Journal of Biosciences and Medicines, 2024, 12, 184-193
https://www.scirp.org/journal/jbm
ISSN Online: 2327-509X
ISSN Print: 2327-5081

Screening and Identifying of Interaction Protein AtL5 in *Arabidopsis thaliana*

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How to cite this paper: Huang, J.Z., Zhong, X.J., Guan, X.Y., Jia, P., Zhang, H.B., Chen, K., Li, Z.J., Chen, G.Y. and Zeng, C.T. (2024) Screening and Identifying of Interaction Protein AtL5 in *Arabidopsis thaliana*. *Journal of Biosciences and Medicines*, **12**, 184-193.
https://doi.org/10.4236/jbm.2024.127017

Received: June 14, 2024
Accepted: July 19, 2024
Published: July 22, 2024

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Abstract

Research background: The *Arabidopsis*-resistance protein L5 (AT1G12290) can trigger cell death in *Nicotiana benthamiana*, which is a characteristic function of an NBS-LRR (Nucleotide-Binding Sites and Leucine-Rich Repeat) protein activation. Purpose: To explore the function and molecular regulatory network of L5. Method: We employed yeast two-hybrid technology to search for interacting proteins of L5, combined with laser confocal microscopy to observe the subcellular localization of these candidate proteins, and analyzed the impact of these proteins on L5 function using an *Agrobacterium* mediated transient expression system. Results: Seven candidate interacting proteins were identified from the *Arabidopsis* cDNA library, including PPA1 (AT1G01050), RIN4 (AT3G25070), LSU1 (AT3G49580), BZIP24 (AT3G51960), BOI (AT4G19700), RING/U (AT4G22250) and PPA3 (AT2G46860). Functional analysis of these candidate interacting proteins showed that they participated in multiple pathways, including biological and abiotic stress, programmed cell death, protein degradation, material metabolism and transcriptional regulation. The results of laser confocal microscopy manifested that RIN4 was only localized on the plasma membrane (PM), and RING/U was mainly associated with the PM. PPA1, PPA3, LSU1, BZIP24, and BOI all emerged nuclear and cytoplasmic localization. The results of the transient assay proclaimed that both BOI and RING/U can inhibit cell death caused by L5. Conclusions: These results indicate that L5 immune receptors may participate in various pathways, and their protein levels and activities are strictly regulated at multiple levels, providing a basis for elucidating the mechanism of L5 immune receptors in *Arabidopsis* resistance.

Keywords

Cell Death, NBS-LRR, *Nicotiana benthamiana*, Yeast Two-Hybrid

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1. Introduction

[Research background] Plant disease resistance response is a complex process in which a series of genes, signaling pathways, and multi gene products are activated. Generally speaking, disease resistant genes activate downstream defense responses after identifying *Avr* genes. Studying the disease resistance response pathway activated by the *Arabidopsis* disease resistance gene L5 (AT1G12290) based on yeast two-hybrid technology is beneficial for elucidating the resistance mechanism of the disease resistance gene, revealing the mechanism of disease occurrence, and laying a theoretical foundation for integrating this gene to cultivate long-lasting and broad-spectrum disease resistant new rice varieties in the future.

Plants have evolved two levels of receptor-based immune systems to battle with multifarious pathogens [1]. Plants employ membrane-localized pattern recognition receptors to monitor conserved microbe-associated molecular patterns (MAMPs), such as bacterial flagellin and fungal chitin, known as MAMP triggered immunity (PTI), which can impede the growth of nonpathogens [2]. Some pathogens suppress PTI by delivering virulence effectors to plant cells, making plants susceptible to diseases. To counter the functions of effectors, plants deploy intracellular nucleotide-binding sites and leucine rich repeat (NBS-LRR) proteins launch the second layer of immunity through recognize effectors and is termed effector-triggered immunity (ETI), resulting in localized hypersensitive-response (HR) cell death [3].

NBS-LRR is mainly divided into two types: CC-NBS-LRR (CNL) and TIR-NBS-LR (TNL) [4]. The function of the NBS-LRR domain has been extensively studied, but the mechanism of NBS-LRR activation and subsequent signal transduction is not yet clear. Some NBS-LRR CC or TIR domains, such as ZAR1, MLA10, L3, L5, SUT1 and L6, are enough to induce cell death when they are transiently expressed in *N. benthamiana* [5]-[10]. The pre-activated NBS-LRR protein has been localized in multiple subcellular compartments. The activation of NBS-LRR is related to dynamic repositioning. In some cases, a small portion of the total NBS-LRR library appears to be repositioned to the nucleus, where it is believed to regulate defense gene transcription [11] [12]. However, the mechanisms regulating these NBS-LRR are far from understood.

[Research theme] L5 is the CC-NBS-LRR immune receptor [8] [13] [14]. The use of yeast two-hybrid (Y2H) system to study the network of protein-protein interactions is a commonly used strategy [15] [16], which also plays an important role in the study of plant pathogen interaction [17]. Using yeast two hybrid technology to identify proteins in *Arabidopsis* that directly interact with L5, revealing the disease resistance signaling pathway directly involved by L5, providing a theoretical basis for the next step of multi disease resistance gene aggregation to cultivate broad-spectrum, persistent, and high resistance rice varieties.

2. Materials and Methods

2.1. Experimental Materials

The *Arabidopsis* and *Nicotiana benthamiana* were grown in a greenhouse at the

Fuzhou Medical University. Briefly, *Arabidopsis* and *N. benthamiana* were sown in sterile vermiculite containing diluted Hoagland solution under a 16 hours light period. Yeast strains Y2HGold and Y187, as well as plasmid vectors pDEST-GADT7 (pGADT7, AD) and pDEST-GBKT7 (pGBKT7, BD), were obtained as gifts from Professor Guangcun He (College of Life Sciences, Wuhan University).

2.2. mRNA Extraction and Reverse Transcription

Total RNA was acquired from 4-week-old wild-type *Arabidopsis* leaves using Trizol reagent and chloroform. Precipitate RNA with isopropanol and dissolve it in RNase-free water. Remove DNA contamination by treating RNA solution with RNase-free DNase at 37˚C for 30 minutes. Use the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific) to synthesize first stranded cDNA from total RNA at 42˚C for 60 minutes. Amplify target CDS from cDNA for cloning and transient expression.

2.3. Plasmid Construction

Simply put, use the cDNA of wild-type *Arabidopsis* leaves as the initial template. Transfer the recovered PCR products into the entry vector PENTR/D through a one-step cloning method. Subsequently, the target fragment was transferred to the expression vector using Gateway cloning technology, such as yeast vector pGBDKT7 and pGADT7 or plant vector pEarleygate101 for YFP-HA or Myc tagged.

2.4. Yeast Two-Hybrid Assay

Linking the CC domain of L5 with the bait vector pGBKT7 for screening 4-week-old wild-type *Arabidopsis* leaves cDNA library constructed in the prey plasmid pGADT7. The transformation, mating, screening, and interaction assays of yeast were strictly carried out in accordance with the corresponding chapters in the Clontech Yeast Protocols Handbook.

2.5. Agroinfiltration in Nicotiana Benthamiana

In a word, *A. tumefaciens* GV3101 carrying the construct was incubated overnight in liquid LB medium containing kanamycin and rifampicin, and suspended in MMS solution [10 mmol MgCl2, 10 mmol MES (pH = 5.6)]. The Agrobacterium suspension was permeated into the leaves of *N. benthamiana*.

2.6. Subcellular Localization

In a nutshell, a laser confocal fluorescence microscope (Leica SP8) was exploited to observe photographs of live cells on the abaxial sides of *N. benthamiana* leaves at 48 h post infiltration (hpi).

2.7. Trypan Blue Staining

N. benthamiana leaves were boiled for 5 min in a 1:1 mixture of absolute alcohol

and staining solution (100 ml lactic acid, 100 ml phenol, 100 ml glycerol, 100 ml H_2O and 100 mg Trypan blue). Leaves were destained in 2.5 g·ml^{-1} chloral hydrate in water.

2.8. Gene Accession Number

The sequence information of the genes used in this article can be identified under GenBank accession numbers AT1G12290 (L5), AT1G01050 (PPA1), AT3G25070 (RIN4), AT3G49580 (LSU1), AT3G51960 (BZIP24), BOI (AT4G19700), RING/U (AT4G22250) and PPA3 (AT2G46860).

3. Results

3.1. Screening of Interaction Proteins of AtL5

The full-length L5 protein (884 amino acids) and its CC domain (160 amino acids) can both cause macroscopic cell death in *N. benthamiana* [8]. Considering that full-length L5 is not conducive to yeast two hybrid screening, we chose its N-terminus as a substitute. In order to avoid false positive clones, we first conducted experiments on whether BD-L5 CC has self-activation activity. We co-transformed two yeast plasmids, BD-L5 CC and AD-T, as well as BD-P53 and AD-T as positive controls, into yeast Y2HGold, respectively. Subsequent results promulgated that whether co-transformed with BD-L5CC and AD-T, or BD-P53 and AD-T, yeast transformants could grow normally on DDO medium (SD/-Trp-Leu), but only the latter could grow ordinarily on TDO/X medium (SD/-Trp-Leu-His plus X-α-gal) and displayed the blue-colony phenotype, implying that the bait vector BD-L5 CC did not have self-activation effect (**Figure 1**).

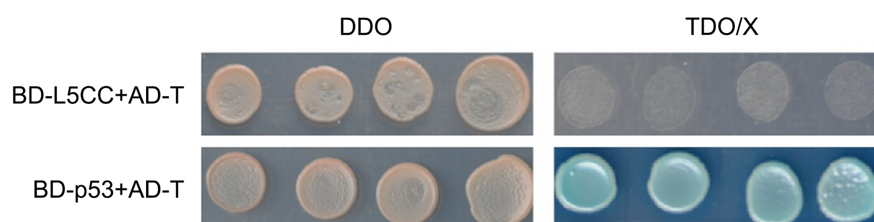

Figure 1. BD-L5 CC had no self-activation effect. BD-L5CC and AD-T, as well as BD-P53 and AD-T combinations, co-transform yeast strain Y2HGold competent cell, respectively. Yeast transformants were grown on the DDO media (SD/-Trp-Leu) and on the TDO/X media (SD/-Trp-Leu-His plus X-a-gal). The pair of p53-T used as a positive control. The blue yeast colonies represent positive interactions.

Seven positive colonies that may interact with L5 CC were screened on TDO/X medium through Y2H library screening and the number of repetitions and functional descriptions of each protein in the screening library are shown in **Table 1**. Functional analysis of these candidate interacting proteins shows that they participate in multiple pathways, including biological and abiotic stress, programmed cell death, protein degradation, material metabolism and transcriptional regulation. PPA1 participated in phosphate-containing compound

metabolic process; RIN4 involved in negative regulation of plant-type hypersensitive response; LSU1 participated in cellular oxidant detoxification, cellular response to sulfur starvation and response to salt stress; BZIP24 involved in DNA-templated transcription, negative regulation of response to salt stress, regulation of DNA-templated transcription and regulation of transcription by RNA polymerase II; BOI participated in proteasome-mediated ubiquitin-dependent protein catabolic process and regulation of programmed cell death; as of now, the functionality of RING/U is unknown; PPA3 involved in phosphate-containing compound metabolic process.

Table 1. The functional analysis of AtL5 binding proteins.

Protein name	Gene locus	Gene description	Repeat times
PPA1	AT1G01050	Encodes a soluble protein with inorganic pyrophosphatase activity that is highly specific for Mg-inorganic pyrophosphate.	4
RIN4	AT3G25070	Encodes a member of the R protein complex and may represent a virulence target of type III pili effector proteins (virulence factors) from bacterial pathogens, which is Primeguard Prime by R protein complex (RPM1 and RPS2 proteins).	5
LSU1	AT3G49580	RESPONSE TO LOW SULFUR gene family member; expressed during sulfur deficiency.	4
BZIP24	AT3G51960	bZIP transcription factor induced by salt stress and promoted salt tolerance. Localized to the cytoplasm and nucleus under control conditions and targeted preferentially to the nucleus under salt stress.	3
BOI	AT4G19700	Encodes BOI (Botrytis Susceptible 1 Interactor). Has E3 ubiquitin ligase activity. It prevents caspase activation and attenuates cell death.	6
RING/U	AT4G22250	RING/U-box superfamily protein.	3
PPA3	AT2G46860	Encodes a protein that might have inorganic pyrophosphatase activity.	4

3.2. The Subcellular Localization Analysis of Interaction Proteins of AtL5

L5 receptor is mainly anchored on the PM due to the presence of acylation sites, and PM localization is crucial for the function of the full-length L5 and its CC domain [8]. Our previous articles have demonstrated that the E3 ubiquitin ligase BOI exhibited a nucleocytoplasmic localization and mediated the ubiquitination degradation of L5 protein outside the nucleus [13] [14]. Furthermore, we employed laser confocal microscopy to observe the subcellular localization of six candidate interacting proteins of L5 one by one. Through the *Agrobacterium* mediated transient expression system in *N. benthamiana* leaves, we that the fluorescence of RIN4-YFP-HA only appeared on the PM, and RING/U-YFP-HA mainly linked to the PM, while PPA1, PPA3, LSU1, and BZIP24 exhibited nuclear and cytoplasmic distribution in *N. benthamiana* leaves (**Figure 2**).

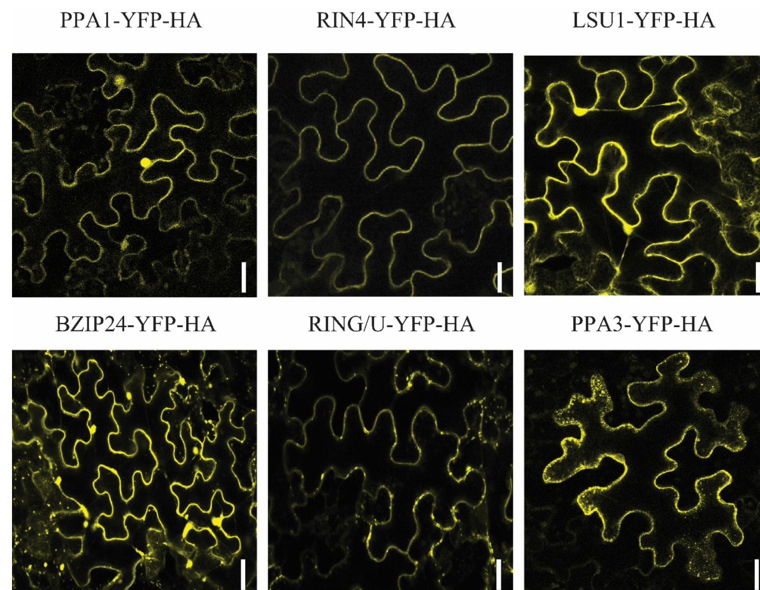

Figure 2. Observation of subcellular localization of proteins interacting with L5 with confocal microscope. These proteins were transiently expressed in *N. benthamiana* (OD_{600} = 0.8). Images were observed at 48 hpi. Scale bars: 25 μm.

3.3. RING/U Can Also Inhibit L5-Mediated Cell Death

The ability of BOI to abolish cell death caused by L5 has been reported [14] (**Figure 3(a)**). We want to know the impact of the other six candidate proteins that interact with L5 on its function. We fused Myc tag at the C-terminus of these proteins and co-expressed them with L5-YFP-HA into *N. benthamiana* leaves. The results of transient expression declared that RING/U could also significantly suppress the cell death activity of L5 protein, as shown in **Figure 3(b)** by trypan blue staining.

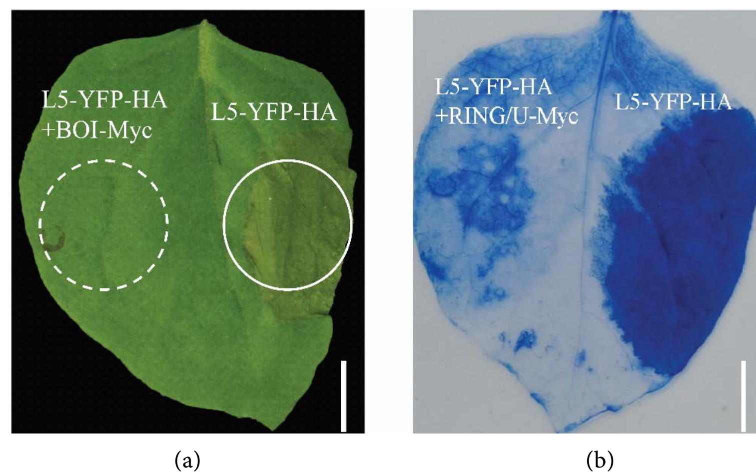

Figure 3. RING/U protein can inhibit cell death triggered by L5 in *N. benthamiana.* (a) L5-mediated cell death was suppressed by the expression of BOI. L5-YFP-HA was used as a positive control. The photograph was taken at 48 hpi. (b) RING/U protein can also restrain cell death caused by L5. Visible cell death was confirmed by trypan blue staining. Picture was obtained at 48 hpi. Scale bar: 1 cm.

4. Discussion

Currently, multiple *Arabidopsis* NBS-LRR genes have been cloned, and these proteins may participate in the same or similar signaling pathways to activate downstream defense responses during disease resistance. However, most current research mainly focuses on the identification of disease resistant genes and their interaction with corresponding effectors, with little understanding of their mediated resistance response pathways. This study constructed a high-quality *Arabidopsis* leaf target cDNA library and screened seven important proteins interacting with L5 using the L5 CC domain as bait (**Table 1**). Further bioinformatics analysis revealed that the selected candidate proteins include transcription factor, E3 ubiquitin ligases, disease resistance related proteins, and abiotic stress-related proteins.

Transcription factors are participated in various biological processes in plants, comprising pathogen defense, seed maturation, plant growth, flower development, aging, light signaling transduction, damage, and response to various environmental stresses. The interaction between L5 and transcription factor BZIP24 indicates that after recognizing effector, L5 enters the nucleus and binds to transcription factor, activating the immune response through transcriptional regulation, suggesting that L5 protein may undergo repositioning. According to reports, some NBS-LRR proteins, such as MLA10 and RPS4, can be repositioned in the nucleus after activation [11] [12]. Recognition of barley disease resistance protein MLA10 with powdery mildew pathogen effector factor A10 induces MLA10 to bind to transcription factor WRKY in the nucleus to initiate disease resistance response [11].

In *Arabidopsis*, plasma membrane localized multifunctional protein RIN4 (RPM1interacting protein 4) plays important role in both PTI and ETI [18]. Previous studies have suggested that RIN4 functions as a negative regulator of PTI [19]. Recent study has shown that RIN4 can also promote the extracellular transport of AtEXO70E2 [20]. In addition, many different bacterial effector proteins modify RIN4 to destabilize plant immunity and several NBS-LRR proteins, including RPM1 (resistance to *Pseudomonas syringae pv. maculicola* 1), RPS2 (resistance to *P. syringae* 2) guard RIN4 [21] [22]. L5 is a CC-NBS-LRR receptor in *Arabidopsis*. The activation mechanism of its disease resistance function is still unclear. Considering that L5 can strike cell death in *N. benthamiana,* we infer an indirect interaction between L5 and the effector. Our previous paper has shown that even if L5-YFP-HA is overexpressed in plants, *Arabidopsis* did not exhibit an autoimmune phenotype [8]. According to the knowledge of the "guardee model", there must be interacting proteins in plants that inhibit L5 activation, similar to the relationship with RIN4-RPS2 [22]. Coincidentally, our yeast two hybrid experiment showed that there is also an interaction between L5 and RIN4. Perhaps in addition to RPS2 and RPM1, RIN4 may also be guarded by L5, and this relationship needs further verification in the future.

The level of NBS-LRR protein is under complex control in plants to balance

defense responses and other processes. In recent years, the ubiquitination 26S proteasome system has become a key function in regulating NLR stability [23]. In *Arabidopsis*, it was found that the stability of two NBS-LRRs, SNC1 and RPS2, is controlled by the F-box protein CPR1 (constitutive expression of pathogenesis-related gene 1) [24] [25]. BOI (Botrytis subseptible1 interactor) is a RING type E3 ligase that mediates the ubiquitination of BOS1 (Botrytis subseptible1), a transcription factor involved in stress and pathogen response [26]. BOI inhibits cell death caused by α-picolinic acid (PA), a toxin produced by some fungi that can lead to cell death, as well as virulent pathogens such as *Pseudomonas syringae pv. tomato DC3000* (Pto DC3000). Our previous article indicated that BOI degrades the protein level of L5 through ubiquitination, which occurs outside the cell nucleus [13] [14]. Furthermore, in addition to the E3 ubiquitin ligase BOI we previously reported, the new interacting protein RING/U has also been shown to suppress L5-induced cell death in *N. benthamiana* (**Figure 3**). In fact, PPA1, PPA3, BZIP24, and LSU1 can all affect the cell death inducing activity of L5 protein to some extent (data not shown), suggesting that the activation of L5 is regulated at multiple levels.

Funding

This study was funded by The Guiding Science and Technology Plan Project of Social Development in Fuzhou City (FKSZ20229003), and The Science and Technology Research Project of Jiangxi Provincial Department of Education (GJJ218112), and The School-level Science and Technology Project of Fuzhou Medical University (fykj202201).

Conflicts of Interest

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

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