Scientific Research Publishing

Journal of Biosciences and Medicines, 2025, 13(10), 375-385
https://www.scirp.org/journal/jbm
ISSN Online: 2327-509X
ISSN Print: 2327-5081

Identification, Structural Analyses of EXO70 Gene Family in *Arabidopsis* and Functional Analysis of EXO70B1 Employing *Nicotiana benthamiana*

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How to cite this paper: Lin, Q., Zeng, J.Y., Jia, P., Chen, K., Zhang, H.B. and Huang, J.Z. (2025) Identification, Structural Analyses of EXO70 Gene Family in *Arabidopsis* and Functional Analysis of EXO70B1 Employing *Nicotiana benthamiana*. *Journal of Biosciences and Medicines*, **13**, 375-385.
https://doi.org/10.4236/jbm.2025.1310030

Received: August 10, 2025
Accepted: October 25, 2025
Published: October 28, 2025

Abstract

The EXO70 gene is a very important component of the exocytosis complex, involving a series of biological processes including plant cell division and immune regulation. The *Arabidopsis thaliana* genome comprises 23 EXO70 paralogs. However, a systematic identification and structural analysis of the EXO70 gene family in *Arabidopsis* is currently lacking. Here, we first analyzed the protein information encoded by these 23 genes and explored their evolutionary relationships. Moreover, tertiary structure prediction results declared that the majority of members within the same subclass exhibited similar protein structures, providing additional insights into the evolutionary relationship of the EXO70 gene. Finally, we only focused on the EXO70B1, which is one of the EXO70 family members, and it will be of interest to study the functional role of the other members as well. EXO70B1 interacts with FLS2 in a bimolecular fluorescence complementation (BiFC) assay. The transient expression results demonstrated that TN2 (TIR-NBS 2)-induced cell death was inhibited by EXO70B1, but not EXO70B2, in *Nicotiana benthamiana*. In summary, the functional divergence between EXO70B1 and EXO70B2 occurs despite their high sequence and structural similarity.

Keywords

EXO70, *Arabidopsis thaliana*, Cell Death, *Nicotiana benthamiana*

1. Introduction

The endometrial system is a unique structure of eukaryotic cells. The transport of

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proteins, lipids, and other substances within the endometrial system and between the endometrial system and the cell membrane is achieved via membrane vesicle transport. Vesicle transport is an extremely important cellular process present in all eukaryotes, and it is directly involved in various biological processes, such as cell polarity establishment, cell secretion, cell growth, cell division, and cell wall formation [1]. It mainly consists of four stages, among which tethering is a key step. A highly conserved protein found in yeast and animals plays an important role in membrane vesicle tethering, known as tethers. The exocyte complex belongs to the tethers and participates in the tethering process between Golgi transport vesicles and the cytoplasmic membrane, and plays an important role as a tether in exocytosis. This complex contains 8 large subunits, namely Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, EXO70, and EXO84 [2] [3]. And EXO70 is a key protein that exercises the function of the exocyst complex. It recruits exosomes on the target membrane and interacts with Rho protein, regulating the assembly and activation of SNARE complexes through SEC6 [4] [5]. In this way, EXO70 mediates polar exocytosis.

In plants, EXO70 regulates pollen tube elongation and polarization, root hair growth, cell wall material deposition, cell plate activation and maturation, defense, and autophagy [6]-[12]. The functional defects of the AtEXO70C2 gene affect the growth of pollen tubes, leading to significant male-specific transmission defects in *Arabidopsis* [13]. Exocyst subunit Exo70B2 is linked to immune signaling and autophagy [14]. Knockout of EXO70B1 leads to damage of light-induced stomatal opening [15]. AtEXO70B1 and AtEXO70B2 participate in plant immune responses by regulating the abundance and localization of FLS2 [16]. Therefore, the role of EXO70 in plant organ development has been differentiated.

The *Arabidopsis* genome comprises 23 EXO70 paralogs [6] [17]. However, a systematic identification and structural analysis of the EXO70 gene family in *Arabidopsis* is currently lacking. Here, we directly conducted evolutionary and structural analysis on the EXO70 family, focusing on the functional differences between EXO70B1 and EXO70B2.

2. Materials and Methods

2.1. Plant Materials

Nicotiana benthamiana and *Arabidopsis thaliana* plants were grown on soil in a greenhouse with a 16 h light/8 h darkness photoperiod and temperatures of 25˚C.

2.2. Plasmid Construction

To create the constructs, cDNA fragments of EXO70B1 or EXO70B2 were amplified and cloned into pEarleygate101-YFP-HA vectors to generate 35S:: EXO70B1-YFP-HA and 35S:: EXO70B2-YFP-HA vectors.

2.3. Transient Expression in *N. benthamiana*, and Fluorescence Microscope

Agrobacterium tumefaciens GV3101 cultures containing the relevant binary con-

structs were centrifuged and suspended in 10 mM MES (pH 5.6), 10 mM $MgCl_2$, 150 mM acetosyringone, and the OD_{600} was adjusted to 0.8. Transient expression was performed by agroinfiltration into *N. benthamiana* leaves. After 2 days, fluorescence was analyzed in infiltrated leaves using a fluorescence microscope (Olympus BX53). YFP-derived fluorescence was monitored through excitation with a 488 nm argon laser.

2.4. *In Vivo* Bimolecular Complementation Assay

In the BiFC assays, the EXO70B1 coding fragment was amplified and cloned into the pEarleygate 201-Yn vector to obtain EXO70B1-YFPn, and the EXO70B2 protein was fused into the pEarleygate 202-Yc vector to acquire EXO70B2-YFPc. Bacterial suspensions containing the BiFC constructs and p19 silencing plasmid were infiltrated into *N. benthamiana* leaves. After the infiltration, fluorescence was observed by a fluorescence microscope after 2-3 days.

2.5. Sequence Alignment and Phylogenetic Analysis

The evolutionary history was inferred employing the Neighbor-Joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in units of the number of amino acid substitutions per site. This analysis involved 23 amino acid sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). Evolutionary analyses were conducted in MEGA-X.

2.6. Gene Accession Number

Sequence information from this paper can be observed in the GenBank data libraries under accession numbers EXO70A1 (AT5G03540), EXO7OA2 (AT5G52340), EXO7OA3 (AT5G52350), EXO7OB1 (AT5G58430), EXO7OB2 (AT1G07000), EXO7OC1 (AT5G13150), EXO7OC2 (AT5G13990), EXO7OD1 (AT1G72470), EXO7OD2 (AT1G54090), EXO7OD3 (AT3G14090), EXO7OE1 (AT3G29400), EXO7OE2 (AT5G61010), EXO7OF1 (AT5G50380), EXO7OG1 (AT4G31540), EXO7OG2 (AT1G51640), EXO7OH1 (AT3G55150), EXO7OH2 (AT2G39380), EXO7OH3 (AT3G09530), EXO7OH4 (AT3G09520), EXO7OH5 (AT2G28640), EXO7OH6 (AT1G07725), EXO7OH7 (AT5G59730), and EXO7OH8 (AT2G28650).

2.7. Protein Structure Prediction

The structural models were retrieved from the AlphaFold Protein Structure Database (https://alphafold.ebi.ac.uk/). AlphaFold is a new machine learning approach that incorporates previous knowledge about protein structure and that, by leveraging multi-sequence alignments into the design of a deep learning algorithm, can predict protein structures with great accuracy [18] [19]. The protein structure from this article can be obtained in the AlphaFold Protein Structure Database under the accession codes EXO70A1 (AF-Q9LZD3-F1), EXO70A2

(AF-F4KG57-F1), EXO70A3 (AF-F4KG58-F1), EXO70B1 (AF-Q9FGH9-F1), EXO70B2 (AF-Q9LMJ4-F1), EXO70C1 (AF-Q9FY95-F1), EXO70C2 (AF-Q9FFX6-F1), EXO70D1 (AF-Q9C9E5-F1), EXO70D2 (AF-Q9SYG5-F1), EXO70D3 (AF-Q9LJH9-F1), EXO70E1 (AF-Q9LIA2-F1), EXO70E2 (AF-Q9FNR3-F1), EXO70F1 (AF-F4K8Y6-F1), EXO70G1 (AF-Q7XYW9-F1), EXO70G2 (AF-Q9C8H6-F1), EXO70H1 (AF-Q8VY27-F1), EXO70H2 (AF-O80625-F1), EXO70H3 (AF-Q9SF50-F1), EXO70H4 (AF-Q9SF51-F1), EXO70H5 (AF-F4IIS8-F1), EXO70H6 (AF-Q9LQP9-F1), EXO70H7 (AF-F4KJ98-F1), and EXO70H8 (AF-Q9SIB0-F1).

3. Results

3.1. Basic Physicochemical Properties and Phylogenetic Tree Analysis

We identified the whole EXO70 gene in *Arabidopsis*. The number of amino acid residues of EXO70 protein ranged from 573 aa (EXO70H8) to 695 aa (EXO70C2), and their molecular weight ranged from 64799 Da (EXO70H8) to 79765.6 Da (EXO70C2). Theoretical isoelectric points are located between 4.64 (EXO70E1) and 8.94 (EXO70A1), which can be roughly divided into two categories: basic proteins (EXO70A1, EXO70A2, EXO70G1, EXO70G2, EXO70H2, EXO70H3, EXO70H6) and acidic proteins (EXO70A3, EXO70B1, EXO70B2, EXO70D1-EXO70D3, EXO70E1, EXO70E2, EXO70F1, EXO70H1, EXO70H4, EXO70H5, EXO70H7, EXO70H8) (Table 1).

Table 1. Analysis of physicochemical properties of proteins encoded by the EXO70 gene family.

Gene Accession	Gene	Length (aa)	Molecular Weight (Da)	Isoelectric Point
AT5G03540	EXO70A1	664	75,674	8.94
AT5G52340	EXO70A2	631	72,081.9	7.95
AT5G52350	EXO70A3	586	67,571.4	6.08
AT5G58430	EXO70B1	624	70,638.9	4.86
AT1G07000	EXO70B2	599	67,714	4.83
AT5G13150	EXO70C1	653	75,005	5.2
AT5G13990	EXO70C2	695	79,765.6	4.66
AT1G72470	EXO70D1	633	71,458.9	5.88
AT1G54090	EXO70D2	622	70,770.4	4.96
AT3G14090	EXO70D3	623	70,545	5.45
AT3G29400	EXO70E1	658	75,225.3	4.64
AT5G61010	EXO70E2	639	73,484.9	5.36
AT5G50380	EXO70F1	683	76,616.5	4.84
AT4G31540	EXO70G1	687	77,695.7	8.62
AT1G51640	EXO70G2	660	76,194.1	7.93
AT3G55150	EXO70H1	636	71,961.3	6.93
AT2G39380	EXO70H2	637	72,179.1	7.97
AT3G09530	EXO70H3	637	72,114	8.55
AT3G09520	EXO70H4	628	70,991.5	6.61
AT2G28640	EXO70H5	605	68,752.6	4.96
AT1G07725	EXO70H6	615	68,401.3	7.77
AT5G59730	EXO70H7	634	71,225.1	5.71
AT2G28650	EXO70H8	573	64,799	6.96

To further clarify the relationship among the EXO70 genes in *Arabidopsis*, a phylogenetic tree was constructed based on the protein sequences encoded by these EXO70 genes. And the results showed that homologous genes belonging to the same subclass are generally distributed on the same main trunk. There are 9 pairs of paralogs on the side branches of the phylogenetic tree, including (Figure 1)

EXO70H1/EXO70H2; EXO70H3/EXO70H4;
EXO70H5/EXO70H6; EXO70E1/EXO70E2;
EXO70D2/EXO70D3; EXO70B1/EXO70B2;
EXO70C1/EXO70C2; EXO70G1/EXO70G2;
EXO70A2/EXO70A3.

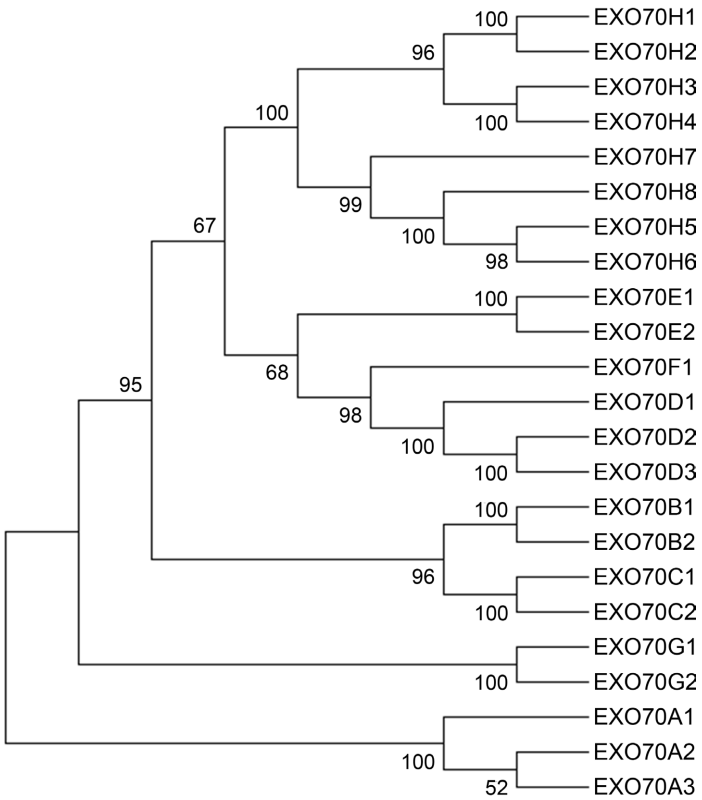

Figure 1. Phylogenetic tree of the EXO70 gene family in *Arabidopsis*. The phylogenetic tree was constructed using MEGA-X based on the neighbor-joining method, with bootstrap repeats of 1000 times.

3.2. Predicted Three-Dimensional (3D) Structures of EXO70s

The 3D structures of proteins encoded by the EXO70 gene family were predicted using the online AlphaFold3 platform. The results revealed that the proteins encoded by the EXO70 family all contained alpha-helix, extended strand, and random coil (Figure 2). Moreover, the majority of members within the same subclass exhibited similar structures, such as EXO70B1/EXO70B2, EXO70C1/EXO70C2, providing additional insights into the evolutionary relationship of the EXO70 gene. Significantly, EXO70A3 seems to be an exception, although in terms of evolution, this protein belongs to the same branch as EXO70A2 and EXO70A1. How-

ever, its protein structure differs significantly from that of EXO70A1 and EXO70A2, while the latter two exhibit a high degree of structural similarity.

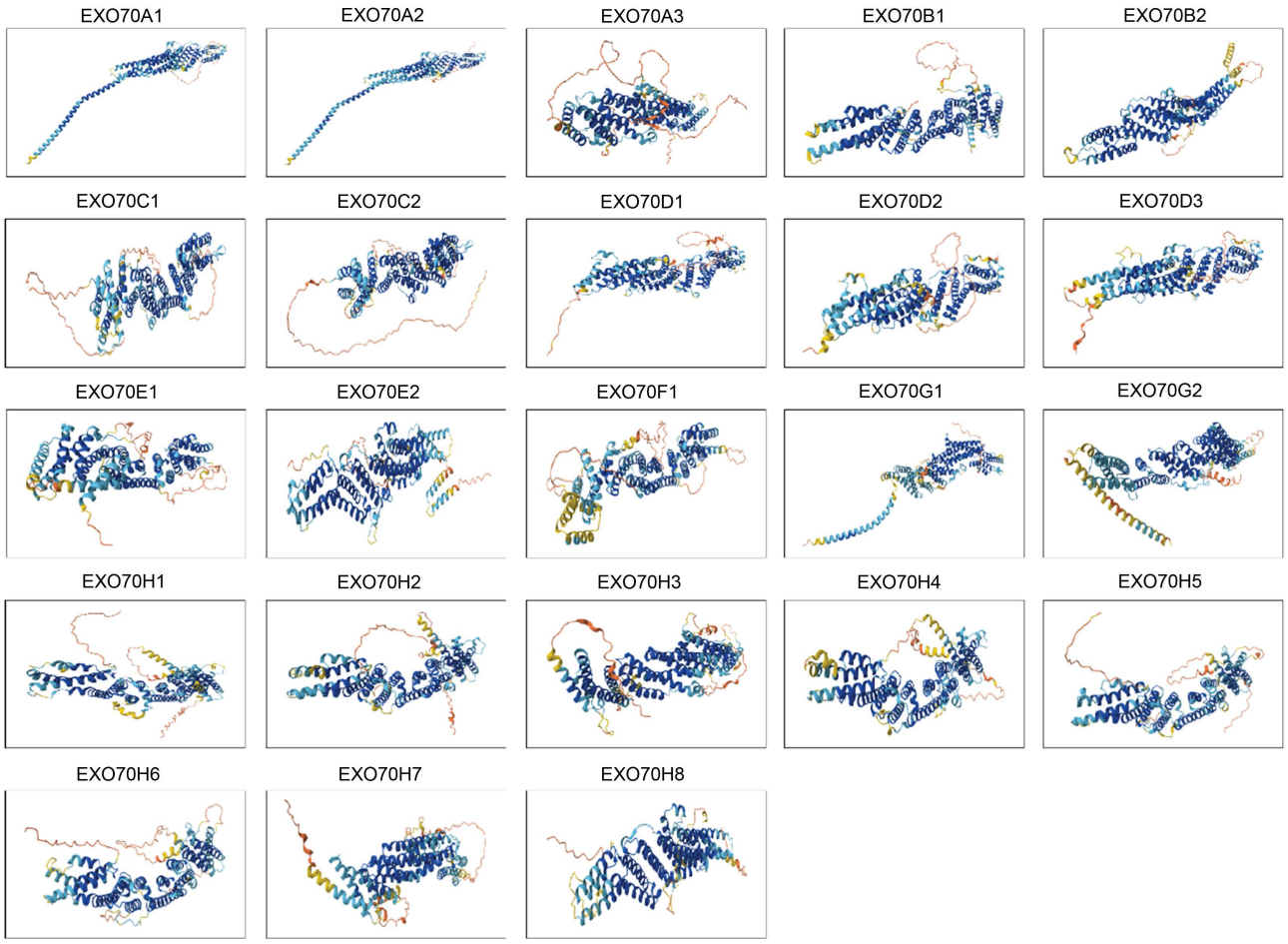

Figure 2. Protein 3D structure prediction model of the EXO70 gene family in *Arabidopsis*.

3.3. EXO70B1 Associates with FLS2

EXO70B1 is a component of the exocyst complex and belongs to the EXO70 protein family, and previous studies indicated that the exocyst subunit EXO70B1 is involved in the immune response to different pathogens in *Arabidopsis* [20] [21]. In this study, we used EXO70B1 in an experimental study on *Arabidopsis* EXO70 genes. In order to examine the localization of EXO70B1, the EXO70B1 fused with the yellow fluorescent protein (YFP) was expressed under the control of the 35S promoter in *N. benthamiana* leaves. We observed clear fluorescence signals in the cytoplasm, nucleus, and plasma membrane (**Figure 3(A)**). This discovery was consistent with the roles of EXO70s in vesicle transport.

Considering that some plasma membrane localization proteins appear as membrane components of transport vesicles. In *Arabidopsis*, FLS2 (flagellin receptor), a PRR protein, is localized on the plasma membrane. We would like to know if EXO70B1 directly interacts with FLS2. We then performed BiFC assays and observed strong YFP fluorescence signals on the plasma membrane in *N. bentham-*

iana leaves only co-transformed with EXO70B1-Yc and FLS2-Yn constructs, but not in the negative controls (**Figure 3(B)**), indicating that EXO70B1 and FLS2 indeed interact with each other. This discovery is consistent with the previous research results of Wang *et al*. [16].

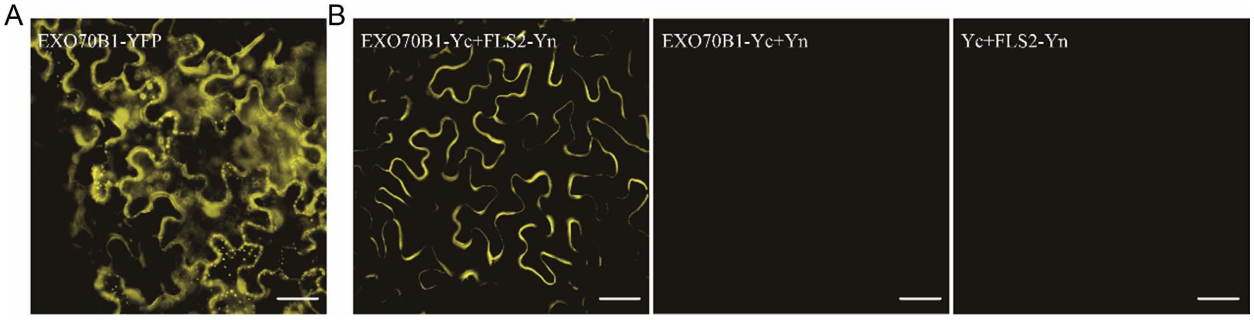

Figure 3. EXO70B1 associates with FLS2. (A) Observation of subcellular localization of EXO70B1 in *N. benthamiana* (OD_{600} = 0.8). (B) BiFCassay. FLS2 was fused to the YFP N-terminus (Yn), and EXO70B1 was fused to the YFP C-terminus (Yc). Different pairs of constructs were co-expressed in *N. benthamiana* (OD_{600} = 0.5, respectively). YFP fluorescence was detected by microscopy. Bar = 20 μm.

3.4. EXO70B1, but Not EXO70B2, Inhibited TN2-Mediated Cell Death in *N. benthamiana*

A previous study had demonstrated that EXO70B1, co-localized with TN2 at the plasma membrane and interacted with TN2 [21], and EXO70B1 could inhibit cell death caused by TN2 in *N. benthamiana* [22]. Considering that the sequences of EXO70B2 and EXO70B1 share the highest similarity, EXO70B1 and EXO70B2 were found to directly interact with FLS2 and hetero-oligomerize [16]. Thus, we further investigate whether EXO70B2 could also abolish the cell-death-inducing ability of TN2. We first observed and discovered that the fluorescence distribution of YFP-labeled EXO70B2 was in the nucleus, cytoplasm, and plasma membrane (**Figure 4(A)**), suggesting that EXO70B2 exhibited a subcellular localization pattern similar to EXO70B1. Using the *Agrobacterium tumefaciens*-mediated transient expression system, it was confirmed that EXO70B1, rather than EXO70B2, could suppress TN2-triggered cell death in *N. benthamiana* (**Figure 4(B)**).

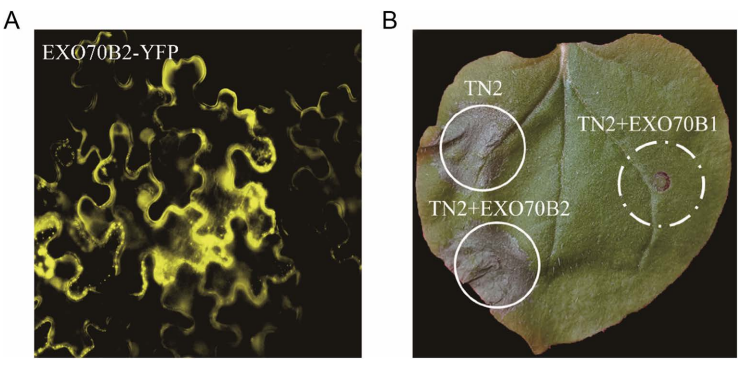

Figure 4. EXO70B1, rather than EXO70B2, inhibited TN2-mediated cell death in *N. benthamiana*. (A) Observation of subcellular localization of EXO70B2 (OD_{600} = 0.8). Bar = 20 μm. (B) TN2-induced cell death was abolished by co-expression with EXO70B1, not EXO70B2.

4. Discussion

Previous studies have reported that EXO70B1, as a component of the exocytosis complex, is involved in plant immunity [23]. FLS2 has been widely recognized as a very important pattern-recognition receptor (PRR) that can directly sense the conserved components of bacterial flagella, thereby initiating primary immunity. Hence, the cell membrane localization of FLS2 is prominent in its functionality. Undoubtedly, the vesicle transport network is important for ensuring the proper accumulation and correct localization of FLS2 at the plasma membrane. Coincidentally, in this article, we confirmed that EXO70B1 could indeed associate with FLS2 on the plasma membrane (**Figure 3(B)**). In fact, besides EXO70B1, there are other secretory complex members that participate in membrane integrity. Kulich *et al*. validated that EXO70H4 is essential for the plasma membrane localization of callose synthases [24] [25]. The EXO70 inhibitor Endosidin2 alters plasma membrane protein composition in *Arabidopsis* roots [26]. Tethering of cellulose synthase complex to the plasma membrane relies on the isoform of EXO70A1 in *Arabidopsis* [27]. Moreover, EXO84b mediates the polarity of PEN3 at the outer lateral membrane [28].

In addition, we found that EXO70B1 and EXO70B2 are highly homologous in sequence, evolutionarily related (**Figure 1**), structurally similar (**Figure 2**), and share the same localization pattern (**Figure 3(A)** and **Figure 4(A)**). Nevertheless, these two proteins represented significant differences in suppressed TN2-mediated cell death in *N. benthamiana* (**Figure 4(B)**). Correspondingly, after *Pto* DC3000 infection of *Arabidopsis*, AvrPtoB ubiquitinated EXO70B1 instead of EXO70B2, and TN2-mediated resistance was activated when EXO70B1 disappeared [22]. After treatment with elf18, the transcription of EXO70B2, but not EXO70B1, was remarkably upregulated [8]. Additionally, immune hub protein RIN4 recruits EXO70B1, not EXO70B2, to the plasma membrane [29]. We speculated that the potential molecular mechanisms underlying the functional differences between EXO70B1 and EXO70B2 may include subtle structural differences, differential post-translational modifications, or interactions with different regulatory partners. The *Arabidopsis* genome encodes a series of exocyst complex members, some of which exhibit functional redundancy and differentiation. Further research is needed to determine whether other members play a role in trafficking or plant immunity with EXO70B1.

Funding

This work was supported by grants from the Science and Technology Research Project of Jiangxi Provincial Department of Education (GJJ2403304 and GJJ218112).

Acknowledgements

The authors thank the teachers and students in our research team for their help and support.

Conflicts of Interest

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

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