CITRX thioredoxin is a putative adaptor protein connecting Cf-9 and the ACIK1 protein kinase during the Cf-9/Avr9- induced defence response

Vladimir Nekrasov, Andrea A. Ludwig, Jonathan D.G. Jones*

The Sainsbury Laboratory, John Innes Centre, Colney Lane, Norwich NR4 7UH, United Kingdom

Received 4 April 2006; revised 23 June 2006; accepted 27 June 2006

Available online 5 July 2006

Edited by Julian Schroeder

Abstract  Tomato Cf-9, a receptor-like protein (RLP), confers resistance to races of the fungal pathogen Cladosporium fulvum that express the Avr9 avirulence gene. CITRX (Cf-resistance to races of the fungal pathogen C. fulvum) was previously identified in a yeast two-hybrid screen as a protein interacting with the cytoplasmic domain of Cf-9 and shown to be a negative regulator of the cell death induced after Cf-9/Avr9 interaction. ACIK1 is a Ser/Thr protein kinase that is specifically required for the Cf-9 and Cf-4 dependent defence response in tomato. In this paper we present data suggesting that CITRX may act as an adaptor recruiting the ACIK1 kinase to the cytoplasmic domain of Cf-9 upon elicitation with the Avr9 peptide. Interestingly, the catalytic activities of both CITRX and ACIK1 are not required for their interaction.

© 2006 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Plant disease resistance; Cf-9; Thioredoxin; Kinase

1. Introduction

Plant cells induce defence responses upon perception of pathogen-derived molecules. General elicitors, such as bacterial flagellin, can activate resistance [1]. However, resistance is often governed by a ‘gene-for-gene’ interaction [2], in which plants carrying a resistance (R) gene specifically recognize a pathogen carrying a corresponding avirulence (Avr) gene. R gene-mediated resistance is typically accompanied by rapid cell death at the site of infection called the hypersensitive response (HR). Proteins encoded by R genes are divided into different classes based on their domain organisation and localisation within the cell [2].

Tomato Cf genes confer resistance to specific races of Cladosporium fulvum, a biotrophic fungus that causes leaf mould in tomato, through recognition of Avr peptides that are secreted into the leaf apoplast during infection. For example, the R gene Cf-9 confers resistance to strains of C. fulvum that express Avr9 [3].

Cf genes encode receptor-like proteins (RLPs), which are type I transmembrane glycoproteins carrying extracellular leucine-rich repeats (eLRRs), a transmembrane region, and a short cytoplasmic domain that has no similarity to known signalling domains. Apart from RLPs, the eLRR protein class includes receptor-like kinases (RLKs), which, in addition to an extracellular LRR region and a transmembrane domain, possess a cytoplasmic kinase domain. eLRR-containing proteins are quite abundant in plants with 56 eLRR-RLPs and 216 eLRR-RLKs encoded in the Arabidopsis genome [4,5]. In addition to Cf proteins, a function has been assigned to several other eLRR-containing RLPs and RLKs. Some RLPs, like Cf-9, RPP27 or Ve1, play a role in defence against pathogens [3,6,7], while others function in meristem development (CLV2) or distribution of stomata (TMM) [8,9]. RLKs carrying eLRRs are also involved in various physiological processes, such as defence response (FLS2, Xa21), meristem development (CLV1), response to brassinosteroid hormones (BR11, BAK1), nodulation (SYMRK), pollination (LePRK) and others (reviewed in [10,11]). Several eLRR RLPs and RLKs were found to interact with other components of signalling complexes. For instance, BR11 interacts with BAK1 [12,13], CLV1 is expected to interact with CLV2 [8], and LePRK2 associates with LePRK1 [14].

Based on the examples mentioned above, it seems that many plant signalling pathways involving eLRR proteins transduce signals through induction of the RLK kinase activity. However, no RLK(s) required for the activation of the Cf-dependent defence response have been identified so far. In this paper we present evidence for physical association between the cytoplasmic domain of Cf-9 and the Avr9/Cf-9 induced kinase 1 (ACIK1). This physical association is mediated by the C-terminal cytoplasmic domain of Cf-9 and was reported as a result of a yeast two-hybrid screen carried out with the Cf-9 interacting thioredoxin (CITRX). CITRX was identified as a result of a yeast two-hybrid screen carried out with the Cf-9 interacting thioredoxin (CITRX). CITRX was identified as a result of a yeast two-hybrid screen carried out with the C-terminal cytoplasmic domain of Cf-9 and was reported to be a specific negative regulator of the cell death induced through Cf-9 [15]. ACIK1 is a cytoplasmic Ser/Thr kinase and its coding gene (ACRE-264) was originally identified as an Avr9/Cf-9 rapidly elicited (ACRE) gene using cDNA-AFLP analysis in Nicotiana tabacum cell cultures [16]. ACIK1 was found to be required for Cf-9/Avr9- and Cf-4/Avr4-mediated HRs but not for the HR or resistance mediated by other R/Avr systems, such as Pto/AvrPto, Rsp/Potato virus X or N/Tobacco mosaic virus [17].
The data presented in this article are consistent with a model, in which Avr9 perception by Cf-9 leads to activation of the ACIK1 kinase that is recruited to the cytoplasmic domain of Cf-9 by the CITRX thioredoxin serving as an adaptor protein.

2. Materials and methods

2.1. Yeast two- and three-hybrid assays

For the experiments using the LexA-based yeast two-hybrid (Y2H) system (Clontech) fusions between the LexA DNA binding domain and either the full length LeACIK1, or its truncated versions (LeACIK1D aa366–464, LeACIK1Δaa1–84, LeACIK1Δaa85–464, LeACIK1Δaa85–365, LeACIK1Δaa1–365) were created by the PCR-amplification of the corresponding region from LeACIK1 cDNA (AF332960) and subsequent cloning of PCR products into pGLDA bait vector. The LexA-fusion of the kinase-inactive mutant LeACIK1D215A was constructed as described above with the only difference that the D215A aa exchange was introduced by a site-directed mutagenesis performed using overlap extension PCR [18] to create a template for the cloning PCR. Prey constructs pB42AD:LeCITRXw.t. and pB42AD:LeCITRXΔaa151–175 were described previously [15]. pB42AD:AtTRX-x was created in the same way as pB42AD:LeCITRXw.t. but in this case primers specific for AtTRX-x cDNA (AAF15952) were used for the PCR. In order to create pB42AD:LeCITRXGPS, C98S and C101S aa exchanges were introduced by site-directed mutagenesis [18]. The interactions were tested in yeast strain EGY48 according to the manufacturer’s instructions (Clontech).

For the assays using GAL4-based Y2H system (Clontech) LeCITRXw.t. and LeCITRXΔaa1–64 were amplified by PCR and cloned into the GAL4 BD-vector pBridge. pBridge:LeACIK1 was created by subcloning LeACIK1 ORF from pGILDA:LeACIK1 into pBridge.

Fig. 1. Cf-9 C-term., LeCITRX and LeACIK1 interact in Y2H and Y3H systems. (A) LeACIK1 amino acid sequence can be subdivided into three domains: N-terminal (N, aa1–84), kinase (K, aa85–365) and C-terminal (C, aa366–464) domain. (B) LeACIK1 interacts with LeCITRX. Both wild-type LeCITRX and the truncated form with first 64aa removed interact with LeACIK1. (C) LeACIK1 interacts with LeCITRX through the N-domain. The intensity of interaction between LeCITRX and different domains of LeACIK1 was measured using a quantitative β-galactosidase assay with ONPG as substrate. (D) LeCITRX with both catalytic cysteines replaced with serines (LeCITRXGPS) and LeCITRX without the last 25 amino acids (LeCITRX Δaa151–175) were tested against LeACIK1 in Y2H assays. (E) LeCITRXΔaa1–64 was compared to LeCITRX w.t. for its ability to interact with Cf-9 C-term. (F) LeCITRXGPS was compared to LeCITRXw.t. for its ability to interact with Cf-9 C-term. AtTRX-x was used as the negative control. Experiments in (B, E) and (C, D and F) were done using GAL4 and LexA Y2H systems respectively. (G) LeACIK1 interacts with Cf-9 C-term. in the presence of LeCITRX in the Y3H system (yeast colonies grown on a – HIS plate). (H) The DB:Cf-9 C-term./LeCITRX/AD: LeACIK1 colonies stop growing on the –HIS medium supplemented with methionine if the linker gene (LeCITRX) is expressed from the methionine repressible MET25 promoter. (I) A model illustrating interactions between Cf-9, CITRX and ACIK1. The region of LeCITRX shown in blue represents the first 64 amino acids.
the reaction mix normalised against the OD 600 of the yeast culture.

For the yeast three-hybrid (Y3H) assays, PCR-amplified LeCITRX ORF was inserted into MCSII of pBridge:Cf-G-term. In the case of both GAL4 Y2H and Y3H systems the interactions were tested in yeast strain AH109 according to the manufacturer’s instructions (Clontech).

The quantitative β-galactosidase assay was performed with ONPG (o-nitrophenyl β-d-galactopyranoside) as substrate according to the protocol described in Clontech “Yeast Protocols Handbook”. The activity of β-galactosidase was estimated based on the OD_{600} of the reaction mix normalised against the OD_{600} of the yeast culture. Three yeast culture samples were processed for each interaction tested and average values were calculated (Fig. 1C).

2.2. Construction of plasmids for the transient expression

pBin19g-LeCITRX construct [15] was used to express LeCITRX-HA. To create a construct for the expression of GUS-HA, the GUS (β-d-glucuronidase) gene from E. coli was amplified by PCR and cloned into the pBin19g vector. LeACIK1-Myc was expressed using a pSR78:LeACIK1 construct. pSR78 contains a cluster of 6 Myc sequences downstream of the XhoI site, under the control of the 35S promoter, in the pBin19 backbone [19]. The LeACIK1 gene was amplified by PCR with primers carrying XhoI (forward) and BamHI (reverse) sites and cloned into the XhoI site of pSR78. To create the construct for the expression of EDS1-Myc, the Arabidopsis EDS1 gene was amplified by PCR using primers carrying CiaI (forward) and BanHI (reverse) sites and digested PCR product was ligated into pBin19 vector, which contains an in-frame cluster of 6 Myc sequences for epitope-tagging downstream of the BanHI site, under the control of the 35S promoter, in the pBin19 backbone [19].

2.3. Agrobacterium-mediated transient expression

Transient expression of proteins in Nicotiana benthamiana was performed as described in [20] with some alterations. In all cases proteins were co-expressed with the suppressor of silencing p19 [21] to boost the protein expression level. Were co-expressed with the suppressor of silencing p19 [21] to boost the protein expression level. In all cases proteins were transiently co-expressed in the presence of 1.5 mM DSP protein cross-linker. B, LeACIK1 can be co-immunoprecipitated with LeCITRX-HA, but not with GUS-HA, in the presence of 1.5 mM DSP.

In the case of GST-tagged proteins the GST tag was removed by thrombin cleavage. The in vitro kinase assay was performed as described in [17] using 0.5 μg recombinant protein kinase and 1.5 μg kinase substrate in a 15 μl volume. Each kinase reaction was started by addition of 15 μl reaction mix to yield a final concentration of 10 mM MgCl2, 10 mM MnCl2, 1 mM CaCl2, 0.925 MBq (2.5 μCi) [γ-32P]ATP (11Tβqmmol; NEN) and 50 μM ATP. After incubation for 30 min at 30 °C, the reaction was stopped by adding 15 μl of SDS loading dye. Samples were loaded onto the same SDS-polyacrylamide gel. After electrophoresis the gel was stained with Coomassie brilliant blue, dried and subjected to autoradiography to detect incorporation of 32P into the kinase substrates.

2.4. Immunoprecipitation and immunoblotting

For co-immunoprecipitation experiments agroinfiltrated N. benthamiana leaves (48 hpi) were infiltrated with 1.5 or 3 mM DSP (dithio-bis(succinimidyl)propionate) protein cross-linker (Pierce). After 30 min. the leaf tissue was frozen in liquid nitrogen. The co-immunoprecipitation experiments were performed as described in [22] with some alterations: the concentration of DTT was reduced to 0.5 mM to stabilise the cross-linker and Nonidet P-40 was omitted from the procedure. The Western blotting was performed with anti-HA and anti-Myc HRP-conjugated monoclonal antibodies according to the manufacturer’s instructions (Roche).

2.5. In vitro kinase assay

MBP-LeACIK1 was prepared as described in [17]. GST-LeCITRX [15], GST-Pto and GST-Pti1K96N [23] were expressed in E. coli BL21 strain and subsequently purified using glutathione sepharose (Amer sham Pharmacia) according to the manufacturer’s instructions. In the case of GST-tagged proteins the GST tag was removed by thrombin cleavage. The in vitro kinase assay was performed as described in [17] using 0.5 μg recombinant protein kinase and 1.5 μg kinase substrate in a 15 μl volume. Each kinase reaction was started by addition of 15 μl reaction mix to yield a final concentration of 10 mM MgCl2, 10 mM MnCl2, 1 mM CaCl2, 0.925 MBq (2.5 μCi) [γ-32P]ATP (11Tβqmmol; NEN) and 50 μM ATP. After incubation for 30 min at 30 °C, the reaction was stopped by adding 15 μl of SDS loading dye. Samples were loaded onto the same SDS-polyacrylamide gel. After electrophoresis the gel was stained with Coomassie brilliant blue, dried and subjected to autoradiography to detect incorporation of 32P into the kinase substrates.

3. Results

3.1. LeACIK1 interacts with LeCITRX in the Y2H system

ACIK1 was previously identified as a protein kinase specifically required for the Cf-9/Avr9 induced defence response [17]. We were interested in finding other protein interactors of ACIK1 that would provide further clues about molecular details of the Cf-9 signalling mechanism. We used the Y2H system to test two ACRE genes, which are required for the Cf9/Avr9 induced HR, ACRE-189 (ACFI, F-box protein) [17] and ACRE-74 (CMPG1, U-box protein) [24], and CITRX as an interactor of the cytoplasmic domain of Cf-9 [15]. As a result, an interaction was only detected between LeCITRX and NtACIK1. The tomato ortholog of tobacco ACIK1, LeACIK1, was also found to interact with LeCITRX (Fig. 1B). We performed mapping of LeACIK1 and LeCITRX domains that are involved in the interaction (Figs. 1A–D). The LeACIK1 amino acid sequence can be divided into 3 parts: the central kinase domain (K, aa1–84) and flanking N-terminal (N, aa85–365) and C-terminal (C, aa366–
464) domains (Fig. 1A). We used the quantitative assay for β-galactosidase activity to assess the intensity of interaction between different domains of LeACIK1 and LeCITRX. In each case the β-galactosidase activity was measured 3 times and an average value was calculated (see Section 2). Data presented in Fig. 1C show that the N-terminal domain of LeACIK1 as well as the kinase inactive mutant LeACIK1D215A interacted with LeCITRX with the intensity similar to LeACIK1w.t. For other LeACIK1 truncations the β-galactosidase activity was close to the basal level. Surprisingly, the “NK” truncation did not show any interaction with LeCITRX although it was well expressed in yeast (Fig. S1). Probably, the kinase domain, in the absence of the C-terminal domain, perturbs the interaction between the N-terminal domain of LeACIK1 and LeCITRX. Based on the data presented, the N-terminal domain of LeACIK1 is involved in the interaction with LeCITRX. Furthermore, the kinase activity of LeACIK1 does not seem to be essential for the interaction.

To test whether the reducing activity of LeCITRX is required for the interaction with LeACIK1, we replaced both cysteines in the catalytic site of LeCITRX (C\(_{G1P_2}\)) with serines (S\(_{G1P_2}\)). LeCITRX\(_{G1P_2}\) interacted with LeACIK1 as efficiently as LeCITRX\(_{w.t.}\) (Fig. 1D). We also tested LeCITRX\(_{G1P_2}\) side by side with LeCITRX\(_{w.t.}\) against the cytoplasmic domain of Cf-9 (CF\(_{C-ter}\)). The data presented in Fig. 1F show that LeCITRX\(_{w.t.}\) and LeCITRX\(_{G1P_2}\) interact with Cf-9 C-term. equally well.

Therefore, it appears that the catalytic activity of LeCITRX is not essential for interactions with either Cf-9 or LeACIK1. ATRX-x [15,25], a thioredoxin related to AtCITRX (Arabidopsis ortholog of LeCITRX), was used as a negative control in this assay. AtTRX-x did not interact with Cf-9 (nor with LeACIK1, data not shown), indicating that the interaction of Cf-9 with LeCITRX is specific.

In order to find out which regions of the LeCITRX amino acid sequence are required for interactions with LeACIK1 and CF\(_{C-ter}\) (Fig. 3). LeCITRX is specifically phosphorylated by LeACIK1 in vitro.

All proteins used in this assay were expressed in E. coli BL21 cells and subsequently purified using either glutathione sepharose (GST-tag) or amylose resin (MBP-tag). Purified LeACIK1 and Pto kinases were subjected to an in vitro kinase assay using [\(^32\)P]ATP as a radioactive label for LeCITRX (C) and Pti\(_{K56N}\) (P) substrates. After the kinase reaction, the samples were separated on a SDS-polyacrylamide gel, and the gel was dried and subjected to autoradiography. Panel (i) shows signals produced due to phosphorylation of the substrates and kinase autophosphorylation activity. Panel (ii) shows the Coomassie-stained gel.

A model summarizing our Y2H and Y3H data are shown in Fig. 1I. According to this model LeCITRX serves as an adaptor protein connecting the LeACIK1 kinase with the cytoplasmic domain of Cf-9.

3.2. LeCITRX and LeACIK1 interact in planta

To examine whether LeACIK1 and LeCITRX also interact in planta, we did a series of co-immunoprecipitations with LeACIK1-Myc and LeCITRX-HA transiently expressed in N. benthamiana leaves. We detected a LeACIK1/LeCITRX interaction by using the DSP protein cross-linker (Fig. 2). Fig. 2A shows that LeACIK1-Myc, but not EDS1-Myc [26]...
used as a negative control, was co-immunoprecipitated with LeCITRX-HA. In a similar experiment (Fig. 2B), LeACIK1-Myc co-immunoprecipitated with LeCITRX-HA but not with GUS-HA, which was used as a negative control. The co-immunoprecipitations shown in Figs. 2A and B were repeated two and three times respectively with similar results.

3.3. LeCITRX is phosphorylated by LeACIK1

Having obtained the evidence for the LeACIK1/LeCITRX interaction, we hypothesized that LeCITRX may be a substrate for LeACIK1. We therefore did an in vitro kinase assay (Fig. 3). In this experiment we compared the efficiency of LeCITRX phosphorylation by MBP-LeACIK1 and GST-Pto kinases. We used kinaseinactive GST-Pti1K96N, a known substrate of Pto [27], as a control. The panel (ii) in Fig. 3 shows that MBP-LeACIK1 phosphorylated GST-LeCITRX ("C") much more efficiently than GST-Pti1K96N ("P"), while Pto showed the opposite result. Both MBP-LeACIK1 and GST-Pto demonstrated autophosphorylation activity in vitro (panel (i) in Fig. 3). The equal intensity of autophosphorylation bands in lanes "P" and "C" in the case of both MBP-LeACIK1 and GST-Pto indicates equal kinase activity used for reactions with GST-LeCITRX and GST-Pti1K96N. The data presented in Fig. 3 are from the same protein gel and from the same exposure time. This experiment was repeated three times with similar results.

4. Discussion

In this paper we demonstrate that LeACIK1 kinase interacts with LeCITRX thioredoxin, both in the Y2H system and in planta. We also show, using the Y3H system, that LeACIK1 interacts with the cytoplasmic domain of Cf-9 in the presence of LeCITRX. Altogether these data suggest that CITRX could serve as an adaptor recruiting ACIK1 kinase to the cytoplasmic domain of Cf-9 during the Avr9-induced defence response. CITRX was previously identified as an interactor of the cytoplasmic domain of Cf-9 and shown to be a negative regulator of the Cf-9/Avr9-induced defence response [15]. Thioredoxins are ubiquitous disulfide reductases that generally serve as electron donors for various enzymatic redox reactions [28]. However, there are also a number of reports on thioredoxins being involved in regulation of cellular processes, such as immune response, proliferation and apoptosis due to the ability of some of these proteins to modulate the activity of certain transcriptional factors (e.g. NF-kB), kinases (e.g. ASK1) and enzymes with tumour suppressor activity (e.g. PTEN) [29–32]. The self-incompatibility response in Brassica species provides another example of a kinase being regulated by a thioredoxin. In this case, the S-locus receptor kinase (SRK), a member of the RLK superfamily, is inhibited by the THL1 thioredoxin in the absence of a ligand [33]. We previously reported that CITRX is a suppressor of the Cf-9/Avr9-induced HR [15]. Results of the kinase assay (panel (i) in Fig. 3), however, indicate that LeCITRX does not inhibit LeACIK1 autophosphorylation activity in vitro. However, we cannot rule out that the in vivo conditions may be crucial for the inhibitory effect. In all examples mentioned above the reducing activity of thioredoxins was found to be essential for their regulatory functions. Interestingly, catalytically inactive mutant LeCITRXG92P interacted with both Cf-9 cytoplasmic domain and LeACIK1 as efficiently as the wild-type (Figs. 1C and D). In agreement with these results, the catalytic activity of LeCITRX was found to be dispensable for the attenuation of the Cf-9/Avr9-induced defence response (Susana Rivas and Jonathan Jones, unpublished). A paradox remains, however, between the proposed role of CITRX as an adaptor for ACIK1 and its function as a negative regulator of the Cf-9/Avr9 induced defence response. As ACIK1 is a positive regulator of the Cf-9 dependent defence response [17], silencing of the CITRX gene, according to the model (Fig. 1), is expected to downregulate the defence response. The effect of CITRX silencing is, however, the opposite: the defence response becomes upregulated [15]. One hypothesis that can explain this phenomenon is that CITRX is the ACIK1 adaptor present in excess. Since it is in excess, the number of CITRX molecules interacting with both Cf-9 and ACIK1 is limited. When CITRX is overexpressed, the number of CITRX molecules associated with both Cf-9 and ACIK1 goes down and this results in a negative effect on the defence response. The virus induced gene silencing (VIGS) is never 100% efficient (around 80% in the case of tobacco rattle virus induced VIGS in tomato [34]). Therefore, according to the proposed model, when CITRX level decreases due to silencing, the number of CITRX molecules interacting both Cf-9 and ACIK1 increases and this results in upregulation of the defence response.

An alternative model is that CITRX is a negative regulator that at the same time acts as an adaptor for the ACIK1 kinase. As it was mentioned above, the VIGS in tomato is around 80% efficient. Therefore, the remaining 20% of LeCITRX in silenced tomato leaves may be still sufficient for it to function as an adaptor for LeACIK1, while its repressor function is abolished. An excellent example of an adaptor protein, which at the same time acts as a repressor of the signalling mechanism, is Tollip (Toll-interacting protein). Tollip is involved in the IL-1R (interleukin 1 receptor), TLR2 and TLR4 (toll-like receptors 2 and 4) signalling in mammalian cells [35,36]. Tollip forms a complex with the IRAK kinase under resting conditions. Upon triggering of the signalling mechanism, Tollip recruits IRAK to the activated receptor. As in the case with CITRX, overexpression of Tollip attenuated the signalling response, while silencing of its coding gene gave the opposite effect [36]. Tollip was shown to be a substrate for IRAK and an inhibitor of its kinase activity at the same time.

LeACIK1 is related to a subgroup of Arabidopsis RLKs called receptor-like cytoplasmic kinases (RLCKs) VII [4,37]. Unlike RLKs, RLCKs lack a transmembrane domain. Therefore, it is possible that, similarly to the Tollip adaptor protein in the IL-1 and Toll-like receptor signalling, LeCITRX serves as an adaptor recruiting LeACIK1 to the cytoplasmic domain of Cf-9 receptor-like protein during early stages of the Cf-9/Avr9-induced defence response.

Acknowledgements: We thank Michael Weaver for the pBIN19g and pBin19d vectors, Maria Santos-Rosa for the pSR78 vector, David Baulcombe’s lab for pi9 suppressor of silencing, Vasilios Andriotis for the Pto and Pti recombinant proteins, and Michal Swiderski, Susana Rivas and Alejandra Rougon for the GUS and LeCITRX constructs. This project was sponsored by the BBSRC (Grant 83/P132372) and Gatsby Charitable Foundation.
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.06.077.

References