

CITRX thioredoxin interacts with the tomato Cf-9 resistance protein and negatively regulates defence

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To identify proteins involved in tomato Cf-9 resistance protein function, a yeast two-hybrid screen was undertaken using the cytoplasmic C-terminus of Cf-9 as bait. A thioredoxin-homologous clone, interacting specifically with Cf-9, was identified and called *CITRX* (Cf-9-interacting thioredoxin). Virus-induced gene silencing (VIGS) of *CITRX* resulted in an accelerated Cf-9/Avr9-triggered hypersensitive response in both tomato and *Nicotiana benthamiana*, accompanied by enhanced accumulation of reactive oxygen species, alteration of protein kinase activity and induction of defence-related genes. VIGS of *CITRX* also conferred increased resistance to the fungal pathogen *Cladosporium fulvum* in the otherwise susceptible Cf0 tomato. *CITRX* acts as a negative regulator of the cell death and defence responses induced through Cf-9, but not Cf-2. Recognition of the Cf-9 C-terminus by *CITRX* is necessary and sufficient for this negative regulation. This is the first study that implicates thioredoxin activity in the regulation of plant disease resistance.

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Introduction

In the study of plant innate immunity, a major goal is to determine how plants perceive and respond to pathogens. Resistance (*R*) genes encode essential components of the plant surveillance system. The major class of *R* genes encodes predicted cytoplasmic proteins, with leucine-rich repeat

(LRR) motifs and a nucleotide-binding domain (NB). This structural conservation indicates that disease resistance to diverse pathogens may operate through conserved mechanisms (Dangl and Jones, 2001; Bonas and Lahaye, 2002). In gene-for-gene interactions, *R* proteins mediate recognition of a pathogen harbouring the corresponding avirulence gene. The specificity of this recognition has led to the idea that *R* proteins evolved as receptors for pathogen-derived molecules (Staskawicz *et al.*, 1995; Ellis *et al.*, 2000). However, despite extensive research, direct physical interaction in an *R*/Avr combination has proven to be the exception rather than the rule (Jia *et al.*, 2000; Deslandes *et al.*, 2003), and experimental evidence suggests that an indirect mode of pathogen recognition is more prevalent (Dangl and Jones, 2001; Mackey *et al.*, 2002; Axtell and Staskawicz, 2003). Although many *R* genes have been cloned, the molecular mechanisms by which *R* proteins trigger resistance remain poorly understood. Therefore, it is essential to identify the cellular components that interact with *R* proteins to mediate signal perception and transduction.

An important class of *R* genes encodes receptor-like proteins (RLPs), type I transmembrane proteins with extracellular LRRs and a short cytoplasmic region. The tomato Cf-9 gene confers resistance to races of the fungal pathogen *Cladosporium fulvum* that carry *Avr9*, encodes an RLP, and confers a hypersensitive response (HR) to *Avr9* peptide in tomato or tobacco (Hammond-Kosack and Jones, 1997). Many Cf-9/Avr9-dependent early events, such as production of reactive oxygen species (ROS; Piedras *et al.*, 1998), activation of mitogen-activated protein kinases (MAPKs; Romeis *et al.*, 1999) and calcium-dependent protein kinases (CDPKs; Romeis *et al.*, 2000a, 2001) and induction of defence-related genes (Durrant *et al.*, 2000), have been described (Romeis *et al.*, 2000b; Rivas and Thomas, 2002).

Cf-9 probably mediates *Avr9* recognition through association with additional proteins. Cf-9 lacks any obvious domain suitable for signal transduction, and association with other proteins could provide this function (Joosten and De Wit, 1999; Rivas and Thomas, 2002). Also, no direct interaction was found between Cf-9 and *Avr9*, suggesting the involvement of the high-affinity binding site for *Avr9*, detected in the membranes of solanaceous plants (Luderer *et al.*, 2001). Cf-9 behaves as if in a membrane-associated protein complex of ~420 kDa (Rivas *et al.*, 2002b). However, although the large apparent mass of this complex cannot be ascribed to association of Cf-9 with additional high-molecular-weight proteins (Van der Hoorn *et al.*, 2003), interaction of Cf-9 with small proteins (<70 kDa) is not ruled out.

A Cf-9-interacting protein was identified in a previous report but the significance of this finding has not yet been validated (Laurent *et al.*, 2000). To identify putative Cf-9 interactors, a yeast two-hybrid screen was carried out with the C-terminal cytoplasmic domain of Cf-9. One interacting clone was isolated encoding a protein with homology to

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thioredoxins. This Cf-9-interacting thioredoxin (CITRX) negatively regulates Cf-9/Avr9-mediated cell death and defence responses in tomato and tobacco. Furthermore, recognition of the Cf-9 C-terminus by CITRX is necessary and sufficient to bring about the regulation of the downstream signalling responses. To our knowledge, this represents the first report of the involvement of thioredoxin activity in plant disease resistance.

Results

Screening of a tomato yeast two-hybrid library

To identify proteins that interact with Cf-9, a Cf-9 C-terminal peptide with the last 33 amino acids was used as bait to screen a tomato prey cDNA library (Zhou *et al*, 1995). The Cf-9 bait construct alone did not activate the transcription of the reporter genes (data not shown).

An initial screening of 10⁶ clones resulted in the isolation of ~72 leucine-prototrophic colonies (data not shown). However, only one of them, which encoded a putative thioredoxin, was validated in subsequent screening steps. The interaction between CITRX and the C-terminal domain of Cf-9, detected by growth in the absence of leucine and β-galactosidase activity, was dependent on growth on galactose medium, indicating that expression of CITRX was required for the activation of the reporter genes (Figure 1). No interaction was detected between CITRX and the Cf-2 C-terminal peptide (last 37 amino acids of Cf-2) or between the Cf-9 C-terminal peptide and several C-terminal deletions of CITRX, suggesting that CITRX C-terminus is required for the interaction.

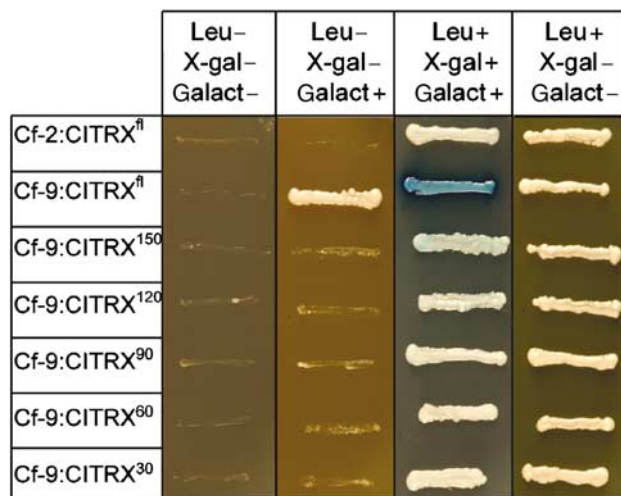


Figure 1 Interaction of Cf-9 C-terminal peptide with CITRX. The C-terminal cytoplasmic domain of Cf-9 was tested for interaction with the full-length CITRX clone (CITRX^{fl}) and several deletion mutants (CITRX¹⁵⁰, CITRX¹²⁰, CITRX⁹⁰, CITRX⁶⁰ and CITRX³⁰, amino acids M1-D150, M1-L120, M1-I90, M1-G60, M1-Y30, respectively). Interaction between the Cf-2 C-terminal domain and CITRX was also tested. Interactions between proteins were assayed after 2 days by growth on synthetic dropout plates lacking leucine (activation of the *LEU2* reporter gene; Leu-) and β-galactosidase activity, detected as blue colour in plates containing X-gal (activation of the *lacZ* reporter gene; X-gal+).

Sequence analysis of CITRX

A BLAST search (<http://www.ncbi.nlm.nih.gov/BLAST/>) with the cDNA clone isolated in the yeast two-hybrid screen identified a putative thioredoxin mRNA from *Lycopersicon esculentum* (*LeCITRX*; accession no. AF261142). Sequence analysis of this clone is shown in Figure 2A. *CITRX* encodes a protein of 175 amino acids with a predicted molecular weight of ~20 kDa and significant homology with thioredox-

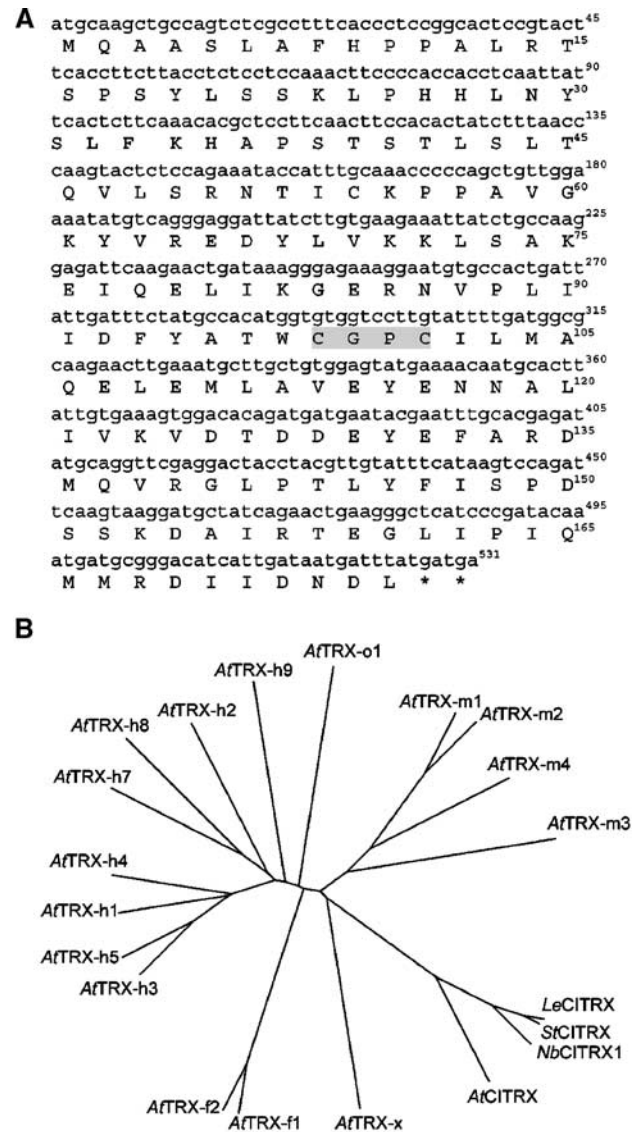


Figure 2 Sequence analysis of CITRX. (A) Nucleotide sequence and deduced amino-acid sequence of *LeCITRX* cDNA isolated from the two-hybrid library. The thioredoxin active site CGPC is shadowed. (B) Phylogenetic tree of *A. thaliana* thioredoxins and plant CITRX homologues. The ClustalW software (<http://clustalw.genome.ad.jp/>) was used to generate the phylogenetic tree of the following thioredoxins: *A. thaliana* thioredoxins *h*: AtTRX-h1 (P29448), AtTRX-h2 (S58123), AtTRX-h3 (S58118), AtTRX-h4 (S58119), AtTRX-h5 (S58120), AtTRX-h7 (AAD39316), AtTRX-h8 (AAG52561) and AtTRX-h9 (AAG51342); *A. thaliana* thioredoxins *f*: AtTRX-f1 (Q9XFH8) and AtTRX-f2 (Q9XFH9); *A. thaliana* thioredoxins *m*: AtTRX-m1 (O48737), AtTRX-m2 (AAF15949), AtTRX-m3 (AAF15950) and AtTRX-m4 (Q9SEU6); *A. thaliana* thioredoxin *x*: AtTRX-x (AAF15952); *A. thaliana* thioredoxin *o*: AtTRX-o1 (AAC12840); CITRX homologues in *A. thaliana* AtCITRX (AF370315), *L. esculentum* *LeCITRX* (AF261142), *N. benthamiana* NbCITRX1 (AY500242) and *S. tuberosum* StCITRX (TC64295).

in proteins. A consensus active site Cys-Gly-Pro-Cys, in which the flanking cysteines are involved in the reduction function (Holmgren, 1989), is present in CITRX. This active site is found in thioredoxins from *Escherichia coli* to mammals. CITRX displays thioredoxin activity *in vitro* (Supplementary Figure 1S). Analysis of *LeCITRX* sequence with the PSORT program (Nakai and Kanehisa, 1992) predicted a cytoplasmic location.

Using RACE localization of CITRX (rapid amplification of cDNA ends), we identified two independent full-length *Nicotiana benthamiana* CITRX sequences (*NbCITRX1* and *NbCITRX2*; GenBank accession nos. AY500242 and AY500243, respectively; Supplementary Figure 2S). A phylogenetic tree was built from the alignment of *LeCITRX*, and its putative orthologues in several plant species (*StCITRX*, *NbCITRX1*, *AtCITRX*), and *Arabidopsis thaliana* thioredoxins (Figure 2B). CITRX encodes a thioredoxin relatively distant from previously described thioredoxins in *A. thaliana* and only related to *AtTRX-x*, which is a plant thioredoxin of the prokaryotic type (Mestres-Ortega and Meyer, 1999).

In vitro interaction between CITRX and Cf-9 C-terminal domains

Binding between CITRX and the C-terminal domains D-G (Cf-9^{D-G}) was investigated in pull-down assays. A maltose-binding protein (MBP) fusion to Cf-9^{D-G} was purified from overexpressing bacteria and incubated with extracts containing glutathione S-transferase (GST)-CITRX. Precipitated proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and GST-CITRX was detected by immunoblot analysis using an anti-GST antibody (Figure 3A, lane 1). GST alone did not interact with MBP-Cf-9^{D-G} (Figure 3A, lane 2). We confirmed the interaction between MBP-Cf-9^{D-G} and GST-CITRX in the reverse experiment after GST-CITRX was purified and incubated with extracts containing MBP-Cf-9^{D-G}. MBP-Cf-9^{D-G} was detected by immunoblot using an anti-MBP antibody (Figure 3B, lane 1). MBP alone did not interact with GST-CITRX (Figure 3B, lane 3). No interaction between GST-CITRX and MBP-Cf-2^{D-G} was detected (Figure 3B, lanes 3 and 2), confirming the specificity of the interaction between CITRX and Cf-9 C-terminus.

Virus-induced gene silencing of CITRX in *L. esculentum*

We performed virus-induced gene silencing (VIGS) of *LeCITRX* using a silencing vector developed by Liu *et al* (2002), based on the tobacco rattle virus (TRV; Ratcliff *et al*, 2001). Silencing of the variable N-terminal region of *LeCITRX* (amino acids 1–88) resulted in a yellowish coloration of the leaves, which was helpful to score visually the efficiency of silencing (Figure 4A). At 3 weeks after silencing, plants were analysed for *LeCITRX* transcript accumulation. mRNA levels were determined by RT-PCR using gene-specific primers. In five independent experiments, *LeCITRX* expression could only be detected in untreated and control TRV:00-inoculated control plants but not, or only very weakly, in *LeCITRX*-silenced plants (Figure 4A).

To analyse the effect of *LeCITRX* silencing on the Avr9-dependent HR, Cf9 tomato plants were silenced with TRV:*LeCITRX* or TRV:00 and subsequently injected with a solution of intercellular fluid (IF) from Avr9-transgenic *N. tabacum* plants (+ Avr9). The HR was observed to be stronger and faster in *LeCITRX*-silenced plants compared to

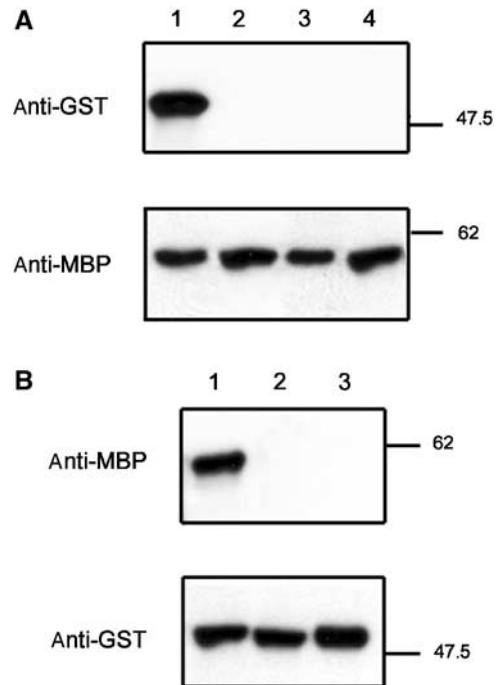


Figure 3 *In vitro* interaction between GST-CITRX and MBP-Cf-9^{D-G}. (A) Pull down of GST-CITRX with MBP-Cf-9^{D-G}, but not MBP-Cf-2^{D-G}. Immunoblot analysis of purified GST fusion proteins after incubation with MBP-Cf-9^{D-G} (lanes 1 and 2) or MBP-Cf-2^{D-G} (lanes 3 and 4) containing amylose beads. GST-CITRX was detected using an anti-GST polyclonal antibody. Lanes 1 and 3: cell extract containing GST-CITRX; lanes 2 and 4: cell extract containing GST alone. Equal loading of MBP-Cf-9^{D-G} and MBP-Cf-2^{D-G} was confirmed by immunoblot using an anti-MBP antibody. (B) Pull down of MBP-Cf-9^{D-G}, but not MBP-Cf-2^{D-G}, with GST-CITRX. Immunoblot analysis of purified MBP fusion proteins after incubation with GST-CITRX-containing glutathione sepharose beads. MBP-Cf-9^{D-G} was detected using an anti-MBP polyclonal antibody. Lane 1: cell extract containing MBP-Cf-9^{D-G}; lane 2: cell extract containing MBP-Cf-2^{D-G}; lane 3: cell extract containing MBP alone. Equal loading of GST-CITRX was confirmed by immunoblot using an anti-GST antibody. Molecular weight markers in kilodaltons are shown on the right.

TRV:00 controls: a complete collapse of the leaf from *LeCITRX*-silenced plants was observed only 1 day after IF injection, whereas a full HR took 2 or 3 days to develop in the control plants (Figure 4B, right leaf panels). No HR was observed when the silenced plants were injected with IF (–Avr9) (Figure 4B, left leaf panels) or when Cf0 tomato plants were used in the same experiment (data not shown). Identical results were observed when the C-terminal conserved region of *LeCITRX* (amino acids 89–157) was silenced (data not shown).

Consistent with the lack of interaction between CITRX and the C-terminus of Cf-2, no significant alteration of the HR was observed when CITRX-silenced Cf2 tomato plants were infiltrated with Avr2 peptide (Figure 4C, left and central panels), indicating that the negative regulatory role played by CITRX is specific to the Cf-9/Avr9-dependent response. To further confirm this idea, *LeCITRX* was silenced in Cf2/9 tomato plants, which express a chimeric protein with the 34 N-terminal LRRs of Cf-2 fused to the three C-terminal LRRs and cytoplasmic C-terminal sequence of Cf-9 (Krueger *et al*, 2002). This chimaera confers an Avr2 response. Significantly, these plants displayed an accelerated HR phenotype after

challenge with Avr2 (Figure 4C, right panel): a complete collapse of the leaf from *LeCITRX*-silenced plants was observed 2 days after treatment with Avr2, whereas a full HR took at least 4–5 days to develop in the control plants. Therefore, CITRX is specifically involved in the negative regulation of RLPs carrying the Cf-9 cytoplasmic tail, consis-

tent with its physical interaction with the Cf-9, but not Cf-2, C-terminus.

To test the effect of *LeCITRX* silencing on Cf-9-mediated disease resistance, TRV:*LeCITRX*- and TRV:00-silenced Cf9 and Cf0 tomatoes were inoculated with *C. fulvum* race 4 GUS. At 3 weeks after *Cladosporium* infection, leaves were stained with X-gluc to score fungal growth. As expected, no X-gluc staining could be observed in Cf9 tomatoes silenced with either TRV:*LeCITRX* or TRV:00 (Figure 4D, right panel). In contrast, Cf0 tomatoes silenced with the control TRV:00 supported extensive fungal growth (Figure 4D, left panel), revealed by X-gluc staining. Unexpectedly, leaves from Cf0 plants silenced with TRV:*LeCITRX* showed significantly reduced staining compared to controls (Figure 4D, central panel), indicating that silencing of *LeCITRX* conferred reduced disease sensitivity in otherwise completely susceptible Cf0 plants.

Overexpression of *LeCITRX* in *N. benthamiana*

We tested the effect of CITRX overexpression on Avr9/Cf-9-mediated HR in *N. benthamiana*. Avr9-transgenic *N. benthamiana* plants were infected with TRV:*NbCITRX* or TRV:00. After silencing, plants were infiltrated with *Agrobacterium* expressing either Cf-9 or Cf-9 and HA-tagged *LeCITRX*. An accelerated HR was observed in TRV:*NbCITRX*-silenced plants (Supplementary Figure 3S-B) compared to the controls. In contrast, the Avr9/Cf-9-dependent HR was significantly compromised in TRV:00-silenced plants when *LeCITRX*-HA was delivered together with Cf-9 (Figure 5). The expression of HA-tagged *LeCITRX* was confirmed by Western blot analysis using an anti-HA antibody (data not shown). The size of CITRX-HA protein is consistent with no cleavage of any possible transit peptide (Figure 7B). The possibility that the reduced HR observed when infiltrating Cf-9 and HA-tagged *LeCITRX* was due to dilution of *Agrobacterium* because of infiltration of two constructs was ruled out by co-infiltration of Cf-9 together with a 35S:GUS construct (data not shown). Cf-9 protein levels were unaffected by the presence or absence of CITRX, so the difference in HR cannot be ascribed to reduced Cf-9 accumulation in the presence of CITRX (data not shown; Figure 7B). Finally, *N. benthamiana* plants overexpressing HA-tagged *LeCITRX* were unaltered in the function of the *N* gene conferring TMV resistance (Supplementary Figure 4S).

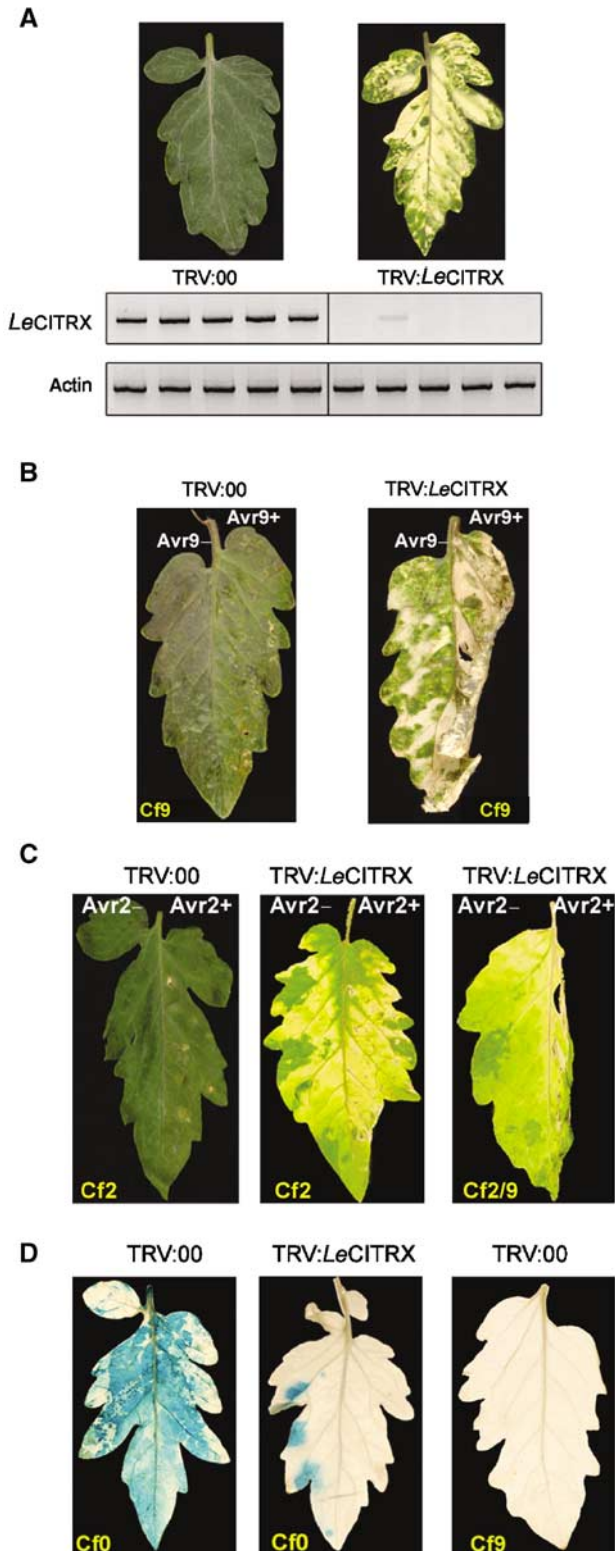


Figure 4 *LeCITRX* is a negative regulator of the Cf-9-dependent cell death and resistance to *Cladosporium* in tomato. Seedlings were inoculated with *A. tumefaciens* expressing TRV:*LeCITRX*, targeting the variable N-terminal region of *LeCITRX* (amino acids 1–88), or control TRV:00. (A) At 3 weeks after silencing induction, leaf discs from five independent plants silenced with either construct were harvested and total RNA was isolated. *LeCITRX* transcript accumulation was analysed by RT-PCR. Actin was used as an internal control for equal cDNA loading. (B) Cf9 tomato plants, silenced with TRV:*LeCITRX* or TRV:00, were injected with IF (+Avr9) (right leaf panels) or IF (–Avr9) (left leaf panels). Pictures were taken 1 day after IF injection. (C) Cf2 (left and central panels) and Cf2/9 chimeric (right panel) tomato plants, silenced with TRV:*LeCITRX* or TRV:00, were injected with Avr2 peptide (right leaf panels) or water (left leaf panels). Pictures were taken 2 days after Avr2 injection. (D) Cf9 and Cf0 tomato plants, silenced with TRV:*LeCITRX* or TRV:00, were infected with *C. fulvum* race 4 GUS, expressing Avr9. Leaves were stained with X-gluc 3 weeks after *Cladosporium* infection, when pictures were taken.

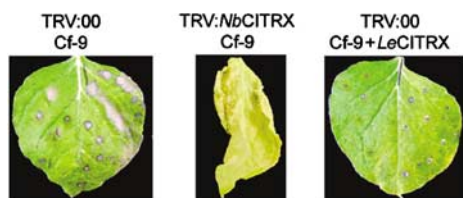


Figure 5 Overexpression of *NbCITRX* induces a delayed *Avr9*/*Cf-9*-dependent HR in *N. benthamiana*. Seedlings of *Avr9*-transgenic *N. benthamiana* plants were inoculated with *A. tumefaciens* TRV:*NbCITRX*, targeting the variable N-terminal region of *NbCITRX* (amino acids 3–66), or control TRV:00. At 3 weeks after silencing induction, plants were challenged with *Agrobacterium* carrying either *Cf-9* or *Cf-9* and HA-tagged *LeCITRX*. Pictures were taken 3 days after agroinfiltration.

Production of H₂O₂

The HR is associated with the production of ROS (Hammond-Kosack and Jones, 1996). The production of H₂O₂ in *Cf9* tomato plants, silenced with either TRV:*LeCITRX* or TRV:00, was monitored over time after a mixture of IF (+*Avr9*) and a solution of diaminobenzidine (DAB) was infiltrated. H₂O₂ accumulated at higher levels in the *LeCITRX*-silenced plants compared to the TRV:00-silenced control (Figure 6A). H₂O₂ did not accumulate when silenced plants were injected with IF (–*Avr9*) (Figure 6A) or in *Cf0* tomato (data not shown). Essentially identical results were obtained in tomato and *N. benthamiana* (data not shown), consistent with the idea that *CITRX* negatively regulates the *Avr9*/*Cf-9*-mediated HR.

Production of ROS was measured in *Cf-2* and *Cf-2/9* chimeric tomato plants after silencing with TRV:*LeCITRX* or TRV:00, followed by treatment with *Avr2*. Comparable ROS accumulation was observed in TRV:*LeCITRX*- and TRV:00-silenced *Cf-2* tomato plants, whereas an increased accumulation of H₂O₂ was detected in the *LeCITRX*-silenced *Cf-2/9* chimeric tomato compared to the control (Supplementary Figure 5S). These data confirm that interaction between *CITRX* and the *Cf-9* cytoplasmic tail is crucial in the negative regulation of the HR exerted by *CITRX*.

Activation of protein kinases

Activation of protein kinases such as MAPKs (Romeis *et al*, 1999) and CDPKs (Romeis *et al*, 2000a, 2001) was previously observed in *Cf9* tobacco after *Avr9* elicitation. MAPK and CDPK activation in *LeCITRX*- and TRV:00-silenced plants was tested over a time course of 30 min after elicitation with IF (+*Avr9*) or IF (–*Avr9*), using in-gel assays with myelin basic protein or histone as substrates. The *Cf9*/*Avr9*-dependent activation of MAPKs was weaker and more transient in *LeCITRX*-silenced plants, in comparison with control plants, whereas activation of CDPKs was stronger (Figure 6B, upper and middle panels). The 70 kDa histone phosphorylation signals were not detectable when the in-gel assay was performed in the presence of 2 mM EGTA (Figure 6B, lower panel), indicating that phosphorylation activity is indeed Ca²⁺-dependent. Our data suggest that *CITRX* preferentially negatively regulates the CDPK, not the MAPK, component of the defence response.

Induction of defence-related genes

LeCITRX-silenced tomato plants were infiltrated with IF (+*Avr9*) or IF (–*Avr9*) and total RNA was isolated at various

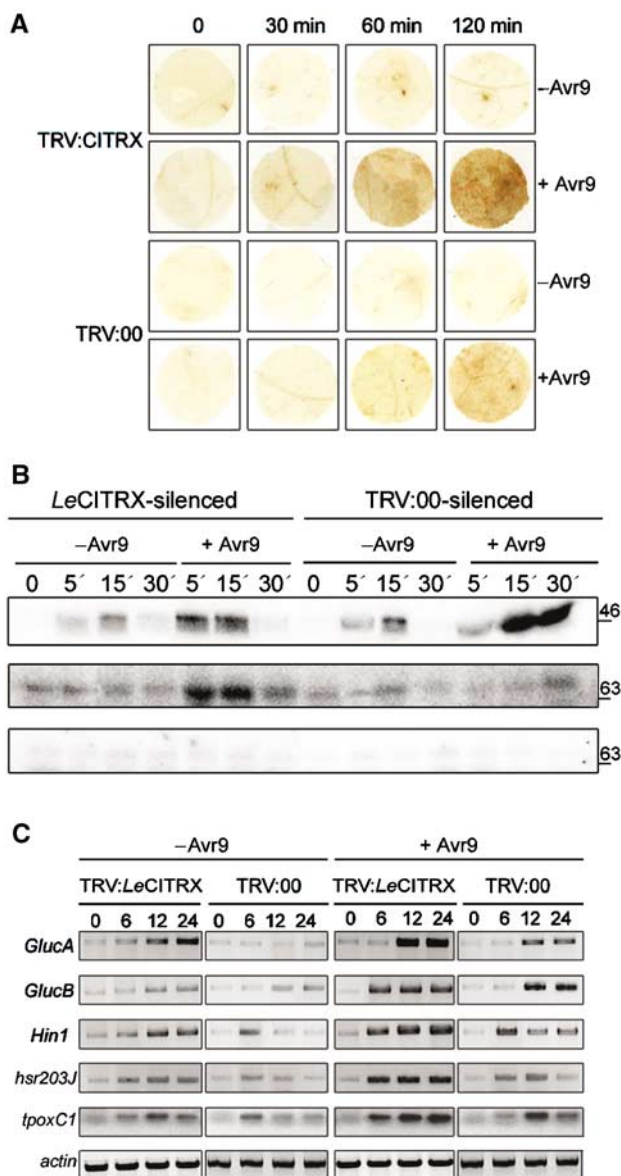


Figure 6 VIGS of *LeCITRX* induces alteration of several *Cf-9*/*Avr9*-induced signalling events. *Cf9* tomato seedlings were inoculated with *A. tumefaciens* expressing TRV:*LeCITRX*, targeting the variable N-terminal region of *LeCITRX* (amino acids 1–88), or TRV:00, and 3 weeks after silencing induction, different treatments were applied. (A) Accumulation of H₂O₂ in *LeCITRX*-silenced plants. A mixture of IF (+*Avr9*) or IF (–*Avr9*) and a solution of DAB was infiltrated in the silenced plants. At the time points indicated, leaf discs were harvested and histochemically analysed for H₂O₂ production. (B) *Avr9*/*Cf-9*-dependent activation of kinases in *LeCITRX*-silenced plants. Silenced plants were infiltrated with a mixture of IF (+*Avr9*) or IF (–*Avr9*). At the time points indicated, leaf discs were harvested and in-gel kinase assays were performed using myelin-binding protein for detection of MAPK activity (upper panel), or histone for detection of CDPK activity (central and lower panels). The in-gel kinase assay in the lower panel was conducted in the presence of 2 mM EGTA. Numbers on the right indicate molecular weight in kilodaltons. (C) Induction of defence-related genes in *LeCITRX*-silenced plants. Silenced plants were injected with IF (+*Avr9*) or IF (–*Avr9*). Leaf discs were harvested at the time points indicated and total RNA was isolated. Expression of several defence-related genes over the time course was analysed by RT-PCR. Actin was used as an internal control for equal cDNA loading.

time points. The transcriptional activation of the following defence-related genes was analysed by RT-PCR: *Hin1*, *hsr203J* and *tpoxC1* markers for HR (Gopalan *et al*, 1996; Hiraga *et al*, 2000), acidic β -1,3-glucanase (*GlucA*) and basic β -1,3-glucanase (*GlucB*) (Niki *et al*, 1998). *LeCITRX*-silenced plants showed an enhanced or earlier induction of all defence markers after injection with IF (+Avr9) compared to the control plants, which showed a weaker and, in the case of *Hin1*, *hsr203J* and *tpoxC1* genes, more transient induction (Figure 6C). Transcriptional activation of these genes was also observed in *LeCITRX*-silenced plants after injection with IF (-Avr9), although the induction was much weaker than after IF (+Avr9) injection, consistent with the previously described overlap between defence- and wound/flooding-related responses (Romeis *et al*, 1999, 2001; Durrant *et al*, 2000).

Expression patterns of *LeCITRX*

LeCITRX transcript levels were analysed in Cf9 tomato over 4 h after elicitation with IF (+Avr9) or IF (-Avr9). RT-PCR with N- or C-terminal *LeCITRX*-specific primers was conducted, using WIPK (Romeis *et al*, 2001) and actin as controls for an induced and a constitutively expressed gene, respectively (Figure 7A). Consistent with its negative regulatory role, a decrease in *LeCITRX* transcript levels was detected after injection of IF (+Avr9) or IF (-Avr9), although a stronger and more sustained mRNA decrease was observed in the case of IF (+Avr9). We propose that downregulation of

CITRX mRNA could attenuate the negative regulation exerted by *CITRX* after elicitation, promoting activation of defence and HR. However, early defence responses, such as MAPK activation, precede significantly the loss of *CITRX* mRNA. Thus, *CITRX* downregulation cannot be the sole mechanism by which negative regulation is relieved.

To analyse *LeCITRX* expression levels after elicitation, Avr9-transgenic *N. benthamiana* plants were infiltrated with agrobacteria expressing TAP-tagged Cf-9 under its own promoter with either a GUS control or HA-tagged *LeCITRX*. The same experiment was conducted using Avr4 *N. benthamiana* leaves as a control. At 2 days after agroinfiltration, proteins were extracted and separated in SDS gels. Consistent with previous experiments, the expression of Cf-9 was identical in the presence or absence of *LeCITRX* in both nonelicited and elicited leaves (Figure 7B, lanes 1–4, top panel). In contrast, elicitation with Avr9 prevented the accumulation of *LeCITRX* (Figure 7B, lanes 2 and 4, lower panel). The size of *CITRX* again corresponds to its full-length size and thus with a cytoplasmic, not chloroplastic, location.

We propose that the negative regulation exerted by *CITRX* could be eliminated after elicitation through downregulation of *CITRX* mRNA and subsequent disappearance of the *CITRX* protein. *CITRX* removal could then enhance the HR and defence responses.

Discussion

We have identified a thioredoxin (*CITRX*) that interacts with the C-terminal cytoplasmic tail of Cf-9. Phylogenetic analysis of *CITRX* suggests that it is unrelated to previously described thioredoxins, being closest to *AtTRX-x*, a plant thioredoxin of the prokaryotic type (Mestres-Ortega and Meyer, 1999). VIGS of *CITRX* induces a yellowish coloration in the leaves, which might suggest a role in chloroplast function. However, several lines of evidence contradict this idea. First, the PSORT program predicted a cytoplasmic location for *CITRX*. Second, analysis of tobacco cells expressing HA-tagged *CITRX* revealed its presence in the soluble fraction (data not shown). Finally, the size of the transit peptide of the described *Arabidopsis* chloroplastic thioredoxins ranges between 82 and 48 amino acids (Mestres-Ortega and Meyer, 1999), which constitutes at least one-third of the non-processed protein. In contrast, HA-tagged *CITRX* is ~25 kDa (Figure 7B), inconsistent with removal of a transit peptide. *CITRX* is thus most likely located in the cytoplasm where it could interact with the C-terminus of Cf-9. Pull-down assays with *CITRX* and the C-terminus of Cf-9 expressed in *E. coli* provided further evidence in favour of this interaction (Figure 3).

Thioredoxins are involved in the regulation of various cellular processes including gene expression, signal transduction, proliferation and apoptosis. Removal of *CITRX* by VIGS induced an alteration of Cf-9-dependent defence responses in both tomato and tobacco. First, the Cf-9 HR was significantly accelerated in the *CITRX*-silenced plants compared to the controls (Figure 4 and Supplementary Figure 3S). Second, silenced plants displayed an increased production of Cf-9-dependent ROS (Figure 6A). Thioredoxins can act as antioxidants in protection against ROS in both prokaryotic and eukaryotic systems. Thioredoxin-deficient chicken and mammalian cells undergo cell death upon repression of a

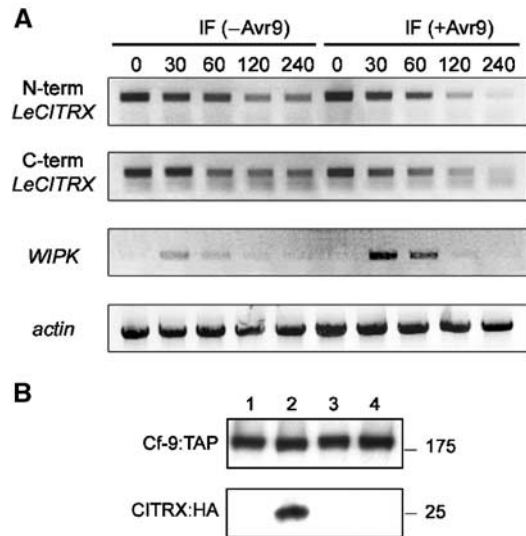


Figure 7 Expression pattern of *LeCITRX* gene and protein in Cf9 tomato plants after elicitation. **(A)** Expression of *LeCITRX* mRNA is downregulated after elicitation. Cf9 tomato plants were treated with IF (+Avr9) or IF (-Avr9). At the time points indicated, leaf discs were harvested and total RNA was isolated and used for RT-PCR with specific primers for the N- or C-terminus of *LeCITRX*, WIPK and *actin*, respectively. Equal cDNA amounts were used as shown by the amplification of the constitutively expressed *actin* gene. **(B)** Elicitation induces disappearance of *LeCITRX* protein. Avr4- (lanes 1 and 2) or Avr9- (lanes 3 and 4) transgenic *N. benthamiana* leaves were infiltrated with *Agrobacterium* expressing Cf-9:TAP and a GUS control (lanes 1 and 3) or Cf-9:TAP and *LeCITRX*:HA (lanes 2 and 4). A 2 dpi protein extracts were prepared and Cf-9:TAP and *LeCITRX*:HA detected by Western blot using PAP and anti-HA antibodies. Molecular weight markers in kilodaltons are shown on the right.

thioredoxin gene and show accumulation of intracellular ROS and activation of caspases (Filomeni *et al*, 2002; Tanaka *et al*, 2002). Also, protein kinase activities are altered after removal of CITRX by VIGS (Figure 6B). In CITRX-silenced plants, CDPK activation was enhanced whereas MAPK activation was reduced compared to the controls. Thioredoxins also inhibit p38 MAPK activation in mammalian cells (Hashimoto *et al*, 1999; Filomeni *et al*, 2002). Expression of several defence-related genes was also accelerated or enhanced after silencing CITRX (Figure 6C). In conclusion, CITRX appears to play a crucial role in the differential regulation of the CDPK and MAPK signalling pathways that govern defence responses in tomato.

VIGS of *CITRX* induces a yellowish coloration in the leaves and this alteration of the leaf appearance might also have an effect on the observed phenotypes. However, CITRX silencing does not affect the HR induced by other pathosystems, namely AvrPto/Pto, PVX coat protein/Rx, AvrBs2/Bs2 and AvrRpt2 (Supplementary Figure 3S-C). Also, CITRX overexpression induces an opposite phenotype to VIGS (Figure 5), suggesting that the acceleration of the HR induced by CITRX removal is specific to the Cf-9/Avr9 interaction and not a secondary effect of the elimination of CITRX expression. Third, two genes, whose removal also confers yellowish coloration of the leaves, were silenced in control experiments. Phytoene desaturase (*PDS*), essential for the production of carotenoids that protect plants from photobleaching (Demmig-Adams and Adams, 1992; Ratcliff *et al*, 2001), and *SULPHUR*, a magnesium chelatase required for chlorophyll production (Kjemtrup *et al*, 1998), were silenced in both Avr9-transgenic *N. benthamiana* and Cf9 tomato plants prior to the delivery of Cf-9 or IF (+ Avr9), respectively. Although *PDS*- and *SULPHUR*-silenced plants showed a similar yellow phenotype to CITRX-silenced plants, no significant difference was observed between the TRV-*PDS*-, TRV-*SULPHUR*- and TRV:00-silenced plants in the timing of the onset of Avr9/Cf-9 HR, nor in the degree of necrosis, whereas the HR in CITRX-silenced plants was always found to be accelerated (data not shown). Similarly, silencing of *PDS* or *SULPHUR* in tomato did not show any effect on either H₂O₂ accumulation or *Cladosporium* growth (data not shown), confirming that the yellowish coloration *per se* does not affect the Cf-9/Avr9-mediated HR and that the specific elimination of CITRX is necessary to accelerate the HR.

Importantly, the negative regulatory role played by CITRX is specific to the Cf-9/Avr9-dependent response. Firstly, no interaction between CITRX and Cf-2 was found, either in yeast two-hybrid or in pull-down assays (Figures 1 and 3). Secondly, in contrast to the accelerated HR phenotype observed in *LeCITRX*-silenced Cf9 tomato after challenge with Avr9, no significant alteration of the development of HR was observed when *LeCITRX*-silenced Cf2 tomato plants were infiltrated with Avr2 peptide (Figure 4C). Thirdly, silencing of *LeCITRX* in plants carrying a Cf2/9 chimaera, which expresses a protein with the 34 N-terminal LRRs of Cf-2 fused to the C-terminus of Cf-9, conferred accelerated HR after challenge with Avr2 (Figure 4C). Finally, increased accumulation of ROS was observed in Cf2/9, but not Cf2, plants after CITRX silencing and elicitation with Avr2 (Supplementary Figure 5S). Taken together, these data show that recognition of the Cf-9 C-terminus by CITRX is crucial for its negative regulation of the Cf-9 HR.

The parallel between Cf-9-mediated defence and *Brassica* self-incompatibility responses is striking. Both are triggered by recognition of small, cysteine-rich, secreted peptides (Avr9 and SCR/SP11). In both cases, the major regulatory components (Cf-9 and SRK) are type I membrane proteins, although SRK contains a cytoplasmic kinase domain that is missing in Cf-9. However, we can speculate that this activity might be provided by an as yet unidentified kinase associated with Cf-9. Third, ARC1 is a positive regulator of the SRK signalling pathway. ARC1 is a U-box-containing E3 ubiquitin ligase that promotes ubiquitination of proteins following self-incompatible pollination (Stone *et al*, 2003). Among the Avr9/Cf-9 rapidly elicited (*ACRE*) genes (Durrant *et al*, 2000), one encodes a U-box protein highly homologous to ARC1; its role in the Cf-9-dependent defence response is currently being investigated. Finally, thioredoxins THL1 and THL2 were found to interact with the kinase domain of SRK (Bower *et al*, 1996). THL1 negatively regulates SRK phosphorylation and activity (Cabrillac *et al*, 2001), as CITRX negatively regulates Cf-9 function. Taken together, these observations warrant further studies to investigate the similarities between tomato Cf-9-mediated defence and *Brassica* self-incompatibility responses.

CITRX silencing also affects resistance to *Cladosporium* in Cf0 tomato. No growth of the fungus could be detected after silencing *LeCITRX* in Cf9 tomato, followed by infection with *C. fulvum* race 4 GUS, as expected after removal of a negative regulator of resistance. However, when *LeCITRX* was silenced in Cf0 tomato, growth of the fungus was significantly compromised (Figure 4D). In *Brassica*, the *in vivo* function of THL1/2 was studied by analysis of transgenic *Brassica napus* cv. Westar carrying an antisense THL1 or THL2 vector under the control of the SLR1 promoter that directs expression to the stigmatic papillae. Although Westar is normally self-compatible, antisense THL1/2 Westar lines showed constitutive partial rejection of pollen (D Goring, personal communication). One possible explanation for these results is that Westar carries a functional SRK that does not normally cause pollen rejection and removal of the THL1/2 inhibitors leads to constitutive upregulation of this SRK causing pollen rejection. Similarly, Cf0 tomato may express a Cf homologous protein that, under normal conditions, does not trigger effective resistance to *C. fulvum*. Silencing of CITRX may result in elevated activity of this Cf homologue, and activation of defence mechanisms. Sequence conservation between different Cf-9 homologues at the C-terminal cytoplasmic tail would allow CITRX to interact with additional Cf homologues other than Cf-9. Also, *C. fulvum* may carry a weak elicitor that does not normally induce sufficient defence responses in tomato to arrest fungal growth. Removal of the negative regulation exerted by CITRX may thus cause enhanced defence responses upon detection of this otherwise insufficiently active elicitor. Finally, although we cannot exclude the possibility that CITRX-silenced plants are weaker than wild-type plants and not fit enough to support *C. fulvum* growth, we think it unlikely since *SULFUR*- and *PDS*-silenced Cf0 plants are unaltered in susceptibility.

Figure 8 shows a model to explain the role played by CITRX in tomato resistance response to *C. fulvum*. In the absence of pathogen, CITRX negatively regulates Cf-9 signalling by influencing the interaction between Cf-9 and a putative additional protein partner. Upon elicitation, this negative

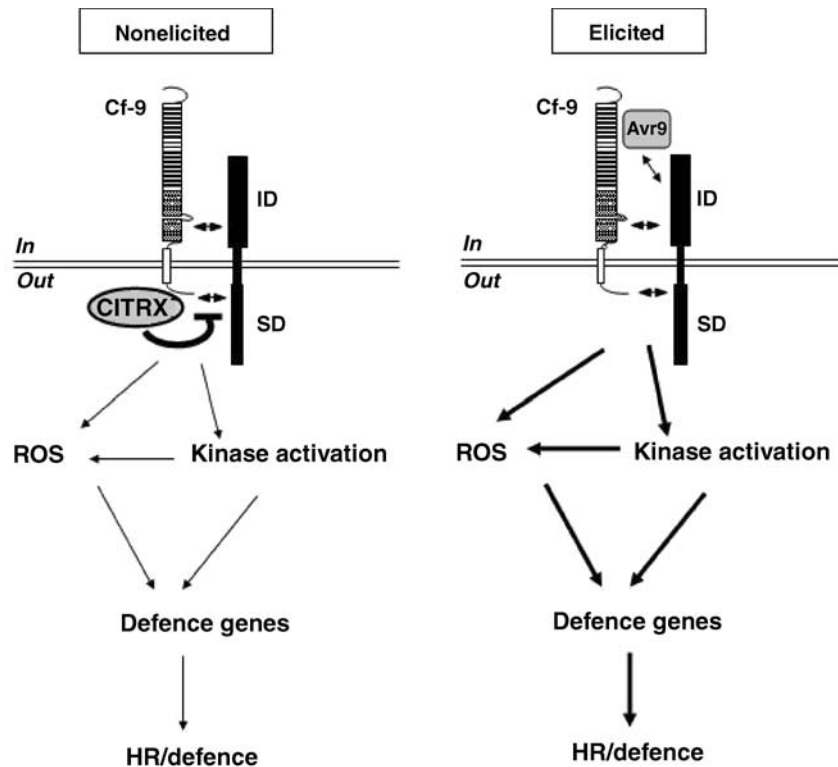


Figure 8 Model for the role of CITRX in the regulation of the Cf-9/Avr9-dependent defence response. Cf-9 is a type I membrane protein that confers recognition of the pathogen-derived Avr9 peptide, most likely through association with a third protein (Joosten and De Wit, 1999; Rivas and Thomas, 2002). Such a protein might have an extracellular domain to interact with Avr9 and/or Cf-9 (ID), and an intracellular signalling domain (SD). Production of ROS is either independent or located downstream of the activation of protein kinases (Romeis *et al*, 1999, 2000a). In the nonelicited situation, CITRX could negatively regulate the interaction between Cf-9 and the putative interacting protein, resulting in the negative regulation of the signalling pathway downstream of the Cf-9/Avr9 recognition event. In the elicited state, probably after recognition of the Avr9 peptide by an additional as yet unidentified protein, the negative regulation exerted by CITRX is eliminated by downregulation of CITRX expression and subsequent disappearance of the CITRX protein. CITRX removal triggers the accumulation of ROS, kinase activation and induction of defence-related genes, leading to the HR and defence responses.

regulation would be eliminated through downregulation of the expression of the *CITRX* gene and reduced accumulation of CITRX protein (Figure 7), leading to the HR and defence responses.

In summary, CITRX is the first identified protein that physically interacts with Cf-9 and regulates the signalling pathways leading to tomato defence responses to *C. fulvum*. Thioredoxins can act as antioxidants in protection against ROS, which are involved in the regulation of a variety of cellular processes. However, to the best of our knowledge, this is the first report of a thioredoxin involved in plant disease resistance. Since the generation of ROS strongly correlates with plant defence, further studies will reveal whether the regulation displayed by CITRX extends beyond the Cf-9/Avr9 interaction and help us fully understand the role played by this and/or other thioredoxin(s) in the regulation of plant defence pathways.

Materials and methods

Yeast two-hybrid experiments

The construction of the prey cDNA tomato library was described previously (Zhou *et al*, 1995).

The Cf-9 C-terminal fragment, encoding the last 33 amino acids of Cf-9 (M⁸³¹ to Y⁸⁶³; Jones *et al*, 1994), was PCR-amplified from plasmid Cf-9DS (Wulff *et al*, 2001), using primers Cf9pGILDA-EcoRIS and Cf9pGILDA-XhoIA, digested, and cloned in the bait

vector pGILDA (Clontech) to produce a protein fusion with the LexA DNA-binding domain. The Cf-2 C-terminal peptide, encoding the last 37 amino acids of Cf-2 (N¹⁹⁷⁶ to F²⁰¹²; Dixon *et al*, 1996), was cloned similarly in pGILDA after PCR amplification from plasmid SLJ8571 (Tang, 1998), using primers Cf2pGILDA-EcoRIS and Cf2pGILDA-XhoIA.

C-terminal CITRX deletions CITRX M1-Y30, CITRX M1-G60, CITRX M1-I90, CITRX M1-L120 and CITRX M1-D150 were made after PCR amplification from the positive clone containing CITRX, using primers CITRXpB42AD-EcoRIS and CITRX M1-Y30XhoIA, CITRXM1-G60 XhoIA, CITRX M1-I90XhoIA, CITRX M1-L120XhoIA and CITRX M1-D150XhoIA, respectively. The amplified fragments were digested and cloned in the prey vector pB42AD (Clontech).

All interactions were tested according to Zhou *et al* (1995).

Construction of GST-CITRX, MBP-Cf-9^{D-G} and MBP-Cf-2^{D-G} and pull-down assays

Full-length *LeCITRX* was amplified by PCR using primers GST-CITRX *Bam*HIS and GST-CITRX *Xma*IA. The purified PCR product was digested and cloned in pGEX-4T-1 (Amersham Pharmacia Biotech), which contains the gene encoding for GST upstream of the *Bam*HI site. Protein purification was performed using glutathione sepharose according to the manufacturer's instructions. Cf-9 domains D-G (D is an acidic domain after the LRRs, preceding Cf-9 transmembrane domain (F) and cytoplasmic tail (G); Jones *et al*, 1994) were amplified by PCR using primers MBP-Cf9 *Eco*RIS and MBP-Cf9 *Pst*IA. The purified PCR product was digested and cloned in pMAL-c2X (New England Biolabs), which contains the gene encoding for MBP upstream of the *Eco*RI site. For amplification of Cf-2 domains D-G (Dixon *et al*, 1996) primers MBP-Cf2 *Eco*RIS and MBP-Cf2 *Pst*IA were used and cloning of the purified PCR product was carried out as described for Cf-9. Protein purification

was performed using amylose resin according to the manufacturer's instructions.

For the pull-down assays, cell lysates containing MBP-Cf-9^{D-G} or GST-CITRX were precleared by incubation for 1 h at 4°C with glutathione sepharose or amylose resin, respectively. For purification of MBP-Cf-9^{D-G} or GST-CITRX, precleared supernatants were incubated for 1 h at 4°C with amylose resin or glutathione sepharose, respectively, followed by three washes with lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 3.3% sucrose (w/v), 1 mM phenylmethylsulphonyl fluoride). Precipitated MBP-Cf-9^{D-G} or GST-CITRX were incubated with precleared extracts containing GST-CITRX or MBP-Cf-9^{D-G} for 1 h at 4°C, followed by three washes with lysis buffer. Interaction between MBP-Cf-2^{D-G} and GST-CITRX was assayed using the same procedure. Proteins were finally separated on 7.5% SDS-polyacrylamide gels and GST-CITRX and MBP-Cf-9^{D-G} or MBP-Cf-2^{D-G} were detected by immunoblot analysis with anti-GST (Amersham Pharmacia Biotech) or anti-MBP (New England Biolabs) polyclonal antibodies, following the manufacturer's instructions.

Construction of gene silencing vectors and virus infections

For silencing in *N. benthamiana*, a 192 bp fragment encompassing the variable N-terminal region of *NbCITRX* (amino acids 3–66) was amplified from *N. benthamiana* cDNA using primers TRV-*NbCITRXv-S* and TRV-*NbCITRXv-A*. For tomato silencing, a 264 bp cDNA fragment encompassing the variable N-terminal region of *LeCITRX* (amino acids 1–88) was amplified with primers TRV-*LeCITRXv-* and TRV-*LeCITRXv-A*. Finally, a 206 bp cDNA fragment containing the C-terminal conserved region of *LeCITRX* (amino acids 89–157) was amplified using primers TRV-*LeCITRXc-S* and TRV-*LeCITRXc-A*. This PRC fragment was used for silencing of the CITRX conserved C-terminal domain in both *N. benthamiana* and tomato plants, since *NbCITRX* and *LeCITRX* share a 94% identity at the DNA level in this region. All PCR products were purified and cloned in *Sma*I-digested pTV00 (Ratcliff *et al*, 2001) or pTRV2 (Liu *et al*, 2002) for *N. benthamiana* or tomato silencing, respectively. *N. benthamiana* and tomato silencing was performed as described (Ratcliff *et al*, 2001; Liu *et al*, 2002).

Construction of HA-tagged CITRX and transient expression of proteins in N. benthamiana

Full-length *LeCITRX* was amplified by PCR using primers CITRX *Clai*S and CITRX *Bam*HI. The purified PCR product was digested and cloned in pBin19g, which contains an in-frame HA sequence for epitope-tagging downstream of the *Bam*HI site, under the control of a 35S promoter, in a pBin19 backbone (Frisch *et al*, 1995). This construct was transformed into *Agrobacterium* for transient expression of HA-tagged CITRX. Transient expression of the various Cf-constructs and detection of the different tagged proteins were performed as described previously (Rivas *et al*, 2002a,b).

C. fulvum inoculation of tomato plants

Paclobotrazol-treated plants (3- to 4-week-old) were inoculated with *C. fulvum* race 4 GUS spore suspensions and scored for resistance or disease sensitivity 3 weeks after infection, as described previously (Balint-Kurti *et al*, 1994; Thomas *et al*, 1997).

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Detection of H₂O₂

H₂O₂ accumulation was detected by endogenous peroxidase-dependent *in situ* histochemical staining using 3,3'-diaminobenzidine (DAB; Sigma) in a protocol modified from Thordal-Christensen *et al* (1997). Leaves were infiltrated *in vivo* with a solution of 1 mg/ml DAB and the plants were kept in the dark. Leaf discs were taken at different time points after infiltration, immediately transferred to a tube containing 1 ml of destaining solution of glycerol/acetic acid/ethanol (1:1:3 (v/v/v)), and cleared by boiling for 5 min in a water bath, followed by further incubation for 1 h on a shaker. Samples were mounted on a slide in 60% glycerol. H₂O₂ was detectable as a reddish brown precipitate.

In-gel kinase assays

MAPK and CDPK activities were determined by in-gel kinase assays with MBP or histone as substrates as described previously (Romeis *et al*, 1999, 2000a).

RT-PCR

Total RNA from two 1 cm diameter leaf discs was isolated using the Tri Reagent method following the manufacturer's instructions (Sigma). First-strand cDNAs were synthesized from 2 µg of total RNA using Superscript II RNase H⁻ Reverse Transcriptase (Invitrogen). Actin was used as an internal positive control for equal cDNA amounts using primers described by Romeis *et al* (2001). cDNAs for several tomato defence-related genes were amplified by PCR using the following primers: Hin1-F and Hin1-R, hsr203J-F and hsr203J-R, tpxoC1-F and tpxoC1-R, GluA-F and GluA-R and GluB-F and Glu-B. Full-length *NbCITRX* and *LeCITRX* cDNAs were amplified using TRV-*NbCITRXv-S* and TRV-*LeCITRXc-A*, and TRV-*LeCITRXv-S* and TRV-*LeCITRXc-A*, respectively. The N- and C-terminal fragments of *LeCITRX* were amplified using TRV-*LeCITRXv-S* and TRV-*LeCITRXv-A*, and TRV-*LeCITRXc-S* and TRV-*LeCITRXc-A*, respectively.

Accession numbers

The GenBank accession numbers of *NbCITRX1* and *NbCITRX2* described in this study are AY500242 and AY500243, respectively.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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