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Structural and Functional Insights into an *Arabidopsis* NBS-LRR Receptor in *Nicotiana benthamiana*

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Abstract

Nucleotide-binding site leucine-rich repeat receptors (NBS-LRR/NLRs) are crucial intracellular immune proteins in plants. Previous article reported a novel NLR protein SUT1 (SUPPRESSORS OF TOPP4-1, 1), which is involved in autoimmunity initiated by type one protein phosphatase 4 mutation (topp4-1) in *Arabidopsis*, however, its role in planta is still unclear. This study employed *Nicotiana benthamiana*, a model platform, to conduct an overall structural and functional analysis of SUT1 protein. The transient expression results revealed that SUT1 is a typical CNL (CC-NBS-LRR) receptor, both fluorescence data and biochemical results showed the protein is mainly anchored on the plasma membrane due to its N-terminal acylation site. Further truncation experiments announced that its CC (coiled-coil) domain possessed cell-death-inducing activity. The outcomes of point mutations analysis revealed that not only the CC domain, but also the full-length SUT1 protein, whose function and subcellular localization are influenced by highly conserved hydrophobic residues. These research outcomes provided favorable clues for elucidating the activation mechanism of SUT1.

Keywords

CC-NBS-LRR, Hypersensitive Response, *Nicotiana benthamiana*, Plasma Membrane Localization

1. Background

1.1. Introduction

In the game process with various pathogens, plants have a two-layered immune

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system, including pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) [1]. PTI and ETI have been demonstrated to interact with each other [2] [3]. PTI is initiated by pattern recognition receptors (PRRs) located on the cell membrane and can resist most pathogens. Some pathogens have evolved a type III secretion system, which transmits effectors to plant cells to inhibit PTI, leading to plant susceptibility. Once the corresponding effectors are directly or indirectly monitored through intracellular immune receptors, which are mostly proteins encoding nucleotide-binding leucine-rich repeat receptors (NLRs), ETI is activated and usually goes with characteristic cell death, known as hypersensitivity response (HR) [4].

Simply put, according to the differences in the N-terminus, NLR receptors can be divided into two categories: TIR (toll/interleukin-1)-NBS-LRR (TNL)and CC (coiled-coil)-NBS-LRR (CNL) [5]. Transient expression of some NLR receptors N-termini in *Nicotiana benthamiana* is enough to generate severe hypersensitive response [6]-[11]. The NBS domain is relatively conserved in NLR receptors, containing many extremely conserved motifs such as P-loop and MHD (Met-His-Asp) motifs, which are considered molecular switches for NLR protein activity [12]. The LRR domain is usually unstable and variable, involving NLR receptor and effector interaction, and auto-inhibition of NLR receptors in the absence of pathogens [13].

Many NLR receptors function on the plasma membrane (PM). Previous studies have shown that RPS5 (*RESISTANCE TO PSEUDOMONAS SYRINGAE* 5), L5 (*AT*1*G*12290) and R5L1 (*RPS*5*-like* 1) all function on the PM, and their N-terminal myristoylation site is important for their function and subcellular localization [11] [14] [15]. Recent reports have shown that ADR1 (*ACTIVATED DISEASE RESISTANCE* 1), NRG1.1 and NRG1.2 (*N Requirement Gene* 1*s* (*NRG*1*s*)) proteins share similar activation mechanisms; when activated, they generated advanced oligomeric permeable thoroughfare related to Ca^{2+} on PM [16] [17]. Notably, the CC domain of barley MLA10 (*mildew locus a* (*Mla*)) has been proven to form homologous dimers, and some conserved hydrophobic residue (I33, L36, and M43) alleles can abolish the self-interaction of CC module [6]. Interestingly, these hydrophobic sites are extremely conserved in many NLR proteins, including RPM1 (*Resistance to Pseudomonas syringae pv. Maculicola* 1). Mutations of the corresponding hydrophobic residues (I31, M34, and M41) can significantly affect the self-association of RPM1 CC and the function of full-length RPM1 [18]. It is currently unclear whether these hydrophobic sites are related to self-interaction, or whether their roles in each NLR are diverse.

Previous article reported a novel NLR protein SUT1, which is involved in autoimmunity initiated by type one protein phosphatase 4 mutation (topp4-1) in *Arabidopsis [19]*, however, its role in planta is still unclear. This study employed *Nicotiana benthamiana*, a model platform, to conduct an overall structural and functional analysis of SUT1 protein.

1.2. Statement of the Problem

What is the activation mechanism of the disease-resistant protein SUT1

(SUPPRESSORS OF TOPP4-1, 1)?

1.3. Justification of the Study

As a model plant, *N. benthamiana* can complete gene expression regulation and protein translation modification within the plant, making it more suitable for functional verification of heterologous genes than *E. coil* and yeast. With the rapid development of *Agrobacterium* mediated gene transient transformation systems, it has become more convenient to express exogenous proteins in *N. benthamiana* instantaneously, rapidly, and in large quantities for functional research. Therefore, *N. benthamiana* is currently widely used in protein subcellular localization, protein interaction, biopharmaceutical production, and other fields.

We employed the *N. benthamiana* transient expression system to deeply analyze the activation mechanism of SUT1 from both structural and functional aspects.

2. Materials and Methods

2.1. Plant Lines and Growth Conditions

The seeds of *Arabidopsis* and *N. benthamiana* are laboratory-owned (Key Laboratory of Chronic Diseases, Fuzhou Medical University, Fuzhou, China), and both model plants are cultured in specific greenhouses at 25˚C with a 16 hours light period.

2.2. Vector Construction

All plasmid vectors in this paper were constructed through the Gateway cloning strategy (Thermo Fisher Scientific). Briefly, using cDNA from wild-type *Arabidopsis* leaves as the initial template. The gel recovery products from multiple DNA fragments, including full-length (FL) SUT1 and its mutants, various SUT1 truncated fragments, and CC domain mutants, were cloned into PENTR/D (Thermo Fisher Scientific) through a step cloning kit (Vazyme, #C112-02), respectively. After complete sequencing, they were transferred to the expression vector pEarleygate101 for YFP (Yellow Fluorescent Protein)-HA tagged through Gateway cloning technology (Thermo Fisher Scientific). The expression plasmids were electro-transferred into agrobacterium strain *GV*3101.

2.3. Transient Expression in *Nicotiana benthamiana*

Agrobacteria (*GV*3101) carrying the constructs were grown overnight in Luria Bertani medium (LB) with suitable antibiotics. Three milliliters of *Agrobacterium* culture were centrifuged and resuspended in MES buffer (10 mM $MgCl_2$, 10 mM MES pH 5.6, and 150 μM acetosyringone). Agrobacteria were incubated at room temperature for 1 h and infiltrated into the leaves of 4-week-old *N. benthamiana* at specific OD_{600} values. Agrobacteria containing the P19 construct were co-infiltrated at an OD_{600} of 0.2. Cell death phenotypes were photographed at indicated time point. The white solid circle represents obvious cell death, and

the white dashed circle represents no visible cell death.

2.4. Total Protein Extraction and Immunoblotting Analysis

Three 8-mm leaf disks were harvested and ground to powder in liquid nitrogen. Total protein was extracted with 100 μL extraction buffer (20 mM Tris-HCl pH 8.0, 5 mM ethylene diamine tetraacetic acid (EDTA), 1% SDS, and 10 mM DL-dithiothreitol (DTT)). Lysate was boiled at 98˚C with 1× protein loading buffer for 10 min. The total protein extract was cleared by centrifuge at 15,000 g for 10 min. Then, the supernatant was separated by 10% SDS–PAGE gels and detected with corresponding antibodies. Antibodies used for immunoblotting include anti-HA (Roche) anti-H^+-ATPase (Agrisera) and anti-β-actin (Abbkine).

2.5. Membrane Fractionation Assays

In brief, sucrose buffer [20 mM Tris (pH 8.0), 0.33 M sucrose, 1 mM EDTA, 5 mM DTT, and 1× Sigma plant protease inhibitor cocktail] was added to the homogenized tissue at a ratio of 5 μL per mg (FW) tissue. The extract was centrifuged at 5000 × g for 10 min at 4˚C; then, the supernatant was transferred to a new tube and designated as total protein (T). Cytoplasmic fraction (C) was prepared by harvesting the supernatant after spinning the total protein fraction at 20,000 × g for 1 h at 4˚C. The total membrane fraction was prepared from the resulting pellet by resuspending in 200 μL of buffer B (Minute™ PM protein isolation kit, Invent Biotechnologies). After centrifugation at 7800 × g for 15 min at 4˚C, the supernatant was transferred to 2 mL Eppendorf tube and mixed with 1.6 mL cold phosphate belanced solution (PBS) buffer mixed by vortexing and spun at 16,000 × g for 1 h at 4˚C to pellet the PM fraction. The pellet was resuspended with sucrose buffer in 4 times less volume than the soluble fraction. The resulting fraction was labeled as the PM-enriched/microsomal fraction. Protein fractions were run on SDS-PAGE gels and analyzed by western blotting.

2.6. Subcellular Localization

In short, a laser confocal microscope (Leica SP8) and a fluorescence microscope (Olympus BX53) were employed to observe images of live cells on the abaxial sides of *N. benthamiana* leaves at 28 or 40 h post-target protein expression. YFP fluorescence was excited at 514 nm.

2.7. 3D Structure of the *Nicotiana benthamiana*

Live images of *N. benthamiana* and specific infection steps refer to the literature [20] and website (https://bio-protocol.org/en/bpdetail?id=2063&type=0).

3. Results

3.1. SUT1 is Anchored to the Plasma Membrane through Acylation

We inferred that SUT1 G2 and C4 sites are necessary for targeting SUT1 to the PM. To verify this hypothesis, we designed a SUT1 version with mutations in the

important sites, SUT1$_{G2AC4S}$. The subcellular localization pattern of SUT1-YFP (Yellow Fluorescent Protein)-HA and SUT1$_{G2AC4S}$-YFP-HA were examined. As expected, we observed that SUT1 exhibited typical membrane localization, while the introduction of G2AC4S resulted in SUT1 mutant exhibiting significant cytoplasmic distribution (**Figure 1(A)**), suggesting that the two acylation sites were prominent in SUT1 PM distribution.

To prove the fluorescence results, we carried out membrane fractionation assays. We employed H^+-ATPase and Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) serve as markers of membrane and soluble fractions, respectively. Immunoblot results indicated that wild-type SUT1 mainly existed in the microsomal fraction containing PM, while on the contrary, acylation deficient variant SUT1$_{G2AC4S}$ was mainly found in the soluble fraction (**Figure 1(B)**). Thus, SUT1 is anchored to the PM through two acylation residues.

3.2. The CC Domain of SUT1 is Enough to Cell-Death Induction

SUT1 containing several domains. In order to further determine the region that can attract cell death, we designed a series of truncated fragments and expressed

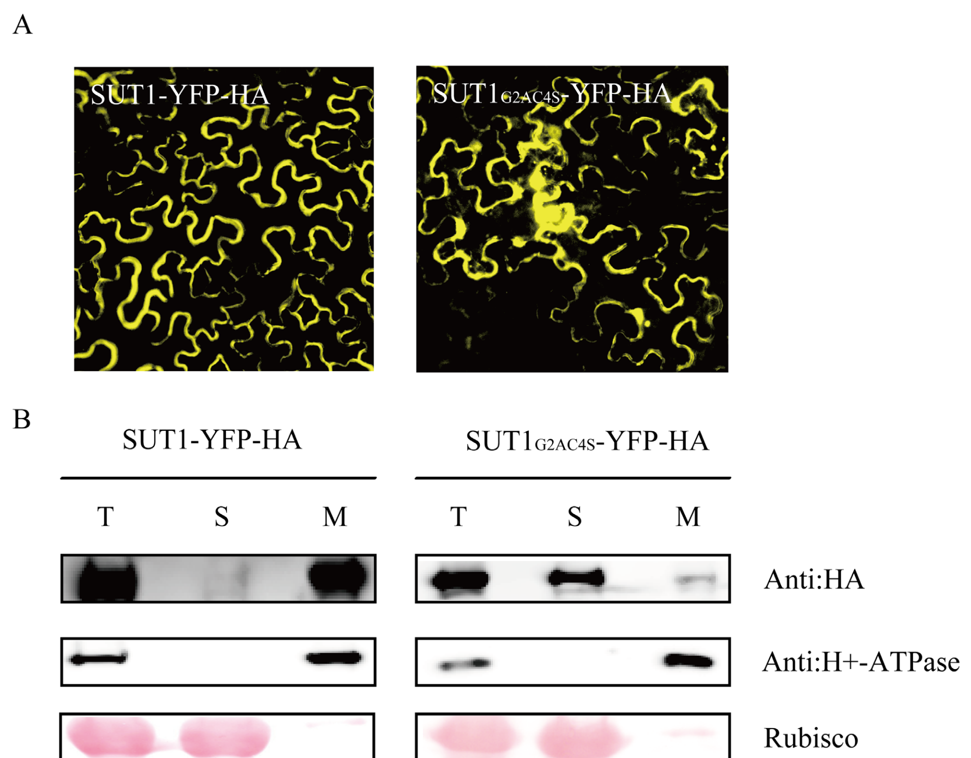

Figure 1. SUT1 (AT5G63020) is anchored to the plasma membrane through acylation. (A) Observation the subcellular localization of SUT1 and SUT1$_{G2AC4S}$ with fluorescence microscope. 35S::SUT1-YFP-HA and 35S::SUT1$_{G2AC4S}$-YFP-HA were instantaneously expressed in N. benthamiana, respectively (OD_{600} = 0.5). Photographs were derived at 40 h post infiltration (hpi). (B) The membrane and cytosol distribution of SUT1 and SUT1$_{G2AC4S}$. Total protein extractions (T) were subdivided into two fractions by high-speed centrifugation: membrane (M) and cytosol (S). Detect the distribution of SUT1 and SUT1$_{G2AC4S}$ using anti-HA antibody. Rubisco is used as a cytosol marker, and H^+-ATPase as membrane marker.

them in *N. benthamiana*, respectively (**Figure 2(A)**). We observed that only the CC domain has cell-death-inducing activity, leading to a significant hypersensitivity response in *N. benthamiana* leaves (**Figure 2(B)**). The immunoblot results showed that all variants exhibited comparable protein abundance, ruling out the possibility that these fragments could not mediate cell death due to incorrect expression (**Figure 2(C)**).

We further analyzed the localization of these fragments employing a fluorescence microscope, and found that any fragment containing two acylation sites exhibited membrane-localized, while fragments lacking these two residues displayed significant cytoplasmic and nuclear distribution (**Figure 2(D)**). Thus, the membrane-localized SUT1 CC module was enough to cause cell death in *N. benthamiana*.

3.3. The Function and Localization of the CC Domain are Affected by Three Hydrophobic Residue Mutations

We found through sequence alignment that these hydrophobic residues are highly conserved in receptors including RPM1, Sr33, and SUT1 (**Figure 3(A)**). Naturally, we introduced alleles of these residues in the CC domain to analyze the impact of these hydrophobic sites on the functionality of the SUT1 CC module. The results declared that both CC (L36E) and CC (L39E) lost their activity to trigger cell death, and CC (I46E) significantly weakened its function (**Figure 3(B)**), although none of the versions affected the protein accumulation of the SUT1 CC domain (**Figure 3(C)**), promulgating that the three hydrophobic residues in CC domain were important for its function.

Furthermore, we investigated the subcellular distribution of these CC variants, and discovered that only CC (L36E) emerged evident cytoplasmic positioning (**Figure 3(D)**). We inferred that these important hydrophobic residues were likely involved in the self-connection of the SUT1 CC domain. Thus, the function and localization of the CC domain are affected by three hydrophobic residue mutations.

3.4. Functional Loss Mutations in the CC Domain Influence the Function of FL SUT1

The motifs in the NBS domain, such as P-loop and MHD, are highly conserved in NLR receptors. To directly verify whether SUT1 protein is a typical CNL receptor, we designed SUT1 (D473V) and SUT1 (K187R/D473V) variants, which correspond to MHD motif mutant, as well as P-loop and MHD motifs double mutant, respectively (**Figure 4(A)**). The results of transient expression in *N. benthamiana* manifested that SUT1 (D473V) provoked a strong hypersensitivity response, while P-loop mutation completely abolished the cell death caused by SUT1 (D473V). Immunoblotting indicated that both proteins mentioned above can be expressed correctly (**Figure 4(B)** and **Figure 4(C)**). In summary, SUT1 protein is a typical CNL receptor.

We further analyze whether the hydrophobic residue mutations that affect the

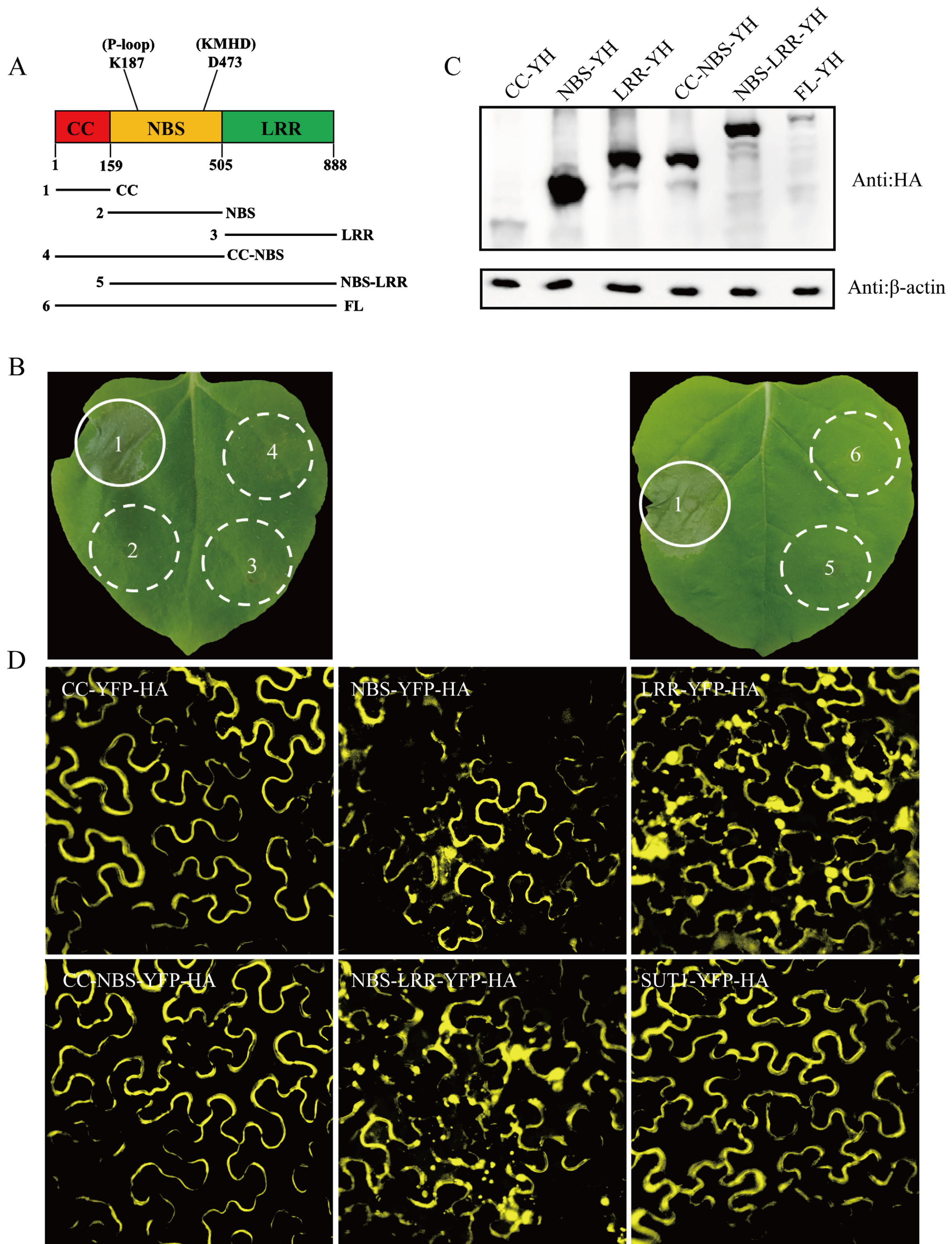

Figure 2. The CC domain of SUT1 is enough to cell-death induction. (A) Schematic diagram of the three structural domains and truncated fragments of SUT1. (B) Analyze whether various truncated fragments of SUT1 can mediate cell death. The constitutive promoter drives the transient expression of the above fragments in *N. benthamiana* (OD600 = 0.5). (C) Detect protein accumulation of six proteins. Proteins were harvested and checked at 28 hpi. β-actin serves as the equivalent load of proteins. (D) Observation the subcellular localization of truncated fragments with fluorescence microscope. Images were recorded at 24 hpi.

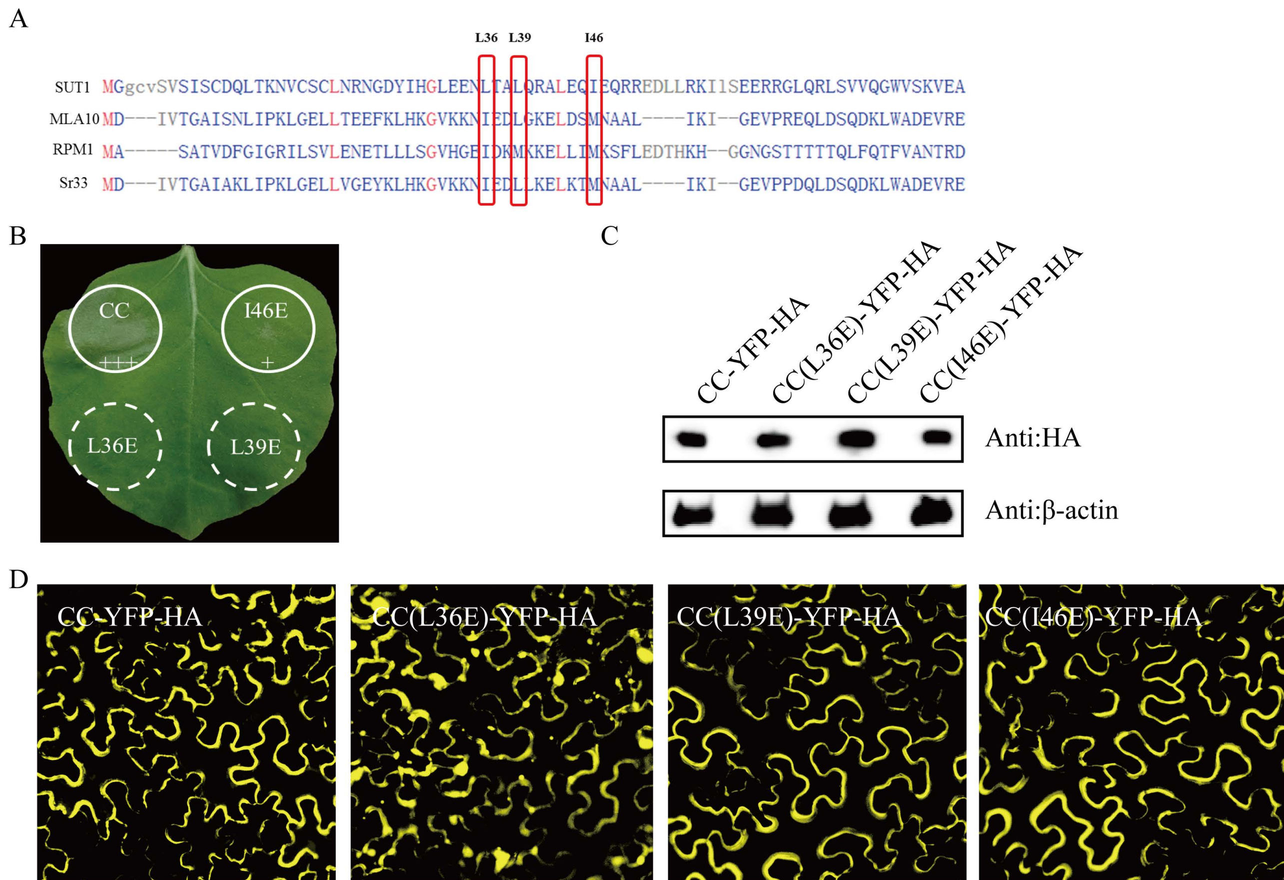

Figure 3. The function and localization of the CC domain are affected by three hydrophobic residue mutations. (A) Alignment of RPM1, Sr33, MLA10, and SUT1 (only partial alignment sequences displayed) indicating conservation of three hydrophobic sites (L36, L39, and I46) important for SUT1 function. (B) Analyzing the effects of three hydrophobic residue mutations on the function of SUT1 CC domain. + represents the severity of cell death. (C) Immunoblotting analysis showed that mutations in the three hydrophobic residues did not affect protein accumulation. (D) Observation the localization of SUT1 CC variants with fluorescence microscope. Images were recorded at 28 hpi.

function of the SUT1 CC domain impact on the function and subcellular localization of the full-length [FL] SUT1. Fluorescence data revealed that all mutations significantly altered the PM localization of wild-type SUT1 (Figure 4(D)). We observed that co-expression of SUT1 and topp4-1, rather than wild-type TOPP4, can induce cell death in *N. benthamiana* (Figure 4(E)). Naturally, we co-expressed three SUT1 alleles with topp4-1 in *N. benthamiana*, and identified that all mutants abolished or compromised the function of wild-type SUT1 (Figure 4(F)). Thus, functional loss mutations in the CC domain influence the function of FL SUT1.

4. Discussion

Previous article reported SUT1 receptor involved in autoimmunity initiated by type one protein phosphatase 4 mutation (topp4-1) in *Arabidopsis* [19]. In this article, we employed *N. benthamiana* to further analyze the activation mechanism of SUT1 from both structural and functional perspectives. The N-terminus

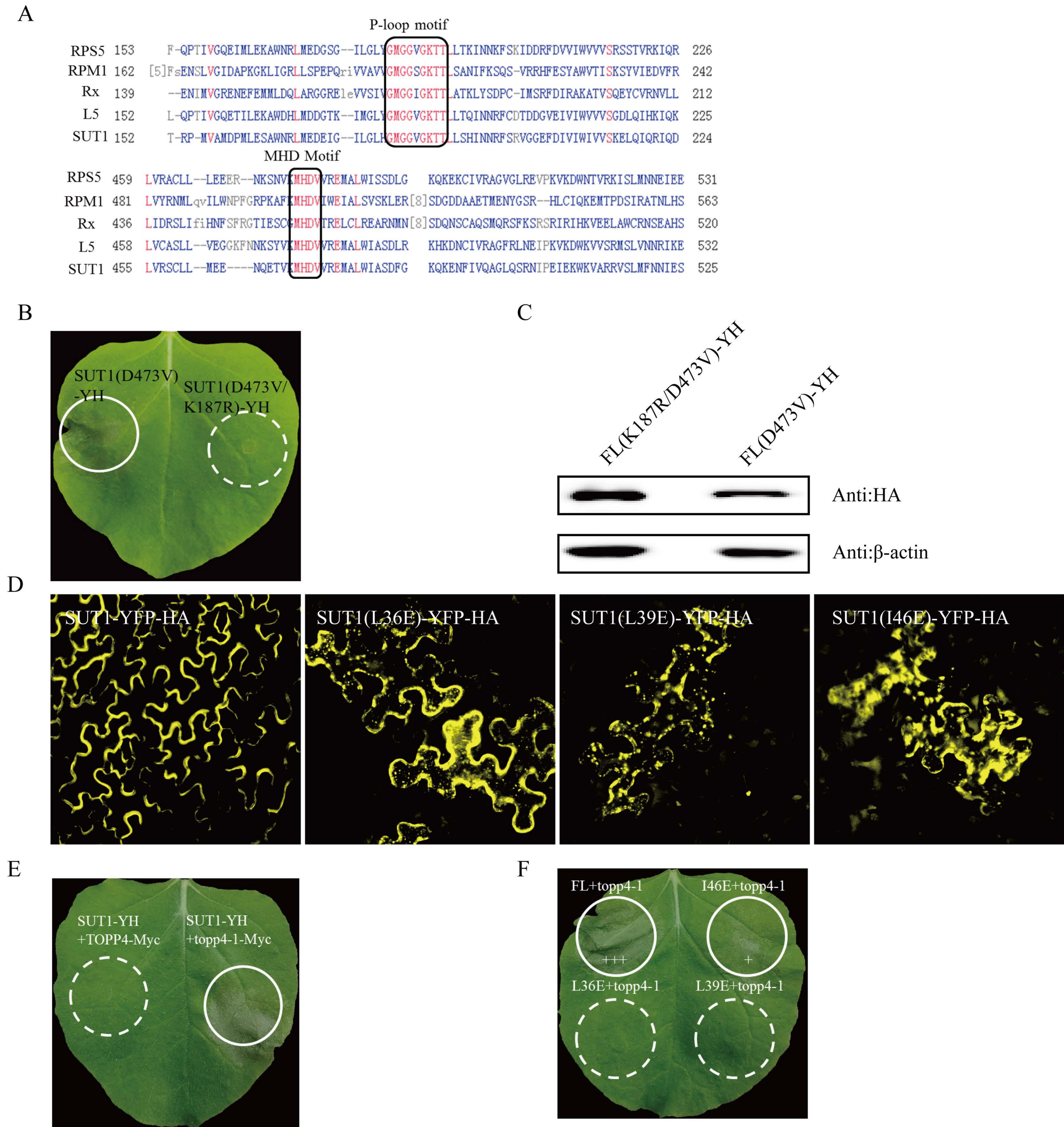

Figure 4. Functional loss mutations in the CC domain influence the function of FL SUT1. (A) Alignment of RPS5, RPM1, Rx, L5, and SUT1 (only partial alignment sequences displayed) showing conservation of P-loop and MHD motifs important for SUT1 function. (B) Dissects of cell-death inducing activity of SUT1 (D473V) and SUT1 (K187R/D473V). (C) Protein blotting showed that both mutants mentioned above can be correctly expressed. (D) Observation of subcellular localization of full-length SUT1 alleles. (E) Co-expression of SUT1 and topp4-1 [TOPP4 (T246M)], rather than wild-type TOPP4, can trigger significant cell death in N. benthamiana. (F) Functional loss mutations in the CC domain influence the function of FL SUT1.

of several SUT1 homologous proteins contains putative acylation sites (G2 and C4) [21]. Proteins modified by myristoylation and palmitoylation would anchor to the plasma membrane (PM). Our results confirmed that SUT1 is anchored to

the PM through acylation (**Figure 1**). The CC domain of SUT1 is enough to cell-death induction in *N. benthamiana* (**Figure 2**), indicating its role as a signaling module for promoting cell death. Nevertheless, not all N-terminals of CNL receptors can initiate cell death, such as RPM1 CC, which cannot spawn hypersensitivity response. In addition, the CC-NBS of RPS5, and the NBS of Rx can touch off cell death in *N. benthamiana*, indicating that NLR receptors have evolved different domains to mediate downstream cell death signaling [14] [18] [22]. Among all SUT1 truncated fragments, only the CC domain has the ability to give rise to cell death, indicating that the activity of SUT1 receptor was strictly regulated via intramolecular interactions. Our results confirmed that functional loss mutations in the CC domain influence the function of FL SUT1 (**Figure 3** and **Figure 4**), hinting the CC module was highly likely to directly participate in the self-inhibition process of SUT1 without pathogens. Coincidentally, previous articles have also found that host cell death undergoing ETI was controlled through the TIR or CC module of the NLR receptors [8] [23] [24]. Previous studies have shown that specific hydrophobic residues located in the CC domain of barley MLA10 are crucial for the formation of homodimers in CC module and the function of full-length MLA10 [6]. Our experimental data indicated that the three conserved hydrophobic residues located in the CC domain may affect the function of FL SUT1 by altering its subcellular localization, spatial conformation, or intramolecular interactions. Subsequent experimental directions should focus on analyzing the protein structure of SUT1 receptor, especially the CC domain, which would facilitate a better understanding of the roles of these three highly conserved sites.

5. Conclusion

In short, this paper utilized *N. benthamiana* to ectopic express the NLR receptor SUT1 in *Arabidopsis*, focusing mainly on analyzing the structure and function of SUT1 protein. It elucidates SUT1 is a typical CNL receptor, and its N-terminal CC domain is sufficient to mediate cell death. In addition, the three conserved hydrophobic residues located in the CC domain can affect the subcellular localization and function of the CC domain and FL SUT1, indicating the complex details of plant immune receptor regulation. These findings provide favorable clues for further understanding the activation mechanisms of SUT1.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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