

Elucidating the Mechanism of Organelle Trafficking in *Arabidopsis thaliana*:
the MYA2 globular tail interacts with AtRabC2a.

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ABSTRACT

Class XI myosins are responsible for mediating cytoplasmic streaming in plant cells. Although it has been demonstrated that the C-terminal globular tail domain of myosin XI binds to a variety of organelles, the mechanism for organelle recognition remains poorly understood. Interestingly, it has recently been revealed that RabGTPases are responsible for docking vesicles on to the tail domain of the related class V myosin in yeast by direct physical interaction. To gain a better understanding of the organelle recognition process in *Arabidopsis*, this research examined an isoform of *Arabidopsis* RabGTPases, AtRabC2a, as a putative key regulator of tail-cargo interaction. To test this hypothesis, Glutathione-S-Transferase (GST) pull-down assays have been performed to investigate whether the tail domain of MYA2, an isoform of myosin XI, directly interacts with AtRabC2a. This research also aims to identify a potential Rab GTPase recognition signal on myosin XI. Specifically, the conserved site of Phe-Leu-Leu-Asp in the tail domains of myosin XI isoforms was proposed to be a Rab GTPase recognition signal. To test the role of this FLLD motif (aa.1552-1555) in MYA2, Leu1553 and Leu1554 were mutated to alanine, and GST pull-down assay between the mutated tail domain and AtRabC2a has been performed. Interestingly, point mutations of two leucine led to severe degradation of the protein, suggesting that this sequence is involved in maintaining stability of the MYA2 tail, rather than regulating interaction with Rab proteins.

INTRODUCTION

Cytoplasmic streaming is believed to serve many important physiological functions. Rapid and constant movement of organelles results in evenly distributed small metabolites that are necessary for cell growth (Shimmen and Yokota, 1994). Movement of organelles and vesicles also leads to hydrodynamic flow that facilitates diffusion of soluble materials (Esseling-Ozdoba, Houtman et al., 2008). It has been demonstrated that cytoplasmic streaming is responsible for intracellular transport of molecules and cell organelles in long cells such as pollen tubes. It also delivers organelles to specific positions in polarized cells. For example, vesicles are transported to the tip of pollen tubes and root hairs after moving through the length of the cell (Cole and Fowler, 2006). It has also been shown that a single myosin gene knockout results in reduced growth of root hairs (Peremyslov, Prokhnevsky et al., 2008).

It has been suggested that rapid movement of organelles in plant cells is mediated by cytoskeletal actin filaments and plant myosins, which are believed to bind to the surface of organelles. Myosins are molecular motor proteins that move along actin filaments by ATP hydrolysis. Myosins are categorized into 24 different sub-families based on the sequence similarity of their conserved N-terminus motor domain. Only class VIII and XI are identified in plant cells, and *Arabidopsis* expresses 13 class XI isoforms and four class VIII isoforms (Foth, Goedecke et al., 2006).

So far, many studies have suggested that myosin XI mediates organelle transport in *Arabidopsis*. Immunostaining studies showed that the globular tail domain of MYA2, an isoform of myosin XI, is co-localized with peroxisomes and unidentified vesicles in *Arabidopsis* (Hashimoto, Igarashi et al., 2005). Also, inactivation of *MYA2* and *XIK* genes in *Arabidopsis*

reduced the movements of Golgi bodies, peroxisomes, and mitochondria and also led to a defect in root hair elongation (Peremyslov, Prokhnevsky et al., 2008). Moreover, recent studies revealed that transiently expression of the tail domains of myosin XI isoforms, including MYA1, MYA2, XIC, XIE, XI-I and XIK, arrests the movement of Golgi apparatus in *Arabidopsis* (Avisar, Abu-Abied et al., 2011). Many studies have also shown that myosin XI is co-localized with organelles such as peroxisome (Li and Nebenführ, 2007), (Hashimoto, Igarashi et al., 2005), Golgi bodies and mitochondria (Avisar, Prokhnevsky et al., 2008), (Reisen and Hanson, 2007). These studies strongly suggest that the globular tail domain of myosin XI interacts with organelles and is responsible for organelle trafficking in *Arabidopsis*.

The domain structure of myosin class XI is well adapted for transporting organelles. All isoforms of myosin XI have a catalytic motor domain at the N-terminus that hydrolyzes ATP and binds to the actin filament. The C-terminal end has a globular tail domain that interacts with cargoes. The tail domain shows diverse structures across all myosin classes, suggesting that it is responsible for determining the physiological role of each class of myosin in cells (Hashimoto, Igarashi et al., 2005). Between the globular tail domain and motor domain, there are six contiguous IQ motifs that are occupied by calmodulin. Binding of calcium to calmodulin appears to cause a rearrangement of the calmodulin and modulate the function of the motor domain. The coiled-coiled domain connects the IQ motif to the globular tail domain. The coiled-coiled region dimerizes two myosin molecules by tightly holding them together (Shimmen T., 2011). Interestingly, it has been suggested that organelle targeting of the tail region enhances dimerization of the coiled-coiled domain, and this dimerization of the coiled-coiled domain also

leads to stabilization of organelle binding of the tail. Therefore, it appears that the coiled-coiled domain and the tail domain are interdependent on each other (Li and Nebenführ, 2008).

The tail domains of myosin XI isoforms have two sub-domains, GT1 and GT2, and each sub-domain can target organelles independently. It has been proposed that the globular tail domain of myosin XI can take different conformations depending on the association of GT1 and GT2, and this conformation change may regulate selection of proper organelles (Li and Nebenführ, 2007). However, the exact mechanism of targeting organelles by myosin XI is not understood. In animals and yeast, it is known that adapter protein called small RabGTPases can recruit proper cargoes to myosin class V tail domains, suggesting that there may also exist similar adaptor proteins that regulate binding of the myosin XI tail to its cargoes. It has previously been demonstrated by yeast two-hybrid that Rab proteins of *Arabidopsis*, specifically AtRabC2a and AtRabD1, weakly interact with the MYA2 globular tail domain, and the pull-down assay experiments suggested that this interaction is mediated in a GTP dependent manner. Moreover, it was also shown that CFP tagged AtRabC2a co-localizes with peroxisomes, suggesting that AtRabC2a may serve as an adapter protein for myosin XI and direct proper cargoes to myosin XI tails (Hashimoto, Igarashi et al., 2008). However, whether this interaction occurs *in situ* is unclear.

The Myosin Vb globular tail interacts with Rab11, Rab11b, and Rab25 on two sites. The first motif is located on its N-terminal end and has an amino acid sequence of LEKNE. The other motif is LLLD, and it is identified in its C-terminus. In myosin XI isoforms, however, the LEKNE sequence is not found. Meanwhile, FLLD, a homolog of LLLD, is located in the C-terminus of all 55 myosin XI isoforms (Shimmen T., 2011). Therefore, it is reasonable to assume

that the myosin XI tail interacts with Rab proteins through this conserved FLLD sequence in its C-terminus, and this interaction may play an important role in selecting proper cargoes for the myosin XI tail.

In the present study, we aimed to provide more concrete evidence that AtRabC2a directly interacts with the MYA2 globular tail domain. We studied whether active and inactive AtRabC2a directly bind to the MYA2 globular tail using GST pull-down assay. Also, we tested whether the conserved FLLD motif (aa.1552-1555) on the C-terminus affects the ability of the MYA2 tail to interact with AtRabC2a by introducing point mutations.

MATERIALS AND METHODS

I. Construction of a plasmid expressing the wild type MYA2 coiled-coiled and globular tail domains fused with 6xHis tag.

pEST11, which is the DNA sequence of the wild type MYA2 coiled-coiled and globular tail domains, was first amplified by PCR with forward primer MYA2-CCF1 and reverse primer M1102RStop. The amplified PCR product was then cloned into the *Bam*HI/*Not*I restriction sites of pET30a(+) vector that encodes 6xHis tag. The recombinant plasmids were transformed into *E. coli* TOP10 cells and the sequences of the plasmids were confirmed by DNA sequencing. The plasmid with the correct sequence was then transformed into *E. coli* BL21(DE3) pLysS cells for protein expression.

MYA2-CCF1: CGAGGATCCGCTCGTGGAGAACTTCGAAAG

M1102RStop: GCAGCGGCCGCAACAATCACAGAGGAAGAGAGC

II. PCR site-directed mutagenesis

To test whether the FLLD motif on the C-terminus end of MYA2 tail regulates the binding of MYA2 tail to AtRabC2a proteins, point mutations were introduced to the FLLD (aa. 1552-1555) motif to yield either AAAA or FAAD using PCR based-mutagenesis. Using the DNA sequence of the wild-type MYA2 coiled-coiled globular tail domain as the template, three PCR reactions were performed. The first PCR reaction used MYA2-AAAA-Rev and MYA2CCF1 primers and the second PCR reaction used MYA2-AAAA-For and pET30R primers. The sequences of the primers are shown below. The two overlapping products were used as templates for the third PCR reaction that used M1102Rstop and MYA2-CCF1 primers to create the full-length DNA sequence of the MYA2 coiled-coiled globular tail domain with the AAAA mutation. The sequence containing the FAAD mutation was constructed using the same procedure, except that MYA2-FAAD-For primer was used instead of MYA2-AAAA-For, and MYA2-FAAD-Rev was used instead of MYA2-AAAA-Rev. The PCR products were introduced into pET30a(+) vector that encodes 6xHis tag and then cloned into *E.coli* Top10 cells for sequencing. The plasmids with desired mutations were transformed into *E. coli* BL21(DE3) pLysS cells for protein expression.

PET30R: TTAATGCGCCGCTACAGGG

MYA2-AAAA-For: TTCCGCCGCGGCGGCTGATGATTCCAGCATTTCCTTT

MYA2-AAAA-Rev:CATCAGCCGCGGCGGCGGAATCACTGTCTGCATCAT

MYA2-FAAD-For: TTCCTTCGCAGCAGATGATGATTCCAGCATTTCCTTT

MYA2-FAAD-Rev:CATCATCTGCTGCGAAGGAATCGCTGTCTCCATCAT

III. Isolation of the recombinant proteins

-Wild-type MYA2 tail and the mutant MYA2 tail with the FAAD mutation

BL21(DE3) pLysS cells carrying the plasmids that encodes the MYA2 tail proteins fused with 6xHis tags were shaken vigorously in 300 ml of 2YT medium[1% trypsin, 1% yeast extract, and 5% NaCl] at 37°C and 250 rpm for six hours until the OD600 reached 0.6. Protein synthesis was induced by adding 0.4 mM isopropyl β -D-thiogalactoside (IPTG), and the cultures were incubated for additional 20 hours at 25°C. After incubation, the bacteria were pelleted by centrifugation at 4°C, 12,000rpm for 15 minutes. The pellet was resuspended in lysis buffer [100 mM NaCl, 10 mM imidazole, 50 mM NaH₂ PO₄, 20 % Glycerol, and 5 mM MgCl₂]. The solution was sonicated for 5 minutes, and then incubated with 1 % trypton for 20 minutes at 4°C. Cell debris was pelleted under the same condition used before, and the lysate, or supernatant, was collected to be used for Ni-NTA purification.

-Mutant MYA2 tail with the AAAA mutation

BL21(DE3) pLysS cells with plasmid encoding the AAAA mutation were shaken vigorously in 2YT medium at 37°C, 250 rpm for three hours until OD600 reached 0.6. After incubation, protein synthesis was initiated by adding 0.2 mM of IPTG, and the culture was incubated either under 16°C for 20 hours or 37°C for three hours. After induction, the pellet was collected and lysed against the homogenization buffer [10 % phosphate-buffered saline (PBS), 10% glycerol, 1 Roche Protease Inhibitors Cocktail tablet/10 ml of the buffer, protease inhibitor mixture, 5 mM dichlorodiphenyltrichloroethane (DDT), 5 mM Ethylenediaminetetraacetic acid (EDTA), and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. The solution was sonicated for 5 minutes, and incubated with 1 % trypton for 1 hour. Cell debris was pelleted by centrifugation at

4°C, 12,000rpm for 15 minutes, and the lysate was collected. Solubility of the target proteins was checked by subjecting the lysate to SDS-PAGE followed by western blot analysis with anti-6xHis antibodies.

-Activated AtRabC2a (RabQL) and inactivated AtRabC2a (RabSN)

BL21(DE3) pLysS cells with plasmid expressing AtRabC2a fused with the GST-tag were shaken vigorously in 2YT medium at 37°C, 250 rpm for three hours until the OD600 reached 0.6. After three hours, expression of the target protein was induced with 0.4 mM IPTG at 20°C for 20 hours. The bacterial cells were pelleted and were lysed in homogenization buffer [10 % PBS, 10 % glycerol, 1 Roche Protease Inhibitor Cocktail tablet/10 ml of the buffer, 1:4,000 protease inhibitors, 5 mM DDT, 5 mM EDTA, and 1 mM PMSF]. Cell debris was pelleted by centrifugation at 4°C, 12,000rpm for 15 minutes, and the lysate was collected and stored in -80°C to be used for GST pull-down assay. Solubility of the target proteins was checked by subjecting the sample to SDS-PAGE followed by immunoblot with anti-GST antibodies.

-Expression of the GST-tag

The GST-tag was also expressed to be used as a negative control. pGEX-T4-1, the vector that expresses the GST-tag, was transformed into *E.coli* BL21(DE3) pLysS cells. The cells were incubated in 2YT medium on an agitation table at 37°C and 250 rpm for three hours, followed by additional three hours of protein synthesis induced by 0.2 mM IPTG. The bacteria cells were pelleted and dialyzed against homogenization buffer [1xPBS, 10 % glycerol, 1 Roche Protease Inhibitor Cocktail tablet/10 ml of the buffer, 1:4000 protease inhibitors, 5 mM DDT, 5 mM EDTA, and 1 mM PMSF]. Cell debris was pelleted by centrifugation under the same condition as above, and the lysate was collected and stored in -80°C to be used for GST pull-down assay.

IV. Purification of MYA2 proteins

The lysates of the wild type MYA2 proteins and the mutant MYA2 proteins were purified through Ni-NTA columns and concentrated using Amicon Ultra-2mL 10K Membrane kit. 600 μ l of Ni-NTA resin (Quagen) was added to each column and washed with washing buffer [300 mM imidazole, 50 mM NaH_2PO_4 , 20 % Glycerol, and 5 mM MgCl_2]. The lysates were added to the columns and incubated for 1.5 hour at 4°C. After incubation, the bead-protein complexes were washed for four times using the same washing buffer used above. The final product was eluted using 500 μ l of elution buffer [500 mM imidazole, 50 mM NaH_2PO_4 , 20 % Glycerol, and 5 mM MgCl_2]. Concentration of the proteins was done by centrifuging the eluted products in the concentration filters (Amicon Ultra-2mL 10K Membrane kit) at 6,500 x g and 4°C for 40 minutes. The concentrated final products were stored in -80°C until use.

V. GST Pull Down assay

The prepared lysates of the GST-tagged proteins and purified MYA2 proteins were used in the following pairs for the pull down assays:

Wild type MYA2+ AtRabC2aSN (GDP-bound active)
 Wild type MYA2 + AtRabC2aQL (GTP-bound inactive)
 Wild type MYA2 + GST-tag
 MYA2 with FAAD mutation + AtRabC2aSN
 MYA2 with FAAD mutation + AtRabC2aQL
 MYA2 with FAAD mutation + GST-tag

For binding assays, GST tagged proteins [350 μ l of AtRabC2aQL, 400 μ l of AtRabC2aSN and 35 μ l of the GST-tag] were incubated with 10 μ l of GST bind resin (Novagen) at 4°C for 1 hour. After 1 hour, the beads with GST-tagged proteins were washed twice using washing buffer [10 % PBS, 1 mM PMSF, and 0.1 % triton]. After washings, 1 mM of GDP was added to the beads that

were incubated with AtRabC2aSN, and 1mM of GTP was added to the beads that were incubated with AtRabC2aQL. 2 µl of purified wild type MYA2 and 2 µl of the mutant with the FAAD mutation were added to the beads and incubated for additional 1.5 hour at 4°C. After incubation, the beads were washed with the elution buffer used for purification, supplemented with 1 mM DDT and 1 mM GTP or GDP. Supernatant was carefully removed and only the beads were subjected to SDS-PAGE with 10% polyacrylamide gels. The gels were transferred to PVDF membranes. The membranes were incubated in 6 % milk solution [6 % milk powder and 10 % 20X Tris Buffered Saline (TBS)] for overnight. For detection, one membrane was incubated in 1 % milk solution [1 % milk powder and 10 % 80X Tris Buffered Saline (TBS)] with 1:15,000 of anti-his antibody (Thermo Scientific His Probe-horseradish peroxidase(HRP)) for 30 minutes. After 30 minutes, the signals detected using chemiluminescent substrate. The other membrane was incubated in 1 % milk solution with 1:15,000 of primary anti-GST antibody for 1.5 hour. After incubation, the membrane was washed with 1 % milk solution for 5 times, and 1:15,000 of IgG anti-mouse secondary antibody was added and incubated for additional 1 hour. After 1 hour, chemiluminescent substrate was used for detection.

RESULTS

The FLLD (aa.1552-1555) motif of the MYA2 tail domain is necessary for stability of the protein

To test the function of the FLLD motif (aa.1552-1555) of the MYA2 globular tail domain, we first introduced quadruple alanine mutations. Despite multiple attempts to induce expression of the mutant protein under various conditions, we failed to obtain soluble mutant protein.

Solubility tests of the quadruple alanine mutant showed that the protein either form inclusion bodies or is not expressed at all, indicating that the FLLD motif may plays an important role in folding and maintaining stability of the MYA2 tail (Fig. 1).

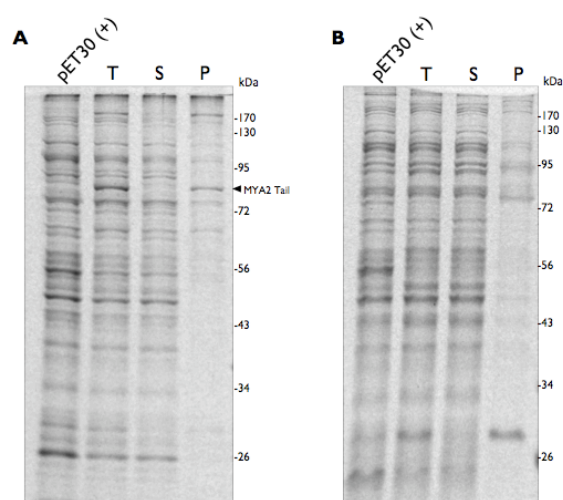


Figure 1. Solubility tests of the mutant with the AAAA mutation. The lysate of the empty pET30(+) vector, total lysate of the mutant proteins before centrifugation (T), supernatant after centrifugation (S), and cell pellets (P) were subjected to SDS-PAGE using 10 % gels. The gels were stained with Coomassie brilliant blue (CBB). (A) The mutant protein synthesis was induced at 37 °C for three hours. The mutant MYA2 tail protein is indicated with an arrow. (B) The mutant protein synthesis was induced at 16 °C for 20 hours. The mutant MYA2 proteins were not expressed.

To further investigate the role of the FLLD motif, Leu1553 and Leu1554 were mutated into alanine. The expression level of the mutant with double alanine mutation was significantly lower than that achieved by the wild type MYA2 tail (Fig. 2). Although it was possible to express the mutant protein, synthesis of the mutant protein was much slower than that of the wild type, and it was also much more susceptible to degradation than the wild-type even after purification (Fig. 3). These results again strongly indicate that the FLLD motif is necessary for maintaining stability of the MYA2 tail.

The Wild Type MYA2 tail interacts with AtRabC2a as well as the GST-tag

To examine whether the wild type MYA2 tail interacts with AtRabC2a proteins, pull-down assays were performed with GST-tagged AtRabC2a proteins and purified 6xHis-tagged MYA2 tail proteins. AtRabC2a proteins fused with GST were bound to GST bind beads (Novagen), and this bead-Rab protein complex was used for the pull down assay with the MYA2 proteins. Beads incubated with the GST-tag alone were used as a negative control.

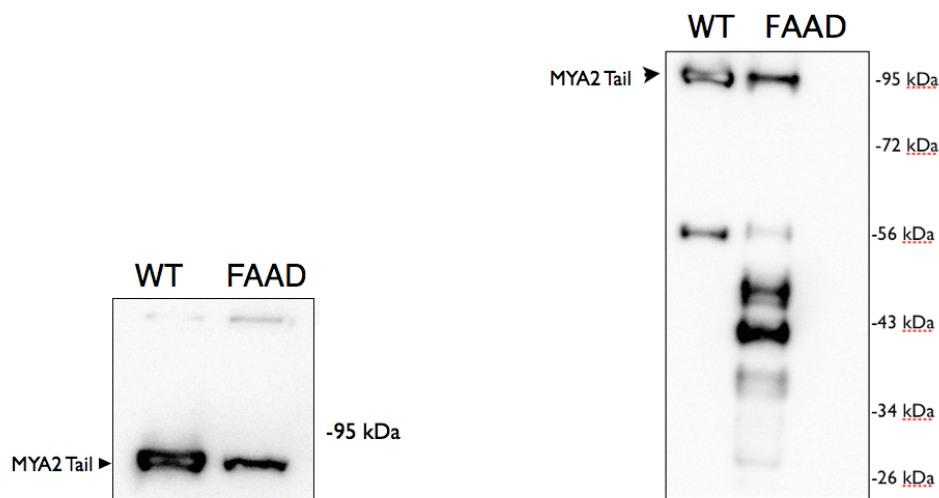


Figure 2. Expression level of the wild type MYA2 tail and the mutant MYA2 tail with the FAAD mutation. The wild type (WT) was expressed at 37 °C for 3 hours and the mutant was expressed at 25 °C for 20 hours. The same amount of each lysate was subjected to SDS-PAGE using a 10% polyacrylamide gel, and then transferred to a PVDF membrane. The signals were detected using anti 6xHis antibody and chemiluminescent substrate.

Figure 3. Western blot of the purified wild type and mutant MYA2 with double alanine mutations. Purified wild type MYA2 proteins (WT) and mutant MYA2 proteins with FAAD mutation (FAAD) were analyzed using western blot with anti 6xHis antibody. The mutant protein shows much more severe degradation than the wild type.

The GST pull-down assay result showed that the wild type MYA2 tail interacts with both active and inactive AtRabC2a (Fig. 4B). Interestingly, the amount of MYA2 tail proteins recovered with beads coated with GTP-bound activated AtRabC2a (RabQL) was greater than the amount recovered from the beads coated with GDP-bound inactive AtRabC2a (RabSN). This indicates that the binding with activate AtRabC2a was stronger than the binding with inactive AtRabC2a. However, nonspecific binding of MYA2 tail to the GST-tag was also strong, which indicates that the binding between MYA2 tail and the Rab proteins could be due to the nonspecific interaction between MYA2 tail and GST tag.

To reduce nonspecific binding with the GST-tag, the pull-down experiment was repeated using 25 µl of the GST-tag protein instead of 35 µl and 0.5 µl of the wild-type MYA2 proteins. Although only a small amount of the wild type MYA2 protein was used, we still detected

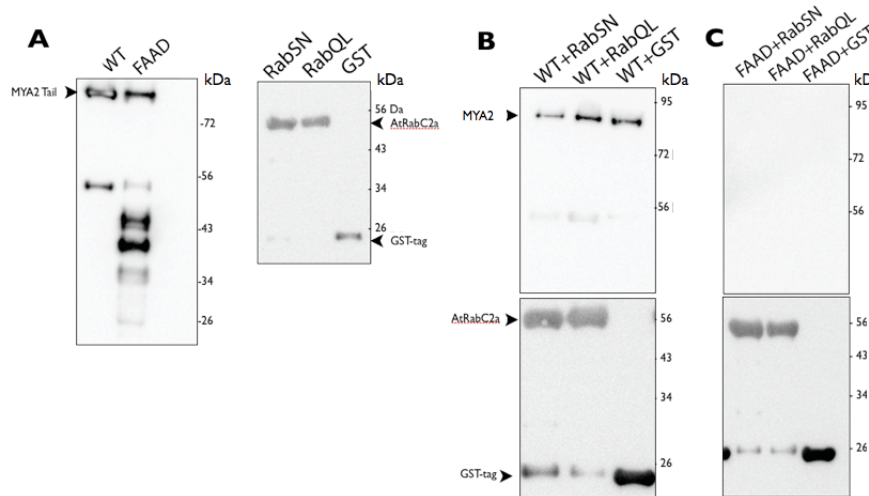


Figure 4. Western blot analysis of the GST pull-down assays of the 6xHis-tagged MYA2 tail proteins with the GST-tagged Rab proteins. (A) The input levels of the wild type MYA2 proteins (WT), the mutant proteins with FAAD mutation (FAAD), activate Rab proteins (RabQL), inactive Rab proteins (RabSN), and the GST-tag (GST). **(B)** The pull-down assays of the wild type MYA2 tail proteins with the Rab proteins. **(C)** The pull-down assay with the mutant MYA2 proteins with the Rab proteins. The pull-down with only the GST-tag was used as a negative control.

interaction of MYA2 with both RabSN and RabQL. Also, we were able to eliminate nonspecific interaction with the GST-tag (Fig. 5B). Therefore, it is convincing to conclude that MYA2 tail directly interacts with AtRabC2a proteins, and the magnitude of interaction depends on the activation state of the Rab protein.

Mutant MYA2 with the FAAD mutation does not interact with AtRabC2a proteins or the GST-tag.

To elucidate the role of the FLLD motif (aa.1552-1555) in mediating the interaction with the Rab proteins, Leu1553 and Leu1554 were mutated into alanine using PCR mutagenesis. The mutant proteins were tagged with 6xHis, and AtRabC2a proteins were tagged with GST. After expression in *E.coli*, the mutant MYA2 proteins were purified using Ni-NTA columns and incubated with AtRabC2a covered GST beads. The beads were sedimented, and the amount of the mutant MYA2 proteins bound to the beads was measured using SDS-PAGE and western blot.

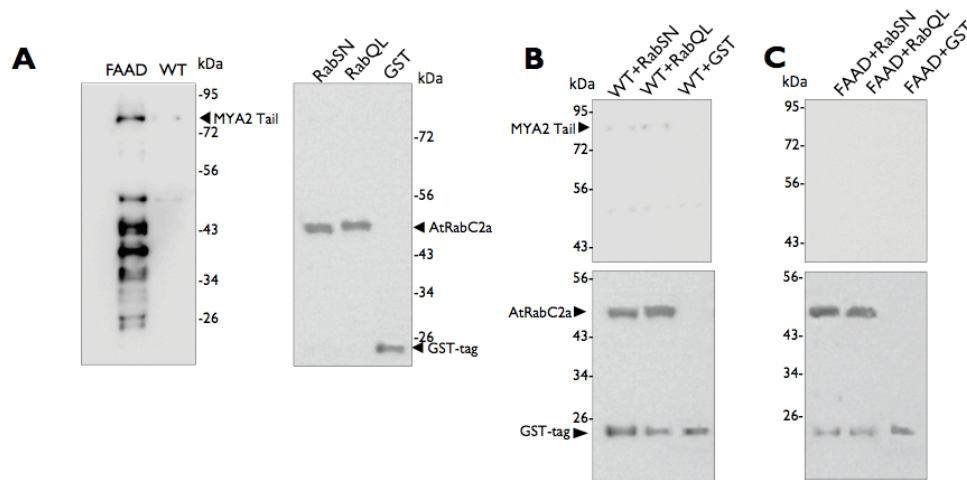


Figure 5. Western blot analysis of the GST pull-down assays of the 6xHis-tagged MYA2 tail proteins with the GST-tagged Rab proteins using a smaller amount of the GST-tag and the wild type MYA2 proteins. (A) The input levels of the wild type MYA2 proteins (WT), the mutant proteins with FAAD mutation (FAAD), activate Rab proteins (RabQL), inactive Rab proteins (RabSN), and the GST-tag (GST) (B) The pull-down assays of the wild type MYA2 tail proteins with the Rab proteins. (C) The pull-down assay with the mutant MYA2 proteins with the Rab proteins. The pull-down with only the GST-tag was used as a negative control.

Point mutations of the FLLD motif of the tail domain seems to abolish its ability to interact with AtRabC2a proteins as well as with the GST-tag (Fig. 4C). Intriguingly, changing Leu1553 and Leu1554 to alanine seems to disrupt the stability of the MYA2 tail by making it more vulnerable to proteolysis (Fig. 2). It was also noted that expressing the mutant MYA2 was much more difficult and slower than expressing the wild type. These results suggest that the abolished ability to interact with AtRabC2a proteins could be due to the reduced stability of the mutant MYA2, rather than due to the disruption of a specific protein-protein interaction.

To confirm lack of interacting ability of the mutant with AtRabC2a proteins, the pull-down experiment was repeated using a greater amount of the mutant MYA2 protein and a smaller amount of the wild type protein. For the pull-down experiment, we applied 2.5 μ l of the mutant instead of 2 μ l used previously, and 0.5 μ l of the wild type instead of 2 μ l. The result was consistent with the previous pull-down assay result (Fig. 5C). We observed a significant degradation of the mutant, and we still did not detect interaction of the mutant with the Rab

proteins or the GST-tag. Therefore, we postulate that the tetrapeptide sequence FLLD plays an important role in stabilizing the protein, rather than mediating interaction with the Rab proteins.

DISCUSSION

Rab GTPases, which belong to the Ras superfamily of small GTPases, have been suggested to mediate organelle targeting of myosin proteins (Hashimoto, Igarashi et al., 2008). Like other regulatory GTPases, the Rab proteins switch between two distinct conformations, GTP-bound and GDP-bound. Conversion from the GDP- to the GTP-bound form by GDP/GTP exchange factor (GEF) activates the protein. Once activated, the protein interacts with Rab effector proteins that are thought to stabilize the active Rab protein and also direct the Rab protein to specific organelles (Pfeffer, 2001). In mammals and yeasts, it has been demonstrated that RabGTPase proteins serve as an adapter for myosin proteins. For instance, yeast myosin, Myo2p (Itoh, Watabe et al., 2002), mammalian myosin Va (Storm, Hume et al., 2002), myosin Vb (Lapierre, Kumar et al., 2001) have been identified to interact with the Rab proteins. The interaction with the Rab proteins is believed to mediate the binding of myosin XI to organelles or cargoes. In *Arabidopsis*, it has been shown that AtRabC2a and AtRabD1 interact with MYA2 globular tail in a GTP-dependent manner using pull-down assay experiments (Hashimoto, Igarashi et al., 2008).

In the present study, we suggest that the MYA2 tail domain directly interacts with AtRabC2a using GST pull-down assay. We also identified a significant difference in the strength of interaction between activate and inactivate states of the Rab proteins, further strengthening the argument that the Rab proteins could function as adapter proteins for MYA2 motors. We also

propose that the FLLD motif (aa. 1553-1555) of the MYA2 tail is necessary for maintaining the stability of the protein.

We examined the interaction of *Arabidopsis* MYA2 tail protein with AtRabC2a by using GST pull-down assay. We were able to detect a significant interaction between the MYA2 tail and the Rab proteins (Fig. 4). However, the nonspecific binding of the MYA2 tail to the GST-tag was also strong, suggesting a possibility that the observed interaction could not be that of MYA2 tail with AtRabC2a. Interestingly, our results also showed that the interaction with activate AtRabC2a was much greater than that with inactivate AtRabC2a (Fig. 4B). The difference in the interaction magnitude suggests that the observed results from the pull-down assays are likely to show a genuine interaction of the MYA2 tail with AtRabC2a. Moreover, when the pull-down experiment was repeated using a smaller amount of the GST-tag for a negative control, we were able to eliminate nonspecific interaction while still observing the association of the MYA2 tail with the Rab proteins (Fig. 5B). Therefore, it is more convincing to conclude that the MYA2 tail directly interacts with AtRabC2a.

We also present a new speculation about the function of the FLLD motif (aa. 1553-1555), which has been proposed as a putative key site for mediating the interaction with Rab proteins (Hashimoto, Igarashi et al. 2008). It has been previously proposed that both N- and C-terminal regions of the MYA2 globular tail domain are necessary for the interaction with the Rab proteins (Shimmen T., 2011). Deletion of either C-terminal or N-terminal amino acids of the tail domain led to failure in interacting with Rab11a proteins, suggesting that both carboxyl and amino ends are needed for the interaction. Two sites on the myosin Vb tail domain were suggested to be responsible for the interaction with the Rab proteins. One site is the LEKNE motif in the N-

terminal end, and the other is the LLLD motif in the C-terminal end (Lapierre, Kumar et al., 2001). In the MYA2 tail domain, the FLLD motif corresponding to the LLLD motif is found within 55 amino acids in the C-terminal end. Interestingly, this motif appears to be conserved in other isoforms of myosin XI in *Arabidopsis* as well as tobacco, with exceptions of myosin XIG and XIJ (Shimmen T., 2011). Because the FLLD motif is located on the C-terminal end of the myosin XI tail and is found in both myosin Vb and other isoforms of myosin XI, it has been postulated that this motif is responsible for selecting proper cargoes or possibly regulating the interaction with Rab proteins.

Contrary to the assumption, however, our study suggests that this motif is more likely to be involved in stabilizing the MYA2 tail domain. When we introduced the quadruple alanine mutation, we failed to express the soluble form of the mutant. The mutant appears to form inclusion bodies or not be expressed at all (Fig. 1). When Leu1553 and Leu1554 were mutated into alanines, we observed a significant degradation of the protein even after purification. (Fig. 3) Although we observed no interaction between the alanine mutants and the Rab proteins, we suspect that the loss of interaction is due to the reduced stability of the mutant proteins.

We postulate that this FLLD motif plays a key role in stabilizing the tail domain by maintaining the interaction between GT1 and GT2 sub-domains. It has been previously proposed that the unstructured region stretching after H13 could be involved in maintaining the proper interaction between GT1 and GT2 (Li and Nebenführ, 2007). Surprisingly, the FLLD motif is located along the unstructured region of GT2 that extends from the C-terminal end of helix H13 (Fig. 6). Based on the structure model, we suggest that the tetrapeptide FLLD is an interacting

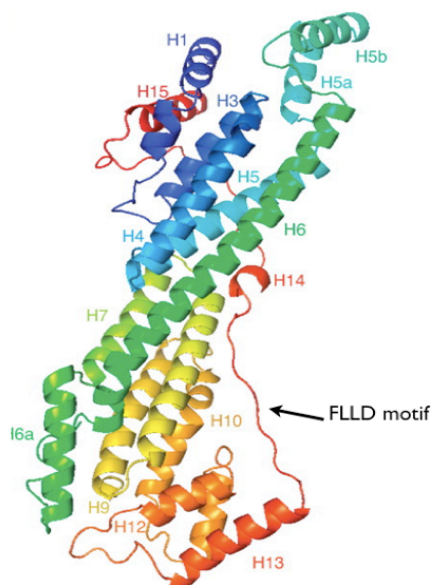


Figure 6. Structure model of the myosin XI-I globular tail (MYA1) (Li and Nebenführ, 2007). Alpha helices are numbered from H1 to H15. the FLLD motif is located on the unstructured region that stretched from the C-terminal end of the helix H13. We speculate that this motif is an interacting partner of the neighboring helix, H8. This sequence-helix interaction could contribute to stabilizing the tail domain by maintaining the proper association of the two sub-domains.

partner of neighboring helix H8, and that this interaction may stabilize the association of GT1 and GT2 sub-domains. Thus, we predict that the tetrapeptide FLLD sequence as an interacting partner of helix H8, and speculate that this sequence-helix interaction plays a crucial role in maintaining proper association of the two sub-domains.

To further characterize the property of the

FLLD motif in maintaining stability of the MYA2 tail, the cleavage sites on the double alanine mutant were marked on the structure model of the MYA1 tail domain (Fig. 7). In this model the GT1 sub-domain

ranges from the helix H1 to helix H6, and the GT2 sub-domain ranges from the helix H6a to helix H15. The size of each dominant degradation product was measured based on the protein marker on the gel, and then normalized based on the predicted size of the protein. The cleavage sites were estimated based on the homology model of the MYA1 coiled-coiled globular tail domain created by Li and Nebenführ (2007). Interestingly, one of the sites were located on the helix H5a, which is near the N-terminal end of GT1 sub-domain. Considering that the cleavage sites were marked based on approximation, it is possible to suggest that the mutation of the FLLD motif leads to dissociation of the two sub-domains. The other cleavage site was found on the helix H3, which is thought to be involved in stabilizing the interaction of the two sub-

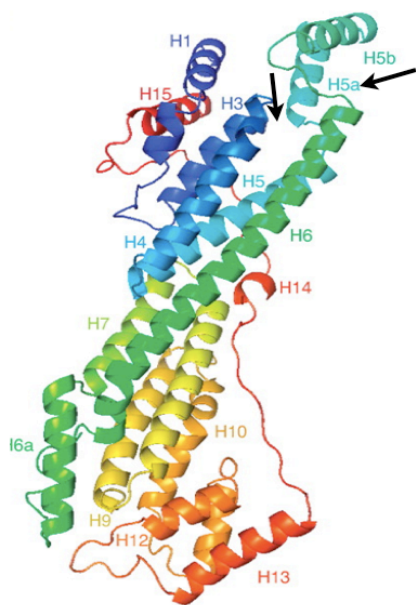


Figure 7. Predicted cleavage sites of the mutant MYA2 proteins with double alanine mutations. The predicted sites are marks on the structure model of the myosin XI-I globular tail (MYA1) (Li and Nebenführ, 2007). Cleavage sites, marked with black arrows, were predicted based on the degradation pattern shown in Figure 3. One site was predicted to be in the N-terminal end of the helix H5a, and the other site is in the helix H3.

domains. Previous research by Li and Nebenführ has proposed that Tyr 1130 and Arg 1137 on the helix H3 are the interacting partners of Phe 1154 of helix H15 by showing that the mutations of these residues disrupted the interaction of the two sub-domains, and also that these residues are conserved in all *Arabidopsis* myosin XI isoforms (Li and Nebenführ, 2007). We suspect that the cleavage on the helix H3 is resulted from the disrupted sub-domain interaction. The loss of the sub-domain interaction could expose Tyr 1130 and Arg 1137, which are involved in the inter-helical interaction,

and cause the helix to be more susceptible to proteolysis.

CONCLUSIONS

In this research, we used GST pull-down assay to demonstrate that the MYA2 globular tail domain interacts with AtRabC2a proteins. We also studied the function of the FLLD motif in the C-terminal end of the tail domain by introducing point mutations to this tetrapeptide sequence. The data suggest that the MYA2 tail directly interacts with AtRabC2a, and this interaction is dependent upon the activation state of the Rab proteins. The FLLD motif (aa. 1552-1555) appears to be involved in stabilizing the tail domain. From the structure model, we speculate that the FLLD motif, which is located on the unstructured region that extends from the C-terminal

end of the helix H13, is an interacting partner of the helix H8. We also suggest that this interaction mediates proper association of the two sub-domains, thereby stabilizing the entire tail domain. Further studies should be done to verify the role of the FLLD motif in detail, and also to investigate whether the interaction of the MYA2 globular tail with the Rab proteins also occur *in situ*.

REFERENCES

- Avisar, D., M. Abu-Abied, et al. (2011). "Myosin XIX is a major player in cytoplasm dynamics and is regulated by two amino acids in its tail." *J Exp Bot* **63**(1): 241-249.
- Avisar, D., A. I. Prokhnovsky, et al. (2008). "Myosin XI-K Is required for rapid trafficking of Golgi stacks, peroxisomes, and mitochondria in leaf cells of *Nicotiana benthamiana*." *Plant Physiol* **146**(3): 1098-1108.
- Cole, R. A. and J. E. Fowler (2006). "Polarized growth: maintaining focus on the tip." *Curr Opin Plant Biol* **9**(6): 579-588.
- Esseling-Ozdoba, A., D. Houtman, et al. (2008). "Hydrodynamic flow in the cytoplasm of plant cells." *J Microsc* **231**(2): 274-283.
- Foth, B. J., M. C. Goedecke, et al. (2006). "New insights into myosin evolution and classification." *Proc Natl Acad Sci U S A* **103**(10): 3681-3686.
- Hashimoto, K., H. Igarashi, et al. (2005). "Peroxisomal localization of a myosin XI isoform in *Arabidopsis thaliana*." *Plant Cell Physiol* **46**(5): 782-789.
- Hashimoto, K., H. Igarashi, et al. (2008). "An isoform of *Arabidopsis* myosin XI interacts with small GTPases in its C-terminal tail region." *J Exp Bot* **59**(13): 3523-3531.
- Itoh, T., A. Watabe, et al. (2002). "Complex formation with Ypt11p, a rab-type small GTPase, is essential to facilitate the function of Myo2p, a class V myosin, in mitochondrial distribution in *Saccharomyces cerevisiae*." *Mol Cell Biol* **22**(22): 7744-7757.
- Lapierre, L. A., R. Kumar, et al. (2001). "Myosin vb is associated with plasma membrane recycling systems." *Mol Biol Cell* **12**(6): 1843-1857.
- Li, J. F. and A. Nebenführ (2008). "Inter-dependence of dimerization and organelle binding in myosin XI." *Plant J* **55**(3): 478-490.
- Li, J. F. and A. Nebenführ (2007). "Organelle targeting of myosin XI is mediated by two globular tail subdomains with separate cargo binding sites." *J Biol Chem* **282**(28): 20593-20602.
- Peremyslov, V. V., A. I. Prokhnovsky, et al. (2008). "Two class XI myosins function in organelle trafficking and root hair development in *Arabidopsis*." *Plant Physiol* **146**(3): 1109-1116.
- Pfeffer, S. R. (2001). "Rab GTPases: specifying and deciphering organelle identity and function." *Trends Cell Biol* **11**(12): 487-491.
- Reisen, D. and M. R. Hanson (2007). "Association of six YFP-myosin XI-tail fusions with mobile plant cell organelles." *BMC Plant Biol* **7**: 6.
- Shimmen, T. and E. Yokota (1994). "Physiological and biochemical aspects of cytoplasmic streaming." *Int. Rev. Cytol* **155**: 97-139.
- Shimmen T., Y. E. (2011). *Plant Myosins. The Plant Cytoskeleton*. B. Liu. New York, Springer: 33-56.
- Storm, M., A. N. Hume, et al. (2002). "A family of Rab27-binding proteins. Melanophilin links Rab27a and myosin Va function in melanosome transport." *J Biol Chem* **277**(28): 25423-25430.