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Dissecting Multiple *Arabidopsis* CC-NBS-LRR Proteins Structure and Localization

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Abstract

NBS-LRR (nucleotide binding sites and leucine rich repeat) protein plays a crucial role as sentries and as defense activators in plants. The structure and function of NBS-LRR proteins are closely related. Previous articles have announced that the activated ZAR1 (HopZ-Activated Resistance 1) forms a pentamer in the plasma membrane, which is a calcium permeable channel that can trigger plant immune signaling and cell death. However, the structure of galore NBS-LRRs in *Arabidopsis* is not yet clear. The functional sites of distinct NBS-LRR in cells may vary. In addition, identifying pathogens and activating defense regions may occur in different subcellular compartments. Therefore, dissecting the specific structure and positioning of NBS-LRRs is an indispensable step in understanding their functions. In this article, we exploit AlphaFold to predict the structure of some designed NBS-LRRs, and utilize *Agroinfiltration* transient expression system, combined with biochemical fractionation, to dissect the localization of these NBS-LRR receptors from *Arabidopsis*. Structural data indicates that the identified NBS-LRRs share analogous conformation. Membrane fractionation assay demonstrates these NBS-LRRs are mainly associated with the membrane. These data show that the Ca^{2+}-permeable channel activity may be evolutionarily conserved in NBS-LRR of *Arabidopsis*, and this study provides some reference clues for analyzing the structure and localization patterns of other plant immune receptors.

Keywords

Arabidopsis, Calcium Permeation Channel, Pentamer, Plasma Membrane

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1. Introduction

The growth and development of plants are influenced by multifarious environmental pressures, including intimidation from pests and pathogens. In order to protect themselves, plants undergo continuous coevolution and complex innate immune monitoring to resist infection. Plant immunity relies on two main types of pathogen perception systems composed of cell surface and intracellular receptors [1]. The first resistance system is composed of pattern recognition receptors (PRRs) that trigger immunity (PTI), which is typically the first response of plants and is activated by PRRs located on the cell surface to recognize conserved microbe-associated molecular patterns (MAMPs) [2]. PRR perceives conserved microbial elicitor molecules, leading to the activation of self-phosphorylation and trans-phosphorylation, as well as PTI reactions, resulting in a series of plant reactions, including the production of reactive oxygen species (ROS), intracellular calcium flow, activation of macrophage activated protein kinase (MAPK), upregulation of defense related genes, and deposition of callose [3] [4]. To promote the spread of pathogens in host plants, specific effectors are released to inhibit PTI. Plants have evolved an alternative immune protective layer called effector triggered immunity (ETI), which utilizes intracellular resistance (R) proteins to recognize the presence of specific effectors and activate defense responses [5]. This reaction is often accompanied by programmed cell death, known as hypersensitivity response (HR) [6] [7].

Most plant R proteins belong to the so-called nucleotide binding sites and leucine repeat rich (NBS-LRR) immune receptors, which share typical modular structures including nucleotide binding sites (NBS) and leucine repeat rich (LRR) domains [8]. According to the presence of toll/interleukin-1 receptors (TIRs) or coiled coil (CC) domains, NBS-LRRs can be divided into two subclasses, called TIR-NB-LRR (TNL) and CC-NB-LRR (CNL) [9]. The N-terminal domain of NLR is usually necessary for identifying different downstream components. The central NBS domain of NLRs acts as a molecular switch, regulating protein activity through nucleotide binding and hydrolysis. The C-terminal LRR domain of NLRs plays a complex dual function of pathogen effector recognition and automatic inhibition through intramolecular interactions in immune signaling [9].

To be effective, NBS-LRRs must exhibit the same subcellular localization pattern as pathogen effectors. They must also be located in positions where they can initiate signals that bring about defense activation. So that, NBS-LRR localization is a hot research area for elucidating how these proteins function. Biochemical fractionation is commonly used to analyze the localization of NBS-LRR [10]-[12]. Accumulating data promulgated that NBS-LRRs exhibited different subcellular distributions. Both *Nicotiana* immune protein N and barley immune receptor MLA10 do not contain classical nuclear localization signals (NLS) and exhibit clear nucleocytoplasmic-localized, and their nuclear components are crucial for their function [13] [14]. Biochemical fractionation experiments

showed that both RPM1 and RPP1-A from *Arabidopsis* are associated with the membrane [15] [16]. RPS4 binds to endo-membranes and co-fractionates with an endoplasmic reticulum marker [17]. Microscopy analyses have shown that L5, SUT1, and PRS5 are anchored to the plasma membrane (PM) due to the presence of acylation sites at the N-terminus [10] [11] [18]. The RGA4 and RGA5 derived from rice are mainly localized in the cytosol [19]. L6 and M proteins are localized on the Golgi and tonoplast, respectively [20]. Remarkably, the localization of NBS-LRR cannot be simply determined based on their sequence. In addition, NBS-LRRs may also undergo repositioning when encountering corresponding effectors [14] [17].

At present, analyzing the subcellular localization of proteins mainly relies on heterologous transient expression systems, such as protoplasts of *Arabidopsis* or rice leaves, onion epidermal cells and *Nicotiana* leaves [21]. *Agroinfiltration* allows for rapid transformation of leaf cells without the need to recycle transgenic lines. Compared to the several months required to produce transgenic lines, *Agroinfiltration* leaf cells can be imaged within 30 - 48 hours after infiltration. It may not be difficult to co-express multiple proteins within the same cell. These advantages have made *agroinfiltration* a popular choice for localized research. Therefore, localization of *Arabidopsis* NB-LRRs can be studied by *Agroinfiltration* transient expression in *Nicotiana benthamiana*.

So far, the structures of some NBS-LRR proteins from plants have been elucidated, including the CNL protein ZAR1 and TNL receptor RPP1 in *Arabidopsis*, Sr35 in wheat, and ROQ1 in *Nicotiana benthamiana* [22]-[25]. CNL and TNL resistosomes form wheel-like structures similar to those of the Apaf-1 apoptosome and the NLRC4 inflammasomes [26] [27]. Nevertheless, there are significant differences in the N-terminal signaling domains among these large protein complexes. Compared with the flexible N-terminal caspase recruitment domain (CARDs) in Apaf-1 apoptosome and NLRC4 inflammasomes, the CC domain in CNL resistosomes or the TIR domain in TNL resistosomes are clear, indicating that different signaling transduction mechanisms may be used in CNL and TNL resistosomes [8].

In this article, we exploited AlphaFold to predict the structure of some designed NBS-LRRs, and utilized *Agroinfiltration* transient expression system, combined with biochemical fractionation, to dissect the localization of these NBS-LRR receptors from *Arabidopsis*. Structural data indicated identified NBS-LRRs share analogous conformations. Membrane fractionation assays attested these NBS-LRRs are mainly associated with the membrane. These research data provided some reference clues for analyzing the structure and localization patterns of other plant immune receptors.

2. Materials and Methods

2.1. Plant Material

Nicotiana benthamiana plants were cultivated in a plant greenhouse with a 16 h

light period at 25˚C.

2.2. Plasmid Constructs

In this article, we directly use the genome of wild-type *Arabidopsis* leaves as a template to amplify fragments of the corresponding NBS-LRR gene by PCR. Conditions for the PCR amplifications were: 35 cycles of a 30 second denaturation step at 94˚C, annealing at 56˚C - 60˚C for 30 seconds, and extension at 72˚C for 1 minute. Then, we recovered the aforementioned fragments separately and cloned them into pENTR/D. After extracting the plasmid, DNA sequencing was performed, and finally, gateway technology was used to connect the correct plasmid to the expression vector pEarleyGate101 fused with the YFP-HA tag. Electro-transfer the expression plasmid into *Agrobacterium tumefaciens* strain GV3101.

2.3. 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 the leaves of 4-week-old *N. benthamiana* at specific OD_{600} values (OD_{600} = 0.8).

2.4. Membrane Fractionation Assays

In short, discard the veins of the leaves of *N. benthamiana*, place approximately 0.5 grams (g) of Agrobacterium infected leaves and 2.5 milliliters (ml) of sucrose buffer in a mortar, thoroughly grind on ice until homogenized, and centrifuge the extract at 5000 × g for 10 minutes at 4˚C; Then, transfer 200ul of supernatant to a new 2 ml EP tube, designated as total protein (T). The remaining supernatant was centrifuged at high speed (20000 g) at 4˚C for 1 hour (h), and the obtained supernatant was collected and designated as cytoplasmic fraction (C). The precipitation section was resuspended with a corresponding volume of 1x protein loading buffer and thoroughly shaken, and labeled as microsomal fraction (M). The above protein components were run on SDS-PAGE gel and analyzed by Western blot.

2.5. Sequence Alignment and Phylogenetic Analysis

The evolutionary history was inferred using the Neighbor-Joining method [28]. The optimal tree with the sum of branch length = 6.78990381 is shown. 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 the units of the number of amino acid substitutions per site. This analysis involved 16 amino acid sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1146 positions in the final data-

set. Evolutionary analyses were conducted in MEGA X [29].

2.6. Gene Accession Number

Sequence information from this text can be found in the GenBank data libraries under accession numbers AT1G12210 (L1/RFL1), AT1G12220 (L2/RPS5), AT1G15890 (L3), AT1G62630 (L4), AT1G12290 (L5), AT1G63360 (L6), AT3G07040 (L7/RPM1), AT3G46530 (L8), AT3G46710 (L9), AT3G46730 (L10), AT3G50950 (L11/ZAR1), AT4G26090 (L12/RPS2), AT4G27190 (L13), AT5G43730 (L14/RSG2), AT5G47250 (L15) and AT5G63020 (L16/SUT1). The protein structure from this paper can be found in the AlphaFold Protein Structure Database under the accession codes L1/RFL1 (AF-Q8L3R3-F1), L2/RPS5 (AF-O64973-F1), L3 (AF-Q9LMP6-F1), L4 (AF-Q9SI85-F1), L5 (AF-P60839-F1), L6 (AF-Q9SH22-F1), L7/RPM1 (AF-Q39214-F1), L8 (AF-Q9M667-F1), L9 (AF-Q9STE5-F1), L10 (AF-Q9STE7-F1), L11/ZAR1 (AF-Q38834-F1), L12/RPS2 (AF-Q42484-F1), L13 (AF-Q9T048-F1), L14 (AF-Q9FG91-F1), L15 (AF-Q9LVT4-F1) and L16/SUT1 (AF-Q8RXS5-F1).

3. Results

3.1. NBS-LRR Proteins from *Arabidopsis* Can be Correctly Expressed in *Nicotiana benthamiana*

Previous studies have proclaimed that the *Arabidopsis thaliana* genome contains approximately 150 NBS-LRR immune receptors [9]. We randomly cloned several CC-NBS-LRRs from *Arabidopsis* and designated as L1-L16. The physiological and biochemical properties of these proteins were characterized, and the general information for the 16 NBS-LRR genes is shown in Table 1. The length of these NBS-LRR proteins ranged from 835 to 985 amino acids, with predicted molecular weights of 95 kDa to 113 kDa. The theoretical isoelectric point of these NBS-LRR proteins ranged from 5.58 to 8.51.

Table 1. The protein data of the CC-NBS-LRR receptor involved in this article.

Protein name	Gene number	Length	Molecular Weight	Isoelectric Point
L1/RFL1	AT1G12210	885	~101 kDa	6.64
L2/RPS5	AT1G12220	885	~101 kDa	7.17
L3	AT1G15890	888	~96 kDa	6.63
L4	AT1G62630	893	~101 kDa	6.75
L5	AT1G12290	884	~100 kDa	6.41
L6	AT1G63360	884	~101 kDa	7.21
L7/RPM1	AT3G07040	926	~106 kDa	8.51
L8/RPP13	AT3G46530	835	~97 kDa	6.49

Continued

L9	AT3G46710	847	~98 kDa	5.95
L10	AT3G46730	847	~98 kDa	8.02
L11/ZAR1	AT3G50950	852	~97 kDa	6.2
L12/RPS2	AT4G26090	909	~104 kDa	6.51
L13	AT4G27190	985	~113 kDa	7.69
L14/RSG2	AT5G43730	848	~96 kDa	5.74
L15	AT5G47250	843	~95 kDa	5.58
L16/SUT1	AT5G63020	888	~102 kDa	6.52

To explore the phylogenetic relationships of these NBS-LRRs, phylogenetic analysis was carried out. The results indicated that NBS-LRRs with similar sequences were clustered on the same branch, such as RPM1/L7, L8, L9 and L10; RFL1/L1, RPS5/L2, and L5. However, NBS-LRRs with lower sequence identities were clustered on the different branches (Figure 1(a)). Ulteriorly, we fused the YFP-HA label at the C-terminus of these genes and transiently expressed them in the leaves of *Nicotiana benthamiana*. The results of immunoblotting indicated that these NBS-LRRs derived from *Arabidopsis* can be correctly expressed in *N. benthamiana* (Figure 1(b)).

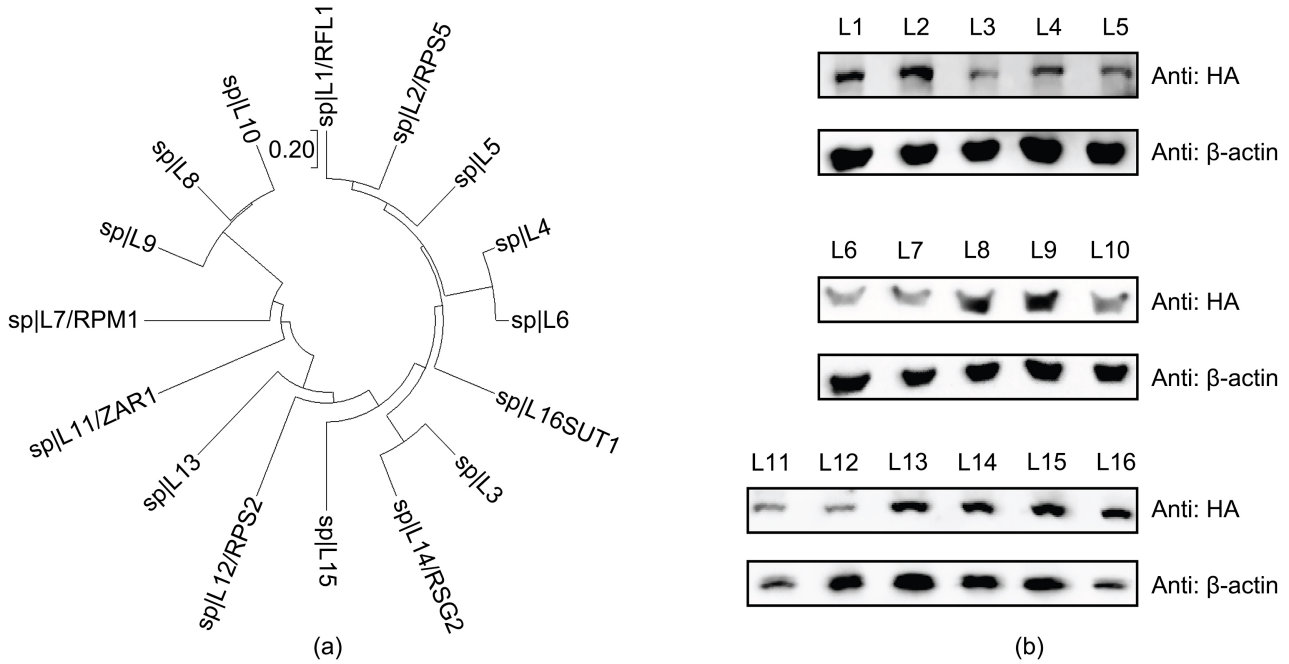

Figure 1. NBS-LRRs from *Arabidopsis* can be correctly expressed in *Nicotiana benthamiana*. (a) Phylogenetic analysis of the deduced amino acid sequences of the designed NBS-LRR proteins. (b) These NBS-LRR receptors can be correctly expressed in *Nicotiana benthamiana*, as confirmed by immunoblotting. The samples were collected and monitored with the anti-HA antibody. *β*-actin was used as the protein loading control.

3.2. Diversity of CC-NBS-LRR Structure

Furthermore, we analyzed structural features of CC-NBS-LRR genes identified in this study. We separately input the complete amino acid sequences of these CC-NBS-LRR immune receptors into the AlphaFold Protein Structure Database (https://alphafold.ebi.ac.uk/) to obtain their structural images. At first glance, the spatial structure of these NBS-LRRs seems to have commonality, meaning that the N-terminus of these receptors all form four-helix bundle conformation (**Figure 2**). Coincidentally, Wang *et al.* found that the activated ZAR1/L11 formed an immune complex called ZAR1 resistosome, in which the oligomeric CC domain was present in a four-helix bundle conformation [22]. In addition, we found that the CC domains of L7/RPM1, L8, L9, and L10 exhibit similar conformations of four-helix bundle to L11/ZAR1, which is consistent with the results of the evolutionary tree (**Figure 1(a)**). Moreover, although the CC domains of the remaining 11 NBS-LRR genes generally exhibit four-helix bundle

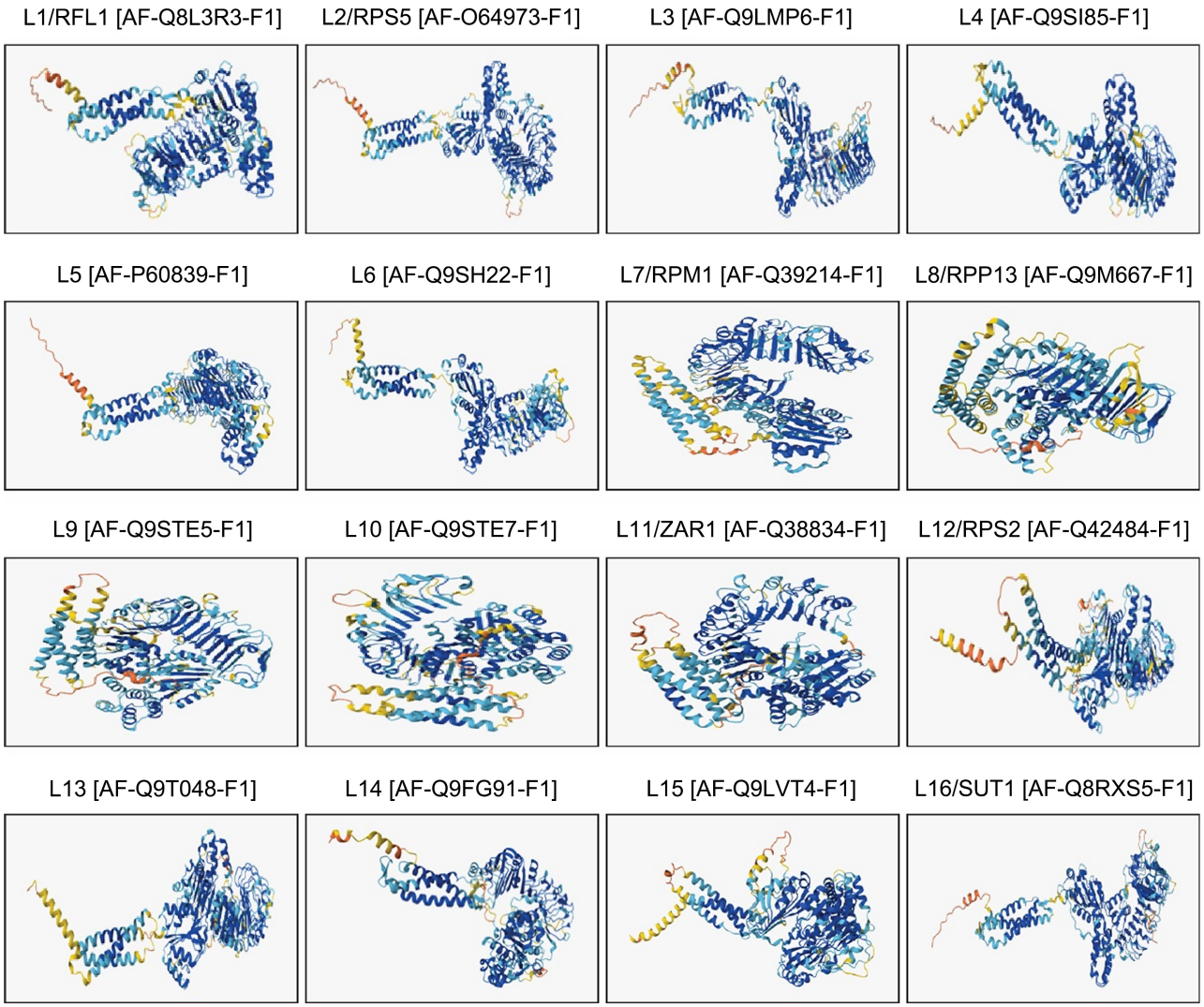

Figure 2. The overall structure of these NBS-LRRs exhibits certain similarities and differences, especially in their CC domains.

conformation, their first helix is independently dissociated (**Figure 2**). Previous studies have shown that the N-terminus of L1, L2, L4, L5, L6, and L16 all contain acylation sites (Gly2 and Cys4), which contribute to associate these immune receptors with membranes [30]. As expected, these receptors have highly similar structures and are closely related in the evolutionary tree (**Figure 2**, **Figure 1(a)**). Surprisingly, L3 is anchored to the plasma membrane due to the presence of acylation sites [31], the protein has shown a closer evolutionary relationship with L14, although the latter does not have a typical acylation site at the N-terminus, and their protein structures seem to be different, especially their CC domains (**Figure 2**, **Figure 1(a)**).

3.3. Diversity of Subcellular Localization of CC-NBS-LRR

Following, the subcellular localization pattern of these NBS-LRRs were examined through biochemical fractionation. We carried out membrane fractionation assays. We employed H^+-ATPase and Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) serves as markers of membrane and soluble fractions, respectively. Immunoblot results indicated that five NBS-LRRs (L2, L3, L6, L7/RPM1, L12/RPS2) are only localized in the microsomal fraction containing plasma membrane. Correspondingly, L3, L7/RPM1, and L12/RPS2 have been confirmed to be anchored to the plasma membrane [12] [31] [32]. Besides, four NBS-LRRs (L4, L5, L11/ZAR1, L16/SUT1) mainly existed in the microsomal fraction containing plasma membrane. We have confirmed that both L5 and L16 are primarily localized on the plasma membrane due to the presence of acylation sites at the N-terminus [10] [11]. ZAR1 has been reported to be localized on the plasma membrane, nucleus, and endoplasmic reticulum [33]. The localization of the remaining seven NBS-LRRs (L1/RFL1, L8/RPP13, L9, L10, L13, L14, L15) is also associated with the membrane. These data indicate that there are certain differences and similarities in the subcellular localization of the designed NBS-LRR proteins.

4. Discussion

Preserving NBS-LRR genomic context has several advantages over expression with a strong constitutive promoter. Sometimes, protein localization is mediated by transport machinery or by retention that is saturable [34]. Therefore, overexpression has the potential to result in mis-localization [35]. Overexpression can also result in protein misfolding and accumulation in insoluble aggregates [36]. In addition, some NBS-LRR transcripts undergo alternative splicing that is necessary for function and depends on the genomic context of the gene [37].

In the ZAR1/L11 resistosome, the N-terminal helix α1 forms a funnel-shaped structure, which is the only exposed part of the CC domain, suggesting that the funnel-shaped structure is important for the function of ZAR1 resistance. Protein fractionation analysis revealed that effector AvrAC induced ZAR1 binds to the plasma membrane [22], consistent with our biochemical experimental results

(**Figure 3**). The activation of ZAR1 in plant cells triggers Ca^{2+} influx, disturbance of subcellular structure and immune response, supporting the plasma membrane channel activity of ZAR1 resistosome [38]. Our structural data indicates that the N-terminal CC domain structure of the designed NBS-LRRs exhibits a four α-helix bundle conformation, and most NBS-LRRs (L1, L2, L3, L4, L5, L6, L12/RPS2, L14, L15, L16/SUT1) have their first α-helix bundle dissociated, which is similar to the conformation of the activated state of ZAR1. These data shows that the Ca^{2+}-permeable channel activity may be evolutionarily conserved in CC-NBS-LRR of *Arabidopsis*.

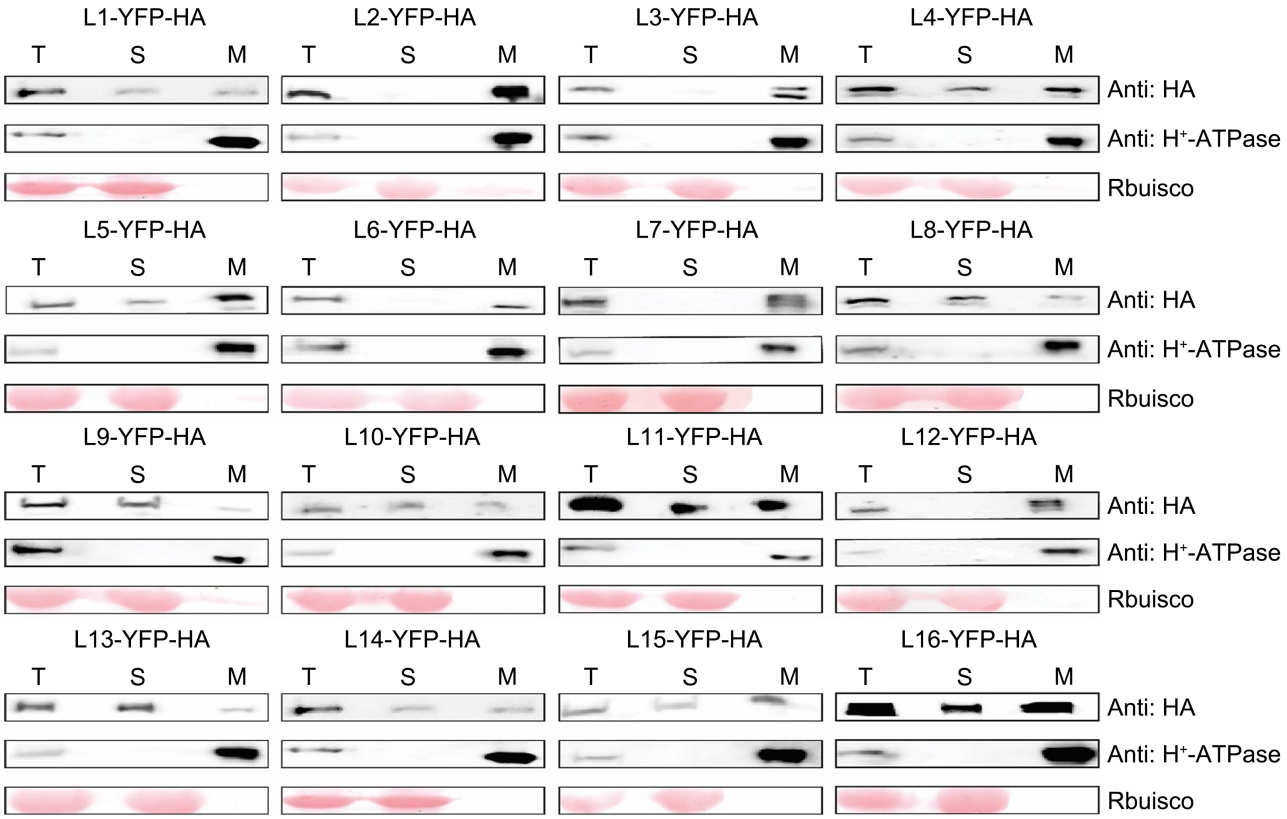

Figure 3. The subcellular localization of the designed NBS-LRRs exhibits both similarities and differences.

We have demonstrated that the localization of 16 designed NBS-LRRs from *Arabidopsis* is not entirely identical, although they are all associated with the membrane (**Figure 3**). Coincidentally, animal NBS-LRRs are also observed in different subcellular positioning, require similar chaperones to maintain pre-activation ability and can be repositioned to get involved various signal complexes during activation. The mammalian CIITA NBS-LRR receptor plays a role in the nucleus [39]. NOD2 exists on the plasma membrane and recruits downstream partners to the plasma membrane; The mutation of its LRR causes NOD2 to appear cytoplasmic and non-functional [40]. NOD1 and NOD2 locally recruit autophagic components to the plasma membrane sites of bacterial infections, while common NOD2 variants associated with Crohn's disease fail to do so [41].

These studies indicate that mammalian NBS-LRRs are located at different positions in cells before activation, and they can be dynamically repositioned, and their cellular localization affects downstream functions. The sum of data from plant NBS-LRR and animal NBS-LRR intracellular immune receptors is consistent with the conceptual role of NBS-LRR protein as a cell homeostasis monitor, which investigates a wide range of cellular defense mechanisms through various subcellular addresses [1].

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Conflict 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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