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ANALYSIS OF AN ACTIN BINDING GUANINE EXCHANGE FACTOR, GEF8, AND ACTIN DEPOLYMERIZING FACTOR IN *ARABIDOPSIS THALIANA*

A Thesis presented

by

ALEKSEY CHUDNOVSKIY

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

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MAY 2010

Molecular and Cellular Biology

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A Thesis Presented

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ABSTRACT

ANALYSIS OF AN ACTIN BINDING GUANINE EXCHANGE FACTOR, GEF8, AND ACTIN DEPOLYMERIZING FACTOR IN *ARABIDOPSIS THALIANA*.

MAY 2010

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Directed by: Professor Alice Y. Cheung

Polarized cell growth is a fundamental biological process that is tightly regulated spatially and temporally. In plants the key systems to study polar cell growth are pollen tubes and root hairs. In recent years a lot of work has focused on elucidating the mechanisms that mediate this process.

The actin cytoskeleton plays a key role in polarized cell growth. Different studies in plant and animal models show that signaling mediated through small GTP-binding proteins is a common theme in actin signaling. In recent years many groups have shown that small GTP binding proteins regulate actin dynamics through the activity of Actin Binding Proteins (ABP).

In this study I explored the function of two ABPs from *Arabidopsis Thaliana*: Actin depolymerizing factor (ADF) and a novel actin-binding guanine exchange factor (GEF).

I used *Arabidopsis* protoplasts as a system to study the function of these proteins. We showed through over-expression of the GFP labeled GEF8 under the constitutively active 35 S promoter, that GEF8 labels the prominent cable like structures inside the cell. Using actin and tubulin binding drugs such as Latrunculin and Oryzalin we showed that GEF8 labels actin cables. Using the Yeast 2 Hybrid system we determined that GEF8 binds actin filaments directly. We established that GEF8 interacts with actin through the unique N terminus of the protein. Finally, using the Basic Local Alignment Search tool we showed that the N terminus of GEF8 is homologous to the Actin Binding Protein 140, a well-established protein marker in Yeast.

ADF is an established key regulator of the actin cytoskeleton. Much is known about ADF regulation in animal systems. In plants it has been shown that the small Rho type GTP binding proteins, called RAC/ROPS, regulate ADF activity and that overexpression of RAC/ROPs causes the inactivation of ADF through the phosphoryaltion on Serine 6. However, little is known about the proteins that transduce the signal from small GTP binding proteins to the ADF.

Here we show some evidence that upon overexpression of Ric 4 (a RAC/ROP effector known to play a role in actin polymerization), ADF gets displaced from the filament. Moreover, ADF is known to be inactivated by phosphorylation at Ser6; the kinase responsible for this phosphorylation has not been identified in plant. We observed that over-expression of Calcium Dependent Protein Kinase 16

(CDPK16) in protoplasts also induced dissociation of ADF from actin cables. These results suggest that both of RIC4 and CDPK16 may play a role in the pathways that regulate ADF activity.

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PART 1

CHAPTER 1

INTRODUCTION

1.1 Small GTP Binding Proteins

The Rho family of small GTP binding proteins (GTPases) are among essential signaling proteins in eukaryotes. They transduce, integrate and amplify different molecular messages in eukaryotic cells. Rho GTPases play important roles in a variety of fundamental biological processes such as polarized cell growth. Examples include pollen tube and root hair growth in plants, axon growth in animals and many others**[1-3]**. Inside the cell this is manifested by intense cytoskeletal rearrangements including activation of different actin and tubulin binding proteins, which affect actin and tubulin cable dynamics**[4]**. In addition, RAC/ROP also mediate defense against pathogens, through the activation of the production of reactive oxygen species (ROS). Plant RAC/ROPs also regulate the 26S proteasome and consequently induction of the hormone auxin-regulated responsive gene expression **[1]**. There are 5 different families of RAS-related small GTPases in mammals: Rab, Arf/Sar, Ran, Ras and Rho. In contrast, plants have a unique family of small GTPases called RAC/ROP (Rho of plants) or RAC because of the homology to animal RAC**[5]**. Here I will concentrate on the RAC/ROP family because it is the primary regulator of actin dynamics, which is the primary topic of this thesis.

Structurally, RAC/ROPs are small proteins with a very conserved GTP binding domain and diverse N and C termini, which may mediate the selectivity to a specific activator or effector. Inside the cell, RAC/ROPs shuttle between the GTP bound active, or "ON", form, which can transduce the signal to effector proteins or GDP bound inactive "OFF" form, which gets recycled to the membrane (Fig1) **[2]**.

1.2 Negative regulators of RAC/ROP signaling

RAC/ROP signaling is fundamental for the cell and therefore has to be tightly regulated. Different highly conserved protein families regulate RAC/ROP activity spatiotemporally (Fig2) **[6]**. Among these families is a Guanine Dissociation Inhibitor GDI. These proteins, as the name suggests, bind the GDP bound inactive form of RAC/ROP, and prevent the dissociation of GDP bound nucleotide thereby keeping RAC/ROPs in the "OFF" mode **[5]**. Recent studies also suggest a role for GDI in spatial regulation of RAC/ROP activity such as recycling of inactive forms from the flank of pollen tubes to the tip where RAC/ROP gets activated, which suggests that GDIs can also play a positive role in RAC/ROP regulation**[7, 8]**. Another key negative regulator of RAC/ROP activity is the GTPase activating protein, GAP. These proteins activate the GTPase activity of RAC/ROPs, which helps to hydrolyze the GTP thereby turning the switch off and inactivating the protein **[5, 7]**.

1.3 Discovery and characterization of plant GEFs

Guanine Exchange Factors (GEFs) are among the key positive regulators of RAC/ROPs. Animal GEFs are characterized by the presence of DH Dbl homology and PH pleckstrin homology domains **[5]**. In plant GEFs these motifs are missing with the exception of Spike, which only recently has been shown to have GEF activity **[9]**. Therefore the identification of GEFs for RAC/ROPs has been extremely challenging. In 2005 Sheila McCormick and colleagues identified a kinase partner protein KPP in tomato and showed in vitro using the Yeast 2 Hybrid system and in germinated pollen tubes using pull down assays that KPP directly interacts with receptor kinase LePRK **[10]**. They also showed that KPP gets phosphorylated by LePRK and therefore is a direct downstream target of the receptor kinase. Overexpression of KPP transiently or in transgenic plants using the pollen specific promoter LAT52 caused the depolarized pollen tip growth, a phenotype which is reminiscent of RAC/ROP overexpression **[5]**. Later on Alfred Wittngoheffer and colleagues using dominant negative form of RAC/ROP as bait pulled out a family of proteins. They showed that in the presence of these newly identified proteins the dissociation of the GDP from RAC/ROP happened more than a thousand times faster. They identified the specific catalytic domain, which was necessary and sufficient for plant RAC/ROPs to mediate the GEF activity. The domain was named PRONE for (Plant specific RAC/ROP nucleotide exchanger)**[11]**. The tomato KPP turns out to be homologous to ROPGEFs from Arabidopsis. Later on the same group made a crystal structure of the PRONE domain of GEF8 alone and in complex with RAC/ROP. They showed that GEF acts as a dimer that simultaneously binds two RAC/ROP molecules and

helps to release the GDP molecule from RAC/ROP **[12]**. Arabidosis has 14 members of the RAC/ROP GEF family. They all share the conserved PRONE domain consisting of C1, C2 and C3 regions which are very conserved among plant species and do not have homologues in other organisms (Fig 3) **[10]**. Some of the members of the GEF family are exclusive to certain tissues. For example GEF8, 9, 11 and 12 are only expressed in pollen (Fig 4) while some others are ubiquitously expressed. While the GTP exchanging PRONE domain is strongly conserved among all members of the family, the N and C termini are quite different even among very close members of the family **[10]**. This feature suggests that these parts may promote specificity of interaction with a particular protein or may promote an extra function for the protein, which is separate from GTP exchange and therefore may provide a function in a different physiological process.

Here we show that pollen specific GEF8 directly interacts with actin filaments in Arabidopsis protoplasts, which strongly suggests that this particular protein has other function beside the conventional GTP exchange.

CHAPTER 2

RESULTS

2.1 GFP tagged GEF8 reveals prominent cable like structures in Arabidopsis protoplasts

We examined the RAC/ROP GEFs that had the highest expression in pollen (Fig 4). Among all Arabidopsis GEFs, GEF8, -9, - 11, and 12 had the highest expression. Moreover, these four proteins were almost exclusively expressed in plant floral organs. In order to assess the localization and functions of these proteins we used a plant protoplasts system, which, contrary to the traditional genetic approaches, allows examining the function of a particular protein in a very short period of time **[13]**. Two week old Arabidopsis protoplasts were transiently transformed with the plasmid DNA carrying the GEF8 fused to the Green fluorescent protein (GFP)(Fig5) under the expression of constitutively active 35S promoter (Fig6). Surprisingly upon the microscopic examination of the transformed protoplasts GFP signal came primarily and almost exclusively from prominent filament/cable like structures (Fig 6). This finding was very surprising and unexpected since GEFs are usually localized to the cell membrane and no GEF actin or tubulin interaction has been described to my knowledge in plants. Moreover, others in the lab (A. Cheung, Y-J Zou) also observed GFP-GEF8 binding to actin cables when expressed in pollen tubes. These interesting observations prompted us to concentrate specifically on GEF8 and to further explore the nature of the cables.

2.2 GFP tagged GEF8 coats actin filaments

In order to accomplish that we took advantage of very well established pharmacologic methods, which utilize small molecules that interfere with cytoskeleton dynamics. We used latrunculin B (LatB), which is a known actin polymerization inhibitor that binds to actin near the nucleotide-binding cleft and prevents it from polymerizing. LatB has been used widely in cytoskeleton studies and shown to inhibit actin driven processes, i.e. pollen tube germination and tube growth**[14]**. LatB at a final concentration of 250 nM was added directly to a microscope slide containing Arabidopsis protoplasts submerged in K3 medium supplemented with 20 percent agarose. A series of images has been taken at 0, 2 and 10 min after treatment. The addition of LatB resulted in the gradual disappearance of the cables suggesting that GFP-GEF8 co-localizes with the actin cables (Fig7). In order to exclude the possibility that GEF8 also co-localized with tubulin cables (analogous to Dictyostelium EB1 protein, which has been shown to interact with microtubules, but also co-localized with actin during cell division **[15]**), we took advantage of another potent cytoskeleton inhibitor, oryzalin, which inhibits microtubule polymerization **[16]**. Upon treatment of Arabidopsis protoplasts with oryzalin (10uM final concentration) for one hour, we did not observe any changes in the cable structure suggesting that GEF8 co-localizes only with actin cables (Fig8). The previous two experiments established that GFP-GEF8 co-localizes with the actin cables in the protoplast system. The question however remained as to whether GEF8 binds directly to actin or whether there is a special adaptor actin binding protein, which mediates GEF8 interaction with the cable.

2.3 GEF 8 directly binds actin in Yeast 2 Hybrid system

In order to investigate whether GEF8 binds directly to actin filaments we took advantage of the Yeast 2 hybrid system. This well established method utilizes yeast genetics in order to investigate novel protein-protein interactions **[17]**. The system takes advantage of the Gal4 transcription factor and various reporter genes, which are introduced into the yeast genome under the Gal4 promoter. In this assay one of the possible interaction partners is fused to the DNA binding domain of the GAL4, while the other interaction partner is fused to the activating domain of GAL4, which brings RNA polymerase to the transcription site of the particular reporter gene under the Gal4 promoter. If the proteins interact the N and C terminal of the GAL4 transcription factor come together to form a functional protein and the expression of the reporter gene is used as readout (Fig 9). We used the His3 gene that is essential for histidine synthesis. If the two proteins interact the HIS 3 gene is transcribed and the yeast will grow on a medium that lacks histidine. We inserted the GEF8 into the pAD vector and actin into the pBD vector, transformed the plasmid DNA into Yeast and in growth media that lacks histidine. In order to exclude the possibility of “false positive colonies”, since some of the proteins are able to activate by themselves the transcription of reporter genes, we also transformed into the yeast Actin-pBD and empty pAD vectors. We observed growth only in the case when we put both actin and GEF8 coding vectors in to the Yeast (table 1). This result showed that GEF8 directly interacts with actin filaments. The next goal was to figure out which region of GEF8 interacts with Actin.

2.4 GEF8 binds actin through the N terminus, which is homologous to actin binding proteins in Saccharomyces Cerevisiae

Since the PRONE domain is very strongly conserved among all GEF members it was not a likely candidate for direct interaction with actin. Therefore, we performed an alignment of 3 pollen specific GEFS in order to look for less conserved domains. Upon close examination we saw that the N-terminal region before the PRONE domain of GEF8 is quite different from its closest relative GEF9 and all other pollen GEFs (Fig 10,11). When we performed a BLAST search for that region, we found some homology in the N termini of GEF8 with ABP 140, which is a well-characterized actin nucleating protein in Saccharomyces cerevisiae (Fig 12) **[18]**. In order to test whether the N terminal region of GEF8 actually binds actin we took advantage of the unique BGL2 restriction site (Fig 13) at nucleotide position 233 of the sequence specifically before the PRONE domain and cloned this region of GEF8 in to the pAD vector. We then transformed them together with Actin pBD vector into the Yeast cells and plated cells without the Histidine supplements (Table 1), just as with the full lengths GEF8. Only the N terminal GEF8 plus actin grew on the –His plates. This finding confirmed in vitro that it is an N terminal region of GEF8 that directly interacts with actin.

2.5 GEF8 colocalizes with the actin binding domain of Fimbrin

In order to better establish the link between GEF and actin we tried to co-localize GEF8 with some other very well established actin marker. One of the possible candidates was fimbrin, which is a very well established Actin marker that is known to bind actin and is widely used in cytoskeleton imaging studies**[19]**. We first transformed Arabidopsis

protoplasts with 35S-RFP-fimbrin actin binding domain ABD just to ensure RFP would not interfere with the fimbrin-actin interaction (Fig. 14). After observing that the fusion construct was functional, we transformed the protoplasts with two constructs: one expressing the 35S-RFP-fimbrin actin-binding domain (ABD) and the other expressing 35S-GFP-GEF8. Upon microscopic examination we observed that Fimbrin colocalized with GEF8 (Fig 15).

2.6 Purification of recombinant GEF8

The big question that we wanted to answer was what is the structure of GEF8 N-terminal that allows it to bind actin filaments? In order to answer this we set a goal of crystallizing GEF8 N terminus and model this fragment on to the available actin crystal structures. To do this we cloned the N terminus of GEF into PET vector and transformed a protease deficient E.Coli BL 21 strain with this construct, and tried to purify it using different affinity chromatography techniques including a polyHis tag, Maltose binding protein (MBP) tag and GST tag. Although the protein was made in large quantities in bacteria (Fig. 15), not cleaved or degraded, upon further purification steps e.g. gel filtration chromatography it always precipitated out of solution. I was therefore not able to continue this project.

CHAPTER 3

DISCUSSION

Actin filaments are an essential part of the cell cytoskeleton, which plays an important role in a variety of cellular processes. These include cell division, cell growth, organelle movement and intracellular transport [20]. One of the most crucial steps in actin polymerization is actin nucleation and therefore it has to be tightly regulated.

There are several actin-nucleating proteins**[21, 22]**. Besides the Arp2/3 complex, which is the best characterized, formins constitute another major family. Formins are defined by the presence of actin binding formin homology (FH2) domain**[22, 23].** They produce actin filaments de novo, initiating them at the cell membrane. They nucleate actin filaments from the fast growing barbed end by capping it and protecting from other proteins, which may inhibit growth. Formins also posses a relatively variable proline reach FH1 homolgy domain which have been shown to bind profilin and profilin actin complexes.

In budding yeast there are only two members of Formin family [24]. The family of formins in Arabidopsis includes 21 members, which strongly suggests that in plants formins play a very important role in actin nucleation. The Arabidopsis family of formins is separated into two clases **[21]**. The first class is different from formins in other species and is characterized by the presence of an N terminal signal sequence, which targets them to the cell membrane, an extracellular domain and a transmembrane domain, just anchoring the cytoplasmic actin-nucleating activity domain at the cell membrane. Not

much is known about the second group of formins in plants. Overexpresion of the FH1FH2 activity domain of Formin 1(AFH1) in pollen tubes induced supernumerary actin cables, and the full length AFH1 induced accumulation of actin cables along the pollen tube cell membrane, ultimately resulting in growth arrest **[23]**.

Another key player in actin regulating machinery is Actin- related protein ARP2/3 **[20]**. It is a seven-subunit protein complex, which is one of the major actin nucleating complexes. Originally it was purified from the protozoan amoeba**[25]**. Subsequently it has been identified in all eukaryotic organisms tested to date. Two major subunits, ARP2 and ARP3, are homologous in structure to the actins and are thought to play a role in the formation of the actin nucleation seed **[26]** which has a high Kd and is an energetically unfavorable process. In contrast to formins, ARP2/3 cannot initiate the formation of the de novo actin filaments. It has to bind to the preexisting filaments and then starts an actin branch at a 70-degree angle to a preexisting actin axis**[20]**. In Arabidopsis, all 7 members of the ARP 2/3 complex are present **[24]**. Despite the fact that in animal system ARP2/3 is probably one of the major players in actin nucleation, it seems to be not as crucial in plants given that only some parts of the plant, especially trichomes and epidermal pavement cell morphology, have an observable ARP2/3 related phenotype. The role of ARP2/3 in pollen tube growth has not been assessed yet. It is known in animal systems that formins and ARP2/3 activation goes through small GTPase signaling**[1]**. It would be very interesting to know whether in pollen tubes activation of these key actin-binding proteins goes through GEF8. The Szymansky group recently showed that the GEF protein SPIKE1 activates SCAR complex **[9].** It seems reasonable to speculate that given

the relatively large size of plant GEFs and GEF8's unique ability to bind actin, it may serve as a scaffold protein which would co-localize RAC/ROP and all other actin polymerizing players directly to the actin cable and activate the actin polymerization. In general intuitively it seems very likely that the major target of GEF8 signaling has to be the actin cytoskeleton, since the location of the GEF8 protein seems to be very well suited for this. Co-expressing GEF8 with different RAC/ROPs and members of Formin and ARP2/3 family proteins tagged with different fluorescent markers and employing confocal microscopy in conjunction with classical biochemical techniques, i.e. pulldowns and co-immunoprecipitation, may address this hypothesis.

Plants also have large families of actin bundling proteins. These include villins, fimbrins, LIMs and elongation factor alpha**[27]**. In contrast to Arp2/3 and formins, which are able to polymerize actin, these proteins do not stimulate assembly of actin filaments. Instead they use their unique structure to bring together the existing actin cables thereby promoting formation of more complex actin networks. Fimbrins for example, have 2 actin binding domains, ABD2 and ABD1. Electron microscopy studies suggest that ABD1 becomes activated only when ABD2 is already bound to actin **[28]**. It's also possible that GEF8 has actin bundling capacity. For example, given that the crystal structure showed that GEF acts as a dimer **[12],** it may be possible that for actin bundling it may employ a similar mechanism to fimbrin. For example, each monomer of GEF8 may use its unique N terminal in order to bring two actin filaments together thereby promoting the formation of more prominent actin cables. Solving the crystal structure of the protein and modeling it on the actin filaments would determine which amino acids

residues are essential for the actin binding. A series of site directed mutagenesis experiments, targeting these residues, would provide insight into the nature of GEF8-actin interaction.

PART 2

CHAPTER 4

INTRODUCTION

4.1 Actin depolymerizing factors

Among actin depolymerizng proteins a key role is played by Actin depolymerizing factor ADF**[21]**. ADF is a small protein that- has been shown to depolymerize actin by enhancing the dissociation of actin monomers from the slow growing pointed end and by severing actin filaments **[29]**. Surprisingly, the plant family of ADF proteins is much larger than for animals. For example Arabidopsis is known to have 12 ADF proteins, while most vertebrates have one or two cofilins **[19],** and yeast only one**[30].** This suggests that in plants cofilins/ADFs probably play a major role in actin dynamics. ADF is a small protein with a conserved Ser3 in animal systems and Ser6 in plants. Substitution of this amino acid to Alanine or Aspartic acid results in either constitutively active or inactive form of the protein. ADF activity can be regulated by different mechanisms. Originally it was shown in a neuronal cell line and in vitro that LIM kinases can phosphrylate ADF, which increased the amount of F-actin in the cells**[31]**. Later on it was shown in Drosophila and mammalian cells that a family of phosphatases called SlingShot (SSH) dephosphorylated cofilin at Ser3 thereby reactivating the protein, which promoted actin depolymerization**[32]**. There is evidence suggesting that in plant systems calcium dependent protein kinases (CDPK) are involved in ADF phosphorylation **[33]**. CDPK represent a very large family of Ser/Thr protein kinases in plants. Arabidopsis has 34 CDPKs, which are subdivided into four distinct groups based on sequence homology.

It is believed that CDPKs arose as a fusion of kinase and calmodulin domains**[34]**. Structurally, CDPK consists of a nonconserved N terminal, a kinase domain, an autoinhibitory region, and a calmodulin domain, which consist of Ca^{2+} binding EF hands. In the inactive state the autoinhibitory domain is bound to the kinase domain, which prevents the kinase from phosphorylating its substrate. During activation Ca^{2+} ions bind the EF hand, which causes structural changes that disrupt binding between the autoinhibitory and kinase domain, which activates the protein**[34]**. Early studies in maize showed that plant extract enriched in CDPK was able to phosphorylate ADF at Ser6 **[35]**, and that this phosphorylation was abolished by the addition of calmodulin antagonist or an antibody specific for the calmodulin domain of CDPK**[33]**. These data strongly suggest that CDPK either directly phosphorylate ADF or at least is involved in the pathway that leads to the ADF phosphorylation.

Here we show some evidence that pollen specific CDPK 16 from Arabidopsis strongly affects the localization of ADF in the Arabidopsis protoplast system and that when overexpressed makes the GFP labaled ADF go to the nucleus, reflecting a default localization of small cytoplasmic proteins known to occur efficiently in plant cells, as opposed to usual actin filament localization.

CHAPTER 5

RESULTS

5.1 CDPK 16 influences the localization of ADF in Arabidopsis protoplasts

A study performed in *Medicago truncatula* using an RNA interference technique revealed that a gene encoding a calcium dependent protein kinase 1 (CDPK1) was involved in root hair growth**[36]**. Silencing CDPK1 resulted in diminished root hair growth. The region of the gene against which the RNAi construct was generated had a high homology to the group IV of Calcium dependent protein kinases whose function are unknown (Fig 16C). This group includes CDPK 16, 18 and 28**[36]**. We used the publicly available Arabidopsis microarray databases in order to investigate the levels of expression of these proteins in the pollen tube (Fig 16B) We speculated based on the previous data that a potential mechanism by which the silencing of this protein inhibits the root hair growth is through the inactivation of ADF, so that when CDPK1 is silenced ADF becomes constitutively active, severs actin filaments and, similarly to ADF inhibition of pollen tube growth, inhibits the root hair growth.

In order to test whether CDPK would have any effect on the ADF localization we used Arabidopsis protoplasts as a system. First we wanted to find out whether pollen specific ADF would show a similar localization pattern as in a pollen tube, i.e. whether it would bind to actin filaments and whether S6D mutant would show a predominantly

cytoplasmic signal, like in pollen tubes**[37]**. For that we transformed 35S-GFP-ADF or 35S-GFP-ADF(S6D) mutants into protoplasts. After observing that ADF labeled the actin filaments and prominent cable like structures were visible inside the protoplasts (Fig 17 A), while ADF (S6D) showed a strong cytoplasmic and nuclear signal (probably due to the small size of the protein which allows it to enter in to the nucleus) (Fig 17 B), we then wanted to determine whether ADF was present in the protoplasts in different states like in the pollen tube**[37]**. This would address whether ectopically overexressed pollen ADF can potentially be phosphorylated in the protoplast system. We transformed protoplasts isolated from three-week-old Arabidopsis seedlings with HA tagged ADF and extracted the total protein sixteen hours after the transformation. We then employed 2D gels followed by western blot with Anti-HA antibody (Fig 17 D). Distinct spots after the isoelectric focusing strongly argue for the presence of multiple forms of the protein, each form having a different charge. After confirming that there are multiple forms of the protein present in protoplasts (Fig 16 C), we transformed protoplasts with both 35S CDPK and GFP labeled ADF. The GFP showed primarily nuclear and cytoplasmic localization suggesting that CDPK 16 inactivates ADF (Fig 18).

5.2 Ric4 impacts ADF localization

Studies from our group have shown that pollen specific ADF from tobacco plays an important role in pollen tube growth**[37, 38]**. Specifically, Christine Chen showed that the concentration of pollen specific ADF is important since even modest overexression of ADF causes pollen tube growth arrest**[38]**. ADF activity is pH dependent. ADF is more active at slightly alkaline pH, which correlates with a region close to the tip of the pollen tubes where pH is alkaline and actin has a mesh like structure suggesting stronger filament severing at that specific region. She also showed that an alanine substitution at Ser6 makes the protein constitutively active and causes the reduced growth of pollen tubes when ADF S6A is overexpressed. On the other hand when serine is substituted to aspartic acid, which mimics the negatively charged phosphate, the protein becomes inactive, consistent with a phosphorylation dependent mechanism of ADF regulation in pollen tubes. When GFP tagged S6D ADF is overexpressed in the pollen tubes it shows strong cytoplasmic as opposed to actin binding localization and does not affect pollen tube growth. Physiologically, the activity of RAC/ROP has been shown to affect the ADF phosphorylation status, since overexpression of RAC/ROP increases the amount of the phosphorylated form of ADF in the pollen tubes, and overexpression of ADF partially rescues the pollen tube swelling RAC/ROP associated phenotype**[37]**. When WT ADF is overexpressed together with RAC/ROP it shows a preferential cytoplasmic signal, suggesting that RAC/ROP inactivates the protein. When phosphorylation resistant S6A mutant of ADF is overexpressed together with RAC1, S6A shows preferential localization to actin cables, suggesting that the phosphorylation is the mechanism by

which RAC/ROP inactivates ADF**[37]**. Although it has been shown that RAC/ROPs regulate ADF activity, the downstream players in between are not known. Here we show some evidence that RAC/ROP acts through the RIC family of RAC/ROP effectors.

The Ric family was identified by using a constitutively active form of RAC/ROP as bait in the Yeast 2 hybrid protein interaction system**[39]**. RICs contain the conserved Cdc42/Rac-interactive binding CRIB motif, which has been shown to be necessary and sufficient for the interaction with RAC/ROP using a series of pull down experiments. In Arabidopsis the RIC family contains eleven members many of which show different expression patterns and belong to 5 different groups based on the amino acid sequence analysis**[39]**. Consequently, its been shown that Ric1 and Ric4 regulate pavement cell morphogenesis**[4]**. Ric4 works downstream of ROP2 and promotes actin polymerization, which mediates outgrowth of the lobes of the pavement cells. RIC1 suppresses this process via activation of microtubules, which control the width of the pavement cells and by inactivation of ROP2**[4]**. In pollen tubes RIC4 and RIC3 has been shown to be the targets of ROP1**[40]**. Using a series of FRET experiments and pollen tube growth assays the authors suggested that pollen tube growth is mediated through the action of two counteracting pathways**[40]**. While Ric4 has a similar action as it has in pavement cells and promotes the polymerization of actin filaments and pollen tube growth, Ric 3 regulates Ca^{2+} influx at the tip of the pollen tube, which causes the actin depolymerization**[40].** This may occur due to the activation of actin sequestering proteins such as profilins. These two processes have the same period, but different phases, partially explaining the differential phases of pollen tube growth. Here we show some

evidence that Ric4 over expression in Arabidopsis protoplasts may inactivate the actin depolymerizing factor shedding light on RIC4-mediated actin polymerization. Although overexpression of Ric4 has been shown to polymerize actin filaments in the pollen tube and in pavement cells, the actual mechanism by which this happens remains largely unknown. We speculated that a possible mechanism through which Ric4 may induce actin polymerization is by inactivating ADF. We used the protoplast system in order to investigate whether Ric4 overexpression would affect ADF localization. In a series of cotransformation experiments, when GFP labeled ADF was transformed into protoplasts with Ric4, GFP signal came primarily from the cell nucleus, instead of the actin cables (Fig 19).

CHAPTER 6

DISCUSSION

A lot of previous data showed that plant ADF gets phosphorylated and that CDPK may play a role in this process**[33, 35]**. Here we showed that pollen specific CDPK 16 may be involved in the phosphorylation of ADF, which is a very intriguing finding given that it remains unknown which specific CDPK phosphorylate ADF. A lot of key questions still remain. Although as was mentioned some previous publications suggest that CDPK directly phosphorylates ADF, it is possible that CDPK is an intermediate player in the pathway and activates another kinase which then phosphorylates ADF. Although the experiments described above show that pollen specific CDPK 16 influences the localization of the ADF, the actual mechanism through which it displaces ADF from the actin filaments remains unknown. Fluorescence microscopy techniques do not address directly whether ADF is a substrate of the CDPK. The nature of the interaction of the kinase with its substrate is usually very transient making it challenging to use biochemical approaches such as protein pulldown assays. Therefore it still remains a challenge to show directly whether ADF gets phosphorylated by CDPK or whether this kinase phosphorylates another substrate, which in turn phosphorylates and inactivates ADF.

It has been shown in a series of papers from Yang's group that Ric4 plays a role in actin polymerization**[4, 40],** although in their model Ric4 would activate actin polymerizing proteins, i.e. formins. Alternatively Ric 4 may inactivate actin-severing

proteins. This scenario seems possible given that both Ric 4 and ADF work through RAC/ROP signaling and overexpression of Ric4 in pollen tubes is reminiscent of the RAC/ROP overexpression, which could be rescued by overexpressing ADF **[37]**. It would be interesting to know whether overexpression of ADF could also rescue the Ric4 related phenotype in the pollen tube. That would strongly suggest that the signaling downstream from Ric4 shuts down ADF.

Previous work has established that Protein receptor kinases work through KPP, which activates RAC/ROP**[10]**. RAC/ROP effectors transduce the signal downstream, potentially to the CDPK, which shuts down Actin depolymerizing factor.

A lot of work still needs to be done in order to establish the whole pathway staring from the cell membrane and going downstream to the final target, the actin cytoskeleton.

CHAPTER 7

METHODS

7.1 Construct generation for protein purification from bacteria

The sequence coding for the GEF8 was cloned into the PET vector downstream of the 6His side. From the PET vector the sequence was digested with BamH1 and Sal1 restriction enzymes. The sequence coding for the GEF8 was ligated to the pMal vector (New England Biolabs) downstream from the maltose binding protein (MBP) and the Factor Xa cleavage site. In order to confirm the insertion, the constructs were transformed into the Dh5 alpha E.Coli strain. Plasmid DNA was purified by minilysis digested with the same restriction enzymes and resolved on a 0.5% agarose gel. After confirmation the construct was transformed to the BL 21 E.coli strain.

7.2 Protein production and purification from E coli

500 ml of LB media was inoculated with BL 21 E.Coli at a 1:100 dilution and grown to the log phase (OD 0.4-0.6) at 600nm. 0.3mM IPTG was added in order to induce the protein expression. After 4 hours of induction with IPTG the culture was harvested and resuspended in 30 ml of extraction buffer (20mM Tris pH 7.5, 200mM NaCl, 1mM EDTA PMSF and a tablet of complete protease inhibitor cocktail from Roshe). 6M urea was used for extraction of the total protein. The extract was sonicated by three 10 sec pulses on a Branson sonifier, each with one minute in between. Debris was spun down

and the supernatant loaded on to the column with amylose affinity resin or talon resin depending on the construct used. After loading, the column was washed 3 times with the extraction buffer in order to get rid of nonspecific binding. The bound proteins were eluted with extraction buffer containing 10mM maltose or different concentrations of imidazole. To check the protein purity protein was resolved on 15% acrylamide gels followed by western blot.

7.3 Western blot

The protein gel was transferred overnight on to a PVDF membrane at 25V at 4 degrees C. In the morning the membrane was blocked for an hour with milk buffer (3.5% dry milk, 0.05% Tween and 100mM NaCl) on an orbital shaker at room temperature. After an hour the PVDF membrane was incubated with primary custom made anti MBP antibody or anti His antibody (Santa Cruz) for 2 hours, then washed 3 times for 5 min each with milk buffer and incubated with secondary goat anti rabbit antibody conjugated to Alkaline Phosphatase for one hour. The membrane was washed 3 times with the buffer (20 mM Tris pH9.5, 10mM $MgCl^2$, 100mM NaCl) incubated in 10ml of the same buffer supplemented with Nitro-Blue Tetrazolium Chloride (NBT) and 5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt (BCIP) until the desired level of development was achieved.

7.4 Construct generation for protoplast transformations

cDNA sequences coding for GEF8, N terminal GEF8, fimbrin ABD, ADF, Ric4, actin CDPK16 were cloned in to the pBuleScript KSII vector under the expression of constitutively active 35S promoter as described earlier [41].

7.5 Protoplast isolation and preparation for the transformation

3-4 week old Arabidopsis seedlings were gently cut with a razor and transferred into a sterile petri dish. Approximately 30ml of K3 enzyme solution (depending on the amount of seedlings in the petri dish) was added to the seedlings and plants were cut extensively in the K3 enzyme medium. Plant cell wall digestion was performed overnight (12 to 14) hours in K3 enzyme solution in a Petri dish in the dark at room temperature in a sterile environment. In the morning the enzyme medium was removed and replaced with k3 medium. After gentle shaking the protoplasts were released and the mixture was passed through the nylon mesh and transferred to a 15ml round bottom glass tube. The protoplast suspension was diluted 4 to 5 times with k3 medium in order to prevent further digestion of the protoplasts and in order to select for intact cells, which would float to the top of the medium. The top layer of the protoplasts was transferred every 2h into a new tube and k3 medium was added. The procedure was repeated 4 times in order to get the most stringent selection of the intact cells and remove residual enzymes. At the final stage, protoplasts were diluted with a protplast suspension medium (0.4M mannitol, 20mM $CaCl^2$, 5mM MES pH5.7)

7.6 Protoplast Transformation

For transformation 3 ug of plasmid DNA was added to a 2 ml microcentrifuge tube. 100 ul of protoplasts were added on top of the DNA and 110ul of PEG was added with gentle mixing. The mixture was incubated for 20 min at room temperature. After that, 1 ml of K3 medium was added to each tube. The tubes were incubated overnight in the dark at room temperature. After 12 to 15 hours the protoplasts were used for subsequent experiments.

7.7 Protein extraction from protoplasts

After 15h of incubation, intact protoplasts banded at the top of the medium. The underlying K3 medium was removed from the protoplasts using peristaltic pump. W5 medium was added to the tube and the protoplasts were spun down in W5 solution (154mM NaCl, 125mM CaCl, 25mM KCl, 2mM Mes pH 5.7) for 10 min. The supernatant was removed and the pellet was resuspended in Laemmli sample buffer and loaded onto the 15% protein gel as described above.

7.8 Protein extraction and preparation for the 2-D gel

Protoplasts were spun down as described above, resuspended and protein was extracted as described [42]. The 2D cleanup kit from BioRad was used afterwards according to the manufacturer's instructions before loading the protein on the immobilized 7 cm pH strips (BioRad pH 7 to 4) according to the manufactur's instructions. The first dimension was run on the IEF focusing system from BioRad. The second dimension was run on a regular 17.5% protein gel as described above.

7.9 Microscopy

All images were acquired as described (38). Briefly, fluorescence images were acquired using a Nikon E800 microscope equipped with a charge-coupled device camera, and the images were analyzed with Photoshop and ImageJ software. Confocal images were acquired using a Zeiss 510 microscope under identical exposure conditions.

7.10 Yeast 2 Hybrid

The Yeast 2 Hybrid was done using the Stratagene system according to the manufacturer's instructions (ref). Constructs coding for actin, GEF8 full length, or GEF8 N terminal were cloned into the pAD or pBD vector according to the manufacturer's instructions. Yeasts were grown to 0.4-0.6 optical density measured at 600 nm wavelength in YPAD full supplemented medium, transformed with appropriate constructs using the PEG method. Briefly, 10 micro grams of sheared salmon sperm DNA was mixed with the construct of interest, cloned into PAD or PBD vectors mixed with PEG and incubated with yeast cells. Transformed cells were plated on plates with SD medium. For selection of cotransformation by pAD and pBD vectors, yeast were plated on the –Leu, -Trp plates since together these two vectors carried the genes essential for the synthesis of these amino acids. To screen for the protein interactions yeasts were plated on –His medium supplemented with 30mM 3AT, which is a chemical that causes histidine starvation and is used to prevent the generation of false positive clones.

APPENDIX

MEDIA RECIPES

K3 media for 1 L

Gamborg's B5 media 100x-5ml (QBiogene)

200x MES (0.1gm/ml)-5 ml

500x myo-inositol (5gm/100ml)-2ml

100x NH3NO3 (25mg/ml)-10ml

100X $CaCl^2$ (75mg/ml)-10ml

100x xylose (25mg/ml)-10ml

0.4M Sucrose-137g

YPAD media

20g Peptone

10g Yeast Extract

900ml-distilled water

5 ml of 1M HCL

20g Difco Bacto Agar, (add for solid plates)

SD media

4.0 gm Difco Yeast Nitrogen Base (w/o amino acids)

12.0 gm glucose

0.50 gm Synthetic Complete Drop Out Mix

600 ml distilled water

10.0 gm Difco Bacto Agar, (add for solidmedium)

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