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J. Biomedical Science and Engineering,
2024, Vol. 17, (No. 10), pp: 171-178

Expression Pattern, Interaction Network, and Functional Analysis of the *Arabidopsis* Botrytis Susceptible1 Interactor

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Keywords: E3 Ubiquitin Ligases, Expression Pattern, Interaction Network, *Arabidopsis*

Received: September 25, 2024 **Accepted:** October 11, 2024 **Published:** October 14, 2024

ABSTRACT

E3 ubiquitin ligases are participated in numerous processes, regulating the response to biotic and abiotic stresses. Botrytis susceptible1 interactor (BOI) is a RING (Really Interesting New Gene)-type E3 ligase that mediates the ubiquitination of BOS1 (Botrytis susceptible1), a transcription factor involved in stress and pathogen responses. Although BOI is an E3 ligase, there are reports to show that BOI interacts with target proteins such as DELLAs or CONSTANS to repress gibberellin responses and flowering without the degradation of the target proteins. In this article, we utilize diversified methods to comprehensively analyze the expression pattern, interaction network and function of *BOI* gene. Firstly, 1800 bp upstream region of *BOI* gene from *Arabidopsis thaliana* (*Arabidopsis*) genome was isolated, and fused GUS reporter gene. The resulting expression cassette was introduced into wild-type *Arabidopsis* through *Agrobacterium*-mediated transformation. The result demonstrated that *BOI* gene was expressed predominantly in leaves, siliques, young roots, and flowering tissues, indicating that *BOI* gene may be involved in multiple processes in plant growth and development in *Arabidopsis*. Besides, eight candidate interacting proteins were obtained from the *Arabidopsis* cDNA library via yeast two-hybrid technology, including EXO70E2 (AT5G61010), WRKY7 (AT4G24240), WRKY11 (AT4G31550), WRKY17 (AT2G24570), UBP20 (AT4G17895), L5 (AT1G12290), SAUR9 (AT4G36110) and TCP21 (AT5G08330). Functional analysis of these candidate interacting proteins manifested that they related to multiple pathways, including biological and abiotic stress, programmed cell death, protein degradation, material metabolism and transcriptional regulation. In addition, the results of the transient assay proclaimed that BOI protein affects the protein stability of EXO70E2 and L5 through its E3 ubiquitin ligase activity. Our

results provide novel clues for a better understanding of molecular mechanisms underlying BOI-mediated regulations.

1. INTRODUCTION

In eukaryotes, the ubiquitin proteasome pathway serves as the main mechanism to degrade and recycle misfolded and damaged proteins [1]. The ubiquitination modification pathway concerns a range of reactions involving ubiquitin activating enzyme E1, binding enzyme E2, and ubiquitin ligase E3, with the substrate primarily dictated by ubiquitin ligase [2]. The plant E3 ubiquitin ligases comprise a large and diverse family of proteins or protein complexes containing either a HECT (homologous to E6-associated protein C-terminus) domain, an RING-finger or U-box domain [3]. The most typical feature of the RING domain family is its RING-finger domain, which is an important factor in its ubiquitin ligase activity. The amino acid sequence of the RING domain is Cys-X_2-Cys-$X_{(9\text{-}39)}$-Cys-$X_{(1\text{-}3)}$-His-$X_{(2\text{-}3)}$-Cys-X_2-Cys-$X_{(4\text{-}48)}$-Cys-X_2-Cys (X represents any amino acid) [4].

Arabidopsis RING E3 ubiquitin ligase (referred to as RING E3 protein) has been reported to be involved in various processes, including the responses to biotic and abiotic stresses [5]. Botrytis susceptible1 interactor (BOI) is an RING type E3 ligase, and mediates the ubiquitination of BOS1 (Botrytis susceptible1), a transcription factor participated in stress and pathogen responses [6]. Although BOI is an E3 ligase, there are reports to show that BOI interacts with target proteins such as DELLAs or CONSTANS to repress gibberellin responses and flowering without the degradation of the target proteins [7, 8]. In this article, we utilize diversified methods to comprehensively analyze the expression pattern, interaction network and function of *BOI* gene. Firstly, 1800 bp upstream region of *BOI* gene from *Arabidopsis thaliana* (*Arabidopsis*) genome was isolated, and fused GUS reporter gene. The resulting expression cassette was introduced into wild-type *Arabidopsis* through *Agrobacterium*-mediated transformation. The result demonstrated that *BOI* gene was expressed predominantly in leaves, siliques, young roots, and flowering tissues. Besides, eight candidate interacting proteins were obtained from the *Arabidopsis* cDNA library via yeast two-hybrid technology, including EXO70E2 (AT5G61010), WRKY7 (AT4G24240), WRKY11 (AT4G31550), WRKY17 (AT2G24570), UBP20 (AT4G17895), L5 (AT1G12290), SAUR9 (AT4G36110) and TCP21 (AT5G08330). Functional analysis of these candidate interacting proteins manifested that they related to multiple pathways, including biological and abiotic stress, programmed cell death, protein degradation, material metabolism and transcriptional regulation. In addition, the results of the transient assay proclaimed that BOI protein affects the protein stability of EXO70E2 and L5 through its E3 ubiquitin ligase activity. Our research provides important clues to elucidate the biochemical functions of BOI.

2. MATERIALS AND METHODS

2.1. Plant Materials and Growth Conditions

The seeds of the wild-type, and transgenic *Arabidopsis* were sterilized and grown in a greenhouse at 22˚C, 16 h light/8 h dark, and 70% relative humidity. *Nicotiana benthamiana* (*N. benthamiana*) plants were grown in a greenhouse at 24˚C, 12 h light/12 h dark, and 70% relative humidity. Transient expression analysis was performed using 5-week-old *N. benthamiana* plants.

2.2. mRNA Extraction and Reverse Transcription

Total RNA was obtained 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. First strand cDNA was synthesized from total RNA using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific) at 42˚C for 60 minutes. Amplify target CDS from cDNA for cloning and transient expression.

2.3. Plasmid Constructs

In brief, using wild-type *Arabidopsis* leaf cDNA 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 or pGWB3 for GUS labeled.

2.4. Histochemical GUS Staining Assay

In a word, after two minutes of vacuum treatment at 80,000 Pa, 4-week-old transgenic *Arabidopsis* plants were stained with 2 mM 5-bromo-4-chloro-3-indoyl-β-d-glucuronide (X-Gluc) in 100 mM phosphate buffer at 37˚C for 24 hours and washed with 70% ethanol to remove chlorophyll.

2.5. Yeast Two-Hybrid Assay

Linking BOI 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 determination of yeast were strictly executed in accordance with the corresponding chapters in the Clontech Yeast Protocols Handbook.

2.6. Agrobacterium Transient Expression Assays

Agrobacteria carrying the constructs were cultivated overnight in LB medium containing Rifampicin and kanamycin. Agrobacterium culture was centrifuged and resuspended in MES buffer, incubated at room temperature for 1 hour and infiltrated into 4-week-old *N. benthamiana* leaves at a specific OD_{600} values.

2.7. Immunoblotting Assay

Total proteins were harvested using extraction buffer (20 mM Tris-HCl pH 8.0, 5 mM EDTA, 1% SDS, 10 mM DTT). The protein samples mixed with the loading buffer were denatured at 100˚C for 10 min. Equal amounts of total proteins were separated through SDS-PAGE and transferred to NC membranes. Immunoblotting was performed using the following antibodies: anti-HA (Roche, #11867423001), anti-Myc (Genscript, #A00704), anti-β-actin (Abbkine, #A01050-2) at a 1:10,000 dilution.

3. RESULTS

3.1. Expression Pattern of *BOI* Gene

The expression localization and stage of *BOI* gene are likely to play a role in specific functions during plant growth. To investigate the *BOI* gene expression pattern, transgenic plants carrying the *GUS* reporter gene under the control of the *BOI* gene promoter, including the region 1.8 kb upstream of the transcription start sites, were generated through *Agrobacterium*-mediated genetic transformation. Several independent transgenic lines were then screened in the T_2 generation for GUS activity at multiple life stages and representative examples are shown in **Figure 1**. The results of the *GUS* reporter gene assay with X-Gluc staining indicated that native expression of the *BOI* gene in *Arabidopsis* was demonstrated in all tissues investigated, containing roots, stems, mature leaves, flowers, and siliques. The results of *BOI* gene promoter triggers *GUS* gene expression were consistent with the observation of expression data collected from The *Arabidopsis* Information Resource (TAIR) website (TAIR - Home (arabidopsis.org)), indicating that *BOI* gene may be involved in multiple processes in plant growth and development in *Arabidopsis*.

3.2. Screening of Interaction Proteins of BOI

Previous articles have shown that the bait vector pGBDKT7-BOI did not have self-activation effect [6, 8]. Eight positive colonies that may interact with BOI were acquired through yeast two-hybrid library

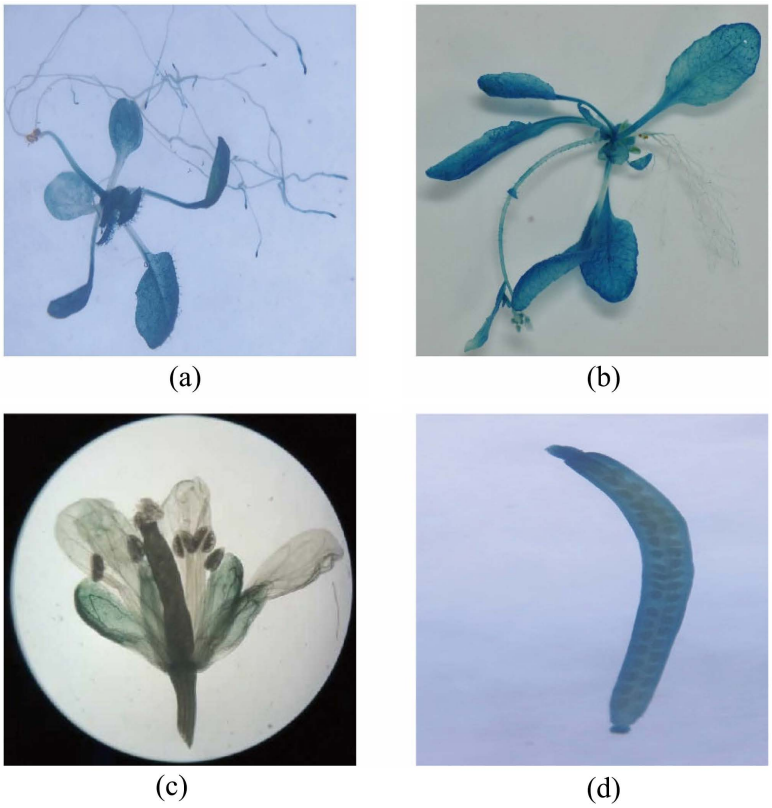

Figure 1. Expression pattern of *BOI* gene. The expression pattern of *BOI* gene was investigated through promoter *GUS* fusion construct in transgenic *Arabidopsis*. The Pro_{BOI}:*GUS* construct led to a homogeneous GUS staining at the multiple life stages. ((a)-(d)) represent roots, mature leaves, flowers, and siliques, respectively. GUS expression in transgenic plants carrying Pro_{BOI}:*GUS* is indicated in blue by staining for GUS activity.

screening and the number of repetitions and functional descriptions of each protein in the screening library are shown in Figure 2 and 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. EXO70E2 participated in regulation of protein targeting and positive regulation of extracellular exosome assembly, which acts upstream of or within immune response and defense response by callose deposition. WRKY7 encodes a Ca-dependent calmodulin binding protein, which sequence similarity to the WRKY transcription factor gene family and involved in regulation of DNA-templated transcription. WRKY11 related to defense response to bacterium, induced systemic resistance, regulation of DNA-templated transcription, regulation of jasmonic acid (JA) mediated signaling pathway and response to bacterium. WRKY17 concerned with regulation of DNA-templated transcription, which acts upstream of or within defense response to bacterium. UBP20 encodes a ubiquitin-specific protease, which involved in regulation of protein stability. L5, a nucleotide-binding site and leucine-rich repeat receptors (NBS-LRR/NLR), localized to plasma membrane. Overexpression triggers cell death, anchored to the plasma membrane due to the presence of the myristoylation site Gly2, which involved in defense response to other organisms. SAUR9 participated in response to abscisic acid, response to auxin, response to brassinosteroid, response to light intensity and response to red or far-red light. TCP21 belongs to the circadian oscillator protein and interacts with bZIP63 to regulate the circadian oscillator's response to sugar. In addition, together with TCP7, 8, 14, 15, 21, 22, and 23, promote the expansion of cells dependent on internal replication in leaves.

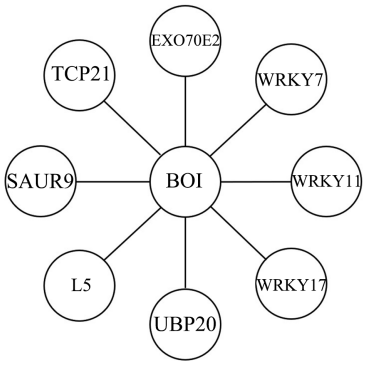

Figure 2. Eight BOI interacting proteins screened using yeast two-hybrid technology.

Table 1. The functional analysis of BOI binding proteins.

Protein name	Gene locus	Functional analysis	Repeat times
EXO70E2	AT5G61010	involved in regulation of protein targeting and positive regulation of extracellular exosome assembly; acts upstream of or within immune response and defense response by callose deposition.	8
WRKY7	AT4G24240	Encodes a Ca-dependent calmodulin binding protein. Sequence similarity to the WRKY transcription factor gene family. Involved in regulation of DNA-templated transcription.	4
WRKY11	AT4G31550	involved in defense response to bacterium, induced systemic resistance, regulation of DNA-templated transcription, regulation of jasmonic acid mediated signaling pathway and response to bacterium.	5
WRKY17	AT2G24570	involved in regulation of DNA-templated transcription, acts upstream of or within defense response to bacterium.	4
UBP20	AT4G17895	Encodes a ubiquitin-specific protease. involved in regulation of protein stability.	3
L5	AT1G12290	NLR protein localized to plasma membrane. Overexpression triggers cell death. Has myristolation site at Gly2 which is required for membrane localization. involved in defense response to others organism.	8
SAUR9	AT4G36110	involved in response to abscisic acid, response to auxin, response to brassinosteroid, response to light intensity and response to red or far-red light.	5
TCP21	AT5G08330	Circadian oscillator protein which interacts with bZIP63 and regulates a response of the circadian oscillator to sugar. Is not required for the sugar-induced circadian phase advance in the morning; regulates a response of CCA1 to sugars. Promotes together with TCP7, 8, 14, 15, 21, 22 and 23 endoreduplication-dependent cell expansion in leaf.	6

3.3. BOI Negative Regulates EXO70E2 and L5 Proteins Stability

We have previously demonstrated that BOI possesses E3 ubiquitin ligase activity, and introducing the H273Y mutation in its RING domain can abolish its E3 ubiquitin ligase activity [9]. Additionally, BOI degrades the L5 receptor through ubiquitination, thereby abolishing the cell death caused by it in *N. benthamiana* [9-11]. Next, we would like to know the impact of BOI on the stability of the other eight candidate interacting proteins. We fused YFP-HA tags at the C-terminus of these candidate proteins and co-expressed them with BOI-Myc in *N. benthamiana* leaves. The results of transient expression indicated that BOI can not only reduce the content of NLR receptor L5, but also negatively regulate the protein abundance of EXO70E2, and its degradation behavior also depends on its E3 ubiquitin ligase activity (**Figure 3(a)** and **Figure 3(b)**).

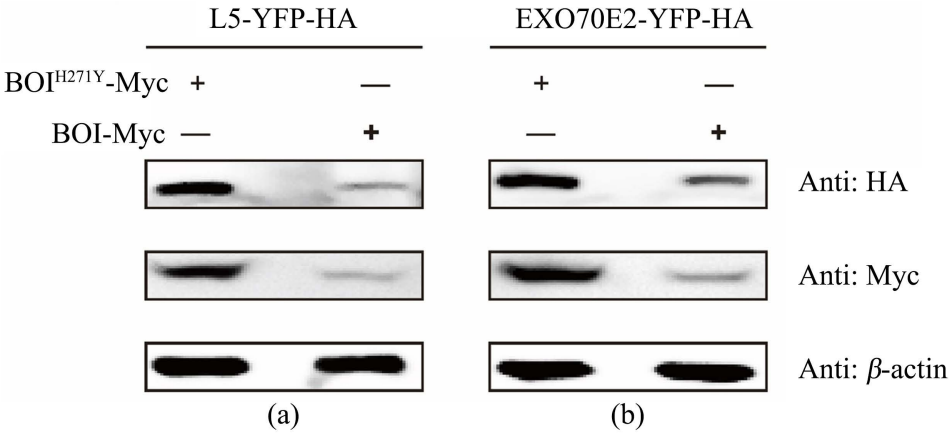

Figure 3. BOI promotes the degradation of L5 and EXO70E2 in *N. benthamiana*. (a) BOI mediates the degradation of EXO70E2. Co-expression of L5-YFP-HA and BOI-Myc or BOI^{H271Y}-Myc in *N. benthamiana* through Agro-infiltration. The protein levels of L5 and BOI were detected at 38 hours post infiltration. L5 and BOI were detected with anti-HA and anti-Myc antibody, respectively. (b) BOI promotes the degradation of EXO70E2.

4. DISCUSSION

E3 ubiquitin ligases play an important role in the ubiquitin-proteasome pathway, which is among the most important protein degradation pathway in eukaryotic organisms [12]. The study of E3 ubiquitin ligases is a topic of immense interest, and the functions of E3 ubiquitin ligases are explored regularly. However, the functions of E3 ubiquitin ligases in *Arabidopsis* were rarely studied. In this research, BOI, an E3 ubiquitin ligase, which was hypothesized to function in response to biotic or abiotic stresses, was selected. The result of histochemical GUS staining assay demonstrated that *BOI* gene was expressed predominantly in leaves, siliques, roots, and flowering tissues (**Figure 1**), indicating that *BOI* gene may be involved in multiple processes in plant growth and development in *Arabidopsis*.

The WRKY family of transcription factors is one of the largest in plants, playing crucial roles in plant growth, development, and response to environmental stresses [13]. WRKYs regulate disease-related processes through various mechanisms, such as enhancing physical barriers, modifying histones, regulating gene expression of pathogenesis-related genes, interacting with ROS signaling, and participating in crosstalk with phytohormones like salicylic acid (SA) and jasmonic acid (JA) [13]. Notably, mutations in *Arabidopsis* WRKY7, WRKY8, WRKY11, and WRKY17 have been shown to enhance basal resistance to virulent strains of the bacterial pathogen *Pseudomonas syringae* [14]. Our research results indicate that BOI interacts with WRKY7, 11, 17, suggesting that BOI may also be involved in the resistance pathway against *Pseudomonas syringae.*

The exocyst complex is an evolutionarily conserved hetero-octameric complex and is required for vesicle trafficking [15]. Previous studies have indicated that the U-box ubiquitin ligase PUB18 targets EXO70B1 and regulates the protein level of EXO70B1 through a proteasome-dependent pathway [16]. AvrPtoB is an E3 ligase isolated from *P. syringae* pv. *tomato* DC3000, which interacts with EXO70B2 and mediates the degradation of EXO70B2 via the 26S proteasome system [17]. Our results indicate that BOI promotes the degradation of L5 and EXO70E2 in *N. benthamiana* (**Figure 3**), but we cannot exclude the possibility that BOI may indirectly affect the protein level of L5 and EXO70E2. The protein content of BOI mutant is significantly higher than that of its wild-type (**Figure 3**), which may be due to the fact that the RING-type E3 ligase can auto-ubiquitinate itself as well as its substrate for proteasome degradation [18, 19].

Our previous studies showed that RIN4 not only alters the subcellular localization of EXO70E2 but also accelerates the transport of EXO70E2 from vesicles to the extracellular and reduces its protein level [20]. However, the functions of EXO70E2 in vesicles transported to the extracellular space remain unclear. Interestingly, RIN4 interacts with EXO70E2 as well as with L5 [11, 20]. As L5, RIN4, BOI, and EXO70E2 are all associated with plant disease resistance, we hypothesize that BOI and RIN4 may be connected to coordinate their interaction with EXO70E2 and L5. Further functional characterization of EXO70E2 is required to clarify the exact relationships between exocytosis and plant immunity.

FUNDING

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

CONFLICTS OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

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