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ORIGINAL ARTICLE

Implication of a pepper *h*-type thioredoxin in type I- and II-nonhost resistance to *Xanthomonas axonopodis*

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Abstract Thioredoxins (TRXs) are distributed ubiquitously in prokaryotic and eukaryotic organisms. Plants have the most complex forms of TRXs. The functional roles of such TRXs have been studied in abiotic stress but their roles in plant defense responses against biotic stresses have been less well studied. Here, we identified an h-type *TRX* gene from pepper, *CaTRXh1*, and characterized its possible effect on Type II nonhost resistance, which entails localized programmed cell death in response to nonhost pathogens. Peptide sequences of CaTRXh1 showed a high degree of similarity with TRXhs from tobacco and *Arabidopsis thaliana*. Southern blot analyses revealed that *CaTRXh1* was present as a single copy in the pepper genome. Intriguingly, leaf infiltration by *Xanthomonas axonopodis* pv. *glycines* 8ra, eliciting a visible type II nonhost hypersensitive response (HR), and its type III secretion-system null mutant 8–13, eliciting a type I nonhost non-HR, both induced *CaTRXh1* at a level similar to that of pathogenesis-related protein 4, an HR marker gene in pepper. More surprisingly, expression of *CaTRXh1* was significantly increased when *X. axonopodis* pv. *vesicatoria* race 3 infiltrated the leaf of a pepper cultivar containing a resistance gene, but not with infiltration of a susceptible pepper cultivar. Taken together, our study suggests that the expression of *CaTRXh1* has a critical role in HR-mediated active defense responses in pepper.

Keywords Biotic stresses · Hypersensitive response · Pathogen-associated molecular patterns · Plant defense responses · *Xanthomonas axonopodis*

GenBank accession number: EF371503.

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Introduction

Plants have developed several layers of defense responses to a broad spectrum of pathogen attacks (Agrios 1997). Innate immunity in plants has recently been reported to be an old and generalized defense response that includes the perception of pathogen-derived molecules, referred to as pathogen-associated molecular patterns (PAMPs) (Nürnberger et al. 2004; Zipfel et al. 2004; Zeidler et al. 2004). PAMPs consist of bacterial flagellin, elongation factor, lipopolysaccharide, etc. In addition to innate immunity, plants have more sophisticated defense mechanisms such as the ‘‘gene-for-gene’’ model. This hypersensitive response (HR) is a highly specific interaction between a plant resistance protein and a pathogen-mediated avirulent protein that results in programmed cell death to arrest pathogen growth in the infected plant tissue (Zeidler et al. 2004; Dangl et al. 1996; Goodman and Novacky 1994). The majority of plant pathogens display constricted host specificity and cannot infect nonhost species. The resistance of plants to most potential pathogens is referred to as nonhost resistance (Heath 2000; Kamoun 2001; Thordal-Christensen 2003; Nürnberger and Lipka

2005). Recently, Mysore and Ryu (2004) have hypothesized the existence of two types of nonhost resistance: type I occurs when pathogen infection does not result in visible cell death or HR, and type II involves HR (Mysore and Ryu 2004; Klement et al. 1999; Dangl et al. 1996). Extensive study of type II nonhost resistance has revealed that many elements may be involved, including active oxygen species (Oh et al. 2006; Apel and Hirt 2004; Goodman and Novacky 1994). However, the gene expression patterns and exact mechanisms underlying both response types have not yet been elucidated.

Thioredoxins (TRXs) are abundant small proteins (~12 kDa) in prokaryotic and eukaryotic cells. They act as protein disulfide oxidoreductases that oxidize two cysteine thiols at the redox active site WCXPC (Holmgren 1985; Gelhaye et al. 2005). Intriguingly, in addition to prokaryotic and animal TRXs, plant genomes encode a large family of TRXs (Holmgren 1985). For example, the *Arabidopsis* genome contains at least 46 *TRXs* (for review, see Meyer et al. 2002). These TRXs range in size from 100 to 578 amino acids, with localization sites as diverse as the plastid, cytosol, nucleus, and mitochondria. The exact function of these TRXs remains unknown. TRXf and m are involved in the regulation of carbon metabolism (Ruelland and Miginiac-Maslow 1999). Plastidal TRXy decreases the expression of 2-cys peroxiredoxins A and B, but does not increase the expression of fructose-1,6-biphosphatase or malate dehydrogenase. TRXy2 has been detected in green leaves, whereas TRXy1 localizes to the root, indicating that TRX does not function only in the plastid (Collin et al. 2003). The *Arabidopsis* TRXh family is the largest TRX family and appears to be localized to the cytosol due to the absence of distinct transit peptides (Gelhaye et al. 2004, 2005). The TRXh gene structure in both monocots and dicots is unique, with two introns at conserved positions (Sahrawy et al. 1996). Recently, the TRXh family has been divided into three subgroups based on their localization and gene structure: Sub1h (h1, h3, h4, and h5), Sub2h (h2, h7, and h8), and Sub3h (h9 and h10) (Gelhaye et al. 2004). Studies of the TRXh family using *gus*-promoter fusions have revealed that induction of the *AtTRXh3* and *AtTRXh5* promoters are not regulated by environmental or pathogenic elicitors, respectively (Reichheld et al. 2002; Laloi et al. 2004). Most studies of TRX have focused on *Arabidopsis* as a model system and legumes such as pea and soybean (Meyer et al. 2002; Lee et al. 2005; Traverso et al. 2007).

Here, we report the isolation of a novel pepper *TRX* gene, *CaTRXh1*, via differential display-polymerase chain reaction (DD-PCR) during a type II-nonhost response producing a visible HR, following infiltration of pepper leaves by *Xanthomonas axonopodis* . To our knowledge, this is the first report demonstrating a specific increase in *TRX* gene expression in nonhost resistance responses in pepper. Our results suggest that TRX can play an essential role in type II-nonhost resistance.

Materials and methods

Plants, pathogens, and chemical preparations

Chili peppers (*Capsicum annuum* L. cv. Bukang) were grown in a growth chamber at 25°C under a 16:8 h light:dark photo-cycle. Healthy leaves from about 1-month-old plants were used for treatment and nucleic acid extraction. Roots, stems, leaves, flowers, seeds, germinating seeds, and seedlings were prepared from healthy pepper plants and frozen immediately in liquid nitrogen for tissue RNA blot analysis. For the pathogen challenge, the incompatible bacterial pathogen *Xanthomonas axonopodis* pv. *glycines* 8ra and its type III-secretion-system null mutant 8–13 (*Xag*; of OD_{600} = 0.4 in 10 mM $MgCl_2$) were pressure-infiltrated into pepper leaves with a needle-less syringe, as described previously (Park and Hwang 1999; Suh et al. 2001). For the salicylic acid (SA) treatment, 5 mM of SA was applied by spraying whole plants. The leaves were harvested at the indicated times and frozen immediately in liquid nitrogen for total RNA extraction. Intact pepper leaves were used for the non-stress treatment (Suh et al. 2001).

Isolation and sequencing of *CaTRXh1*

In order to better understand the molecular and cellular defense mechanisms that result from the interaction between the plant pathogen and its nonhost during the HR, we inoculated hot pepper (*Capsicum annuum* cv. Bukang) leaves with the soybean pustule pathogen, *Xanthomonas campestris* pv. *glycines* 8ra. A pool of genes that were induced or repressed by infection with this pathogen had been isolated previously using a DD-PCR technique (Yi et al. 2004). Approximately one-half of the isolated genes had no sequence similarities in existing databases, and the majority of the identified DNA fragments encoded enzymes involved in primary or secondary metabolic pathways. One of the fragments was found to have significant sequence homology with *CaTRXh1* from diverse plant species. Pepper cDNA libraries were constructed from seven different plant tissues (leaves infiltrated with *Xag* suspension cells, main and axillary roots, flowers, fruit, placentas, and anthers), and the cDNAs were amplified in *E. coli* after in vivo excision, as previously described (Yi et al. 2004). The 5′ partial nucleotide sequences and deduced polypeptides obtained are given in the chili pepper EST database (http://www.genepool.kribb.re.kr). The chili

pepper EST cDNA database was searched to identify the annotated *CaTRXh1* cDNA clones (GenBank accession number: EF371503). *CaTRXh1* cDNAs in pBluescript SK(-) were sequenced with T7 primer to determine the full-length cDNA sequences.

Genomic DNA blot analysis

Genomic DNA from pepper plants was prepared as described previously (Yi et al. 2004). Total DNA (20 μg) was digested with *Eco*R I or *Xba* I. The digested DNAs were separated by size on a 0.8% (*w*/*v*) agarose gel. Southern transfer was carried out according to the standard method (Sambrook et al. 1989). Probe labeling, blot hybridization, and washing conditions were as previously described (Choi et al. 1996; Yi et al. 2004). The membranes were exposed to an imaging plate and scanned using BAS-1800 (Fujifilm, Japan).

Isolation of total RNA and Northern blot analysis

To investigate the expression kinetics of the *CaTRXh1* transcripts during inoculation with *X. axonopodis* pv. *glycines* 8ra and 8–13 and *X. axonopodis* pv. *vesicatoria* race 1 and race 3, total RNAs were isolated from hot pepper leaves at 0, 4, 8, and 12 h after inoculation, and Northern blot analysis was performed using ^{32}P-labeled *CaTRXh1* as the probe, as previously described (Yi et al. 2004). HR cell death in pepper leaves appeared at approximately 15 h post-infiltration, whereas no visible symptoms were detected from leaves infiltrated with buffer (0.9% NaCl). A pathogenesis-related protein 4 (PR-4) probe was included as a positive marker for pathogen inoculation. The experiment was repeated three times with similar results.

Results and discussion

Sequence analysis of *CaTRXh1*

In an attempt at molecular characterization of *CaTRXh1* in pepper plants, a search of the pepper EST database (http://www.genepool.kribb.re.kr) was conducted for pepper homologues of any of the known *CaTRXh1* sequences. Full-length cDNA clones of pepper *TRX* were identified and designated as *CaTRXh1*. The deduced amino-acid sequences for CaTRXh1 from the pepper in this study and other plant species were aligned and compared (Figs. 1, 2a).

The polypeptide encoded by *CaTRXh1* contained 124 amino-acid residues with a calculated molecular mass of 14 kDa and an isoelectric point (pI) of 5.01. The CaTRXh1 protein sequence showed a high degree of similarity to TRXh1 proteins from other plant species, due to the presence of highly conserved domains (Fig. 2a). Phylogenetic analysis was carried out with the deduced amino-acid sequences from *CaTRXh1* and eight *TRXh* family members from tobacco, *A. thaliana*, and the Brassicaea (Table 1). The pepper TRXh sequence was most similar to the tobacco TRXh, NtTRXh1 (86%). The sequence identities between CaTRXh1 and *Arabidopsis* TRXh1, 2, 3, 4, 5, 7, 8, and 9 are were 64, 49, 61, 59, 56, 41, 42, and 41%,

Fig. 1 Nucleotide and deduced amino-acid sequences of *CaTRXh1*

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1    GGCACGGGATTTGTTTGGTGTTTTCTGTTTTTTGAATTTCGAATTTTCCGAAAATCTGTG

61   AGAATGGCTGCTACTTCATCTGAAGAAGGACAAGTTTTTGGTTGCCACAACGTTGAGGAG
        M  A  A  T  S  S  E  E  G  Q  V  F  G  C  H  N  V  E  E   19

121  TGGGATCAGCACTTCAAGAAGGGTGTCGAGACTAAGAAATTGGTGGTGGTGGATTTTACC
      W  D  Q  H  F  K  K  G  V  E  T  K  K  L  V  V  V  D  F  T   39

181  GCATCCTGGTGCGGTCCTTGCCGTTTTATTGCCCCAATTCTTGCTGACATTGCTAAGAAG
      A  S  W  C  G  P  C  R  F  I  A  P  I  L  A  D  I  A  K  K   59

241  ATGCCCCATGTCATATTCCTCAAGGTTGATGTTGATGAACTAAAGACTGTTGCAGAGGAA
      M  P  H  V  I  F  L  K  V  D  V  D  E  L  K  T  V  A  E  E   79

301  TGGAATGTGGATGCTATGCCAACATTTGTCTTCTTTAAAGATGGCGAAGAAGTGGATAGG
      W  N  V  D  A  M  P  T  F  V  F  F  K  D  G  E  E  V  D  R   99

361  GTTGTTGGTGCCCAGAAAGAGGAGTTGCAGGCGGCCATACTTAAGCATGTTGGTGCTCCT
      V  V  G  A  Q  K  E  E  L  Q  A  A  I  L  K  H  V  G  A  P   119

421  GCTACTGTGACAGCTTGAGCCATAATCAAGGATATGATATTCTGGTGTTTAGTATTGTCC
      A  T  V  T  A  *                                            124

481  TTTTGTAATAAGTCTGGATGGCTGTGTGTTTTATTATCCACTATCTCTCTCTTGTTTTTG

541  TGAACCCTTTTGGTGCTTGAATTCTGATATTTGTGCAGATGGCCTTACTGGTGTAAGGTT

601  ATATGCTCATTTCTATGAAAAAAAAAAA
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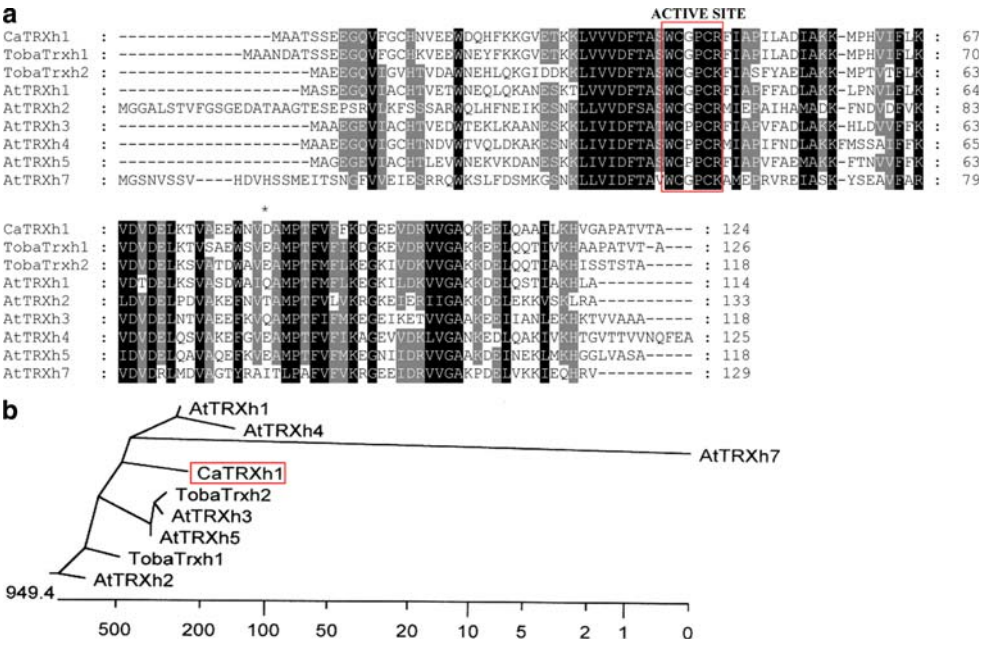

Fig. 2 Alignment of deduced amino-acid sequences and phylogenetic tree analysis of TRXh sequences from plant species. **a** Alignment of TRXh sequences was performed using the T-Coffee program (Notredame et al. 2000). *Overlines* indicate the consensus tetramerization and dimerization domains. *Black boxes* denote identical amino acids and *grey boxes* denote highly conserved amino acids. *Dashes* indicate gaps in the sequences to allow for maximal alignment. The active site is indicated. **b** The phylogenetic tree was constructed as described for Fig. 2a

Table 1 Amino-acid sequence identities of CaTRXh1 and plant h-type thioredoxin family members

Plant TRX	Identity with CaTRXh1 (%)
NtTRXh1	86
AtTRXh1	64
NtTRXh2	62
AtTRXh3	61
AtTRXh4	59
BraTHL2	58
BraTHL1	57
AtTRXh5	56
AtTRXh2	49
AtTRXh8	42
AtTRXh7	41
AtTRXh9	41

CLUSTAL W software was used to align *A. thaliana* AtTRXh1 (P29448), AtTRXh2 (S58123), AtTRXh3 (S58118), AtTRXh4 (S58119), AtTRXh5 (S58120), AtTRXh7 (AAD39316), AtTRXh8 (AAG52561), and AtTRXh9 (AAG51342) and *Nicotiana tabacum* NtTRXh1 (U59379) and NtTRXh2 (Z11803)

respectively. Cabbage BraTHL1 and BraTHL2 shared 57 and 58% identity, respectively, with CaTRXh1 (Table 1). The deduced amino-acid sequences contained the typical TRXh active-site sequence WCG/PPCR/K (Fig. 2a). Phylogenic analysis revealed that CaTRXh1 was in the TRXh subgroup Sub1h with *Arabidopsis* TRXh1, 3, 4, and 5 (Fig. 2b) (Meyer et al. 2002). Sahrawy et al. (1996) have suggested that the *TRXh* gene family includes two intron markers in the thioredoxin domain. However, the genomic structure of *CaTRXh1* is not currently available.

Southern blotting and tissue-specific expression of *CaTRXh1*

Genomic DNA gel-blot analyses were performed to determine the copy number of *CaTRXh1* in the pepper genome (Fig. 3a). Hybridization with the full-length *CaTRXh1* cDNA yielded a single band (Fig. 3a, left lane). Since digestion with *Eco*R 1 yielded two bands, the Southern blot was re-assessed with a specific *CaTRXh1* 3′-end probe. With this probe, a single band was observed with either *Eco* RI- or *Xba* I-digested DNA (Fig. 3a). These results indicate that *CaTRXh1* is present as a single copy in the pepper genome.

Tissue-specific Northern blot analyses were carried out to determine whether *CaTRXh1* is developmentally regulated (Fig. 3b). *CaTRXh1* had low expression in the root, but was more highly expressed in the leaf and seedling. The highest expression of *CaTRXh1* was detected in the stem, flower, seed, and germinating seed (Fig. 3b). Taken together, these results indicate that the expression of *CaTRXh1*mRNA is developmentally regulated in pepper plants and that the expression patterns are distinct in different tissues. Using eight *Arabidopsis TRXh* promoters in *gus*-fusion constructs, Reichheld et al. (2002) reported that the expression patterns of all *AtTRXh* genes, except *AtTRXh8*, were the same in almost all tissues. In tobacco, the *NtTRXh1* and *NtTRXh2* genes were expressed only in dividing cells (Marty and Meyer 1991). In contrast, *CaTRXh1* mRNA from pepper, which, like tobacco, is in the Solanaceae family, was detected in all examined organs. In pea, *PsTRXh1* gene expression was greater in leaf tissue than in stem, root, flower, or seed (Traverso et al. 2007).

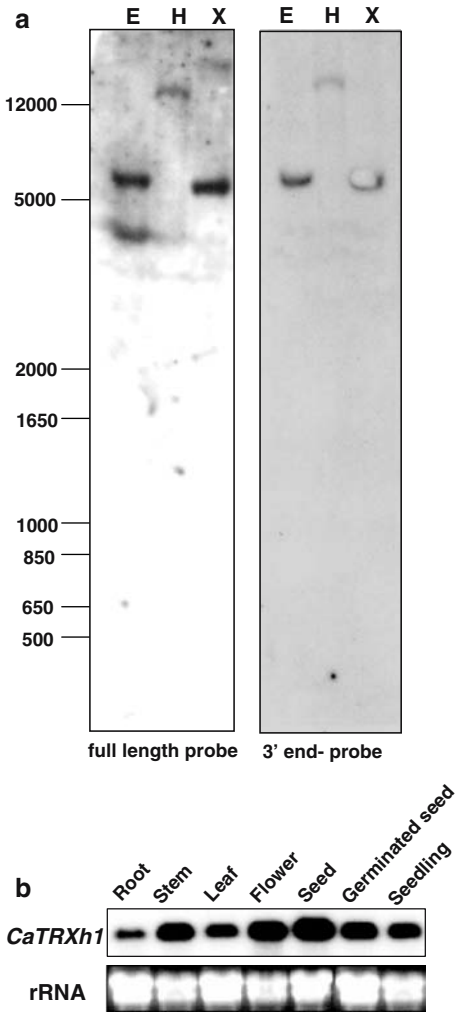

Fig. 3 Genomic DNA gel blot analyses of *CaTRXh1* and RNA expression of *CaTRXh1* in the different tissues. **a** Genomic DNA (20 μg) purified from chili pepper was digested with *Eco*R I (E) or *Xba* I (X), separated on an 0.8% (*w/v*) agarose gel, and hybridized with ^{32}P-labeled probes corresponding to the full-length *CaTRXh1* cDNA and the 3′-end, as described in the Materials and methods. **b** *CaTRXh1* RNA levels were monitored in the pepper root, stem, leaf, flower, seed, geminated seed, and seedling, as described in the Materials and methods

Our results indicate that *CaTRXh1* gene expression is more similar to the *AtTRXh1* expression pattern than to that of tobacco or pea *TRXh1*s. Further characterization of *CaTRXh1* will be required to determine its protein localization in each tissue and its biochemical role in responding to abiotic stresses.

Expression patterns of *CaTRXh1* during types I- and II-nonhost resistance responses against *X. axonopodis*

The mRNA expression patterns of *CaTRXh1*genes were examined during nonhost (incompatible) pathogen responses of pepper plants (Fig. 4a). The *CaTRXh1* mRNA levels in pepper plants began to increase 8 h after inoculation with *X. axonopodis* pv. *glycines* 8ra (*Xag* 8ra), which elicited the HR on pepper leaves within 24 h after infiltration (Fig. 4a). As a positive control, *PR-4* expression was specifically induced after pathogen infection, demonstrating that the pathogen challenge was adequate (Fig. 4a). To assess whether CaTRXh1 was involved in type I-nonhost resistance, we evaluated its gene expression after inoculation with *X. axonopodis* pv. *glycines* 8–13, which is deficient in its capacity to elicit any visible lesion. No differences were observed between the expression-pattern responses of *CaTRXh1* and *PR-4* to *Xag* 8ra and 8–13 (Fig. 4a).

To analyze whether the induction of *CaTRXh1* mRNA was specific to incompatible plant–microbe interactions, we inoculated two nearly isogenic pepper lines, *C. annuum* cv. ECW2DR (*BS2*) and *C. annuum* cv. ECW (*bs2*) with a natural pepper pathogen *X. axonopodis* pv. *vesicatoria* race3 (*avrBS2*). A large accumulation of *CaTRXh1* transcript was detected in the incompatible (*C. annuum* cv. ECW2DR) interaction, but not in the compatible (*C. annuum* cv. ECW) interaction (Fig. 4a, right panel). Likewise, a high level of *PR-4* mRNA was detected only in the incompatible interaction. In both cases, the induced transcripts were detected within 4 h of pathogen infiltration and were associated with the HR in pepper.

Northern blot analyses were also carried out to determine whether the application of the defense-inducing chemicals, methyl jasmonate (MJ), ABA, SA, and ethylene (ET) affected *CaTRXh1* mRNA expression levels in the pepper plants (Fig. 4b). Infiltration with SA, MJ, and ET gradually increased the expression of *CaTRXh1*. No change was detected with ABA application in pepper. *CaTRXh1* mRNA expression was slightly increased by ABA. Thus, *CaTRXh1* expression was unaffected by inoculation with a nonhost pathogen or by application of SA in pepper.

Of the eight *AtTRXh*s, only the *AtTRXh5* gene can be implicated in resistance against biotic stress. *AtTRXh5* gene expression was up-regulated after inoculation with a bacterial pathogen, *Pseudomonas syringae*, and a flagellin-derived peptide, Flg22; whereas the expression level of its paralogue, *AtTRXh3*, remained unchanged (Reichheld et al. 2002). Further study of the role of *AtTRXh5* indicated that the gene was induced by infection with type II-nonhost pathogens before the appearance of a visible HR phenotype (Fig. 4). The transcription factor *WRKY6* also regulated the *AtTRXh5*-mediated defense response against flg22 and oxidative stress (Nürnberger and Lipka 2005). Our sequence comparisons of *CaTRXh1* and *AtTRXh* family members showed that *AtTRXh1*, *3*, and *4* sequences are more homologous than other *AtTRXh* genes including *AtTRXh5*. To date, no report has associated TRXh1 with biotic stress. To our knowledge, this is the first report of a

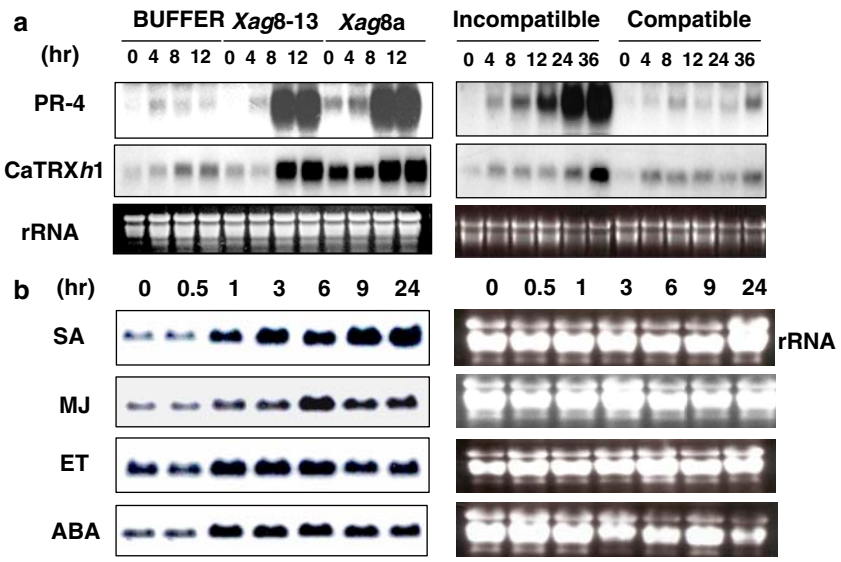

Fig. 4 Expression of *CaTRXh1* after treatments with an incompatible bacterial pathogen and chemical inducers. **a** Pepper leaves (8-week-old) were inoculated by syringe-infiltration with buffer (0.9% NaCl) or *X. campestris* pv. *glycines* 8ra (5×10^7 CFU/ml) or 8–13 suspension cells and harvested at the indicated times. The resistant *C. annuum* cv. ECW2DR (*BS2*) (Incompatible) and susceptible *C. annuum* cv. ECW (*bs2*) (Compatible) cultivars were inoculated by syringe-infiltration with *X. campestris* pv. *vesicatoria* race3 (avrBS2, 5×10^7 CFU/ml) and the inoculated leaf tissues were harvested at the indicated time points. An equal amount (20 μg) of total RNA from each sample was loaded in each lane. Ethidium-bromide staining of the gel prior to transfer indicated equal loading of the RNA. **b** Whole plants were sprayed with stock solutions of 5 mM SA and prepared for RNA sampling at the indicated times. RNA blot analyses were performed as described in the Materials and methods

TRXh1 gene that is expressed specifically in response to an incompatible (nonhost) pathogen, but not in response to a compatible pathogen. Our results using the type III-secretion-system null mutant of *X. axonopodis* pv. *glycinea* indicate that the PAMP-elicited plant innate–immunity–signaling pathway can be upstream of the CaTRXh1-related defense mechanism (Fig. 4a) (Mysore and Ryu 2004). The specific role of CaTRXh1 remains unknown. Furthermore, the question of whether *CaTRXh1* is unregulated or down-regulated during the HR remains to be answered. The interaction between tomato Cf-9 and Avr9 from *Cladosporium fulvum* is a good example of the gene-for-gene hypothesis between plants and fungi. Cf-9-interacting thioredoxin (CITRX), that was identified through a yeast two-hybrid screen, negatively regulates the HR following Cf-9/Avr9 interaction (Rivas and Thomas 2005; Nekrasov et al. 2006). We also cannot rule out the possibility that CaTRXh1 may be a negative regulator in type II-nonhost resistance-related HR formation.

In conclusion, we have identified and studied the expression pattern of a pepper CaTRXh1 that is specifically elicited during the nonhost resistance response to the bacterial pathogen *X. axonopodis*. Comparison analysis based on amino-acid sequences and Northern blot analysis shows that CaTRXh1 has similar traits to *Arabidopsis* AtTRXh5 and indicates that CaTRXh1 has a unique role among the TRXs of Solanaceae plants in the response to attack by bacterial pathogens. In the future, we will employ RNAi or virus-induced gene-silencing techniques to examine the detailed role of CaTRXh1 in disease resistance and to address whether CaTRXh1 confers resistance against type I and II pathogens.

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