PROTEIN STRUCTURE REPORT

The crystal structure of a TIR domain from *Arabidopsis thaliana* reveals a conserved helical region unique to plants

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Abstract: Plants use a highly evolved immune system to exhibit defense response against microbial infections. The plant TIR domain, together with the nucleotide-binding (NB) domain and/or a LRR region, forms a type of molecule, named resistance (R) proteins, that interact with microbial effector proteins and elicit hypersensitive responses against infection. Here, we report the first crystal structure of a plant TIR domain from *Arabidopsis thaliana* (AtTIR) solved at a resolution of 2.0 Å. The structure consists of five β -strands forming a parallel β -sheet at the core of the protein. The β -strands are connected by a series of α -helices and the overall fold mimics closely that of other mammalian and bacterial TIR domains. However, the region of the α D-helix reveals significant differences when compared with other TIR structures, especially the α D3-helix that corresponds to an insertion only present in plant TIR domains. Available mutagenesis data suggest that several conserved and exposed residues in this region are involved in the plant TIR signaling function.

Keywords: plant; immunity; infection; structure

Introduction

The plant Toll/IL-1 receptor/plant disease resistance gene (TIR) domains play an integral role in its immune system forming part of a defense mechanism against microbial infection. TIR domains in plants exist as a component of a family of multido-

main proteins known as resistance (R) genes. These R proteins also contain a nucleotide-binding (NB) domain and/or a leucine-rich repeat (LRR) region.¹ One of the best characterized systems is the tobacco plant resistance protein N, with a domain arrangement TIR-NB-LRR, being implicated in the defense against tobacco mosaic virus (TMV) infection.²

The TIR domains are not unique to the plant kingdom. Extensive studies have shown the important role of TIR domains in the Toll-like receptor (TLR) pathway in initiating the innate immune response in animals. Upon sensing microbial pathogen-associated molecular patterns (PAMP), TLR extracellular LRR regions will form homo- or heterodimers, leading to the activation of their cytoplasmic TIR domains. This brings about the recruitment of

Additional Supporting Information may be found in the online version of this article.

Abbreviations: AtTIR, *Arabidopsis thaliana* TIR domain; IL-1, interleukin-1; TIR, toll/IL-1 receptor/plant disease resistance gene; TLR, toll-like receptor.

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Figure 1. Crystal structure of AtTIR and comparison with other TIRs. (A) Stereodiagram depicting the backbone trace for the AtTIR protein structure. The electron density for the region between residues 45 and 58 was not observed and therefore not modeled. (B) Structural superposition of AtTIR with mammalian and bacterial TIR domains. The crystal structure of AtTIR (blue) is superimposed with other known TIR domain structures, such as human TLR1 (red), human MyD88 (green), and bacterial PdTIR (yellow) according to a DALI24 structural alignment. The structures overlap well especially in the α -sheet region at the core of the protein. Note that AtTIR contains an extension of the α D-helix region when compared with the others.

TIR-containing adaptor proteins such as the myeloid differentiation primary response gene 88 (MyD88) and MyD88 adaptor-like (MAL, also known as TIRAP) via heterotypic receptor–adaptor TIR interactions. Several TIR structures have been solved including the ones from TLR1, TLR2,³ TLR10,⁴ and MyD88.⁵ Bacteria too have developed virulence factors containing the TIR domain. Proteins such as TlpA from *Salmonella enterica*,⁶ TcpB from *Brucella melitensis*, and TcpC in *Escherichia coli*⁷ act as inflammation blockers impairing the TLR signaling of the host. Recently, the crystal structure of a bacterial TIR domain from *Paracoccus denitrificans* (PdTIR)⁸ was solved and its structure closely resembles that of mammalian TIRs.

Although the structure and function of TIR domains involved in the TLR pathway have been extensively studied, the signaling properties of TIR domains in plants are not well understood. In the genome of *Arabidopsis thaliana*, it is predicted that there are 94 TIR-NB-LRR proteins,⁹ and several are well characterized. *A. thaliana* TAO1 protein has been shown to contribute to disease resistance against *Pseudomonas syringae* infection.¹⁰ In the studies of *A. thaliana* RPS4 protein, transgenic plants expressing RPS4-TIR elicited inducer-dependent cell death, indicating a role for the TIR domain in cell death signaling.¹¹ Here, we report the first crystal structure of a plant TIR domain, the protein NP_177436 from *A. thaliana*

(AtTIR). The 3D structure reveals a unique feature: the presence of an extended α D-helix containing the residues mapped to perform its signaling function.

Results

Expression and purification of AtTIR

The *A. thaliana* NP_177436 protein (AtTIR) is a 176 amino acids long polypeptide chain that contains only a TIR domain in its architecture. AtTIR was expressed as a soluble His-tag protein and purified by Ni-affinity chromatography. The N-terminal His-tag was removed using thrombin and the digested protein was subjected to a final purification step using a gel filtration column. The purified protein appeared as a single band on SDS-PAGE and its molecular weight was verified using MALDI-TOF mass spectrometry. Analysis of a native PAGE showed that the protein appeared as two bands in the absence of reducing agent, but behaved as single homogenous band in the presence of 10 mM β -mercaptoethanol or 10 mM DTT (data not shown). Therefore, usage of a reducing agent was introduced throughout the purification process. Incorporation of selenomethionine into the protein was verified using MALDI-TOF, and the results showed that three selenomethionines were detected in the molecule, which is in agreement with the presence of three methionine residues in its amino acid sequence.

Table I. Crystallographic Data and Refinement Statistics

Data collection	Peak	Inflection	Remote	Native
Wavelength	0.979	0.979	0.918	0.979
Resolution (Å)	50–2.5	50–2.5	50–2.5	50–2.0
R_{sym}	8.2 (30.4)	7.9 (31.5)	8.2 (35.6)	8.1 (52.7)
$I/\sigma I$	17.2 (4.6)	18.1 (4.4)	16.7 (3.3)	23.9 (3.0)
Completeness	99.9 (99.3)	100 (99.7)	99.7 (98.0)	99.7 (97.1)
Redundancy	3.9 (3.6)	3.9 (3.7)	3.8 (3.1)	7.4 (5.4)
Refinement				
Resolution (Å)	50–2.0			
$R_{\text{work}}/R_{\text{free}}(\%)$	0.168/0.208			
R.M.S. bond lengths (Å)	0.005			
R.M.S. bond angle (°)	1.10			
Number of atoms				
Protein	1231			
Ligand/ions	1			
Water	125			
Average B -factors (Å ²)				
Protein	32.0			
Ligand/ions	39.6			
Water	38.8			
Ramachandran analysis				
Most favored (%)	92.8			
Additionally allowed (%)	7.2			
Generously allowed (%)	0			
Disallowed (%)	0			

Crystal structure of AtTIR

We have obtained diffracting crystals at room temperature using the hanging drop vapor diffusion method for both native and selenomethionine-labeled AtTIR. Native crystals were able to diffract up to 2.0 Å resolution at the SSRL synchrotron. The phases were obtained using multiwavelength anomalous diffraction (MAD) data with selenomethionine crystals diffracting up to 2.5 Å resolution. The protein crystals belong to the space group $P4_12_12$ with one molecule in each asymmetric unit. This is consistent with the observed protein elution volume on a calibrated size exclusion chromatography column, which indicated its monomeric state in solution. The structure was refined against a native dataset using the CNS¹² software with a final R -work and R -free values of 0.168 and 0.208, respectively. Ramachandran plot analysis of the deposited coordinates found no residues in the disallowed region.

The crystal structure of AtTIR reveals a compact globular fold resembling those observed in mammalian and bacterial TIR domain proteins. The structure is composed of a β -sheet comprising five parallel β -strands, each connected with a series of α -helices. The naming of secondary structure elements and loops follows the nomenclature used in the TLR1-TIR and PdTIR structures. The first 10 N-terminal residues after the thrombin cleavage did not show any electron density, and thus the protein structure was modeled from residue Thr-7 onward [Fig. 1(A)]. Electron density between residues 45 and 58, comprising the BB-loop and α B-helix, was not observed and therefore not modeled. Crystallo-

graphic data and structure refinement statistics are described in Table I.

The structure of AtTIR resembles that of other mammalian and bacterial TIR domains

AtTIR structure was compared with known TIR domain structures including the human TLR1 and MyD88 as well as the bacterial PdTIR. Although AtTIR only shares less than 20% sequence identity with these proteins, the overall structure closely resembles the typical fold of a TIR-domain. A DALI¹³ search revealed that AtTIR has the highest structural similarity with human TLR1-TIR with a Z -score and rmsd values of 10.6 and 2.6 Å, respectively. AtTIR also possesses high structural similarity with human MyD88 and PdTIR with Z -scores of 9.6 and 9.5, respectively. Such high Z -score values show that these TIR domains across the animal, bacterial, and plant kingdoms share the same structural fold [Fig. 1(B)].

Although the 3D fold of AtTIR is highly superimposable with other TIR domains, there is a noticeable difference at the region of the α D-helix where AtTIR displays an insertion. In plants, the α D-helix region is composed of three α -helices named α D1-, α D2-, and α D3-helix. The sequence alignment shows an addition of about 20 residues in this region in AtTIR when compared with mammalian and bacterial TIR domains [Fig. 2(A)]. These extra residues are observed in other plant TIR domains, including those in *Vitis vinifera* predicted protein XP_002269819, *Nicotiana tabacum* N protein, and *A. thaliana* RPS4. This inserted

Figure 2. Mapping of functional residues on the AtTIR structure. (A) Multiple sequence alignment of TIR domains. Amino acid sequences of plant AtTIR, RPS4, *Vitis vinifera* XP_002269819, N protein, bacterial PdTIR as well as human TLR1 and MyD88 were aligned using ClustalW14. The secondary structure elements of TIRs with known structure are labeled with blue (s-strand) or red (α -helix). The cartoon on top depicts the secondary structure elements for AtTIR. Functional residues identified in mutagenesis studies^{2,11} are highlighted in bold. Note the distinctive α D3-helix region that is present only in plant TIRs, but absent in the bacterial and mammalian sequences. (B) Ribbon diagram depicting (in wireframe) the location of the mutagenized functional residues identified in the RPS4 and N protein. Note the clustering of residues at the α D3-helix and its surrounding region (labeled in red) as well as the residues near BC-loop region (labeled in blue).

sequence folds into the α D3-helix, which is unique to the plant TIR domains, displaying several highly conserved positions such as residues Glu-131, Trp-136, Arg-137, and Ala-139 (numbering for AtTIR).

Mapping of functional residues of plant TIR domains on the AtTIR structure

Several mutagenesis studies have pointed out a few residues important for the TIR function in plants defense against infection. Deletion studies in the tobacco plant N resistance protein have shown that the TIR domain plays an essential role in providing protection against TMV infection.² Single point mutation of D46H or I63M leads to a complete loss-of-resistance response against TMV. Furthermore, substitutions at positions 12, 67, 82, 138, 141, and 142 of the N protein generated a partial loss-of-function phenotype. This further causes systemic hypersensitive response and spread of the TMV infection.²

The crystal structure of AtTIR allows us to map the relative location of these functional residues in the context of the 3D structure. Sequence alignment between AtTIR and the tobacco N protein was car-

ried out using the software ClustalW¹⁴ and the corresponding positions were identified [Fig. 2(A)]. The mapping of these residues on the crystal structure revealed that they cluster in two surface areas. The first one consists of residue Tyr-9 at the N-terminal end and residues Ile-60 and Arg-64, which are located in the BC-loop. The second region groups Val-133, Trp-136, and Arg-137, all part of the unique α D3-helix, with α C-helix residue Trp-79 spatially located just adjacent to them [Fig. 2(B)].

Another independent mutagenesis study on *A. thaliana* RPS4 protein has revealed similar residues involved in the function of TIR domains in plants. Mutation of α C-helix residue W84A results in a gain of function phenotype causing an increase in cell death.¹¹ Mutations at RPS4 residues Arg-135, Lys-137, and Lys-140 (all positively charged) located in the α D3-helix resulted in loss of function and reductions in cell death,¹¹ whereas the mutation of E134K caused a gain of function. Furthermore, the strictly conserved α D3-helix tryptophan residue is always followed by a positively charged Arg or Lys. The residues along α D3-helix and the electrostatic potential

map around this region are shown in Supporting Information Figure S1.

To determine the degree of exposure of these residues in the structure, their solvent-accessible surface area was assessed using the GETAREA software.¹⁵ Of all the amino acids mentioned in the mutagenesis studies, only the equivalent AtTIR residues Trp-79, Glu-131, and Leu-134 are highly exposed on the surface of the protein and accessible to the solvent indicating that their side chains may be involved in the interaction with a signaling partner. On the other hand, residues Tyr-74, Cys-80, Glu-83, Val-133, and Trp-136 are mainly buried and inaccessible to the solvent. Therefore, any mutational data concerning these positions have to be interpreted with care as these replacements may contribute to substantial internal conformational changes and lead to the destabilization and unfolding of the domain.

All in all, we predict that the cluster of positively charged residues in the α D3-helix region plays a role in the function of TIR domains possibly participating in a protein–protein interaction with its binding partner. This helix is highly conserved and unique among the plant TIRs and is not found in other TIR domains, neither in mammals (TLR1, MyD88) nor in bacteria (PdTIR).

Discussion

TIR domains, functioning as a protein–protein interaction platform, are present in proteins across the bacteria, plant, and animal kingdoms. In mammals, TIR domains are found not only as the cytosolic portion of TLRs but also in their adaptor molecules such as MyD88 or TIRAP.¹⁶ The important role of TIR domains in the signaling cascade eliciting innate immune responses is linking the activated receptor with downstream kinases via heterotypic TIR–TIR interactions. In plants, the TIR domain of the tobacco resistance protein N is involved in the recognition of the TMV p50 effector protein by forming a complex with the N-receptor interacting protein (NRIP1), suggesting a novel protein–protein interaction role for the TIR.^{1,17,18}

Several structural and mutational studies have pointed to the BB-, DD-, and EE-loop regions as mediators of the homo- or heterodimerization function of TIR domains in bacteria and mammals.^{3,4,8} The most noteworthy is the BB-loop region characterized by the Φ x PG sequence motif, which is involved in the homodimerization of human TLR10 TIR domain⁴ and in the interaction between the TIR domains of human TLR2 and MyD88.³ However, mutagenesis studies in plants have shown only limited evidence of functional residues in this region, which lacks the otherwise universally conserved PG motif. Incidentally, the electron density corresponding to the BB-loop residues of AtTIR was not visible,

probably because of the intrinsic flexibility of this particular sequence [Fig. 1(A)].

As it is the case for several other TIR domain structures that have been solved, AtTIR behaves as a monomer in solution. However, under crystallization conditions, different TIRs have shown different dimer interfaces in the crystallographic cell, suggesting BB-loops in TLR10-TIR⁴ and DD- and EE-loops in PdTIR⁸ as the homodimerization surface. In the AtTIR crystal structure, only one molecule was observed in the asymmetric unit. Although the oligomerization of TIR domains plays an important role in transducing the signal of the mammalian TLR pathway, it has yet to be proven that such interaction has any effect in plants.

The structure of AtTIR has revealed that the plant TIR possesses the same overall fold seen in other TIR domains from bacterial and mammalian proteins [Fig. 1(B)]. AtTIR shares about 43, 46, and 32% sequence homology with the TIRs of the tobacco N protein, *V. vinifera* XP_002269819 and *A. thaliana* RPS4, respectively. With the availability of the crystal structure of AtTIR, modeling of other plant TIR domain structures can now be carried out reliably.

The AtTIR structure shows a unique extension of the α D-helix area of about 22 residues folded into the α D3-helix. The α D3-helix is absent in bacteria and mammalian TIR domains, but the related sequence can be found in all other plant TIRs (Fig. 2). Mutagenesis studies on the N and RPS4 proteins have shown that multiple residues in this region are important for the function of the plant TIR. Therefore, this suggests that plant TIRs may be involved in a novel signaling interaction unlike the receptor–adaptor TIR heterodimerization observed in the mammalian TLR pathway. The discovery that key functional residues (like Trp-79 or Glu-131) are strictly conserved and highly exposed forming part of the distinctive α D3-helix or its surroundings points toward a crucial role played by this region in eliciting the plant infection resistance response as well as in cell death signaling, via the interaction with a protein partner.

Materials and Methods

Protein expression and purification

The cDNA encoding the *A. thaliana* NP_177436 protein (AtTIR) was subcloned into the pET-28a plasmid (Novagen) and verified by DNA sequencing. The plasmid was transformed into *Escherichia coli* Rosetta strain cells (Novagen) for protein expression. Overnight cultures of the bacterial cells were grown in 2 L of Luria Broth, supplemented with 50 μ g/mL of kanamycin. Protein expression was induced with 0.3 mM final concentration of isopropyl β -D-thiogalactoside (IPTG) at 15°C for an overnight duration.

Bacterial cells were harvested using centrifugation and cell pellets were kept at -20°C until further analysis. Selenomethionine-labeled protein was obtained similarly by growing the bacteria cells in M9 media with an addition of 60 mg/L of selenomethionine 15 min before IPTG induction.

For protein purification, bacterial cell pellets were solubilized in 20 mM Tris pH 8.5, 0.3M NaCl, 1 mM PMSF, and 10 mM β -mercaptoethanol and lysed using sonication. Cell lysates were loaded into a His-Trap HP column (GE Healthcare) equilibrated with 20 mM Tris pH 8.5, 0.3M NaCl, and 10 mM β -mercaptoethanol followed by washing with similar buffer in the presence of 25 mM imidazole. Purified protein was eluted straight into a Sephacryl S200 HiPrep 16/60 gel filtration column equilibrated with 20 mM Tris pH 8.5, 0.1M NaCl, and 10 mM β -mercaptoethanol. His-tag was removed from the protein by digestion using thrombin (5 U/mg, Sigma-Aldrich) for 1 h at room temperature. The protein sample was reloaded into the same gel filtration column for final purification. The molecular weight of the protein samples and incorporation of selenomethionine were examined using MALDI-TOF mass spectrometry.

Crystallization and X-ray diffraction experiments

Purified protein was buffer-exchanged with 10 mM Tris pH 8.5 and 10 mM DTT (dithiothreitol) using an Amicon Ultra (Millipore) centrifugation filter device before crystallization trials. Native crystals were obtained at room temperature using the hanging-drop vapor diffusion method, in a 3 μL total drop volume containing 1 μL of protein solution (at a concentration of 5.2 mg/mL) and 2 μL of the crystallization buffer. Selenomethionine-labeled crystals were obtained similarly except that the protein concentration was at 2.75 mg/mL. Crystallization buffer was optimized to 0.1M sodium cacodylate pH 6.5, 1.0M sodium citrate, and 5% glycerol for the native crystals and 0.1M sodium cacodylate pH 6.5 and 1.0M sodium citrate for the selenomethionine-labeled protein. Crystals were observed at full size after 3 days and were cryoprotected with 0.1M sodium cacodylate pH 6.5 and 1.4M sodium citrate before freezing in liquid nitrogen for data collection. All data sets were collected at the Stanford Synchrotron Radiation Laboratory (SSRL) beamline 9-2 equipped with MAR325 detector. HKL-2000 software package¹⁹ was used to index, integrate, and scale the diffraction data.

Crystal structure solution and refinement

Selenium sites were located in the selenium methionine crystals by using the SOLVE software²⁰ with a figure of merit of 0.51 and data up to 2.5 Å resolution. Initial phases and models were obtained using RESOLVE²⁰ and the models were improved manually with the Coot program.²¹ The crystals

belonged to the space group $P4_12_12$ with one molecule per asymmetric unit. Model refinement was performed with the CNS software¹² against native data up to 2.0 Å resolution. Ramachandran plot analysis was carried out with the PROCHECK software²² observing no residues in the disallowed region. The coordinates and structure factors were deposited in the PDB with accession code 3JRN. All structure diagrams were generated with the UCSF Chimera software.²³

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