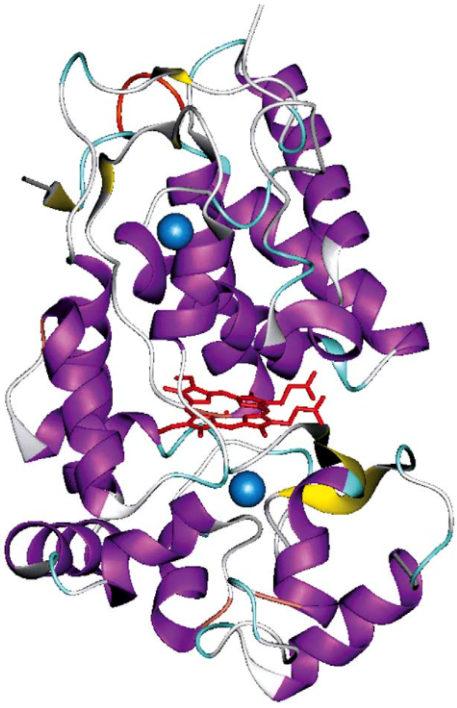


Fig. S1 Three-dimensional representation of the X-ray crystal structure of horseradish peroxidase isoenzyme C. The heme group (red color) is located between the distal and proximal domains which each contain one calcium atom (blue spheres). α-Helical and β-sheet regions of the enzyme are shown in purple and yellow, respectively. This figure is copied from Veitch (2004).

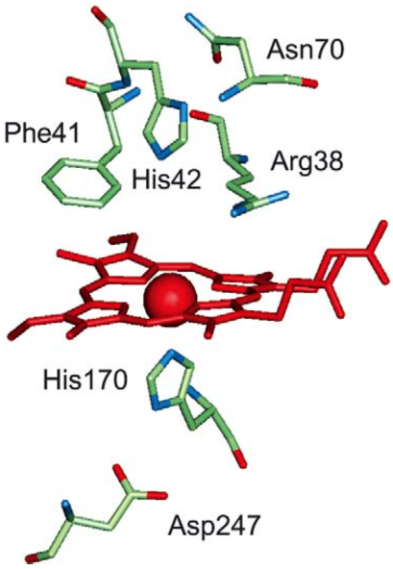


Fig. S2 Key amino acid residues in the heme-binding region of HRPC. The heme group and heme iron atom are shown in red. This figure is copied from Veitch(2004).

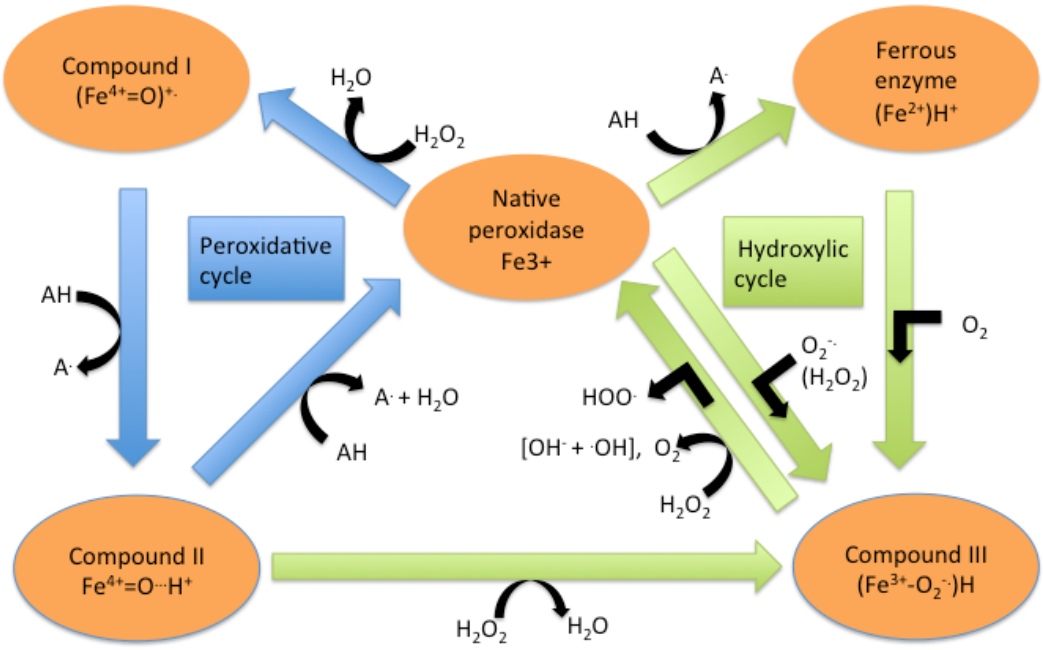


Fig. S3 Class III peroxidase reaction cycles. The hydroxylic cycle (represented in red) can generate oxygen species (ROS) such as ·OH and HOO· by different routes. The peroxidative cycle (represented in blue) can oxidize various substrates, represented by AH and A. for the reduced and oxidized forms, respectively. This figure is modified from Passardi et al. (2004).

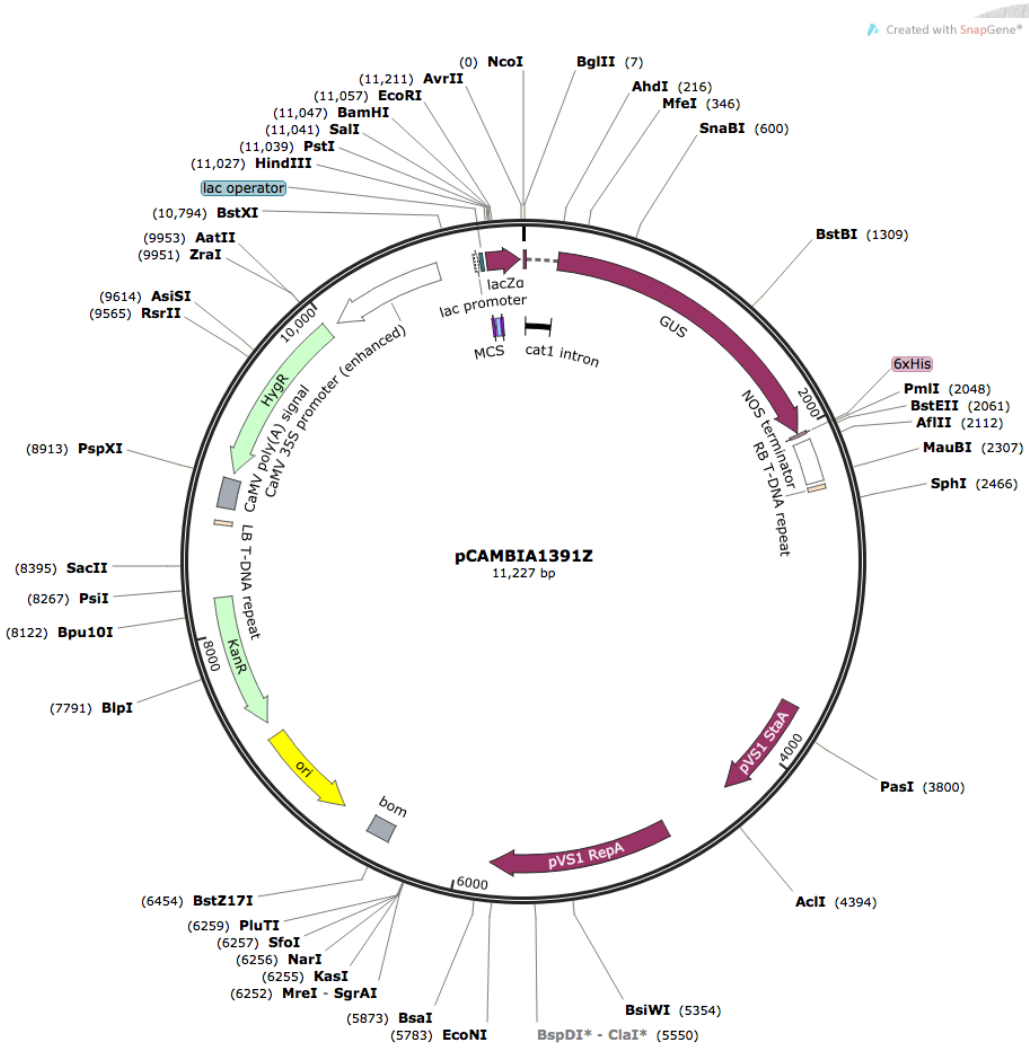


Fig. S4(a) The map of pCAMBIA 1391Z. These maps are copied from Snap Gene Website(http://www.snapgene.com/).

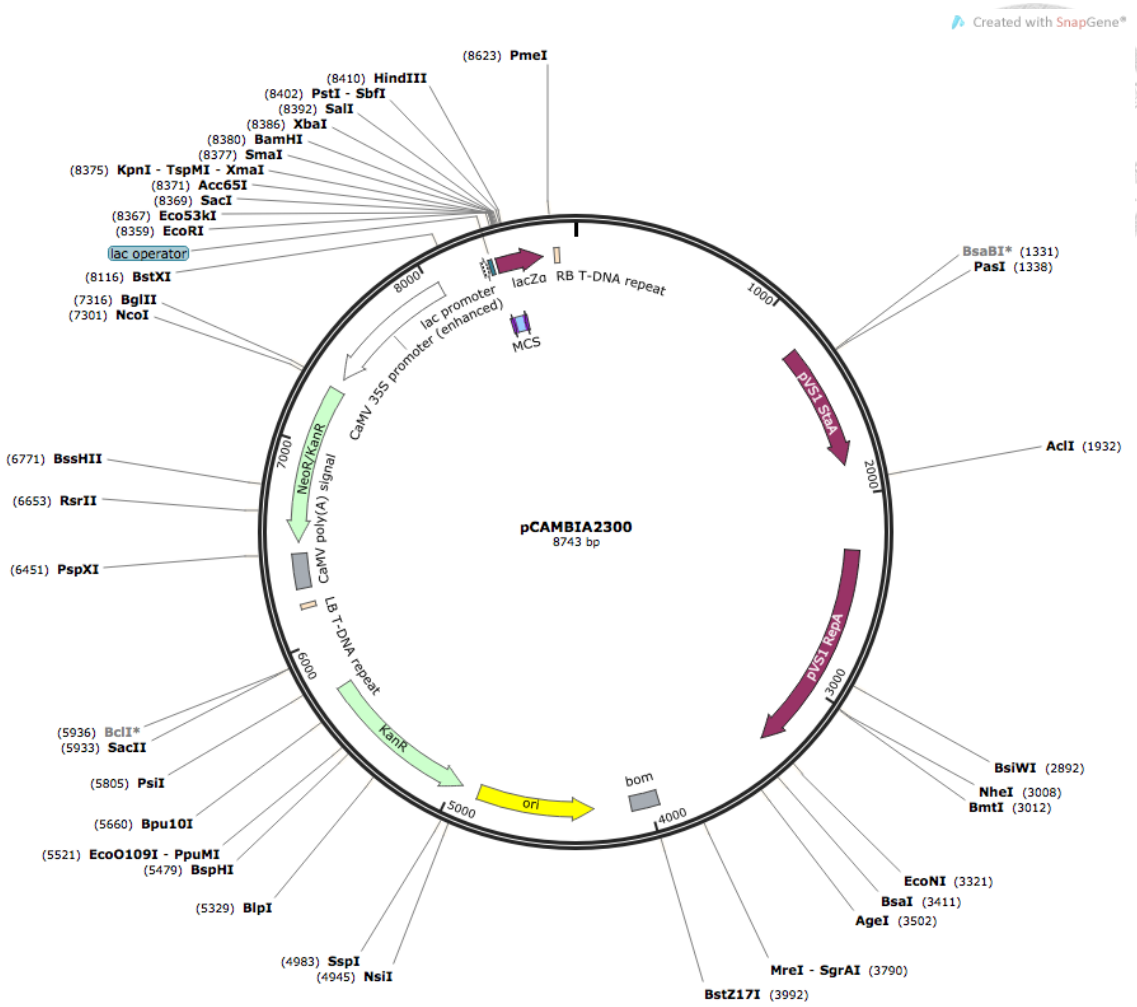


Fig. S4(b) The map of pCAMBIA 2300. These maps are copied from Snap Gene Website(http://www.snapgene.com/).

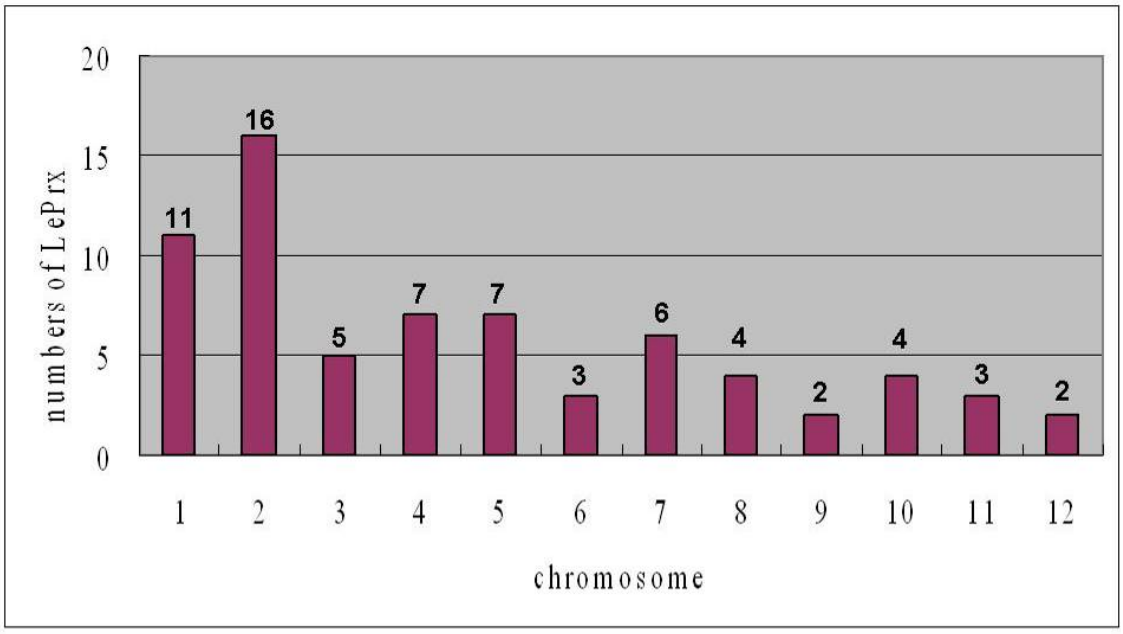


Fig. S5 The numbers of *LePrx*s located in the 12 chromosomes. This data is transferred from Table 1.

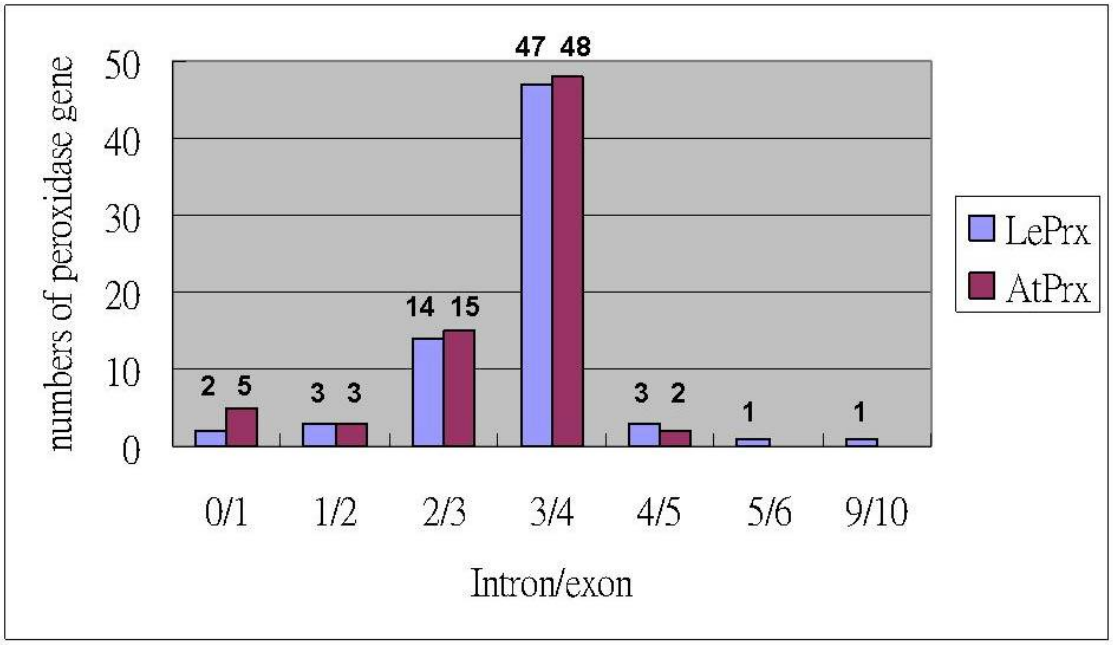


Fig. S6 The numbers of various types (intron/exon) of *LePrx*s and *AtPrx*s. This data is transferred from Table 1.

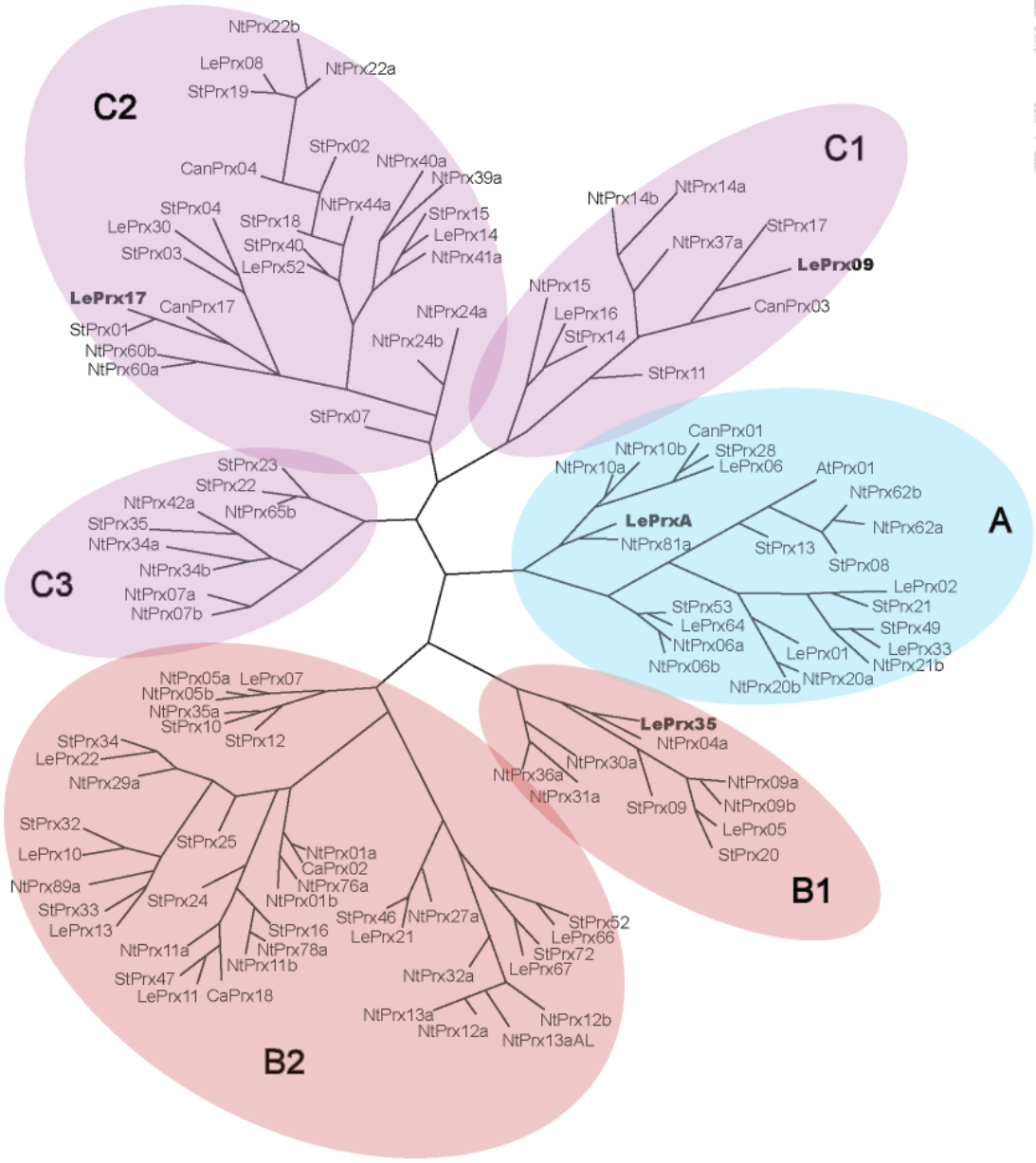


Fig. S7 Phylogenetic tree of LePrx09, LePrx17, LePrx35, LePrxA and class III peroxidases in *Solanaceae* crop species. The un-rooted tree was constructed with PRODIST and KFITCH methods in the PHYLIP package. Full-length sequences of peroxidases of tomato (Le), tobacco (Nt), potato (St) and pepper (Can) were extracted from the PeroxiBase database. *Arabidopsis thaliana* peroxidase, AtPrx01, was included as an outgroup in the tree.

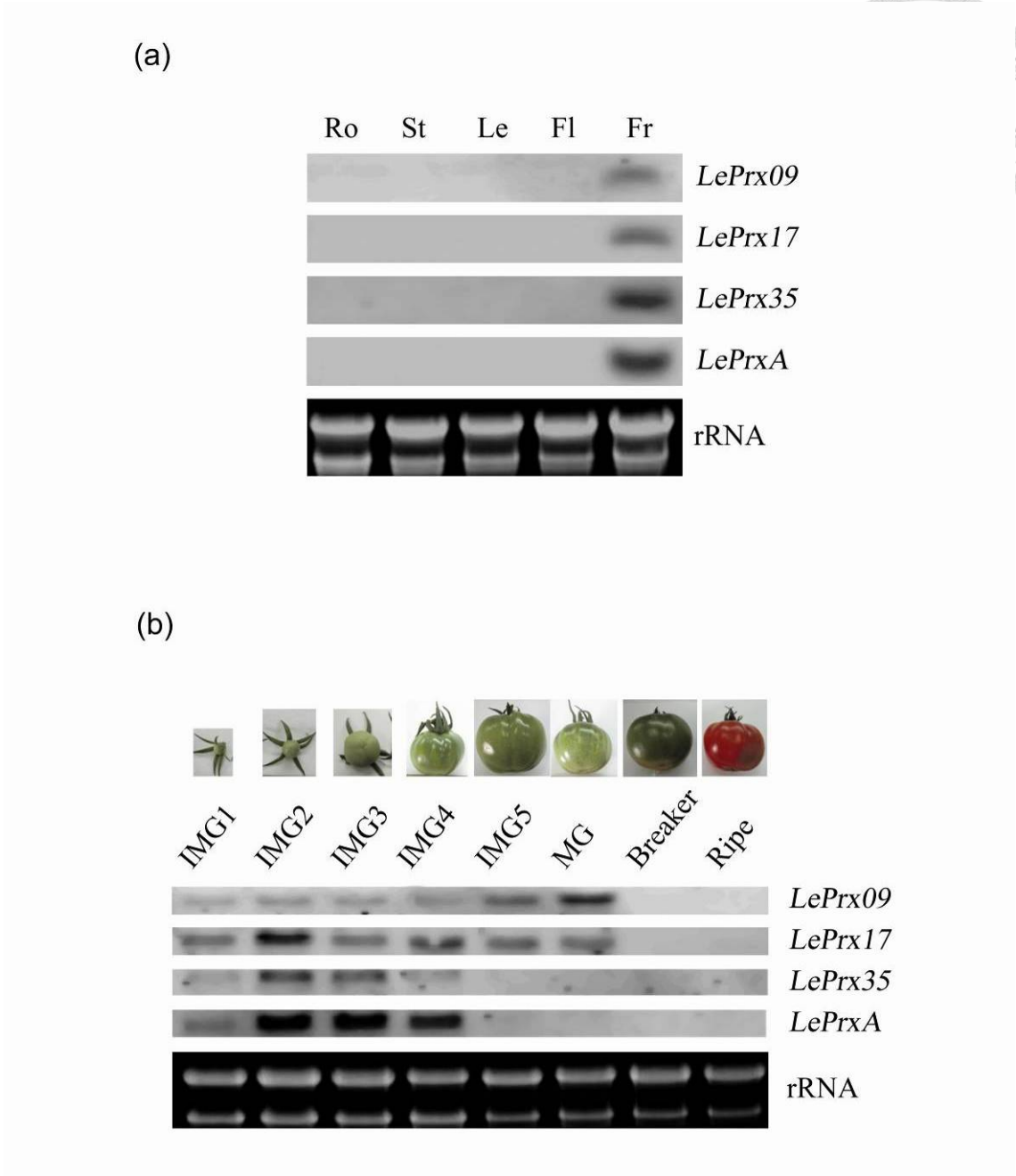


Fig. S8 Organ-specific expression patterns of the four *peroxidase* genes. (a) Four *LePrxs* displayed fruit-specific expression profiles. Ro: Root; St: Stem; Le: Leaf; Fl: Flower; Fr: Fruit. (b) Expression patterns of four *LePrxs* at various growth stages of tomato fruits. The diameters of immature fruits were: IMG1, 0.5 to 1 cm; IMG2, 1.0 to 1.5 cm; IMG3, 2.0 to 2.5 cm; IMG4, 3.5 to 4.0 cm; and IMG5, 6.0 to 7.0 cm. MG: mature green stage. Breaker stage represents tomato fruits with the top turning to red color. At the ripe stage the fruits were completely ripe.

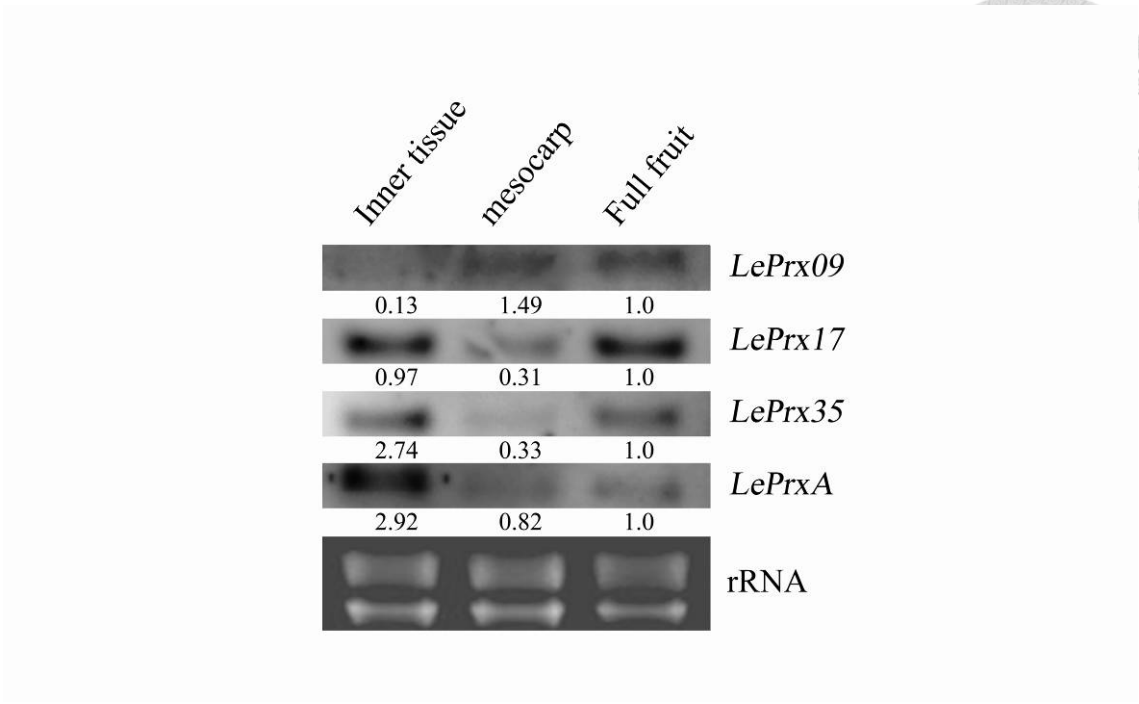


Fig. S9 Expression patterns of four *LePrxs* in the mesocarp and inner tissues of tomato green fruits.

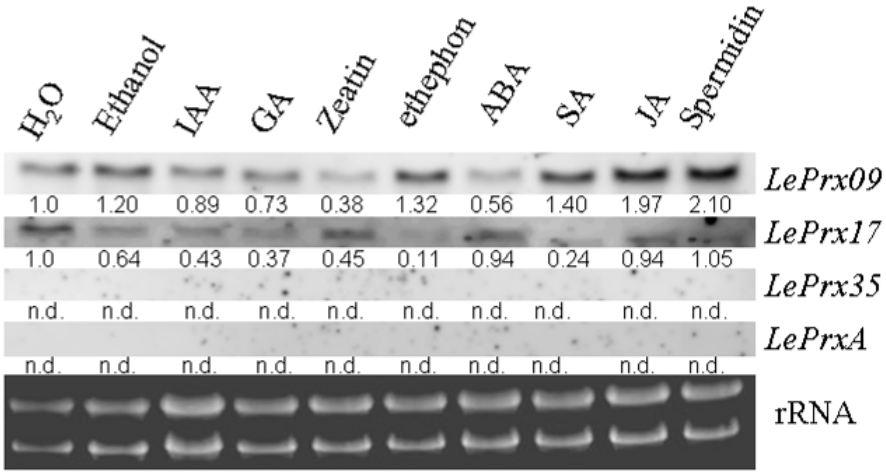


Fig. S10 Expression patterns of four *LePrx*s in immature fruits following hormone treatments. Detached IMG3 fruits were placed in solutions of various hormone (IAA, GA, zeatin, ethephon, ABA, SA and JA), at final concentration 20 μM, for 6 hours. The fruits were placed in H_2O as a control for IAA, GA, zeatin, ethephon, and ABA or 1 μM ethanol as the control for SA and JA treatmentsFor RNA gel blot analysis.

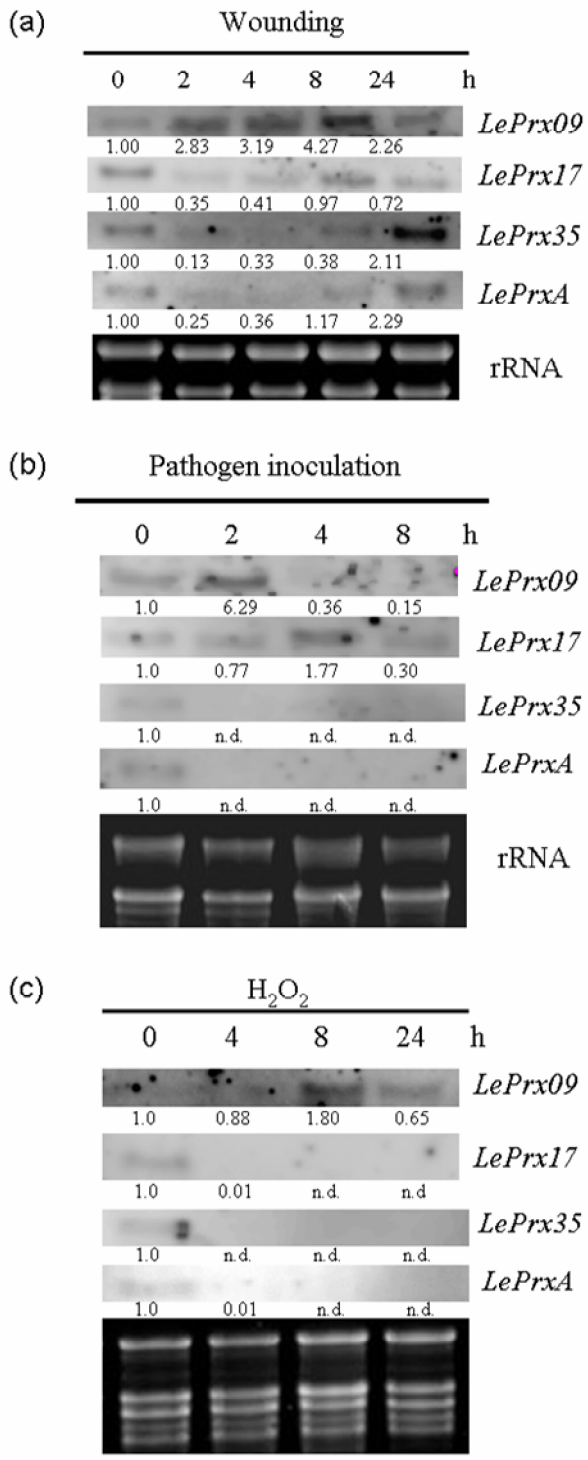


Fig. S11 Response of four *LePrxs* in immature fruits to environmental stresses. (a) The expression of four *LePrx*s in tomato fruits 2 to 24 h after wounding. (b) The expression of four *LePrx*s in tomato fruits in the absence and presence of Alternaria solani for 2 to 8 h. (c) The expression of four *LePrx*s in tomato fruits treated with 10 μM H_2O_2 for 4 to 24 h.