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Screening, Validation and Functional Research of *Arabidopsis* TIR-NBS 2 Interaction Proteins

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Abstract

The *TIR-NBS* 2 (*TN*2) gene from *Arabidopsis thaliana* (*Arabidopsis*), which encodes a TIR (the Toll and Interleukin-1 Receptor)-type of nucleotide binding site (NBS) receptor protein (TIR-NBS) that can cause cell death in the model plant *Nicotiana benthamiana* (*N. benthamiana*). Nevertheless, the mechanism of TN2 signal initiation is still unclear. This research performed yeast two-hybrid and bimolecular fluorescence complementation (BIFC) assays to investigate interactions between proteins of TN2, and analyzed the influences of these interactors on TN2 function using *N. benthamiana*. EXO70B1, SOC3 and CPK5-VK were identified as interacting proteins of TN2 based on yeast two-hybrid and BIFC methods. Functional annotations of these interacting proteins indicate their involvement in multiple pathways, including exocytosis, positive regulation of abscisic acid-activated signaling pathway, regulation of stomatal closure, response to water deprivation, defense response, signal transduction and intracellular signal transduction. The transient assay results proclaimed that EXO70B1 can suppress cell death triggered by TN2 and TN2-TIR. These outcomes suggest that TN2 receptor may be participated in various pathways, and the protein level and activity are strictly controlled at multiple aspects, providing novel clues for elucidating the molecular mechanism of TN2 immune receptor in *Arabidopsis* resistance.

Keywords

TIR-NBS, Yeast Two-Hybrid, BIFC, Cell Death, *N. benthamiana*

1. Introduction

Plants rely on two levels of innate immune system to ward off pathogen attacks [1] [2]. The first layer is basal immunity triggered by membrane-localized pattern

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recognition receptors (PRRs), which can recognize highly conserved microbial molecules such as bacterial flagellin or fungal chitin, known as pattern-triggered immunity (PTI) [3]. In order to overcome PTI, pathogens secrete effector proteins into plant cells through a type III secretion system to inhibit plant immunity. Once pathogens suppress PTI and enter host cells, the second layer of immunity is then activated. The intracellular nucleotide-binding leucine rich repeat receptors (NB-LRR/NLRs) activate the second layer of immunity by directly or indirectly recognizing effectors released by pathogens, known as effector-triggered immunity (ETI) [4]. ETI typically leads to rapid and intense reactions, commonly referred to as hypersensitivity reactions (HR), displaying local host cell death [5].

Based on this N-terminus motif NLRs are classified into three subclasses CC (coiled-coil)-NLRs (CNLs) or TIR (Toll/interleukin-1 receptor)-NLRs (TNLs) and RPW8-NLRs (RNLs) [6]. Many NLR receptors function on the plasma membrane [7] [8]. In addition, some NB-LRRs have been described in plant genomes, which lack some NB-LRR specific domains and only contain TIR, TIR-NB, CC, and CC-NB domains [9]. Studies using overexpression of plant resistance genes, implying that the CC, TIR and NBS domains themselves might be sufficient to cause HR and to initiate plant defense responses [10]-[19].

The genome of *Arabidopsis* contains 21 genes encoding TN proteins [20], the functions of most TN proteins, including TIR-NBS 2 (TN2), are not particularly clear. Yeast two-hybrid system and BIFC technology are widely used for screening target protein interactors and verifying protein interactions in planta, respectively. The model plant *N. benthamiana* combined with *Agrobacterium*-mediated transient expression system is frequently applied to clone plant NLRs genes or plumb the function of plant NLRs receptors [21]. In this study, we utilized TN2 TIR domain (TN2-TIR) as a bait to screen for interacting proteins in the *Arabidopsis* leaf cDNA library, and demonstrated through BIFC technology that EXO70B1, SOC3, and CPK5-VK all interact with TN2 on the plasma membrane. Further transient expression results indicate that EXO70B1 can inhibit cell death of TN2 and TN2-TIR in *N. benthamiana.* In summary, our research findings provide valuable reference clues for understanding the activation mechanism of TN2.

2. Materials and Methods

2.1. Plant Materials and Cultivation Conditions

The *N. benthamiana* seeds were sown in pots (8 cm × 10 cm) containing vermiculite soaked with half-strength Hoagland nutrient solution in a growth chamber at 25˚C with a 16/8 hours (light/dark) cycle and relative humidity of 75% - 80%.

2.2. Plasmid Construction

The full-length coding sequence of *Arabidopsis* TN2 (AT1G17615) was acquired with the Phanta DNA polymerase (Vazyme, Nanjing, China) and inserted into a modified pUC19 vector using a ClonExpress II One Step Cloning Kit (Vazyme). The gateway-compatible pEarleyGate101 plant binary expression vectors was used

to produce C-terminal YFP-tagged constructs. The gateway-compatible pGADT7 and pGBKT7 were employed to generate plasmids for the yeast two-hybrid experiment. The gateway-compatible pEarleyGate201-YN and pEarleyGate202-YC were applied to manufacture constructs for the bimolecular fluorescence complementation (BIFC) assay. All plasmids were further validated by Sanger sequencing.

2.3. Arabidopsis cDNA Library Construction

To construct a cDNA library, 4-week-old *Arabidopsis* leaves were collected. cDNA library, including primary and secondary libraries, were constructed by Shanghai Ouyi Biomedical Technology Co., Ltd. Finally, we transformed the plasmids used in the secondary library, randomly detected the fragment sizes of 30 positive clones using universal primers, and sequenced them. We found that the average insertion size was 1.2 kb, and the repeat rate was only 2/30. We determine that the cDNA library meets the requirements for yeast two hybrid screening.

2.4. Yeast Two-Hybrid Assays

Linking the TIR domain of TN2 with the GAL4 BD into the pGBKT7 for screening the *Arabidopsis* leaves cDNA library constructed in the GAL4 AD into the pGADT7 vector. After transformation of yeast with the appropriate constructs, strictly follow the corresponding chapters in the Yeast Protocols Handbook (Clontech) for mating and interaction determination.

2.5. Agroinfiltration in Nicotiana Benthamiana

Agrobacterium tumefaciens GV3101 carrying the construct was incubated overnight in liquid LB medium containing kanamycin and rifampicin, and adjusted to an appropriate OD_{600} using the infiltration buffer (10 mM $MgCl_2$, 10 mM MES, 200 μM acetosyringone, pH 5.6). *A. tumefaciens* cells were infiltrated into approximately 4-week-old *N. benthamiana* leaves using a syringe without needle.

2.6. Bimolecular Fluorescence Complementation (BIFC)

For bimolecular fluorescence complementation (BIFC) assays, ORF of TN2 was amplified and subcloned in frame with the YFP^N into the pEarleyGate201 vector, and ORFs of EXO70B1, SOC3 and CPK5-VK were amplified and subcloned in frame with the YFP^C into the pEarleyGate202 vector, respectively. The resulting constructs were then transformed into *A. tumefaciens* strain GV3101 for transient assays. About 4-week-old *N. benthamiana* leaves were employed to perform infiltration as described previously. After 28 hours of infiltration, yellow fluorescent protein (YFP) fluorescence was imaged using a Leica SP8 laser scanning confocal microscopy system (Leica Microsystems, Wetzlar, Germany).

3. Results

3.1. Screening for Arabidopsis TN2-Interacting Proteins

The full-length TN2 protein and its TIR domain can cause severe cell death in *N.*

benthamiana [22]. Considering that full-length TN2 may not be conducive to yeast two-hybrid screening, we chose its N-terminus as a bait substitute. Yeast two-hybrid screening identified 3 proteins as having potential interactions with TN2-TIR (Table 1). The identified proteins were predicted as being involved in exocytosis, positive regulation of abscisic acid-activated signaling pathway, regulation of stomatal closure, response to water deprivation, defense response, signal transduction and intracellular signal transduction. EXO70B1 is involved in exocytosis, positive regulation of abscisic acid-activated signaling pathway, regulation of stomatal closure and response to water deprivation. SOC3 participates in defense response and signal transduction. CPK5 is involved in intracellular signal transduction.

Table 1. TN2-TIR interacting proteins from *Arabidopsis* leaf cDNA library identified by a yeast two-hybrid screening.

Protein name	Gene locus	Description	No. of clones
EXO70B1 (Exocyst subunit EXO70 family protein B1)	AT5G58430	A member of EXO70 gene family, putative exocyst subunits, conserved in land plants. *Arabidopsis thaliana* contains 23 putative EXO70 genes, which can be classified into eight clusters on the phylogenetic tree. Targeted by AvrPtoB to manipulate the defense molecule secretion machinery.	5
SOC3 (suppressors of *chs1*-2 (*soc*))	AT1G17600	SOC3 is a TIR-NB-leucine-rich repeat (TNL) protein. Mutants suppress loss of chs2 phenotype of auto-activation of immunity. When the TIR domain of SOC3 interacts with CHS2 the binding results in temperature activation of cell death, the suppressors inhibit this interaction.	3
CPK5-VK (Calmodulin-domain protein kinase 5)	AT4G35310	CPK5-VK, the truncated variant consisting only of the variable and kinase domains, which displays constitutive, calcium-independent kinase activity.	6

3.2. Validation of the Protein Interactions between TN2 and EXO70B1, SOC3 and CPK5-VK, Respectively

To further confirm the interaction between TN2 and EXO70B1, SOC3 or CPK5-VK, we carried out BIFC assays in planta. *Agrobacterium tumefaciens* containing TN2-nYFP and EXO70B1-cYFP, TN2-nYFP and SOC3-cYFP, TN2-nYFP and CPK5-VK-cYFP constructs were co-infiltrated into *N. benthamiana* leaves, respectively, and strong yellow fluorescence signals were observed on the plasma membrane of *N. benthamiana* epidermal cells under a confocal microscope. However, the combinations of TN2-nYFP and cYFP produced no fluorescence signal (Figure 1). These data indicated that TN2 physically interacted with EXO70B1, SOC3 and CPK5-VK on the plasma membrane in vivo, respectively.

3.3. EXO70B1 Inhibits TN2-Induced Cell Death in *N. benthamiana*

In order to further explore the relationship between TN2 and EXO70B1, SOC3 and CPK5-VK, we expressed these proteins in *N. benthamiana* plants. We fused Myc tag at the C-terminus of these proteins and co-expressed them with TN2-TIR-YFP-HA or TN2-YFP-HA into *N. benthamiana* leaves. The expression of TN2 or TN2-TIR provoked a violent and rapid cell death. This TN2- or TN2-TIR-

triggered cell death was inhibited by co-expression with EXO70B1, rather than SOC3 and CPK5-VK (Figure 2(A)). TN2- or TN2-TIR-induced cell death was also abolished when we co-expressed TN2 or TN2-TIR with EXO70B1 using different *Agrobacterium* GV3101 concentrations (Figure 2(B)). These data suggested that EXO70B1 may maintain TN2 in an inactive state, while TN2 triggers cell death only in the absence of EXO70B1.

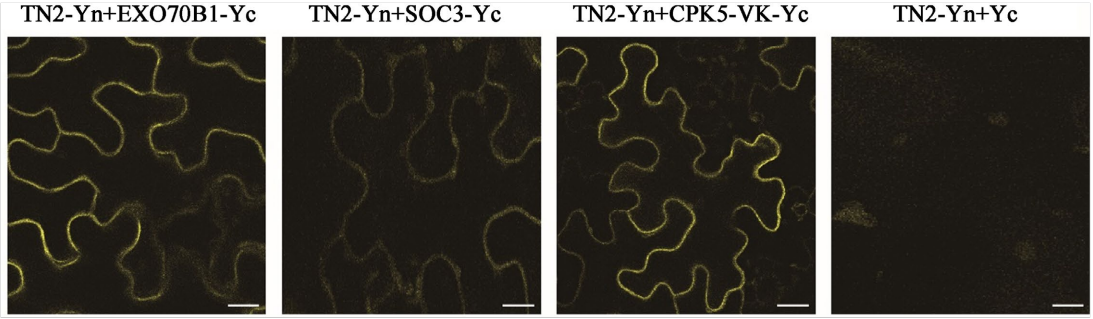

Figure 1. BIFC assay of the interactions of TN2 with EXO70B1, SOC3 and CPK5-VK. The photographs were taken using the green channel (YFP fluorescence) and their combination under a confocal microscope. Scale bars = 20 μm.

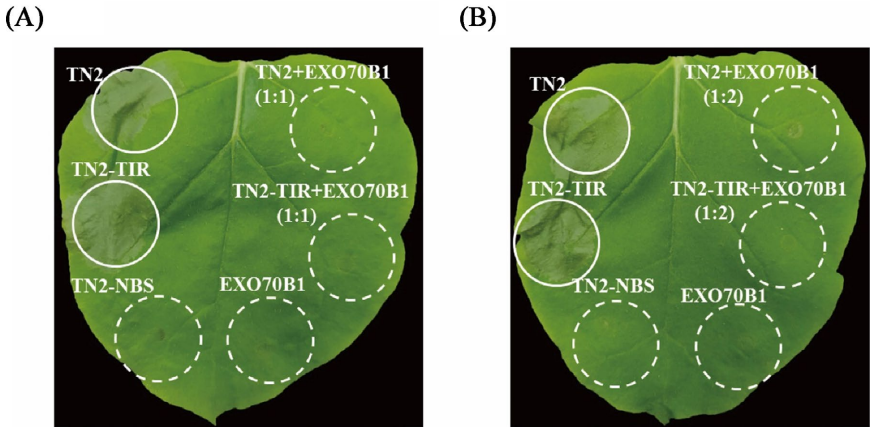

Figure 2. EXO70B1 inhibits TN2- or TN2-TIR-induced cell death in *N. benthamiana*. (A) Only the full-length TN2 receptor and its N-terminal TIR domain can cause hypersensitivity response (HR) in *N. benthamiana*, and the cell death-mediated by both can be completely inhibited by the interacting protein EXO70B1. Solid line circle indicates cell death, and dashed line circle indicates no cell death at the infiltrated region. (B) TN2- or TN2-TIR-induced cell death was also abolished when we co-expressed TN2 or TN2-TIR with EXO70B1 using different concentrations. Pictures were photographed 3 days post-infiltration. The experiments were repeated three times, with similar results obtained.

4. Discussion

TN2 is one of the 21 TIR-NBS receptors in the *Arabidopsis thaliana* genome [22]. Although the specific function of TN receptors is not yet clear, some evidence suggests that they can positively regulate the immune system of plants. For example, ectopic expression of the full-length or truncated TIR domain of some NLR proteins in the absence of pathogens can trigger an HR in *N. benthamiana* [19]

[23] [24]. Previous studies have shown that the deficiency of TN2 receptor can partially inhibit cell death caused by the bon1/bon3 double mutant [25]. In this study, we demonstrated that transient expression of TN2 or TN2-TIR alone induces strong and rapid cell death in *N. benthamiana*, which is suppressed by co-expression of EXO70B1, not SOC3 and CPK5-VK (**Figure 2**). This suggests that EXO70B1 specifically suppresses the activation of TN2, indicating that EXO70B1 associates with TN2 and may maintain TN2 in an inactive status.

EXO70B1 belongs to the EXO70 protein family and encodes a component of the vesicle transport machinery, playing a critical role in anchoring and binding vesicles to specific sites on the plasma membrane [26]. In vesicle trafficking, exocytosis plays an important role in maintaining membrane integrity and promoting membrane remodeling to cope with changes in environmental conditions [27]. In this study, we found that TN2 interacts with EXO70B1 on the plasma membrane (**Figure 1**). Previous studies have found that RIN4 recruits EXO70B1 into the plasma membrane, and AvrRpt2 can release RIN4 and EXO70B1 into the cytoplasm [28], declaring that the localization of EXO70B1 on the plasma membrane may be essential for plant immunity.

SOC3 encodes a typical TIR-type NLR (TNL) receptor. Liang *et al.* found that SOC3 can guard the homeostasis of the E3 ligase SAUL1 by interacting with CHS1 (TN1) and TN2, respectively. Overaccumulation of SAUL1 is monitored by the SOC3-TN2 pair, while SAUL1's disappearance is guarded by the SOC3-CHS1 pair. SOC3 forms a head-to-head genomic arrangement with CHS1 and TN2, indicative of transcriptional co-regulation [29]. This complex cooperative interaction may expand the recognition range of NLR and increase its functional flexibility, which can partially explain the overwhelming occurrence of NLR gene clustering in higher plants.

Generally speaking, functional redundancy in multi-gene families is an unavoidable challenge for studying specific genes within them. CPK5 belongs to the CPK family in plants, and previous studies have shown that in the absence of functional CPK5 instead of CPK4, CPK6, and CPK11, EXO70B1-mediated cell death and enhanced resistance to the *Golovinomyces cichoracearum* phenotype are lost [30], indicating that the specific function of CPK5 cannot be achieved through its functionally redundant homologs. In addition to CPK5, EXO70B1-mediated autoimmunity also requires the atypical truncated NLR resistance protein TN2 [26]. Therefore, the tripartite interaction between EXO70B1, CPK5, and TN2 controls cell death. The activity of CPK5 kinase and its membrane binding are both essential, although the exact mechanism is not fully understood. Whether other CPK family members are also involved in similar interactions and whether this mechanism represents innovation that only exists in *Arabidopsis* remains an unresolved question.

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Conflicts of Interest

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

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