Zipcode RNA-binding Proteins and Membrane Trafficking Proteins Cooperate to Transport Glutelin mRNAs in Rice Endosperm

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Short Title: Glutelin mRNAs are transported on endosomes

One-sentence summary: Glutelin mRNAs are transported on endosomes through the direct interactions of two RNA-binding proteins with two membrane trafficking factors.

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ABSTRACT

In rice (Oryza sativa) endosperm cells, mRNAs encoding glutelin and prolamine are translated on distinct cortical-endoplasmic reticulum (ER) subdomains (the cisternal-ER and protein body (PB)-ER), a process that facilitates targeting of their proteins to different endomembrane compartments. Although the cis- and trans-factors responsible for mRNA localization have been defined over the years, how these mRNAs are transported to the cortical ER has yet to be resolved. Here, we show that the two interacting glutelin zipcode RNA-binding proteins (RBPs), RBP-P and RBP-L, form a quaternary complex with the membrane fusion factors N-ethylmaleimide-sensitive factor (NSF) and the small GTPase Rab5a, enabling mRNA transport on endosomes. Direct interaction of RBP-L with Rab5a, between NSF and RBP-P, and between NSF and Rab5a were established. Biochemical and microscopic analyses confirmed the colocalization of these RBPs with NSF on Rab5a-positive endosomes that carry glutelin mRNAs. Analysis of a loss-of-function rab5a mutant showed that glutelin mRNA and
the quaternary complex were mis-targeted to the extracellular paramural body structure formed by aborted endosomal trafficking, further confirming the involvement of endosomal trafficking in glutelin mRNA transport. Overall, these findings demonstrate that mRNA localization in plants co-opts membrane trafficking via the acquisition of new functional binding properties between RBPs and two essential membrane trafficking factors, thus defining an endosomal anchoring mechanism in mRNA localization.

INTRODUCTION

Localization of mRNAs is a universal mechanism to efficiently drive protein targeting in eukaryotes and prokaryotes. The targeting of mRNAs facilitates the accumulation of the locally translated proteins to specific cellular compartments and, hence, is an essential mechanism in establishing cell polarity, patterning, and fate determination as well as protein sorting (Herbert and Costa, 2019; Hughes and Simmonds, 2019; Tian et al., 2019b, 2020).

mRNA localization occurs as a multi-step process. After transcription, cis-acting elements (RNA zipcodes) are recognized and bound by trans-acting factors, mainly RNA-binding proteins (RBPs) to form a primary mRNA–nucleoprotein (mRNP) complex. After export to the cytoplasm, the mRNP complex undergoes extensive remodeling with recruitment of new factors and detachment of others enabling cytoskeletal-based transport to the destination site (Blower, 2013; Weis et al., 2013; Tian and Okita, 2014).

Although extensive knowledge on mRNA localization has been acquired by studies in Drosophila, yeast and mammalian cells, only a few examples have emerged from higher plants. The best defined model in plants is storage protein mRNA localization in developing rice (Oryza sativa) endosperm cells, where mRNAs encoding glutelin and prolamine are recognized by zipcode RBPs and transported to two distinct cortical endoplasmic reticulum (ER) subdomains, the cisternal-ER, and protein body-ER (PB-ER), respectively (Chou et al., 2019; Tian et al., 2019b). Translation of prolamine mRNAs on the PB-ER results in the assembly of prolamine intracisternal granules that form an ER-derived protein body I (PB-I), while glutelin precursors are exported to the Golgi and then transported to protein storage vacuoles (PSVs) for processing and storage.
(Chou et al., 2019; Tian et al., 2019b). Although several cytoskeleton-associated RBPs required for mRNA localization have been identified (Doroshenk et al., 2009, 2012), information on how these mRNAs are transported to distinct ER subdomains remains elusive.

Emerging evidence from fungal model systems reveals the intimate link of mRNA transport with membrane trafficking (Schmid et al., 2006; Jansen et al., 2014; Haag et al., 2015; Niessing et al., 2018). Several mRNAs from *Saccharomyces cerevisiae*, *Candida albicans*, and *Ustilago maydis* are co-transported with mobile ER or shuttling endosomes (Schmid et al., 2006; Jansen et al., 2014; Haag et al., 2015; Pohlmann et al., 2015; Niessing et al., 2018). *ASH1* as well as other mRNAs are co-transported on tubular ER that moves to the emerging bud or daughter cell in *Saccharomyces cerevisiae*. This process is mediated by the RBPs She2p and She3p, with She2p having membrane binding properties and She3p serving as an adaptor protein linking the mRNP-cER to Myo4P protein (Schmid et al., 2006; Niessing et al., 2018). The *cdc3* mRNA is transported on shuttling endosomes in the smut fungus, *Ustilago maydis*, a process requiring localization of the RBP Rrm4 on the endosomes and the interaction of a membrane-associated linker protein Upa1 with Rrm 4 (Pohlmann et al., 2015; Niessing et al., 2018). Specific adaptor proteins appear to be needed to hitch mRNPs on endosomes for active transport over long distance. More recently, neuronal RNA granules have been shown to hitchhike on moving lysosomes using annexin11 as a tether (Liao et al., 2019). Although co-transport of mRNAs with membranous compartments was proposed to be a common mechanism in higher eukaryotes (Jansen et al., 2014), whether the mechanism is utilized by higher plants remains to be determined.

Previous studies suggested that endocytosis and membrane trafficking likely play a role in mRNA localization in plants. For example the loss-of-function of the small GTPase Rab5a and its cognate guanine nucleotide exchange factor (GEF) resulted in defects in endocytosis and membrane trafficking and the mis-targeting of glutelin proteins to the prolamine containing PB-I as well as to the extracellular paramural body (PMB) in rice endosperm cells (Fukuda et al., 2011; Wen et al., 2015). As storage protein targeting is regulated by their mRNA localization in rice endosperm cells, the mis-targeting of glutelin proteins in the mutant suggests a relationship between endosomal
transport and glutelin mRNA localization in rice. The extracellular distribution of glutelin mRNAs within PMBs from a mutant expressing a defective GEF (Yang et al., 2018) further supports the possible involvement of endosomal trafficking in glutelin mRNA transport. However, direct evidence depicting the co-transport of glutelin mRNAs with shuttling endosomes and how endosomal trafficking are engaged in glutelin mRNA localization have yet to be established. Such mis-targeting of glutelin mRNAs in rice lines expressing mutant Rab5a and GEF may simply be a consequence of pleiotropy.

Recent studies (Tian et al., 2018; Tian et al., 2019a) identified two RNA-binding proteins, RBP-P and RBP-L, which contain two and three RNA recognition motif (RRM) domains, respectively. These RBPs specifically bind to the glutelin zipcode mRNA sequences and regulate glutelin mRNA localization. In this study, using these two glutelin zipcode RBPs as entry points, we identified their interacting partners, N-ethylmaleimide-sensitive factor (NSF) and the small GTPase Rab5a, which participate in endosomal membrane trafficking. The four proteins may form a quaternary complex carrying glutelin mRNAs for active transport on endosomes to the cortical ER membrane. The identification of these key linker proteins that enable endosome-mediated mRNA transport in rice endosperm cells provides new insights on how mRNAs can be distributed to specific locations in eukaryotes.

RESULTS

RBP-P interacts with membrane fusion factor NSF

Previous studies (Doroshenk et al., 2014; Tian et al., 2018; Tian et al., 2019a) established that the glutelin zipcode RNA-binding proteins RBP-P and RBP-L, which interact with each other, are essential for localization of glutelin mRNAs to the cisternal-ER, as mutations in these RBPs led to the mis-localization of glutelin mRNAs. To obtain additional insight into how glutelin mRNAs are transported to the ER, we performed immunoprecipitation-mass spectrometry (IP-MS) studies using affinity-purified anti-RBP-P antibody (Figure 1A). Tandem mass spectrometry of a major polypeptide band observed in the IP generated with anti-RBP-P but not with control anti-GFP identified a major interacting protein as N-ethylmaleimide sensitive fusion protein (NSF).
To determine whether RBP-P interacts directly with NSF, yeast two-hybrid studies were carried out. In such two-hybrid screening, the two proteins of interest were fused to activating domain (AD) and DNA-binding domain (BD) domain of yeast GAL4 transcription factor, respectively. Interaction of these proteins restore GAL4 that, in turn, activate the transcription of histidine and adenine reporter genes. While no background interaction of RBP-P and NSF with complementary empty vector was observed, yeast cells carrying both RBP-P and NSF genes activated the reporter genes and survived in selection medium lacking histidine and adenine (Figure 1B). These results indicate that RBP-P and NSF interact under stringent binding conditions.

To further substantiate the interaction between RBP-P and NSF, bimolecular fluorescence complementation (BiFC) analyses using tobacco (Nicotiana tabacum) BY-2 cells was performed. In addition to supporting an interaction between protein pairs, BiFC also provides intracellular information on where this interaction occurs. RBP-P and NSF were fused to two complementary non-fluorescent fragments of enhanced yellow fluorescent protein (EYFP), nEYFP and cEYFP, respectively, and co-transformed into live BY-2 cells. If the two proteins interact, the two EYFP fragments are brought in close proximity to reform the native protein structure enabling emission of yellow fluorescence and, in turn, direct visualization of the protein complex in live cells (Kerppola, 2006; Miller et al., 2015). EYFP fragments lacking one of the putative protein partners were used as negative control. As shown in Figures 2A-2C, while no interaction was detected between RBP-P or NSF with the empty EYFP fragment controls, cells expressing both RBP-P and NSF fused to EYFP fragments emitted bright yellow fluorescence, indicating that RBP-P interacted with NSF. Closer examination reveals that RBP-P/NSF complexes were distributed to the cytoplasm as intensely bright clusters together with more loosely diffuse structures (Figure 2C).

NSF is a soluble hexameric ATPase commonly found in the cytoplasm of eukaryote cells (Mastick and Falick, 1997; Zhao et al., 2007; Zhao et al., 2010; Guo et al., 2017), which predominantly plays a major role as a chaperone in intracellular membrane fusion events. Through its interaction with the adaptor protein, soluble NSF attachment protein (SNAP), NSF binds to SNARE (soluble NSF activating protein receptor) complexes and utilizes the energy of Mg\textsuperscript{2+} -dependent ATP hydrolysis to disassemble the SNARE
protein complex, and thus facilitating the recycling of SNARE proteins for further cycles of membrane fusion (Zhao et al., 2007; Chang et al., 2012; Ryu et al., 2015). During this process, SNAP serves as chaperone by stimulating the ATPase activity of NSF and dissociating from NSF after ATP hydrolysis (Zhao et al., 2007). This transient interaction between NSF and SNAP is only detected under conditions where a nonhydrolyzable ATP is used as a substrate (Hanson et al., 1995; Barnard et al., 1997; Chang et al., 2012). Alternatively, the addition of EDTA to chelate Mg\(^{2+}\) and thus inhibit ATPase activity has been used to detect the transient interaction between NSF and SNAP (Hanson et al., 1995; Barnard et al., 1997; Chang et al., 2012; Li et al., 2018).

Based on the established properties of NSF, we performed Co-IP experiments to assess the formation of RBP-P/NSF complexes in rice endosperm cells. Rice seed lysates supplemented with 1 mM ATP and 8 mM MgCl\(_2\) or EDTA were incubated with affinity purified anti-RBP-P and NSF antibodies (Figure 2D) or anti-GFP antibody, the latter used as a negative control. No proteins were captured by the control GFP antibody, revealing the reliability of the IP experiments. Interestingly, RBP-P and NSF were co-precipitated in the presence of Mg\(^{2+}\)-ATP but not when EDTA was added to the seed lysates (Figure 2D). This result indicates that formation of the RBP-P/NSF complex in rice endosperm cells requires Mg\(^{2+}\)-ATP, a condition distinct from those complexes involving the stable interaction between NSF and SNAP (Hanson et al., 1995; Barnard et al., 1997; Chang et al., 2012; Li et al., 2018). The differences in binding properties, as well as the absence of SNAP in RBP-P IPs (Figure 2D), indicate that the events of membrane fusion requiring NSF-SNAP are not required for formation of the RBP-P/NSF complex. On the other hand, the interaction between NSF with RBPs suggests that NSF may function in mRNA metabolism by its interaction with RBP-P.

**RBP-P indirectly interacts with Rab5a through NSF**

The interaction of the glutelin zipcode trans-factor RBP-P with NSF supports a close relationship between glutelin mRNA transport with membrane trafficking. Previous studies demonstrated that Rab5a, an evolutionarily conserved key GTPase involved in the biogenesis of early endosomes and membrane trafficking in the cytoplasm (Woodman, 2000; Saito and Ueda, 2009; Ito et al., 2018), is also required for glutelin mRNA
localization (Doroshenk et al., 2010) and vesicular membrane transport between the
Golgi and protein storage vacuole in rice endosperm cells (Wang et al., 2010; Fukuda et
al., 2011). Therefore, we investigated whether the abovementioned RBP-P/NSF complex
is associated with Rab5a to regulate the endosomal transport of mRNAs.

We first performed a transient expression study to confirm the association of rice
Rab5a (Figures 2E-2F, Supplemental Figure 1) with endosomes. FM4-64 is a lipophilic
dye that initially labels the plasma membrane and, subsequently, internalizes with
membrane vesicles transported along the endocytic pathway (Vida and Emr, 1995; Ueda
et al., 2001). In this experiment, BY-2 protoplasts expressing GFP-Rab5a were treated
with FM4-64 for 15 min before observation. As shown in Figure 2F, red fluorescence
derived from FM4-64 was evident on the plasma membrane and in internalized
endosomes. GFP-Rab5a displayed a similar distribution pattern and co-localized with the
internal FM4-64 labeled vesicles (Figure 2F), an observation confirming that Rab5a is
associated with endosomes.

We then studied the relationship of Rab5a with the abovementioned RBP-P/NSF
complex. Co-IP studies were carried out using affinity-purified antibodies to Rab5a,
RBP-P and NSF. All three proteins were found in IPs generated by anti-Rab5a, anti-RBP-
P and anti-NSF (Figure 2D), suggesting that they form a multi-protein complex in rice
endosperm cells. Protein complex formation apparently required active ATPase
hydrolysis as all three protein interactions could only be simultaneously detected in the
presence of Mg\(^{2+}\) and ATP and not when EDTA and ATP were included (Figure 2D).

To further characterize this protein interactome and the formation of their complexes,
BiFC (Figures 2G-2J) studies were performed. Interaction between NSF and Rab5a was
observed in the tested cells, and their complexes existed in the cytoplasm. By contrast,
Rab5a does not interact with RBP-P (Figure 2J) and, therefore, the association of RBP-P
with Rab5a in the Co-IPs (Figure 2D) is mediated through the interaction between NSF
and Rab5a.

Rab GTPases are highly conserved small proteins and share two conserved regions
that are required for conformational switching between active and inactive states of GTP
hydrolysis (Figure 2E, Supplemental Figure 1). The switch regions are disordered in an
inactive conformation and adopt a well-defined conformation in the active stage
Amino acid substitutions within the switch regions affects their conformational switching. While the G45D mutation in switch 1 inhibits the conformation switching to an active GTPase stage and restricts Rab5a in a GDP-bound form, a Q70L mutation in switch 2 locks it in a constitutively active GTP bound form (Lee et al., 2009). Only the GTP-bound form of Rab5 are membrane-bound during endosomal trafficking (Yuan and Song, 2020).

To investigate whether the NSF-Rab5a complex is associated with active endosomes, the interaction of NSF with the two structural forms of Rab5a containing G45D or Q70L, was analyzed by BiFC (Figures 2K-2L). Although NSF directly interacts with both GDP- (Rab5a\textsuperscript{G45D}) and GTP- (Rab5a\textsuperscript{Q70L}) forms of Rab5a, their complexes are observed as two distinct populations. When bound to the GTP-fixed Rab5a (Q70L) form (Figure 2L), the NSF complex was distributed as endosomal punctate structures. By contrast, the NSF complex constituted by GDP-fixed Rab5a (G45D) showed a diffuse distribution throughout the cytoplasm (Figure 2K). These results are consistent with the view that the GTP-fixed Rab5a (Q70L) form is membrane bound while the GDP-fixed Rab5a (G45D) is mainly soluble.

The direct interaction between NSF and Rab5a provides an indirect link to RBP-P and, in turn, RNA localization. To confirm the association of the RBP-P/NSF complex to Rab5a-positive endosomes, we performed a three-way co-localization test consisting of RBP-P and NSF as BiFC interacting partners in BY-2 cells expressing Rab5a tagged with the red fluorescence protein (RFP) (Figures 2M-2N). Fluorescence analysis of BY-2 cells showed that RFP-Rab5a\textsuperscript{WT} was distributed as diffuse signals throughout the cytoplasm and as punctate structures (endosomes) near the plasma membrane (Figure 2M, middle panel). By contrast, RFP-Rab5a\textsuperscript{Q70L} was present only as punctate structures (Figure 2N, middle panel) indicating that the activated GTPase form of Rab5a is associated with endosomes. The fluorescence distribution pattern seen for RFP-Rab5a\textsuperscript{WT} is consistent with membrane-free Rab5a being distributed in the cytoplasm and active Rab5a associated with endosomes (Yuan and Song, 2020).

As earlier seen in Fig. 2C, BiFC-linked RBP-P/NSF complexes are distributed as fluorescent punctate structures in BY-2 cells (Figure 2M and N). These RBP-P/NSF complexes co-localized with RFP-Rab5a-associated endosomes in the cytoplasm,
especially with membrane-associated GTP-fixed (Q70L) Rab5a (Figures 2M-2N). To further confirm that the RBP-P/NSF complexes are associated with endosomes, we used FM4-64, which specifically labels these small membrane compartments (Figure 2O). The fluorescent signals from the RBP-P/NSF complexes co-localized with endosomal compartments labeled by FM4-64, providing direct evidence that RBP-P/NSF complexes co-localize with endosomes. Consistent with the abovementioned Co-IP analysis (Figure 2D) where RBP-P, NSF and Rab5a may co-assemble into a multi-protein complex in rice endosperm cells, the BiFC results suggest that RBP-P is associated with endosomes through a NSF-mediated interaction with Rab5a.

It should be noted that not all RBP-P/NSF complexes co-localized with Rab5a-linked endosomes (Figures 2M-2N, open arrowheads). Similarly, the location of several Rab5a-active endosomes (Figures 2M-2N, open arrowheads) do not coincide with RBP-P/NSF complexes. The lack of overlap in the distribution of a population of RBP-P/NSF complexes and Rab5 endosomes suggests the multiple roles of these proteins in RNA localization and membrane trafficking.

**RBP-L is involved in the RBP-P/NSF/Rab5a complex**

We had previously demonstrated that RBP-P co-assembles with RBP-L to form a protein complex that is essential for storage protein mRNA localization (Doroshenk et al., 2014; Tian et al., 2018; Tian et al., 2019a). To determine whether RBP-L is also involved in interacting with NSF or Rab5a, BiFC studies were conducted (Figures 3A-F). Such analysis showed that RBP-L interacts directly with Rab5a but not with NSF (Figures 3A-3C). The RBP-L/Rab5a WT complex is observed as two distinct populations. One population is distributed as endosomal punctate structures with the bulk located close to the plasma membrane. The co-localization of RBP-L/ Rab5a WT with endosomes is supported by their close association with FM4-64 labeled endosomal compartments (Figure 3D). A second population of RBP-L/ Rab5a WT is viewed as a diffuse cloud around the nucleus. Similar to the RBP-P/NSF/Rab5a complex, the interaction of RBP-L with Rab5a was not dependent on the functional state of Rab5a as it interacts with both the GTP-bound Rab5a Q70L and GDP-bound Rab5a G45D (Figures 3E and 3F), although the distribution patterns are distinct. RBP-L/ Rab5a G45D is distributed mainly as a diffuse
cloud around the nucleus and near the plasma membrane while RBP-L/\(\text{Rab5a}^{Q70L}\) are observed predominantly as discrete endosomal punctate structures.

To further determine whether RBP-L is associated with the RBP-P/NSF/Rab5a complex, we performed Co-IP with paraformaldehyde-fixed seed extracts to optimize the capture of any potential dynamic endosome-associated complexes formed \textit{in vivo}. Such results showed that, irrespective of the antibody used in the initial immunoprecipitation, RBP-P, RBP-L, NSF and Rab5a were detected in IPs generated by all four antibodies (Figure 3G). By contrast, SNAP is only present in IPs obtained with anti-NSF and anti-Rab5 but not in IPs generated by antibodies to RBP-P and RBP-L (Figure 3G). These results are consistent with the Co-IP results depicted in Figure 2D where SNAP is present in IPs generated with antibodies to NSF and Rab5a in the presence of EDTA but not in the presence of \(\text{MgCl}_2\). Overall, these results are consistent with the view that NSF-Rab5a complexes exist as two separate populations. One NSF-Rab5a population together with SNAP participates in Rab5-mediated endosomal fusion (Woodman, 2000; Zhao et al., 2007), where NSF and SNAP disassemble the SNARE complex formed by Rab5-mediated membrane fusion. A second NSF-Rab5a complex contains RBP-P and RBP-L and is independent of SNAP.

To further address whether RBP-P and RBP-L are attached to endosomes together, sequential IPs were performed (Figure 3H). In this analysis, the RBP-P/NSF/Rab5a complexes precipitated by anti-RBP-P antibodies were next subjected to an additional IP using anti-RBP-L. All four proteins were detected in the second IP generated by anti-RBP-L, suggesting that RBP-P, RBP-L, NSF and Rab5 co-assemble to form a quaternary protein complex.

The quaternary complex attaches to active endosomes carries glutelin mRNAs

To investigate whether the quaternary complex binds glutelin mRNA, we performed RNA-IP analysis to detect the \textit{in vivo} association of the complex with glutelin mRNAs (Figure 3I). In this analysis, antibodies to RBP-P, RBP-L, NSF and Rab5a were utilized to capture the associated RNA-protein complexes, and the RNA was subsequently isolated from the IP fractions and subjected to RT-PCR using specific primers to amplify glutelin transcripts. Compared with the negative empty-resin control and anti-GFP
antibody, glutelin mRNAs were highly enriched in IPs generated by all four antibodies (Figure 3I). The mRNA amount associated with NSF and Rab5a was lower than that of RBP-P and RBP-L, a result consistent with the roles of these proteins in membrane fusion events. Overall, these results support the view that the quaternary complex contains glutelin mRNAs.

Based on these results, a working model of cytosolic glutelin mRNA transport is proposed in Figure 4. The mRNP complex containing glutelin mRNA, RBP-P and RBP-L is bound to Rab5a-associated endosomes through a 4-way interactome, i.e. the direct interaction of RBP-P with RBP-L, of RBP-L with Rab5a, of NSF with RBP-P, and of Rab5a with NSF. The GTP-bound Rab5a is associated with endosomes (Yuan and Song, 2020) as suggested by distribution of this activated Rab5a form as endosomal punctate structures (Figures 2N and 3F).

**Loss-of-function of Rab5a results in mis-targeting of glutelin mRNAs**

To provide evidence in support of this model, we analyzed a rab5a mutant EM960 (Fukuda et al., 2011) expressing a GDP-fixed (G45D) Rab5a (Figure 5A). Similar to the phenotype shown in the EM956 mutant lacking Rab5a (Fukuda et al., 2011) or a mutant line expressing a defective Rab5a effector GEF (Wen et al., 2015), normal endosomal trafficking is disrupted in the endosperm cells of GDP-fixed rab5a mutant and leads to the formation of PMBs (Figures 5B-5C), an aborted endosome complex containing mis-sorted endomembrane proteins. These extracellular PMBs, which contain numerous electron-dense vesicles, are located in the space between the invaginating plasma membrane and the cell wall in the mutant endosperm cells (Figures 5B-5C).

To investigate the co-localization of RBP-P, RBP-L, and NSF with Rab5a and the subcellular localization of their complex in rice endosperm cells, we performed double immuno-fluorescence labeling on thin sections of rice developing seeds using antibodies raised against each of the four proteins. Although the bulk of these proteins were evidently independent of Rab5, there was ample evidence for co-localization of RBP-P, RBP-L, and NSF with Rab5a. The co-localization of these proteins with Rab5a was apparent as punctate structures in the cytoplasm, particularly in the cortical region underneath the plasma membrane (Figures 6A, 6C and 6E), an intracellular location.
enriched in Rab5a-mediated endosome activity (Chavrier et al., 1990; Fischer von Mollard et al., 1994). To directly assess the co-localization of these proteins, the fluorescence intensity profiles of these proteins were quantified along a specific linear distance (Figure 6, right panels). The fluorescence signals for the proteins examined overlapped substantially indicating that RBP-P, RBP-L and NSF co-localized to Rab5a-labeled endosomal compartments in rice endosperm cells. The independent distribution of RBP-P, RBP-L and NSF with Rab5a was also evident in the BiFC/RFP double labeling (Figures 2M-N), which is indicative of their roles in other cellular processes. This view is also supported by the Co-IP results (Figures 2F, 3H) where IPs by antibodies to RBP-P, RBP-L and NSF contained only a small proportion of the total Rab5a amounts.

The RBPs retain their co-localization with Rab5a in the rab5a mutant (Figures 6B and 6D), an expected observation as RBP-L as well as NSF interact with both the GDP- and GTP-fixed Rab5a forms (Figures 2K-2L, Figures 3E-3F). These protein complexes exist as punctate structures within the PMBs in the rab5a mutant, (Figures 6B, 6D and 6F). Hence, disruption of membrane trafficking in the rab5a mutant displaces not only endosomal proteins to the extracellular PMBs but also RBP-mRNA complexes.

Transmission electron microscopy (TEM) analysis further confirmed the co-localization of RBP-P and RBP-L with Rab5a on endosomes in wildtype and aborted endosome vesicles within PMBs in rab5a mutant (Figure 7). In wild type, Rab5a-mediated endosomes were observed as electron-dense vesicles with an irregular shape likely due to endosomal fusion (Figures 7A-7E). Co-localization of RBP-P and RBP-L with Rab5a was observed on those endosomes, further suggesting that mRNP complexes, carrying glutelin mRNAs bound by RBP-P and RBP-L, are transported on endosomes. In the rab5a mutant line, normal endosomal trafficking is disrupted resulting in the displacement of mRNA-associated endosomes to the extracellular PMBs (Figures 7F-7J).

To further address whether glutelin mRNAs are associated with the Rab5a-mediated endosomes, in situ reverse transcription (RT)-PCR on developing endosperm sections was performed to locate glutelin mRNAs. In this experiment, Rhodamine B dye was used to specifically stain the PB-ER (Muench et al., 2000) while glutelin mRNAs were labeled with Alexa488-dUTP by in situ RT-PCR using glutelin-specific primers (Figure 8). In wild type, glutelin mRNAs are localized on the cisternal-ER separate from PB-ER
(Figure 8). In rab5a mutant cells, however, they were mainly distributed to the PMBs and with smaller amounts associated with the PB-ER. These results indicate that normal glutelin mRNA localization is disrupted in the rab5a mutant. This result resonates with the retention of the complexes formed by RBP-P, RBP-L, NSF and Rab5a in PMBs (Figures 6 and 7), further supporting a Rab5a-dependent endosome transport of glutelin mRNAs.

Taken all together, the results described here support the view that glutelin mRNAs are transported to the cisternal-ER membrane via their hitchhiking on endosomes. In the rab5a mutant, however, glutelin mRNA-containing mRNP complexes are not transported to their normal location but are mis-targeted to the PB-ER and displaced to the extracellular PMBs because of the disruption in normal endosomal trafficking.

**DISCUSSION**

The study of rice storage protein mRNA localization as a model has provided considerable information on why and how plant mRNAs are localized to specific ER compartments. Although zipcode cis-elements of glutelin and prolamine mRNAs and several key RNA-binding proteins have been identified (Hamada et al., 2003; Washida et al., 2009; Doroshenk et al., 2012; Washida et al., 2012; Tian et al., 2018; Tian et al., 2019a), the mechanism by which glutelin and prolamine mRNAs are transported to distinct subdomains of the ER membrane has yet to be established. Here, we report that rice endosperm cells employ Rab5a and NSF, proteins traditionally known for their essential roles in endosomal trafficking and membrane fusion events, as adaptor proteins linking mRNA-protein complexes to endosomes for active transport of glutelin mRNAs.

Active transport of mRNAs along cytoskeleton networks by hitchhiking on motile, membrane-bound organelles has been reported in mammalian and fungi cells. In human axons, the Ras GAP SH3 domain binding protein 1 (G3BP1)-labeled RNA granules were found to co-localize and co-traffic with moving lysosomes along microtubules (Liao et al., 2019). The membranous amyotrophic lateral sclerosis (ALS)-associated phosphoinositide-binding protein, annexin A11 (ANX11), functions as a molecular tether between RNA granules and lysosomes (Liao et al., 2019). In addition to mRNAs,
neuronal precursor miRNAs are transported to the tip of growing axons by hitchhiking on late endosomes/lysosomes (Corradi et al., 2020).

Endosomal transport of mRNAs has been defined in the filamentous fungus *Ustilago maydis*, where mRNAs are transported throughout the growing hyphae via endosome trafficking on microtubules (Baumann et al., 2012; Gohre et al., 2012; Vollmeister et al., 2012; Pohlmann et al., 2015; Niessing et al., 2018). mRNP complexes carrying *cdc3* mRNAs are attached to endosomes via the adaptor protein Upa1, which associates with the endosomal membrane through its FYVE domain and two RNA binding proteins, Pab1 and Rrm4, through its PABP-associated motif 2 (PAM2) and PAM2-like (PAM2L) domains, respectively (Pohlmann et al., 2015).

Unlike the abovementioned examples in mammalian and fungi cells, higher plants apparently adapted the two membrane trafficking factors, NSF and Rab5a, to mediate transport of storage protein mRNAs. The linking of glutelin mRNP complexes to endosomes is mediated by the four-way interactions of NSF and Rab5a with the two glutelin zipcode RNA-binding proteins, RBP-P and RBP-L. These interacting proteins likely form a quaternary complex enabling endosomal transport of glutelin mRNAs. Such protein to protein interactions are accomplished through a gain in binding properties by Rab5a and NSF, and the glutelin zipcode mRNA binding proteins, RBP-P and RBP-L, which allow for the heterotypic interaction of a RNA binding protein with a membrane fusion factor (RBP-P/NSF and RBP-L/Rab5a). The interaction of an RBP with a membrane trafficking factor, *i.e.* between RBP-P with NSF and between RBP-L and Rab5a, as well as between NSF and Rab5a are unexpected findings as such interactions have not been reported in other organisms. It remains unclear whether the interactions of these membrane trafficking factors to RBPs and even between themselves is an inherent property or whether they are unique to higher plants. While Upa1-mediated endosomal mRNA transport is proposed as evolutionarily conserved in fungi (Muller et al., 2019), further investigation is needed to assess whether endosomal mRNA transport by the NSF-Rab5a-RBP machinery is a widespread phenomenon in transporting mRNAs among higher plants and other eukaryotic organisms.

It appears that the recognition of NSF and Rab5a with RBPs is highly selective. These non-random interactions highlight the diverse binding capabilities of NSF, Rab5a,
as well as for RBP-P and RBP-L in rice endosperm cells. Both RBP-P and RBP-L contain RNA recognition motifs (RRM) with the former having two RRM motifs and the latter having three RRM motifs. Due to the conservation of the RRM motifs, the recognition of NSF and Rab5a to RBP-P and RBP-L, respectively, is likely via the unique N- and C-terminal regions that flank the RRM motifs. Indeed, our preliminary results (Supplemental Figure 2) show that the N-terminal end of RBP-P is essential for its interaction with NSF. Through the selective recognition of NSF and Rab5a to other specific RBPs, these membrane trafficking factors could serve as the core components enabling endosome-coupled mRNA transport with the RBPs specifying the mRNA species.

NSF is homo-hexamer with three domains: the N-terminal domain (NSF-N) that is required for SNAP-SNARE binding; the ATP-binding domain 1 (NSF-D1) responsible for ATPase activity; and the ATP-binding domain 2 (NSF-D2) responsible for hexamerization (Tagaya et al., 1993; Zhao et al., 2007). The NSF-N domain is the main protein-protein interaction site for NSF binding to other proteins. NSF-N domain is likely responsible for its interaction with the N-terminus of RBP-P in rice as well (Supplemental Figure 2). Sequence alignment of NSF homologues from rice, Arabidopsis, *Drosophila*, yeast and human show that the N-terminal region displays considerable sequence diversity among these species (Supplemental Figure 3). Further structural studies are required to investigate their binding mechanism and whether this kind of interaction occurs in other species.

Rab5a interacts with a large number of proteins, including its regulators (activating effectors and inhibitors), linkers to molecular motors, membranous factors, components of membrane fusion complexes, protein kinases and phosphatases (Herve and Bourmeyster, 2018; Pylypenko et al., 2018). Many of these interacting proteins function as Rab5a effectors to activate endosomal transport and tightly control the specific functions of Rab5a in membrane composition and modification, cytoskeleton regulation, and intracellular trafficking (Herve and Bourmeyster, 2018; Pylypenko et al., 2018). Although NSF has been reported to interact with several Rab5a effectors to drive membrane fusion during endosomal docking (McBride et al., 1999; Grosshans et al., 2006), no direct relationship between Rab5a with NSF or with a RNA-binding protein
had been established until now. These findings will stimulate future research efforts on identifying new alternative roles of Rab5a and NSF beyond membrane fusion.

Although the GTPase activity of Rab5a is not required for the apparent formation of the quaternary protein complex as the four proteins still co-localized in the GDP-fixed (G45D) Rab5a line (Figures 6-7), Rab5 GTPase activity is required for proper mRNA transport. Endosome formation and membrane trafficking are dependent on Rab5 GTPase activity (Woodman, 2000; Zeigerer et al., 2012). Null Rab5a activity results in aborted endosomal transport in the mutant, which, in turn, disrupts glutelin mRNA transport on endosomes and causes its mis-localization to the PB-ER as well as displacement to the extracellular PMBs (Figure 8). Therefore, the active GTPase activity of Rab5a is essential for the transport of glutelin mRNAs on endosomes. This also raises the question on whether NSF and RBP-L act as Rab5a effectors to modulate the role of Rab5a in mRNA transport and localization. While this hypothesis needs further examination, other effectors, functionally equivalent to Rabaptin-5 and EEA1 that interact with both NSF and Rab5a (McBride et al., 1999; Grosshans et al., 2006), may also be involved. These effectors, including the abovementioned GEF, may allow further stabilization of the linkage of mRNP complex to active endosomes and regulate endosomal mRNA transport via the cytoskeleton (Figure 4).

The identification and study of rice lines expressing mutations in Rab5a were instrumental in establishing its involvement in both RNA and membrane trafficking in developing rice endosperm. While Rab5 is an essential growth factor, loss of Rab5a only had a slight effect on rice growth and development as its activity is complemented by other Rab5 activities. Rice expresses three other Rab5 genes, Rab5b, Rab5c, and Rab5d (Supplemental Figure 4). Like Rab5a, Rab5c is a conventional type, while Rab5b and Rab5d are plant-specific type homologous to the Arabidopsis plant-specific Rab5F1/ARA6. During rice growth and development, the loss of Rab5a is offset by Rab5c and possibly by Rab5b and Rab5d. Although the other Rab5 isoforms can compensate for Rab5a, they are only able to partially fill this role as rab5a rice lines grow slower and flower later than normal, likely due to their significant lower expression compared to Rab5a (Supplemental Figure 5). At the grain filling stage where there is massive protein transport from the ER via the Golgi to the storage vacuole, the reduced...
expression of these other Rab5 isoforms fails to maintain normal membrane trafficking
resulting in the secretion of storage proteins, the formation of PMBs, and mis-localization
of glutelin mRNAs.

Efforts to identify rice lines expressing defective NSF have not been productive,
likely because of the importance of its ATPase activity in membrane fusion. Mutations in
NSF were reported to induce severe defect and cell lethality in several organism
(Boulianne and Trimble, 1995; Golby et al., 2001; Mohtashami et al., 2001; Horsnell et
al., 2002; Zhao et al., 2007). For example, Drosophila expresses two NSF isoforms,
d NSF1 and d NSF2. d NSF1 is dominant in the adult central nervous system while d NSF2
is broadly distributed at the larval/adult stages of development, respectively (Boulianne et
al., 1995). While d NSF1 null mutants perish as pharate adults, d NSF2 deletion mediates a
recessive lethal phenotype, which is not even rescued by the addition of a d NSF2
transgene (Golby et al., 2001; Mohtashami et al., 2001). In yeast, loss-of-function
mutation of the NSF gene, Sec18, resulted in a dominant lethal phenotype (Horsnell et al.,
2002). These studies suggest that NSF mutations are pleiotropic and cause severe growth
problems. In Arabidopsis, even a subtle mutation of NSF caused severe abnormal Golgi
morphology (Tanabashi et al., 2018). Given that NSF is coded by a single gene copy in
the rice genome, mutations that affect NSF activity will likely confer a strong lethal
phenotype in rice.

Although beyond the scope of this study, NSF mutations that affect binding to RBP-P
would be a viable approach to obtain further insight on its role in mRNA trafficking. As
discussed earlier, the NSF-N domain is likely responsible for its interaction with the N-
terminus of RBP-P. Selected residues in the NSF-N region can be replaced by amino
acids that alter charge or conformation and then tested for its protein-interactive
properties by yeast 2-hybrid analysis. Mutations in NSF that abolish its interaction with
RBP-P but not with Rab5a would be potential sites for genetic alteration by CRISPR
technology and, thereby, disrupting glutelin mRNA transport but maintaining normal
function in membrane vesicle transport.

Overall, this study provides evidence on how glutelin mRNPs are able to
hitchhike on trafficking endosomes in rice endosperm cells by exploiting the binding
properties of RBPs and membrane trafficking factors, NSF and Rab5a. These findings
will provide the basis for future research on membrane trafficking-mediated mRNA transport and the unique functions of NSF and Rab5a in this cellular process.

MATERIALS AND METHODS

Plant materials and growth conditions. The rice wildtype (Oryza sativa japonica variety TC65) and rab5a mutant line EM960 (Fukuda et al., 2011) were potted in Sunshine Brand #1 soil (Sungro Horticulture) and grown in walk-in growth chambers with a diurnal cycle of 12 h light/12 h dark at 27°C and a lighting intensity of 400 to 700 μmol m⁻² s⁻¹ using a combination of metal halide and high pressure sodium lamps.

Antibodies. Full length cDNAs encoding RBP-P, RBP-L, NSF, SNAP, BiP and GFP were cloned into pET30a for His-tagged fusion protein expression. The His-tagged proteins were then purified and used to immunize New Zealand White rabbits for antibody production. Anti-Rab5a rabbit and mouse antibodies were obtained from an earlier study (Fukuda et al., 2011). For immunofluorescence labeling, Alexa Fluor-488 labeled goat anti-mouse IgG antibody (Invitrogen, Cat # A32723) and Alexa Fluor-594 labeled goat anti-rabbit IgG antibody (Invitrogen, Cat # A32740) were used as secondary antibodies. For immunogold labeling, EM-grade 10 nm-gold-conjugated goat anti-mouse IgG (Electron Microscopy Science, Cat # 25128) and EM-grade 15 nm-gold-conjugated goat anti-rabbit IgG (Electron Microscopy Science, Cat # 25112) were used as secondary antibodies.

Immunoprecipitation (IP) analysis. Most IP experiments were conducted as previously described (Doroshenk et al., 2014) except that incubations with antibody were conducted in the presence/absence of 1mM ATP/GTP, 8 mM MgCl₂ or EDTA. To maximize capture of transient mRNP complexes on endosomes, developing seeds were treated with 1% paraformaldehyde (PFA). Detailed procedures for IP-MS, the identification of NSF protein, and PFA fixation for the enhanced IP are described below.

IP-MS analyses. Affinity purified RBP-P or GFP antibodies were prepared using immobilized metal affinity chromatography based on irreversibly oxidized Co(III)-IDA resin as described previously (Crofts et al., 2010) and crosslinked to Protein A/G agarose resin using the Pierce Crosslink Immunoprecipitation Kit according to the manufacturer’s instructions (Thermo Fisher Scientific). Protein extraction and co-IP experiments were
performed at 4°C unless indicated. Two g of dehulled mid-developing wild type rice seed harvested 12-14 days after flowering were frozen in liquid nitrogen and grounded to a powder. Proteins were extracted in 6 ml of IP buffer (20 mM Tris-HCl pH 7.5, 0.15 M NaCl, 1 mM EDTA, 0.5% v/v NP40) containing 1X protease inhibitor cocktail and 0.5X phosphatase inhibitor (Sigma-Aldrich). The crude extract was clarified by twice centrifuging at 12,000 g for 10 min. The resulting supernatant was gently rotated overnight with agarose resin to eliminate non-specific interactions. 700 µL of the unbound protein fraction were added to columns containing RBP-P or GFP antibodies crosslinked to Protein A/G resin or naked resin and incubated approximately 7 hours with rotation. The columns were washed 5 times with IP buffer and once with 1X conditioning buffer, and bound proteins were eluted with 50 µL IgG elution buffer (Thermo Fisher Scientific). The antibody conjugated Protein A/G resin columns were regenerated by washing with 1X Coupling Buffer (Thermo Scientific) followed by IP buffer. Fresh, pre-cleared rice seed extract was added and incubated overnight. The columns were washed and proteins eluted as above.

Eluted proteins from a total of five co-IP experiments using anti-RBP-P, anti-GFP antibodies, or naked resin were pooled, precipitated with addition of 100% trichloroacetic acid (TCA) to a 20% final concentration. The TCA precipitate was washed with acetone and then resuspended in 30 µL SDS sample buffer containing 4 M urea and 5% v/v β-mercaptoethanol. Protein samples were resolved on 10% SDS-PAGE gels and stained using a silver nitrate staining protocol (Chevallet et al., 2006). Because the protein profile of the minus antibody control IP looked very similar to the GFP Co-IP, proteins from only the GFP and RBP-P co-IPs were analyzed by mass spectrometry. Gel slices corresponding to similar positions within each lane were excised, in-gel trypsin digested, and subjected to liquid chromatography-tandem mass spectrometry as described previously (Doroshenk et al., 2009). Proteins were identified by searching the *Oryza sativa* NCBI non-redundant database (134548 sequences) using Mascot (www.matrixscience.com) as previously described (Doroshenk et al., 2009).

**IP with 1% PFA fixation.** Antibodies raised against RBP-L, RBP-P, NSF, or Rab5a were affinity purified as abovementioned. 20µL of resuspended protein A Magbeads slurry (25% w/v slurry, GenScript) in a 1.5 mL tube was washed twice with 1X PBS and
then incubated overnight at 4 °C under rotation with 30 µg affinity-purified antibody (diluted to 500 µL by 1x PBS). Antibodies were cross-linked to protein A Magbeads using the above-mentioned Pierce Crosslink Immunoprecipitation Kit.

Dehulled developing rice seeds were fixed with 1% paraformaldehyde (PFA) in PBSM (1.76 mM KH$_2$PO$_4$, 10 mM Na$_2$HPO$_4$, 136 mM NaCl, 2.6 mM KCl, 5 mM MgCl$_2$, and 10% glycerol) under vacuum for 30 min. The reaction was stopped by vacuuming in quenching buffer (0.333 M Tris and 10% glycerol) for 10 min. Ten to twenty aleurone-layer peels collected from the fixed seeds were ground to a fine powder using an ice cold mortar and pestle in 800 µL of lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM EDTA, 0.5% NP-40, 0.1% Triton X-100, 1x proteinase inhibitor cocktail, 100 µg/ml cycloheximide, 20 units/mL of RiboLock RNase Inhibitor (Thermo Fisher Scientific) and then centrifuged at 1000 g for 5 min to remove starch followed by centrifugation at 12,000 g for 10 min. The clear supernatant was added to the prepared Magbeads for incubation overnight at 4°C with gentle rotation. After washing twice with lysis buffer, the bound fractions were eluted with IgG elution buffer (ThermoFisher Scientific) and neutralized by addition of 1/10 volume of 1 M Tris-HCl (pH 8.8). The neutralized elution samples were analyzed by immunoblotting.

**In situ RT-PCR.** In situ RT-PCR on developing rice seed sections was performed as previously described (Washida et al., 2009). Specific primers Glutelin-F 5’-

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CCCTCAAGCATACAGGCGTG-3'
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and Glutelin-R 5’-

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CGCTCTCTTGATTGCACCTTGCC-3'
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were used in the PCR to amplify glutelin RNAs.

**Construction of vectors.** Gene sequences of RBP-P, NSF and their truncated forms were cloned into pGAD T7 and pGBK T7 vectors and used as preys and baits, respectively, in Y2H analyses for detection of protein-protein interaction. BiFC vectors of pSAT1-nEYFP-C1 and pSAT1-cEYFP-C1-B for N- and C-terminal EYFP fusion, respectively, were obtained from the Arabidopsis Biological Resource Center (https://www.arabidopsis.org). The cDNA sequences of RBP-P, RBP-L, NSF, Rab5a and its mutant forms (Rab5a$^{Q70L}$ and Rab5a$^{G45D}$) were cloned into pSAT1-nEYFP-C1 or pSAT1-cEYFP-C1-B vectors for BiFC analysis. GFP or RFP-fusion vector driven by double 35S promoters was constructed by replacing the N-terminal EYFP in pSAT1-
nEYFP-C1 with GFP or RFP cDNA sequences. The cDNA sequences of NSF and Rab5a were then cloned into GFP or RFP-fusion vector to obtain RFP protein fusions.

**RNA-IP, Yeast two hybrid (Y2H), and BiFC assays.** Experimental procedures for these studies were performed as previously described (Doroshenk et al., 2014; Tian et al., 2018; Tian et al., 2019a). Briefly, for RNA-IP analysis, developing rice seeds, collected 10–14 days after flowering, were subjected to 1% PFA fixation and extracts prepared and used for IP as mentioned above. The elution samples obtained from IP were incubated at 70 °C for 45 min to reverse the RNA-protein crosslinks followed by RNA extraction using TRIzol (Invitrogen), cDNA synthesis using M-MLV reverse transcriptase (Promega), and PCR with 20–25 cycles of amplification using glutelin and ACTIN specific primers (Glutelin-F and Glutelin-R as mention in *in situ* RT-PCR, Actin-F 5’-TCCATCTTTGCGATCTCTCAG-3’, and Actin-R 5’-GTACCCGACATCACGCTCAGTCT-3’). The antibodies used in the RNA-IP were affinity-purified as aforementioned. In Y2H analysis, synthetic dropout (SD) growth media without leucine and tryptophan (SD/-Leu/-Trp) was used to screen positive transformants and selection media without leucine, tryptophan, histidine, and adenine (SD/-Leu/-Trp/-His/-Adenine medium) with supplement of 3 mM 3-amintriazole (3-AT) (SD/-Leu/-Trp/-His/-Adenine/+ 3-AT) or 40 mg/L X-α-Gal was used to verify protein-protein interaction.

BY-2 suspension cells were used for BiFC analyses. In brief, BY-2 cells were treated with cell wall digestion buffer (1% cellulase (Onozuka RS, PhytoTechnology Laboratories), 0.05%pectolyase (Seishin Pharmaceutical, Japan), 0.2% Driselase (Sigma-Aldrich), 20 mM KCl, 10 mM CaCl₂, 20 mM MES hydrate, and 0.5M sucrose, pH 5.7) at room temperature for 3 h. After washing with W5 solution (154 mM NaCl, 125 mM CaCl₂, 5 mM KCl, 5 mM glucose, pH 5.8-6.0), the BY-2 protoplasts were subject to PEG-mediated transformation with the abovementioned vectors of pSAT1-nEYFP-C1 and pSAT1-cEYFP-C1-B as described previously (Tian et al., 2018). After culture at 26°C for 16 hours, the BiFC fluorescence images were observed using a Leica SP-8 confocal microscope. Negative controls using empty vectors were also examined to check the reliability of the transformation procedure. The localization pattern of target proteins or complexes was determined by examining at least 5 different protoplast cells.

To confirm the involvement of Rab5a and the corresponding complexes in endocytic
pathway, protoplast incubation was treated with the endocytic tracer FM4-64 (Invitrogen) at a final concentration of 10 μM for 15-30 mins before observation.

**Microscopy.** Light microscopy was performed on 10 μm thick sections of developing rice seed samples embedded in LR-white resin. The sections were positioned on Leica X-tra slides, stained by 1% Toluidine blue and observed using an Olympus BH-2 Light microscope. Co-localization test of RBP-P, RBP-L, and NSF with Rab5a in rice endosperm cells was performed through double-immunolabeling using the rabbit anti-RBP-P, RBP-L, NSF antibodies and mouse anti-Rab5a antibodies (see Section of Antibodies) on 1 μm thick LR-white sections as described previously (Fukuda et al., 2011), and observed under a Leitz Epi-Fluorescent Microscope with Leica DFC425C Camera. Fluorescence intensity of green and red signals was analyzed by plot profile tool in FUJI (ImageJ) software. Transmission electron microscopy analysis was performed as previously described (Tian et al., 2018).

**ACCESSION NUMBERS**

Sequence data from this article can be found in the GenBank/EMBL data libraries under NCBI accession numbers shown in the legends of Supplemental Figures 3 and 4.

**SUPPLEMENTAL DATA**

**Supplemental Figure 1.** Sequence information of Rab5a in rice.

**Supplemental Figure 2.** Possible binding domains of RBP-P with NSF revealed by yeast two hybrid (Y2H) analysis.

**Supplemental Figure 3.** Protein sequence alignment of NSF.

**Supplemental Figure 4.** Protein sequence alignment of Rab5 isoforms.

**Supplemental Figure 5.** Expression profile of Rab5 isoforms in rice plants.

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**AUTHOR CONTRIBUTIONS**

L.T. designed the study; K.A.D. identified NSF as interacting partner of RBP-P through IP-MS; L.T. discovered interaction of RBPs and NSF with Rab5a and conducted BiFC, RNA-IP, yeast two hybrid, light microscopy and TEM analyses; L.T. and L.Z. conducted co-IP analysis; M.F. conducted immunofluorescence microscopy; L.T. constructed vectors; L.T. and H.W. conducted *in situ* RT-PCR; T.K. provided *rab5a* mutant; T.W.O. supervised the project; L.T. and T.W.O. wrote the manuscript.
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FIGURE LEGENDS

Figure 1. Identification of NSF as an interacting partner of RBP-P. (A) Precipitation of NSF by RBP-P antibody as revealed by IP-MS. Left panel, immunoblot (IB) analysis to test the IP reliability; right panel, silver stained SDS-polyacrylamide gel of eluted samples from ($\alpha$)-GFP and RBP-P IPs. Input, starting material of rice lysate; Ub, unbound fraction from IPs; B, bound fraction (eluted samples) from IPs. Blue asterisk (*) indicates a modified form of RBP-P. The bands indicated by red and black arrows were excised for MS analysis and NSF was identified as a specific protein precipitated by anti-RBP-P but not by anti-GFP. (B) Interaction between RBP-P and NSF revealed by yeast two hybrid. Yeast colonies co-transfected with pGBK and pGAD constructs were labeled 1-4 as described in the upper Table. --, empty vector. Yeast cells carrying the corresponding genes were grown on SD/-Leu/-Trp medium as growth control and SD/-Leu/-Trp/-His/-Ade/+ 3-AT selection medium to detect their interaction. Note that only the yeast cells carrying both NSF and RBP-P survived on the strict selection medium (lower panel), suggesting that RBP-P interacts with NSF.

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(A) Schematic representation of the Rab5a mutation site in the rab5a mutant. A G134A base substitution within the Rab5a gene resulted in a G45D amino acid replacement. (B) Formation of PMBs (white asterisks) was observed in endosperm cells of rab5a mutant through light microscopy observations on seed sections stained with 1% Toluidine Blue. Scale bar, 25 μm. (C) Ultrastructure of PMBs formed in rab5a mutant due to aborted endosomal trafficking in comparison to wildtype (WT) endosperm cells. Cell wall and PMB boundaries are indicated by magenta and green dashed lines, respectively. SG, starch granules; orange *, protein body I; blue *, protein storage vacuoles. Scale bar, 1 μm.
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