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Structural Requirements of RIN4 for Regulation of EXO70E2 Plasma Membrane Localization in Plant Cell

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Abstract

Protein localization is essential for maintaining cellular organization and function. EXO70E2, a plant-specific EXO70 family member, participates in exocyst-associated trafficking and unconventional secretion pathways. Our previous study demonstrated that RIN4 promotes EXO70E2 extracellular transport; however, the structural basis underlying this regulation remains unclear. Here, we investigated the structural requirements of RIN4-mediated EXO70E2 localization regulation using fluorescence localization analysis and RIN4 mutants. EXO70E2-YFP showed punctate intracellular localization when expressed alone, whereas co-expression with RIN4 promoted its redistribution toward the plasma membrane-associated region. The CCC/SSS mutation impaired RIN4-mediated EXO70E2 localization regulation, whereas T166 mutations showed limited effects. DTT-mediated perturbation further reduced EXO70E2 membrane accumulation. These findings indicate that the conserved cysteine cluster region and structural integrity of RIN4 contribute to EXO70E2 localization regulation, extending our understanding of RIN4-mediated control of plant trafficking-associated protein localization.

Keywords

RIN4, EXO70E2, Exocyst, Subcellular Localization, Plasma Membrane, Protein Trafficking, Unconventional Secretion

1. Introduction

Protein localization is essential for maintaining cellular organization and functional activity. In plant cells, precise regulation of intracellular trafficking controls

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secretion, cell polarity, development, and adaptation to environmental changes [1] [2]. Therefore, understanding the mechanisms regulating protein localization is important for elucidating how plant cells coordinate complex biological processes. The exocyst complex is a conserved vesicle-tethering machinery that mediates the targeting of secretory vesicles to specific plasma membrane domains before membrane fusion. In plants, the exocyst complex participates in diverse cellular processes, including cell growth, cytokinesis, and polarized secretion [3] [4]. Compared with animals and fungi, plants possess a greatly expanded EXO70 gene family, suggesting that individual EXO70 members may have evolved specialized regulatory functions during plant evolution [5] [6]. Although different EXO70 proteins exhibit distinct localization patterns and biological functions, the mechanisms controlling the intracellular distribution of specific EXO70 members remain largely unknown.

EXO70E2 is a specialized member of the plant EXO70 family involved in unconventional secretion pathways [7]. Previous studies demonstrated that EXO70E2 participates in the formation of exocyst-positive organelles (EXPOs), which contribute to non-classical extracellular secretion in *Arabidopsis* [8]. These findings indicate that EXO70E2 represents an important connection between exocyst activity and specialized secretion processes. However, the regulatory mechanisms controlling EXO70E2 intracellular localization remain poorly understood.

RIN4 (RPM1-interacting protein 4) is a conserved plant regulatory protein that has been extensively studied because of its central role in plant immunity. RIN4 was initially identified as a target of bacterial type III secretion system effectors and was shown to interact with multiple pathogen-derived proteins, including AvrRpm1, AvrB, and AvrRpt2, thereby regulating immune receptor activation and plant defense responses [9]-[11]. In addition to its immune-related functions, RIN4 contains conserved structural regions associated with its molecular activity and membrane localization [12], suggesting that RIN4 may participate in additional membrane-associated cellular processes. Increasing evidence indicates that plant immune signaling and intracellular trafficking pathways are closely interconnected [13]. Regulatory proteins involved in defense responses may also influence trafficking-associated processes, thereby coordinating cellular signaling and spatial organization [14]. However, whether immune-associated proteins regulate trafficking-related components through specific structural features remains unclear.

Our previous study demonstrated that RIN4 promotes EXO70E2 extracellular transport, establishing a functional relationship between RIN4 and EXO70E2-associated trafficking processes [15]. However, this previous work mainly focused on the functional consequence of RIN4-mediated EXO70E2 transport, whereas the structural basis underlying this regulation remained unresolved. In this study, we investigated the structural requirements of RIN4 involved in EXO70E2 localization regulation using EXO70E2-YFP fluorescence analysis, RIN4 structural mutants, and DTT-mediated structural perturbation. We found that RIN4 promotes

redistribution of EXO70E2 from intracellular punctate structures toward the plasma membrane-associated region. The conserved cysteine cluster region contributed to this regulatory activity, whereas alteration of the T166 residue showed limited effects. Furthermore, disruption of RIN4 structural integrity reduced EXO70E2 membrane accumulation. These findings provide new evidence that specific structural features of RIN4 contribute to the regulation of trafficking-associated protein localization in plant cells.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

The cultivation method of *Nicotiana benthamiana* is as described earlier [16] [17]. In short, *N. benthamiana* Plants were grown under controlled conditions in a growth chamber with a 16 h light/8 h dark photoperiod at 24˚C. Fully expanded leaves from 4 - 5-week-old plants were used for *Agrobacterium*-mediated infiltration experiments.

2.2. Plasmid Construction and Generation of RIN4 Mutants

The coding sequence of *Arabidopsis thaliana* EXO70E2 was fused with yellow fluorescent protein (YFP) to generate the EXO70E2-YFP fluorescence reporter construct for subcellular localization analysis. The full-length RIN4 coding sequence was cloned into an expression vector pEarleyGate101 for transient co-expression assays. To facilitate both fluorescence imaging and protein expression analyses, specific tags were utilized: N-terminal YFP-tagged RIN4 (YFP-RIN4) constructs were generated for all live-cell confocal imaging experiments, whereas N-terminal T7-tagged RIN4 (T7-RIN4) constructs were used exclusively for immunoblotting assays to assess protein stability. To investigate the structural regions of RIN4 involved in EXO70E2 localization regulation, different RIN4 mutants were generated by site-directed mutagenesis. The T166A and T166D mutants were constructed to examine the contribution of the conserved T166 residue. In addition, the CCC/SSS mutant was generated by substituting conserved cysteine residues within the cysteine cluster region with serine residues. All recombinant constructs were confirmed by sequencing before subsequent experiments.

2.3. *Agrobacterium*-Mediated Transient Expression Assay

The method of *Agrobacterium*-mediated transient expression assay is as described earlier [18]. Simply put, the recombinant plasmids were introduced into *Agrobacterium tumefaciens* GV3101 cells and transiently expressed in *N. benthamiana* leaves. Bacterial cultures were harvested and resuspended in an infiltration buffer containing 10 mM MES (pH 5.6), 10 mM $MgCl_2$, and 150 μM acetosyringone, and adjusted to a final optical density (OD_{600}) of 0.5. For co-expression assays, Agrobacterium strains harboring different constructs were mixed at a 1:1 volume ratio prior to syringe infiltration. All downstream imaging and biochemical extractions were performed at 48 hours post-infiltration (hpi). For EXO70E2

localization analysis, EXO70E2-YFP was expressed alone or co-expressed with wild-type RIN4. To investigate the structural requirements of RIN4 function, EXO70E2-YFP was co-expressed with different RIN4 variants, including T166A, T166D, and CCC/SSS mutants. After infiltration, plants were maintained under appropriate growth conditions to allow protein expression, and fluorescence signals were subsequently analyzed by confocal microscopy.

2.4. Confocal Microscopy Analysis

The subcellular localization of EXO70E2-YFP and RIN4-associated constructs was analyzed using confocal laser scanning microscopy (Leica TCS SP8). YFP was excited at 514 nm (argon laser) with emission collected between 525 - 550 nm. Pinhole size was set to 1 Airy unit, and images were acquired using a 20× water-immersion objective. Fluorescence signals were collected from epidermal cells of infiltrated leaves. Fluorescence localization patterns were analyzed systematically across three independent infiltration experiments. For each experimental condition, a minimum of 50 randomly selected cells expressing the fluorescent constructs were evaluated (total n = 150 cells per condition). EXO70E2-YFP localization patterns were compared among cells expressing EXO70E2-YFP alone, EXO70E2-YFP with wild-type RIN4, and EXO70E2-YFP with different RIN4 mutants. The localization patterns were classified according to fluorescence distribution, including intracellular punctate structures and plasma membrane-associated accumulation. Specifically, fluorescence was classified as "peripheral" if the signal tightly localized with plasma membrane, and as "punctate" if the signal was predominantly observed in distinct intracellular vesicles.

2.5. DTT-Mediated Structural Perturbation Assay

To examine whether the structural integrity of RIN4 contributes to EXO70E2 localization regulation, DTT-mediated perturbation assays were performed. Leaf discs expressing RIN4-associated constructs were submerged in a solution of 5 mM DTT for exactly 30 minutes prior to imaging. Leaves expressing RIN4-associated constructs were treated with DTT, and subsequent changes in EXO70E2-YFP localization were monitored by confocal microscopy. The effects of DTT treatment were evaluated by comparing EXO70E2-YFP distribution patterns between untreated and DTT-treated samples. Because DTT may influence cellular redox homeostasis and multiple cellular processes, these experiments were interpreted as an assessment of the contribution of RIN4 structural integrity to localization regulation rather than direct evidence of specific structural modification.

2.6. Protein Extraction and Immunoblot Analysis

To verify protein stability, total proteins were extracted from infiltrated leaves 48 hpi using a standard extraction buffer. Equal amounts of total protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. Immunoblot analysis was performed using an anti-T7 primary antibody to detect T7-RIN4 var-

iants, ensuring that altered EXO70E2 distribution could not be attributed to unequal expression or degradation of mutant proteins.

3. Results

3.1. RIN4 Promotes Redistribution of EXO70E2 toward the Plasma Membrane-Associated Region

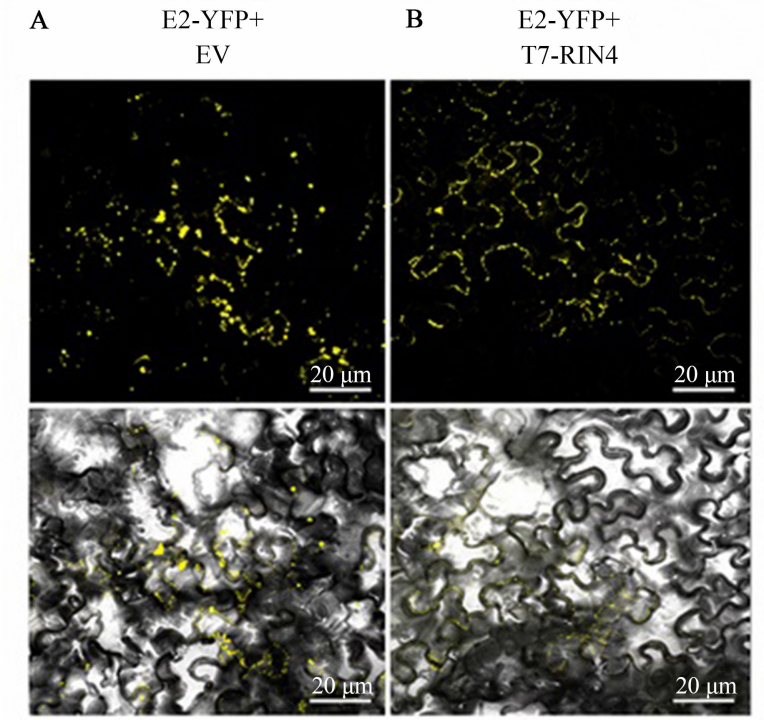

Figure 1. RIN4 promotes redistribution of EXO70E2 toward the plasma membrane-associated region. (A) Confocal images showing EXO70E2-YFP exhibited predominantly intracellular punctate localization. (B) Confocal images showing RIN4 expression promoted EXO70E2-YFP redistribution toward the cell periphery.

Our previous study demonstrated that RIN4 promotes EXO70E2 extracellular transport [15]. However, whether this regulatory effect is associated with changes in the intracellular distribution pattern of EXO70E2 remains unclear. Therefore, we first examined the subcellular localization of EXO70E2 in the presence or absence of RIN4 using fluorescence microscopy. When EXO70E2-YFP was transiently expressed alone in *Nicotiana benthamiana* cells, fluorescence signals were predominantly detected as intracellular punctate structures (**Figure 1(A)**), indicating that EXO70E2-YFP mainly accumulated in intracellular compartments under these conditions. To determine whether RIN4 affects EXO70E2 localization, EXO70E2-YFP was co-expressed with wild-type RIN4. Compared with cells expressing EXO70E2-YFP alone, cells co-expressing RIN4 showed increased fluorescence accumulation at the cell periphery and reduced intracellular punctate distribution (**Figure 1(B)**). These observations indicate that RIN4 regulates the subcellular distribution of EXO70E2 by promoting its re-

distribution toward the plasma membrane-associated region. To quantify this redistribution, we calculated the proportion of cells exhibiting clear PM localization and measured the membrane-to-cytosol fluorescence ratio. Peripheral EXO70E2 signal was predominant in 82% of cells co-expressing wild-type RIN4, compared to only 14% in the empty-vector control. Together with our previous finding that RIN4 promotes EXO70E2 extracellular transport, these results suggest that RIN4-mediated transport regulation is associated with changes in EXO70E2 localization.

3.2. The Conserved CCC Region of RIN4 Is Required for Plasma Membrane Localization and EXO70E2 Redistribution Regulation

After confirming that RIN4 promotes EXO70E2 redistribution toward the plasma membrane-associated region, we next investigated the structural basis underlying RIN4 localization and regulatory activity. RIN4 is a plasma membrane-associated protein, and the conserved cysteine cluster (CCC: Cys203-Cys205) region has been implicated in its membrane anchoring [19]. Therefore, we examined whether disruption of the CCC region affects RIN4-dependent regulation of EXO70E2 localization. To assess the contribution of the T166 residue, EXO70E2-YFP was co-expressed with RIN4-T166A or RIN4-T166D mutants. Both T166A and T166D variants retained the ability to promote EXO70E2 redistribution toward the plasma membrane-associated region, showing localization patterns comparable to wild-type RIN4 (**Figure 2(A)**). These results indicate that alteration of T166 has limited effects on RIN4-mediated EXO70E2 localization regulation under the tested conditions. We next analyzed the role of the CCC region. The CCC/SSS mutant was generated by replacing conserved cysteine residues within the CCC region with serine residues. To ensure that any observed differences in EXO70E2 localization were not artifacts of unequal expression or altered protein stability, we evaluated the protein levels of all RIN4 variants in planta. Immunoblot analysis confirmed that wild-type RIN4, the T166 mutants, and the CCC/SSS mutant accumulated at comparable levels (data not shown). Therefore, the failure of the CCC/SSS mutant to redirect EXO70E2 to the PM is directly attributable to the loss of functional membrane anchoring rather than protein degradation.

Compared with wild-type RIN4, the CCC/SSS mutation disrupted RIN4 plasma membrane localization, resulting in reduced membrane-associated RIN4 accumulation (**Figure 2(B)**). Consistent with the impaired RIN4 membrane localization, cells co-expressing EXO70E2-YFP with RIN4-CCC/SSS showed reduced EXO70E2 accumulation at the cell periphery and increased retention of intracellular punctate fluorescence. These results indicate that the CCC region is required for proper plasma membrane targeting of RIN4. Loss of CCC-dependent membrane localization compromises the ability of RIN4 to promote EXO70E2 redistribution, suggesting that plasma membrane association of RIN4 is an important prerequisite for regulating EXO70E2 localization.

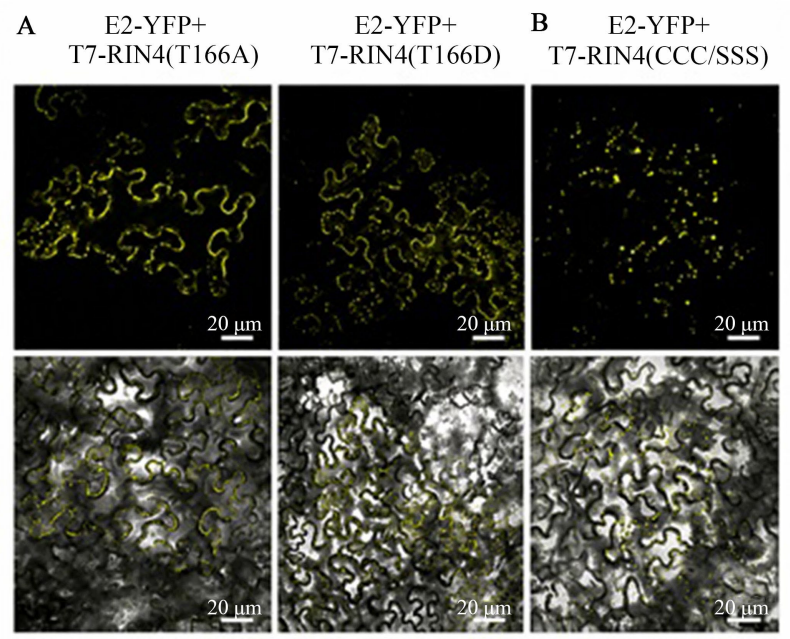

Figure 2. The conserved CCC region of RIN4 is required for plasma membrane localization and EXO70E2 redistribution regulation. (A) EXO70E2-YFP localization in cells co-expressing RIN4-T166A, or RIN4-T166D. Both T166 mutants retained the ability to promote EXO70E2 redistribution toward the plasma membrane-associated region. (B) CCC/SSS mutation disrupted RIN4 plasma membrane localization and reduced RIN4-mediated EXO70E2 redistribution.

3.3. RIN4 Structural Integrity Contributes to EXO70E2 Localization Regulation

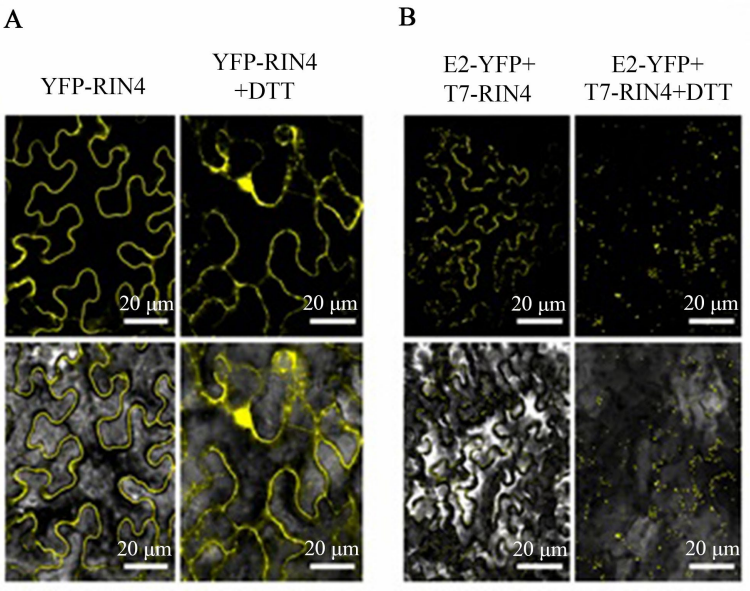

Figure 3. RIN4 structural integrity contributes to EXO70E2 localization regulation. (A) Changes in RIN4-associated fluorescence distribution following DTT treatment. (B) DTT treatment reduced EXO70E2 accumulation at the cell periphery and increased intracellular punctate fluorescence. Scale bars = 20 μm.

The CCC/SSS mutant analysis suggested that specific structural regions of RIN4 contribute to EXO70E2 localization regulation. To further investigate whether the structural state of RIN4 affects this activity, DTT-mediated perturbation experiments were performed. Previous studies have shown that DTT treatment can perturb intracellular protein trafficking and membrane association by altering disulfide-dependent protein conformation [20]. Under untreated conditions, co-expression of RIN4 promoted EXO70E2-YFP accumulation at the cell periphery. Following DTT treatment, changes in RIN4-associated fluorescence distribution were observed (**Figure 3(A)**). We subsequently examined EXO70E2-YFP localization after DTT treatment. Compared with untreated cells, DTT-treated samples showed reduced peripheral accumulation of EXO70E2-YFP and increased intracellular punctate fluorescence patterns (**Figure 3(B)**). These observations indicate that disruption of RIN4 structural integrity affects its ability to regulate EXO70E2 localization. Together with the CCC/SSS mutant analysis, these results support the contribution of RIN4 structural features to EXO70E2 distribution regulation.

4. Discussion

Protein localization is a dynamic process that depends on coordinated regulation of protein targeting, membrane association, and intracellular trafficking [21]. In this study, we further characterized the mechanism underlying RIN4-mediated regulation of EXO70E2 distribution by analyzing the contribution of specific structural features of RIN4. Our results demonstrate that RIN4 promotes the redistribution of EXO70E2 from intracellular punctate structures toward the plasma membrane-associated region, and this activity is dependent on the conserved cysteine cluster region of RIN4. These findings extend our previous observation that RIN4 promotes EXO70E2 extracellular transport [15] by providing additional evidence that RIN4 regulates EXO70E2 trafficking at the level of intracellular localization.

The CCC region of RIN4 emerged as an important determinant of its regulatory activity [19]. Mutation of the conserved cysteine residues impaired RIN4 plasma membrane association and consequently reduced its ability to promote EXO70E2 redistribution. These results suggest that proper membrane targeting of RIN4 is a prerequisite for its regulation of EXO70E2 localization. Rather than acting as a general regulator of protein trafficking, RIN4 may influence specific trafficking events through its own spatial positioning within the cell. This finding provides a mechanistic explanation for how a membrane-associated regulatory protein can modulate the localization behavior of another trafficking-associated component.

In contrast, modification of the T166 residue had limited effects on RIN4-mediated EXO70E2 localization regulation. Although T166 has been associated with regulatory modification and immune-related functions of RIN4 [12] [22] [23], the present results indicate that this residue is not a major determinant of EXO70E2 redistribution under the experimental conditions used. These observations fur-

ther suggest that different structural regions of RIN4 may contribute independently to distinct biological activities, with the CCC region being particularly important for functions requiring proper membrane association.

Chemical perturbation experiments using DTT further supported the importance of maintaining the appropriate structural state of RIN4 for EXO70E2 localization regulation. Following DTT treatment, the accumulation of EXO70E2 at the cell periphery was reduced, suggesting that disruption of cellular redox conditions or protein structural stability may interfere with RIN4-dependent regulation. Our observation that short-term DTT treatment abolishes RIN4-mediated EXO70E2 re-localization is consistent with established models of cysteine-cluster-dependent membrane anchoring in plant cells. Furthermore, the specific DTT concentration and exposure time used in our study have been previously validated to effectively disrupt lipid-anchoring modifications of peripheral membrane proteins without inducing acute cellular stress. However, because DTT can influence multiple cellular processes, including protein conformation and intracellular trafficking [20] [24] [25], these results should be interpreted as evidence supporting the requirement for an appropriate regulatory environment rather than direct evidence of a specific structural modification of RIN4.

5. Conclusion

Together, this study provides new insight into the structural basis of RIN4-mediated regulation of trafficking-associated protein localization. The CCC-dependent membrane association of RIN4 represents a critical factor enabling EXO70E2 redistribution, whereas other regulatory residues such as T166 appear to have limited contributions in this process. These findings expand the functional understanding of RIN4 beyond its established roles in immune signaling and suggest that immune-associated proteins may also participate in regulating the spatial organization of cellular trafficking components. Further studies identifying the molecular interaction interface between RIN4 and EXO70E2 will be important for understanding how RIN4 selectively controls the localization and transport behavior of EXO70E2.

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Author Contributions

Conceptualization, J.H.; formal analysis, J.H.; methodology, J.H. and K.C.; investigation, J.H., and K.C.; data collection, J.H. and K.C.; validation, J.H. and K.C.; supervision, J.H.; writing—original draft preparation, J.H.; funding acquisition, J.H. and K.C. All authors have read and agreed to the published version of the manuscript.

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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