Characterization of RD20 as a potential regulator of Heterotrimeric G protein signaling in *Arabidopsis thaliana*

Mohammad-Reza Ehdaeivand

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By: Mohammad-Reza Ehdaeivand

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Signed by the final examining committee:

Dr. Selvadurai Dayanandan	Chair
Dr. Luc Varin	Examiner
Dr. Reginald Storms	Examiner
Dr. Madoka Gray-Mitsumune	Examiner
Dr. Patrick Gulick	Supervisor

Approved by Chair of Department or Graduate Program Director

Dean of Faculty

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Abstract

Heterotrimeric G proteins are known to be important components of regulatory pathways in animals and fungi and the elucidation of G protein signaling in plants is an emerging area of investigation. The study of signaling and regulatory control of plants responses to environmental stress at the molecular level is an area of wide interest and the plant growth regulator abscisic acid is known to play a major role in the plant response to environmental stress. This work reports on the characterization of the stress induced caleosin gene *RD20/CLO3*. Here the interaction of the caleosin protein Responsive to Dehydration 20, RD20/CLO3 with the alpha subunit (GPA1) of the heterotrimeric G protein complex in Arabidopsis was investigated at protein level and the N-terminal domain of RD20 protein was found to interact with GPA1.

Characterization of the effect of *RD20/Clo3* on plant stomatal density and index indicate that the gene is necessary for the ABA mediated reduction of stomatal proliferation. In addition, methods to improve the level of protein expression in *E. coli* and the subsequent purification of RD20 and GPA1 were developed. The purified protein will be used in future studies to investigate the role of RD20 as a potential GTPase Accelerating Protein for GPA1.

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Table of Contents

List of figures

List of tables

Recognition of shared work in this analysis.

Though the work presented in the thesis was carried out by myself, development of a number of the experimental methods used in this thesis were carried out as shared work with Sabrina Brunetti. Sabrina Brunetti and I contributed equally to the development of the protein expression protocol, the protein purification methods, and stomatal density and index determination methods.

Introduction

Heterotrimeric G protein

Adaptations and responses to environmental stress have been recognized in plants at the level of growth and development, and complex changes in gene expression have been characterized for plants response to abiotic stresses, particularly responses to water stress, low and high temperatures and mineral toxicity of soils. In plants it is known that members of the heterotrimeric G protein complex play different roles in the response to environmental stress related plant hormone abscisic acid (ABA) (Liu et al. 2013). In the model for heterotrimeric G protein signaling established in animals and fungi three G protein subunits, α, β, and γ, are associated with a 7 transmembrane domain G protein coupled receptor (GPCR) (Urano & Jones 2014). Ligand binding to the receptor causes conformational changes in the GPCR protein which then can be recognized by the α subunit of the heterotrimeric G protein and thereby activate it with the exchange of bound GDP with GTP (Urano & Jones 2014). Concomitant with the GDP/GTP exchange, the α subunit disassociates from the β, and γ subunits (Urano & Jones 2014). After activation, the α subunit and the β-γ duplex are able to modulate downstream effectors such as cAMP and PLC (Urano & Jones 2014). The α subunit is deactivated by hydrolysis of bound GTP, a regulatory step that can be mediated by regulators of G protein signaling (RGS) proteins that accelerate the inherent GTPase activity of the Gα subunit; these GTPase accelerating (or activating) proteins are also known as GAPs (Urano et al. 2013, Urano & Jones 2014). Though heterotrimeric G proteins have been identified in plants, emerging data indicate that the regulation of their activity is different than that in animal and fungal species (Urano & Jones 2014).

The human genome contains 23 genes that code for Gα subunits while the *Arabidopsis thaliana* genome has only one gene encoding a Gα subunit GPA1 (Chen et al. 2006). In human genome there are 5 Gβ and 12 Gγ genes (Jones & Assmann 2004) while in Arabidopsis there is only one Gβ, (AGB1) and three Gγ genes (AGG1, AGG2 and AGG3) (Urano and Jones 2014). There are twenty one RGSs encoding genes in human while in plant only one RGS has been identified (Jones & Assmann 2004). The α subunits of the heterotrimeric G proteins in Arabidopsis and animals have similar crystal structures (Jones et al. 2012). However the kinetic properties of the Gα in plants are markedly different from those in animals (Jones et al. 2012). Unlike animal Gα's, the plant Gα protein has a high inherent GTP for GDP exchange activity and a relatively low level of inherent GTPase activity (Urano & Jones 2014). The rate of nucleotide exchange by the Arabidopsis Gα is approximately 144 times higher than exchange rate in animal's Gα while the hydrolysis rate in the Arabidopsis Gα is one thirtieth that of the animal Gα (Urano & Jones 2014). The Arabidopsis Gα's GDP to GTP exchange rate is two orders of magnitude higher than that of GTP hydrolysis; the Arabidopsis Gα is estimated to be 99% in the GTP bound state, when it is not associated with an RGS (Johnston et al. 2007). The high intrinsic GEF activity of plant Gα's implies that plant Gα proteins are self-activating and unlike animal and yeast Gα's, do not require GDP/GTP exchange factors (GEFs) for activation and that GTPase accelerating proteins (GAPs) likely play a critical role in regulating Gα activity (Urano & Jones 2014).

How the signals are received and processed and how they can affect G protein signaling in plant still requires further study and there is a growing body of research that addresses such questions. One of the proteins reported to regulate G protein signaling is the Regulator of G protein Signaling 1 (RGS1), a protein with seven transmembrane domains, which is closely associated with the heterotrimeric G protein complex in Arabidopsis (Urano et al. 2012). RGS1 accelerates

GTPase activity of Gα about 100 fold and plays an important role in regulation of heterotrimeric G protein; it would be expected to maintain Gα in an inactive state (Urano et al. 2012). RGS1 plays a role in glucose sensing; *rgs1* mutant lines are insensitive to high concentrations of D-glucose and D-fructose that inhibit seed germination, inhibit leaf development, increase anthocyanin levels and lower chlorophyll level in the leaves of wild type plants (Chen & Jones 2004). The presence of the seven membrane domains in RGS1 is reminiscent of G protein coupled receptors (GPCR) though RGS1 has not been shown to have receptor like activity.

The structure of the heterotrimeric G proteins have been extensively analyzed through crystallography and three domains referred to as switch I, II and III and the N-terminal helical domain are important for the α subunit to be able to bind to β and γ subunits (Temple & Jones 2007). In the animal and fungi models, all three switches are also necessary for Gα to bind RGS's and make the conformational changes necessary to accelerate GTP hydrolysis (Temple & Jones 2007). The sites of interaction between RGS1 and Gα in Arabidopsis (GPA1) are conserved between plant and animal Gα proteins (Temple & Jones 2007). The GPA1 P-loop is important for nucleotide recognition and a myristoylation site near the N terminus of Gα is important for plasma membrane localization of the protein (Temple & Jones 2007). The Gβ subunit interacts with Gγ subunit with its N terminal alpha helix domain (Temple & Jones 2007). The WD40 repeat motif region of Gβ, is important for 7-bladed propeller structure (Temple & Jones 2007). The WD40 repeats, also known as transducin repeats and are known to mediate protein-protein interaction (Gachomo et al. 2014). Protein trafficking, cell division, flowering and light signaling are among known biological roles of WD40 repeat proteins (Gachomo et al. 2014). The Gγ subunits of Arabidopsis (AGG1 & AGG2) have N terminal coiled-coils which interact with the beta subunit and have C terminal CAAX box (cysteine, two aliphatic amino acids and any terminal amino acid)

which serve as a myristoylation site (Temple & Jones 2007). The CAAX box has shown to be essential for anchoring to the plasma membrane in animal and yeast Gγ's (Temple & Jones 2007).

Modern bioinformatics methods that predict folding patterns based on helix alignment methods, can align the template to known classes of GPCRs with higher accuracy than previous methods (Taddese et al. 2014). These methods showed similarities between the putative Arabidopsis receptor GCR1 and GPCRs in animals in that it has eight amphipathic helixes and conserved serine and threonine residues (Taddese et al. 2014). However, no GPCRs have been identified in plants (Urano & Jones 2014). GRC1 interacts with GPA1 (Pandey & Assmann 2004) but there is no evidence to demonstrate that it has GEF activity (Urano & Jones 2014, Taddese et al. 2014). Interestingly *gcr1* mutant plants are more drought tolerant and some ABA response genes are constitutively up-regulated in a *gcr1* mutant line (Pandey & Assmann 2004).

Caleosins

The calcium binding protein Clo3 from wheat has been shown in our laboratory to interact with GA3 (Gα in wheat) (Khalil et al. 2011) and similar work with Arabidopsis homologs in our lab has shown similar interactions (Zhe Jun Wang MSc. Thesis, 2009). Clo3 is a member of the caleosin gene family that has 7 members in Arabidopsis and 11 members in the diploid genome of wheat (Khalil et al 2014). Members of the gene family are characterized by a single EF hand Ca^{2+} binding domain in the N terminal half of the protein, followed by an amphipathic α helix that is thought to be a membrane associated domain, followed by a proline knot and a C terminal domain (Chen et al. 1999).

Responsive to Dehydration 20, RD20/CLO3, a member of the caleosin gene family in Arabidopsis, is induced by drought and abscisic acid (ABA) (Takahashi et al. 2000). RD20 contains a single EF-hand helix-loop-helix calcium binding motif and has been shown to bind calcium (Takahashi et al. 2000). *RD20* is expressed in siliques, leaves, flower and stem (Takahashi et al. 2000) and Arabidopsis *rd20* mutant lines are drought sensitive and they show increased levels of transpiration that was speculated to be caused by altered regulation of stomatal closing in response to drought (Aubert et al. 2010). Another member of caleosin gene family, AtClo4, has been reported to be a negative regulator of ABA signaling (Kim et al. 2011).

RD20 is an ABA response gene and plants under higher levels of ABA showed elevated expression level of the gene while *RD20* was highly expressed in guard cells (Aubert et al. 2010). In plants carrying mutations in essential ABA signaling genes, expression of *RD20* is not induced by dehydration conditions while in wild type plant, level of expression for the gene, *RD20*, is more than 20 times higher than the mutant plants which shows that expression of *RD20* is regulated by the ABA signaling pathways in plants (Aubert et al. 2010).

Previous study of the RD20*/CLO3* gene showed that the gene is expressed in plants above ground tissues and that *rd20* mutant lines are more sensitive to drought than wild type plants (Aubert et al. 2010).

Stomata

Stomata are small pores found on the surface of leaves and other plant organs. They consist of two guard cells which can cause the pore to open or close in response to environmental conditions and in response to fluctuations of hormone levels (Pillitteri & Dong 2013). Stomata

allow entry of CO_2 into the plant tissues for photosynthesis, and as a consequence of gas exchange, facilitate water loss from the plant in a process referred to as transpiration (Pillitteri & Dong 2013). Stomata can also control plants temperature through evaporative cooling (Pillitteri & Dong 2013).

In Arabidopsis, there are more stomata on the lower leaf epidermis than the upper epidermis which shows that the distribution is not random (Pillitteri & Dong 2013). Density of stomata is different in different tissues and the stomata are normally separated from each other by at least one cell (Pillitteri & Dong 2013). Stomatal density and index are controlled by genetic and environmental factors such as humidity, temperature and light (Berger & Altmann 2000). Several genes are known to affect stomatal distribution, formation and development, such as *Too Many Mouth (TMM)* and *Erecta-Like 1 and 2 (ERL1 &ERL2)* which are important for spacing and stomatal patterning (Pillitteri & Dong 2013) and genes such as *SPEECHLESS (SPCH)* and *Epidermal Patterning Factor1 (EPF1*) are important for differentiation and patterning, respectively (Pillitteri & Dong 2013).

It is well known that plants need water to grow and stomatal development and pattern formation play important roles in controlling transpiration (Lawlor 2009). Although there are still many questions about the mechanism of how external stresses such as drought or salt can affect photosynthesis (Lawlor 2009) it is known that plants need to balance their CO_2 uptake with water loss for survival and optimal growth (Nilson & Assmann 2010). Because of the intrinsic links between CO_2 uptake, photosynthesis and water loss, transpiration plays an important role in plant biomass accumulation (Nilson & Assmann 2010). Transpiration Efficiency (TE), which is measured as the ratio of biomass accumulation per amount of water loss, can be affected by the regulation of stomatal aperture and stomatal density (Nilson & Assmann 2010). The *gpa1* mutant line in Arabidopsis was shown to have higher transpirational efficiency and this was attributed to

lower stomatal density than wild type plants (Nilson & Assmann 2010). Abscisic acid (ABA) which is a drought related hormone (Pantin et al. 2013) that also regulates plant growth (Finkelstein & Somerville 1990), has been shown to trigger stomatal closure (Pantin et al. 2013). The *gpa1* mutant lines have a wild type response to ABA stimulation of stomatal closing but they are insensitive to ABA inhibition of stomatal opening and experiments with detached leaves reported the mutant to have higher rates of water loss (Wang et al. 2001). In whole plant experiments *gpa1* mutants showed higher TE, but this was found to be attributable to their decreased stomatal density and not to altered regulation of stomatal aperture responses (Nilson and Assmann 2010).

Environmental factors such as light intensity and CO_2 can change stomatal density, as an illustration, both high CO_2 concentration and low light exposure decrease stomatal density (Caspar et. al 2013). It is also known that low concentrations of ABA in plants leaf leads to higher stomatal density while higher ABA concentration on leaf causes lower stomatal density and stomatal index (Caspar et. al 2013).

Previous work in our laboratory indicated that RD20 can affect stomatal density and change stomatal density in response to ABA treatment in 6 weeks old leaves of *Arabidopsis thaliana* (Justin Wright MSc Thesis, 2014). It was shown that *rd20* mutant line has higher stomatal density compared to wild type plant and ABA treatment increases stomatal density in *rd20* mutant line (Justin Wright MSc Thesis, 2014).

Purpose of the study

One objective of my work was to identify which regions of RD20/CLO3 are responsible for interaction with the GPA1 protein by assaying interactions using sub clones of *RD20/CLO3* which produce protein truncations. Secondly, the effect of *RD20* on stomatal development in response to ABA treatment was investigated. In addition I developed methods to achieve higher levels of purified GPA1 and RD20 proteins than our lab had previously been able to produce in order to test the GAP activity of RD20/CLO3 *in vitro*.

The proteins that interact with GPA1 may act as GTPase accelerating Proteins (GAPs) or they may regulate the signaling associated with the G-proteins by either inhibiting or by stabilizing the interaction among the three subunits of heterotrimeric G protein complex. As an initial step to assay how regulators of G protein signaling bind to members of the complex, four different truncated versions of *RD20* were cloned into plant expression vectors and were assayed for interaction with GPA1 using Bimolecular Fluorescent Complementation (BiFC) assay. This study is an important primary step for testing G protein complex interacting proteins and downstream signalling that are affected by proteins that interact with any subunit of the G protein complex.

In this study the role of RD20 in stomatal density and stomatal index was also investigated. Stomatal density can be decreased or increased by changes in environmental conditions, hormone treatment and genotype (Caspar et al. 2013). I investigated whether *RD20* plays a role in the ABA signaling that controls stomatal density. In this study the effect of exogenous ABA treatment on *rd20* mutant line was tested to investigate role of RD20 in stomatal density in response to ABA treatment.

In the third part of this work, I described the optimization of conditions by which both His-tagged RD20 and GPA1 proteins are expressed and purified from *E.coli*. This work was undertaken to achieve high levels of protein expression to facilitate further purification of GPA1 and RD20 with size exclusion or anionic exchange chromatography and to facilitate testing the potential role of RD20 as a GTPase accelerating protein (GAP) for GPA1.

Materials and Methods

Bimolecular Fluorescent Complementation assay (BiFC)

Wild tobacco, *Nicotiana benthamiana*, used for Bimolecular Fluorescence Complementation (BiFC) assays were grown in 10 cm pots using a soil mixture containing equal amounts of peat moss, black soil and vermiculite. Plants were grown in the green house maintained at 22°C and with supplementary lighting to provide with 16 hours of light per day.

The experiment was performed with some modification on Walter et al. 2004. All plasmid constructs for transient gene expression were first transformed to *Agrobacterium tumefaciens* strain AGL1 using electroporation. An overnight culture of one colony was grown in 10 ml LB with appropriate antibiotic at 30°C overnight. The required amount of the culture to reach OD 600 nm of 0.5 were centrifuged in 4000 g for 20 minutes and the pellet was re-suspended in agro-infiltration solution (47 ml H_2O, 200 µM of 2.5 M $MgCl_2$ and 75 µl of 100 mM acetosyringone) to an OD 600 nm of 0.5.

Leaves of plants approximately 16-25 day old were infiltrated with a mixture of cultures of *Agrobacterium* carrying different combinations of experimental expression plasmids by applying the mixed cultures with a syringe without a needle to the lower side of the leaf. Infiltrations included cultures that contained one or two experimental samples, as well as a cellular marker and a strain carrying the P19 suppressor of transgene suppression (Voinnet et al. 2003). Positive controls was the co-infiltration of full length GPA1 and RD20 in BiFC expression vectors; negative controls were single BIFC constructs, i.e. constructs with the N-terminal of C-terminal YFP partial protein fusion, GPA1 or RD20, without the potential interacting partner.

Three for four days after infiltration the samples of leaf tissue were taken as described by Walter et al 2004 and observed with Nikon Eclipse TiE inverted epifluorescence microscope in the Centre for Microscopy at Concordia (CMAC). Photos were taken with 20X primary lens using LED RFP and GFP light sources at 555 nm and 480 nm excitation wavelengths respectively. GFP emission was 535/25 and RFP emission was 630/75. The images were taken using Photometrics CoolSNAP KINO CCD camera and processed using X3 and Imaje-J software.

Cloning

Truncated versions of RD20/CLO3 protein used for BiFC were generated by PCR reactions with the primers listed in Table 1 using a full length cDNA clone as a template. The reaction mixture included 0.2 mM dNTP, 2 mM $MgCl_2$, in 1X Taq buffer (Fermentas), 10 µM Forward Primer, 10µM Reverse Primer and 2.5 units of Taq Polymerase.

The N-terminal portion of RD20 without proline knot included amino acids 1-90 of RD20 protein. N-terminal construct of RD20 with proline knot spanned amino acids 1-126. The two C-

terminal constructs of RD20 spanned amino acids 116-236 and 124-236 for C-terminal domains with and without proline knot, respectively.

All constructs were cloned with some modification using Gateway technology method (Invitrogene:https://www.invitrogen.com/content/sfs/manuals/gatewayman.pdf) in which constructs were initially cloned by *in vitro* recombination of the experimental PCR product into either of two entry vectors, pDONR201 or pDONR207. Subsequently genes were transferred by *in vitro* recombination into plant expression vectors that expressed the gene as a fusion to the N terminal half of the yellow fluorescent protein (YFP) in the vector pBatTL-B-sYFP-N, or cloned the protein as a full length green fluorescent protein (GFP) in the vector pK7WGF2.

Stomatal Density and Index

Stomatal density and index are two different ways of quantifying stomata; stomatal density is typically reported as the number of stomata per mm^2, and stomatal index is the number of stomata divided by total number of cells (stomatal cells + non stomatal cells) (Tanaka et al. 2013). The Arabidopsis *rd20* and *gpa1* homozygous mutant lines in the Wassilewskija (WS) ecotype genetic background as well as wild type WS plants were sown on a soil mixture containing equal amounts of peat moss, black soil and vermiculite. Seeds sown on moist soil were, stratified (cold treated) at 4°C for three days and then moved to the greenhouse and maintained at 22°C with supplemental light used to extend the day length to 16 hours. Plants were treated with ABA as previously described by Nilson & Assmann (2010) with some modifications. Starting two days after germination, plants were sprayed three times a week with 25 μM ABA in 0.05% ethanol. The control plants were sprayed with 0.05% ethanol.

The first and second leaves from 15 day old plants were cut from the plant and fixed overnight in 9:1 mixture of ethanol and acetic acid. The samples were washed successively with 70%, 50% and 20% ethanol for 30 minutes each and then transferred to deionized water for 30 minutes. Each leaf was stained with 0.5% toluidine blue (TBO) for 5 minutes and washed three times with water. Photographs were taken from an area on each leaf halfway between the leaf tip and the base, and half way between the midrib and the leaf margin using the 40X magnification using primary lens of ZEISS Axioplan microscope. The images were taken using 3-1C 1.4 megapixel color cooled CCD camera. The first and second leaf from three plants were used for each genotype and three adjacent photos were taken per each leaf. Three plants per each genotype were used in this study.

Protein Expression and Purification

Full length cDNA clones of RD20/CLO3 and GPA1 with *E. coli* optimized codons and cloned into the *E. coli* expression vector pRsetA were ordered from Life Technology/Gene Art and transformed into *E. coli* BL21-DE3 cells using electroporation. Both RD20 and GPA1 proteins were expressed in *E. coli* cultures using the auto induction method (Studier, 2005) as follows: A 5 ml aliquot of an overnight culture was added to 800ml of ZY media (8 gram tryptone, 4 gram yeast extract in 800ml water) supplemented with 16 ml of 50X inducing solution (25% glycerol, 2.5% glucose and 10% a-lactose) and 16 ml of 50 X buffer M (1.25 M Na_2HPO_4, 1.25 M KH_2PO_4, 2.5 M NH_4Cl and 0.25 M Na_2SO_4) and 1.6 ml of 1 M $MgSO_4$ was added to the culture and incubated at 37°C for 8 hours then transferred to 18°C for 16 hours. The proteins were purified using the method of Pandey et al. in 2009 with some modification.

The overnight cultures of both GPA1 and RD20 were centrifuged for 30 minutes at 10,000 x g to collect the cell pellet. The bacterial pellet was re-suspended in the lysis buffer (0.25% NP40, Bacterial Protein Extraction Reagent (B-PER) reagent (Thermo Scientific), DNase I (Sigma-Aldrich) and Proteinase inhibitor (Sigma-Aldrich) for GPA1 and with the same buffer with addition of 1% tween for RD20 and incubated at room temperature for 15 minutes. The lysate was centrifuged for 25 minutes at 10,000 x g. The supernatant was incubated with equilibrated Nickel beads (GOLDBIO) in the lysis solution for 1 hour. The incubated sample on the nickel column was washed 5 times with three column volumes of wash buffer 1 containing 50 mM Tris HCl pH 7.5, 200 mM NaCl, 15mM Imidazol, 10% glycerol for GPA1 and with addition of 0.25% Tween 20 for RD20. The column was washed for three times with three column volumes of wash buffer 2 which contained 50 mM Tris-HCl PH 7.0, 300 mM NaCl, 30 mM Imidazol and 12% glycerol for GPA1 and with addition of 0.25% Tween 20 for RD20. The protein was eluted with 50 mM Tris-HCl pH 7.0, 300 mM NaCl, 300mM Imidazole and 12% glycerol for GPA1 and with addition of 0.1% Tween 20 for RD20.

Table 1. Primers used for cloning.

N terminal RD20 Forward Primer- 5' GGGGACAACTTTGTACAAAAAAGCAGGCTTC***ATGGCAGGAGAGGCAGAGGCTTT**
N Terminal RD20 with PK Reverse Primer 5' GGGGACCACTTTGTACAAGAAAGCTGGGTA**GTCGATATAAACCGGCAATAATGGTGATG**
N Terminal RD20 no PK Reverse Primer-5' GGGGACCACTTTGTACAAGAAAGCTGGGTA**ACCAAGGTCACGAAATCCCTTATAAGTCT**
C Terminal RD20 with Proline knot Forward Primer- 5' GGGGACAACTTTGTACAAAAAAGCAGGCTTC**ATG**GGA**GTGCCATCACCATTATTGC** *****
C Terminal RD20 Reverse Primer- 5' GGGGACCACTTTGTACAAGAAAGCTGGGTA**GTCTTGTTTGCGAGAATTGGCCCT**
C-Terminal RD20 No PK RD20 Forward Primer- 5' GGGGACAACTTTGTACAAAAAAGCAGGCTTC**ATG**GGA**ATCGACAACATACACAAAGCC**

The underlined sequences are gateway ends used to clone the sequences. The highlighted sequences are the extra start codons added to the C terminal fragments. The bolded regions are gene specific sequences. N terminal RD20 No Proline Knot primer set amplified amino acids 1-90 of the RD20 protein. N Terminal domain of RD20 with proline knot amplified the region of amino acids number 1-126 and included the membrane associated domain and proline knot primer. C Terminal RD20 with Proline knot primer set amplified amino acids number 116-236. C-Terminal RD20 No PK primer set amplified amino acids number 124-236.

Table 2. Plasmid constructs used for BiFC.

Construct name	Description
Tonoplast Marker; T-rk CD3-975	C-terminus of c-TIP, an aquaporin of the vacuolar membrane fused to the fluorescent protein
Endoplasmic Reticulum Marker; ER-rk CD3-959	Expresses the signal peptide of wall-associated kinase2, fluorescent at the N-terminus of protein and ER retention signal, His- Asp -Glu-leu, at the C- terminus.
Mitochondria Marker; Mt-rk CD3-991	The first 29 amino acids of yeast cytochrome C oxidase IV.
GPA1; Gα-pBatTL-B-sYFP-C	The α subunit fused to C terminal of YFP protein
P19	Protein of Tomato bushy stunt virus

The constructs provided by Dr. Gulick's lab

Table 3. List of the Constructs made for BiFC.

Construct name	Description
RD20 C- Terminal with proline knot pK7WGF2	Contains amino acids 116-236 of the RD20 protein fused to full length GFP
RD20 N-Terminal no proline knot pBatTL-B-sYFP-N	Contains amino acids 1-90 of the RD20 protein fused to N terminal half of YFP protein
RD20 C-Terminal no Proline knot pBatTL-B-sYFP-N	Contains amino acids 124-236 of the RD20 protein
RD20 C- Terminal with proline knot pBatTL-B-sYFP-N	Contains amino acids 116-236 of the RD20 protein fused to N terminal half of YFP protein
RD20 N-Terminal with proline knot pBatTL-B-sYFP-N	Contains amino acids 1-126 of the RD20 protein fused to N terminal half of YFP protein

Results

Protein-protein Interaction

BiFC assays with truncated versions of RD20 indicated that the N-terminal domain of RD20 protein interacts with GPA1. N-terminal portions of the protein up to amino acid 90 which included the EF calcium binding domain, was sufficient for interaction with GPA1 when the constructs were co-expressed in *N. benthamiana* leaf epidermal cells (Figure. 1. A). The larger N terminal truncation construct (amino acids 1-126), which included the membrane associated domain and proline knot in addition to the calcium binding domain, also showed interaction with GPA1 (result not shown). In contrast, the 120 aa C terminal portion of the RD20 protein, from amino acid 116 to the C terminal end, including most of the proline knot, did not show any interaction with the G protein (Figure 1. B). An additional construct for C-terminal domain without proline knot (amino acid 125-236) also did not interact with GPA1 (result not shown). Since the lack of BiFC signals with C-terminal fusions could be due to the mislocalization of the fusion proteins, additional expression assays were performed using the C-terminal portion of the RD20 protein with proline knot (amino acids 116-236) fused to full length GFP (Figure 2). Previous work in our lab showed that full length RD20 protein fused to GFP localized in endoplasmic reticulum and tonoplast and that it interacts with GPA1 in the endoplasmic reticulum (Zhe Jun Wang MSc thesis 2009). The C-terminal RD20-GFP fusion also localized to the endoplasmic reticulum and tonoplast (Figure 2). Therefore, it can be concluded that the lack of interaction with GPA1 was not due to mislocalization.

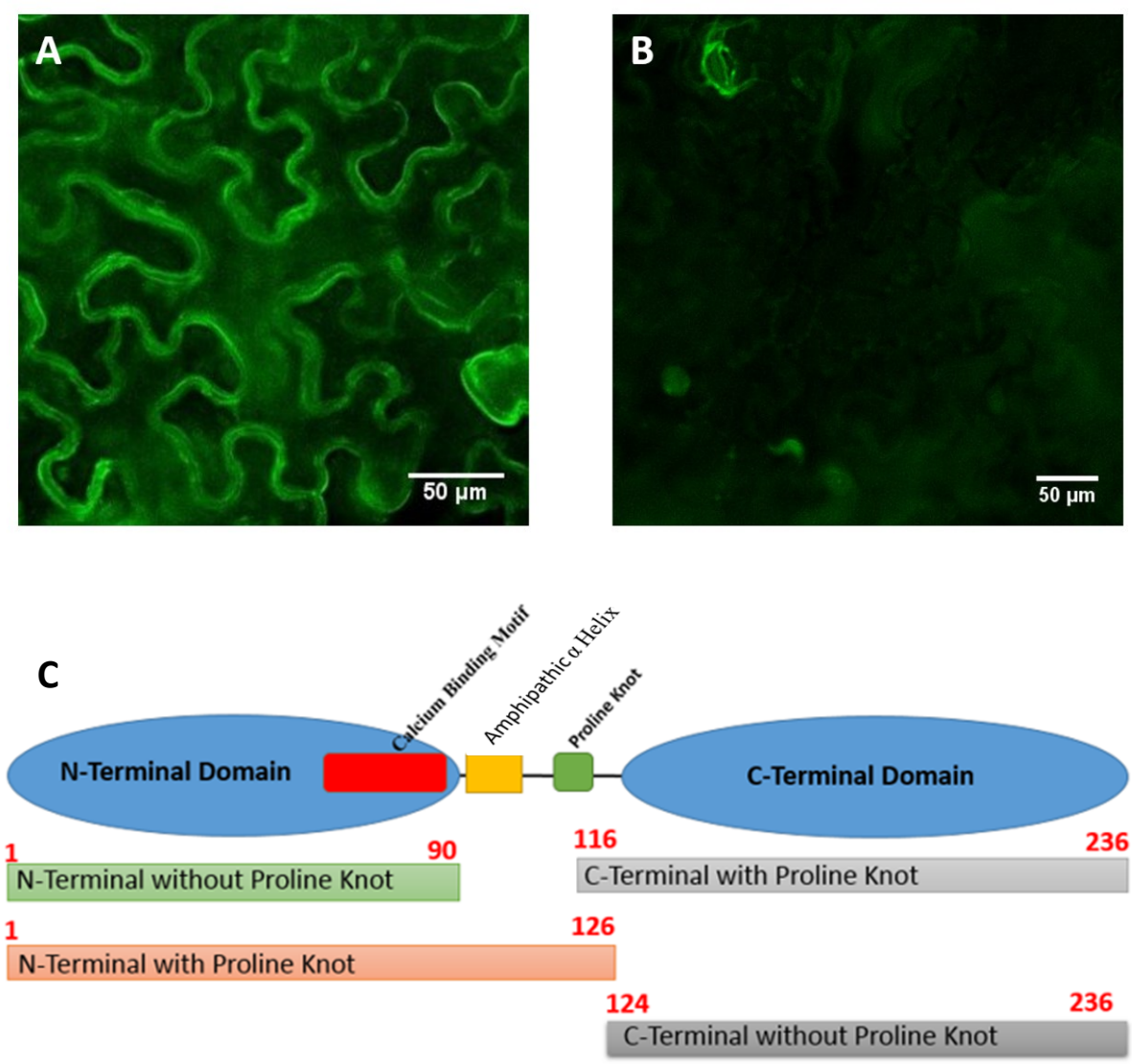

Figure 1. Protein-protein interaction assayed by BiFC in *Nicotiana benthamiana* leaf. A) N-terminal domain of RD20 protein without proline knot fused to N-YFP interaction with GPA1 fused to C-YFP. **B)** C-terminal domain of RD20 protein with proline knot fused to N-YFP with GPA1 fused to C-YFP. **C)** Truncated constructs shown in the figure. The numbers on each construct indicates the amino acid numbers. Images were taken under 20X magnification primary lense. The scale bar represents 50 µm on the image.

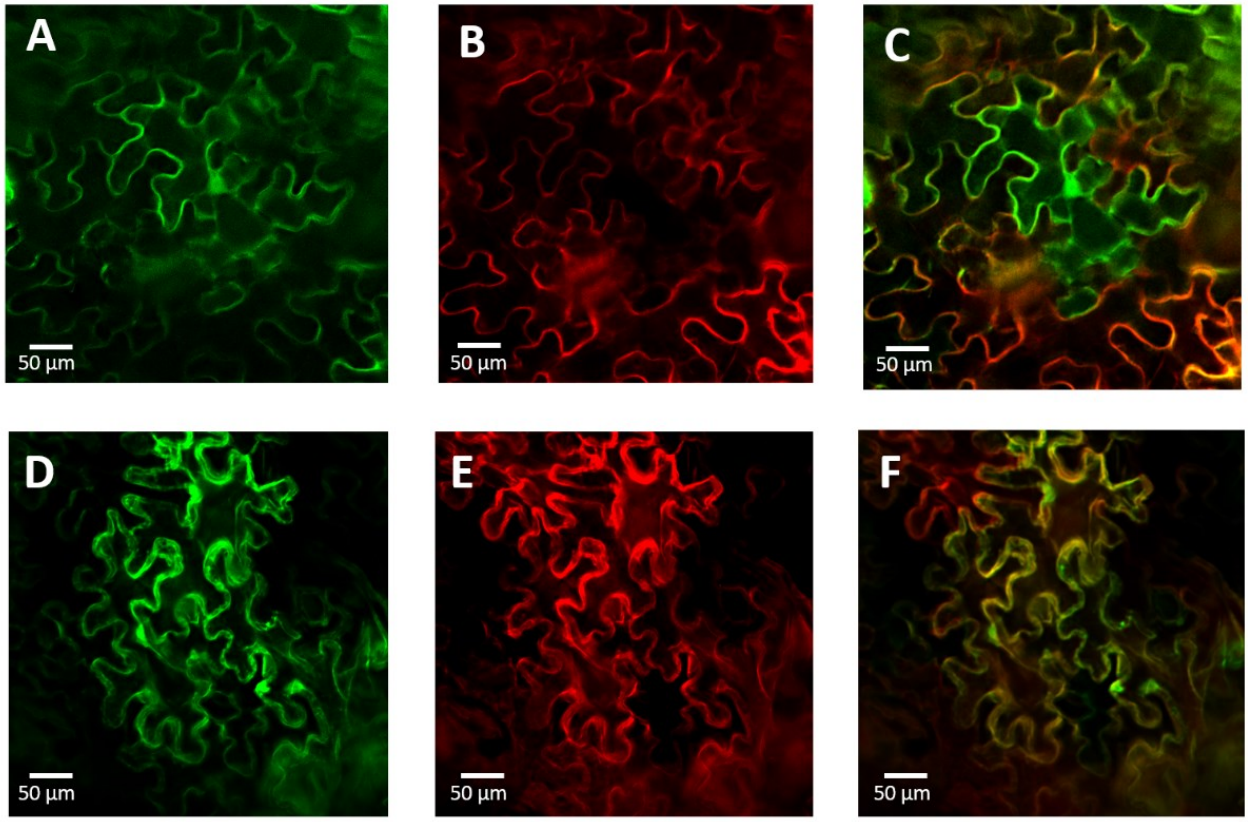

Figure 2. Localization of RD20 C-Terminal in *Nicotiana benthamiana* leaf. A) Truncated C terminal portion of RD20 with proline knot fused to full length GFP. **B)** Tonoplast marker. **C)** Overlapping image showing both Truncated C terminal portion of RD20 with proline knot fused to full length GFP and Tonoplast marker. **D)** Truncated C terminal portion of RD20 with proline knot fused to full length GFP. **E)** Endoplasmic Reticulum Marker. **F)** Overlapping image showing both Truncated C terminal portion of RD20 with proline knot fused to full length GFP and Endoplasmic Reticulum Marker. The scale bar represents 50µm on the image.

Stomatal Density

ABA treatment significantly decreased the stomatal density and stomatal index in wild type Arabidopsis plants (Figure 3), an effect that had been previously reported in several studies (reviewed in Caspar et al. 2013). Analysis of the mutant lines *rd20* and *gpa1* indicate that both of these genes affect stomatal development and the plant's response to ABA treatment. The mutant line *rd20* showed lower stomatal density and stomatal index than WS wild type plant in control conditions (Figure 3). The mutant line, *rd20,* showed no change in stomatal density after ABA treatment and had a higher stomatal index. The number of non-stomatal cells was lower in ABA treated *rd20* plants (Table 6). The *gpa1* mutant line had lower stomatal index than the wild type and also lower stomatal density as was previously reported by Nilson & Assmann (2010). The *gpa1* mutant line did not show a statistically significant change in stomatal density in response to ABA, (though it did have a small decrease in density) suggesting that GPA1 also plays a role in the ABA regulation of stomatal development. The *rd20* and *gpa1* mutants showed opposite effects; *rd20* showed an increase in stomatal index in response to ABA, whereas *gpa1* showed a decrease, albeit a statistically insignificant change.

The distribution of stomata on the lower leaf epidermis in *Arabidopsis thaliana* is not uniform. Different stomatal density and indices were observed in different parts of the leaf. There are more stomata at the areas closer to the petiole (leaf stem) and less stomata closer to the leaf tip. There is also more variance in stomatal density at the two ends of the leaf when leaves of the same developmental stages on different plants are compared. For accurate comparison of stomatal density and index, it is important to take microscopic images from sites on the leaves that are less variable and to take the measurements from the same areas of different leaves. Considering that the age of the leaf, the leaf number and leaf size can affect stomatal density. In this study leaves

of the same age, number and size were selected for the stomatal density and index assays (*N.B.* Leaves emerge from the meristem in a sequential and ordered fashion, leaf number indicates the order of leaf's emergence. Leaves with different leaf number have different size at maturity).

The representative images show that both *rd20* and *gpa1* have reduced stomatal density relative to wild Type plants in control conditions (Figure 4). In these experiments the first and second true leaves on 15 days old plants were selected for determining stomatal density parameters, leaves at this age were approximately 1 cm long.

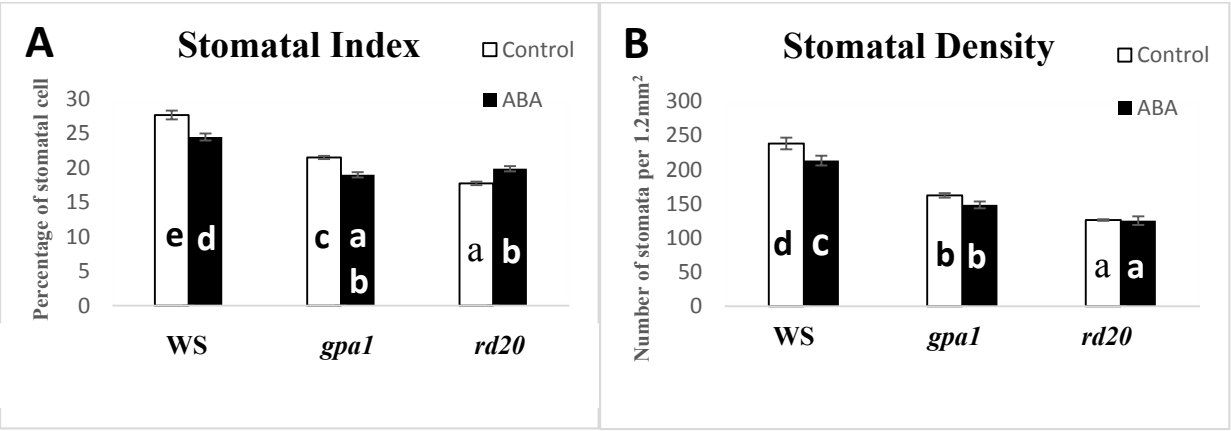

Figure 3. Stomatal Density and Stomatal Index in *gpa1* and *rd20* mutants. A) Stomatal Index for each genotype both control plants (white bar) and ABA treated plants (black bars) are shown. The number on the Y axix coresponds to the percentage of stomata per total numbers of cells. **B)** Stomatal Density. The X axis is showing genotypes and the Y axis is showing nubers of stomata per 1.2mm^2. ABA treated plants are shown by black bars and control plants are shown by white bars**.** Letters represents values from Duncans multiple comparison test. The error bars on the graphs are standard erors.

Table 4. 2-way Anova Table for Stomatal Density.

Source	df	F	Sig.
Intercept	1	4428.700	.000
Treatment	1	6.839	.010
Genotype	2	135.370	.000
Treatment * Genotype	2	1.866	.161

Dependent Variant: Stomatal Density

Table 5. 2-way Anova Table for Stomatal Index.

Source	df	F	Sig.
Intercept	1	12572.451	.000
Treatment	1	9.481	.003
genotype	2	130.579	.000
Treatment * genotype	2	18.646	.000

Dependent Variant Stomatal Index

Table 6. Number of stomata and non-stomatal cells per 1.2mm^2

Genotype	Treatment	Non-Stomatal cells	Stomatal cells per (Stomatal Density)	Stomatal Index
WS	Control	627	238	27.6
WS	ABA	660	213	24.4
gpa1	Control	594	162	21.4
gpa1	ABA	631	148	18.9
rd20	Control	585	126	17.7
rd20	ABA	507	125	19.8

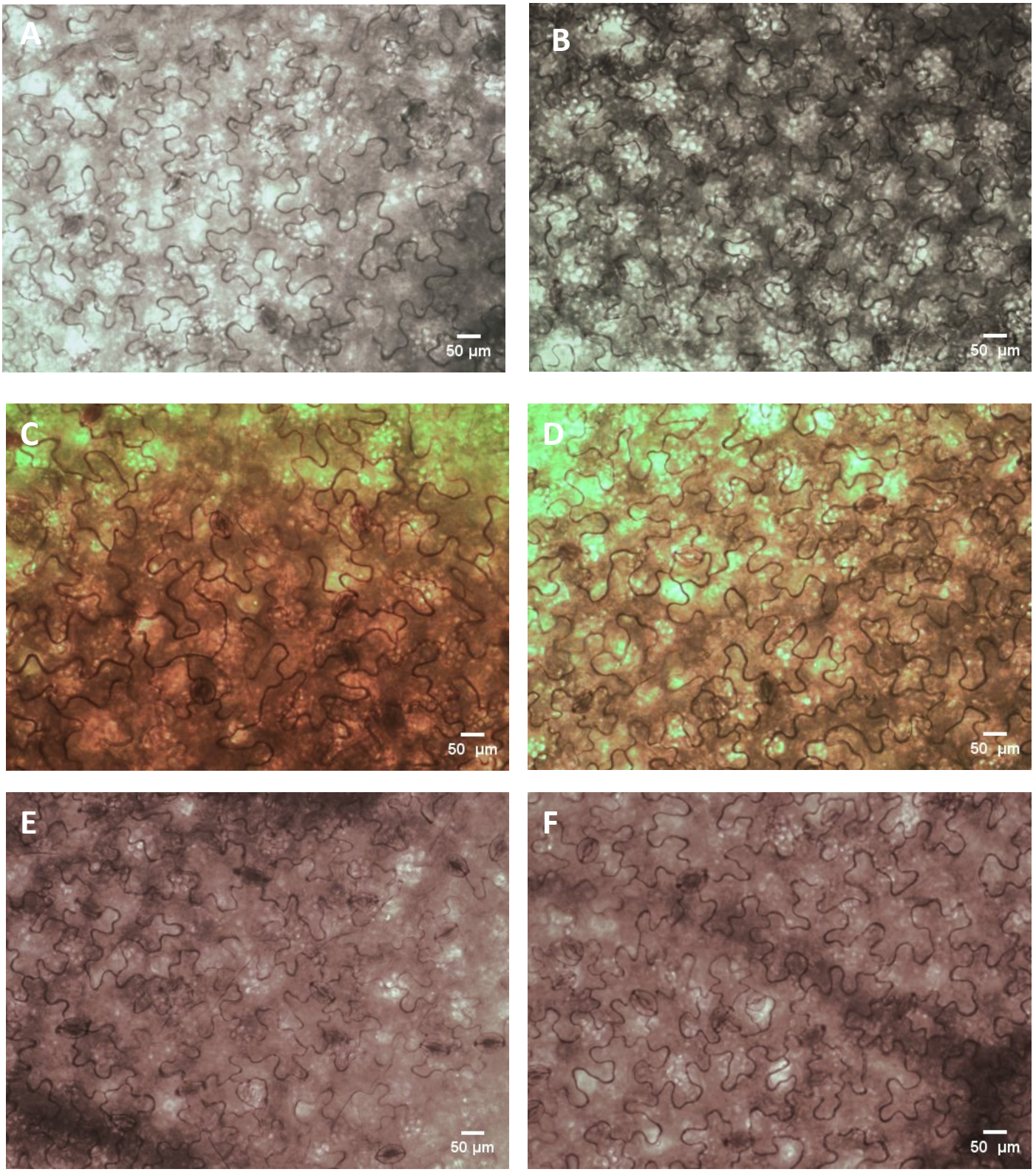

Figure 4. Representitive images for Stomatal Density and Index. A) Leaf of 15 days old *gpa1* mutant plant treated with ABA. **B)** *gpa1* mutant plant Control. **C)** Leaf of 15 days old *rd20* mutant

plant treated with ABA. **D)** *rd20* mutant plant Control. **E)** Wild Type (WS) treaed with ABA. **F)** Wild type plant control. The scale bar represents 50μm.

Protein Purification

High levels of expression were achieved for both proteins, RD20 and GPA1, by changing the codon bias of the clones and by modifying the conditions of culture in *E. coli* BL21-DE3 (Figure 5). A number of parameters were tested in an attempt to increase the levels of expression of the protein and marked increases production of soluble protein was achieved. We found that higher levels of both RD20 and GPA1 proteins, could be achieved by using the auto-induction method at 18°C (Studier 2005) which relies on lactose as an inducer instead of conventional IPTG induction protocol (data not shown). Different methods of lysis such as sonication, French Press and different lysis chemicals were used to lyse the bacteria before other parameters were changed, but these did not result in higher levels of protein and resulted with most protein being insoluble and being recovered mostly in the pellet. The proteins were plant proteins and their expression in *E. coli* may result in binding to a membrane and rendering the protein insoluble. They may make inclusion bodies and aggregate in the cell. Both *RD20* and *GPA1* genes were resynthesized with optimized codon by Life Technology/ Gene Art. The change of codons improved protein production significantly and resulted with most of the protein being recovered in the soluble fraction. Although the experimental proteins were the dominant component after elution from nickel beads (Figure 5), there were numerous other non-specific protein bands observed on the gel.

Different concentrations of IPTG and different temperatures were used for inoculating the sample but these procedural changes did not affect expression yield and solubility. With various expression tests used in this study, the protein was mostly detected in the bacterial cell pellet which suggested aggregation of the protein and formation of inclusion bodies. The constructs were also transformed and expressed in a different *E. coli* cell line (BL21 DE3+ PRIL) in which formation of inclusion body was also presence and the protein was mainly recovered in the bacterial cell pellet after lysis. Different incubation times after induction with IPTG were assayed; both proteins were expressed but the protein was not soluble and the yields were relatively low.

To increase probability of a more highly purified protein from nickel affinity column, another washing step with 40mM imidazole for the RD20 samples was tested. The additional wash step was predicted to elute some non-specific binding of proteins to nickel beads but it was seen that it did not increase the purity and it reduced the yield of RD20 by half in the final elution. The inclusion of Tween 20 in the lysis buffer increased the solubility of RD20 and resulted in higher protein yield.

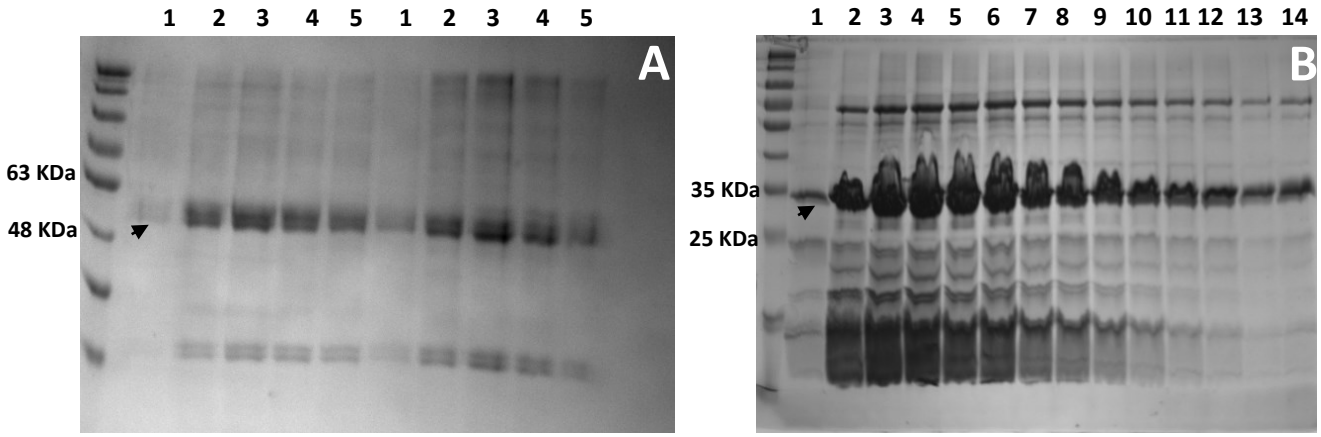

Figure 5. SDS page gel showing purified GPA1 and RD20. A) Purified GPA1 protein shown by arrow. The numbers on the top of the gel presents the different elution fractions collected from the Nickel bead columns. Numbers 1-5 are from two different cultures. **B)** Purified RD20 protein shown by arrow. The numbers on the top of the gel presents different elution fractions of RD20 protein. Expected molecular weight for RD20 and GPA1, including the 3 KDa added from the vector are 29.6 KDa and 47.5 KDa, respectively. The differences observed and expected in sizes are due to absence of DTT in the sample.

Discussion

Protein-Protein interaction

Both protein truncation constructs that included N-terminal domain of RD20 showed interaction with GPA1. The construct which expressed N-terminal portion of RD20 up to amino acid 90 showed that the N terminal domain containing the calcium binding EF hand is sufficient for the interaction (Figure 1A). Considering that interacting sites between G protein subunits α, β and γ are known (Temple & Jones 2007), investigating the interacting domains of RD20 proteins that interact with GPA1 is an important initial step in understanding the domains of GPA1 that may interact with RD20 and initialize understanding RD20's potential role as a GAP protein or as a competitive or synergistic binding partner in the G-protein complex.

The C terminal domain of RD20 did not interact with GPA1. Neither constructs expressing amino acids 124-236 (without proline knot) and 116-236 (with proline knot) of RD20 protein showed any interaction with GPA1 (Figure 1B). It can be concluded that membrane associated domain and proline knot and the C terminal part of the protein are not important regions of the protein for interaction with GPA1. In addition, the C-terminal site of the protein with proline knot is sufficient to localize the protein in ER and tonoplast (Figure 2). The protein was also expressed in other organelles but the overlapping images showed most of the localization in endoplasmic reticulum. In our lab it was reported that full length RD20 protein is also localized in ER and tonoplast (Zhe Jun Wang, thesis 2009). Localization of the truncated C-terminal domain protein also showed localization to the same two organelles. This suggests that the C-terminal domain may be sufficient for localization of the RD20.

Since there is no X-ray crystallography data available for RD20, it is not possible to propose protein model for RD20 at this point. Based on Chen et al. 1999, caleosins are divided by central domain which consists of an amphipathic α helix and proline knot (Chen et al. 1999). N-terminal domain of the protein contains calcium binding motif while C-terminal has phosphorylation site (Chen et al. 1999). Considering the proposed model for caleosins in which the protein structure is divided into N terminal and C terminal domains separated by a membrane associated domain (Chen et al. 1999) it is possible that they play different roles, that of protein-protein interaction and protein localization.

Stomatal Density

The study on stomatal density showed that ABA treatment decreases stomatal density in Arabidopsis leaves and that *RD20* is required for this response. The significant decrease in stomatal density observed in wild type plants after ABA treatment was not seen in the *rd20* mutant line. The mutant line *rd20* also had significantly lower stomatal density than wild type plants in control conditions which indicates that RD20 also plays a role in stomatal initiation in non-stress conditions in Arabidopsis. Nilson and Assmann showed that *GPA1* also plays a role in stoma development, in that *gpa1* mutants have decreased stomatal density relative to the wild type plant (Nilson & Assmann 2010). The protein-protein interaction data reported here indicate that GPA1 and RD20 may act in the same regulatory pathways and the stoma initiation regulatory network is a promising model to further study the consequence of the GPA1 RD20 interaction. Only by comparing stomatal densities observed for both *gpa1* and *rd20* mutant lines it is not possible to know if they are acting in the same pathway. If double mutant plants show additive effect by

having lower stomatal density than the individual mutant lines, it would indicate that the proteins act in different pathways. It would be important to investigate this possibility further by comparing single and double mutant lines or other forms of double gene suppression such as RNAi lines. In this study *rd20* mutant line had lower stomatal density compared to wild type which is in contradiction with the previous study in our lab which showed higher stomatal density for *rd20* mutant line (Justin Wright MSc Thesis, 2014). The difference in the two experiments was mainly age of the leaf. In Arabidopsis, older leaves have lower ABA concentration, and are suggested to have higher sensitivity to ABA, at least in the regulation of stomatal aperture (Caspar et al. 2013). Here effect of the genotype and treatment were investigated on first and second leaf of Arabidopsis. Since leaves of different ages have different sensitivities to ABA (Caspar et al. 2013) different result may have been seen if older leaves were measured in the study. The differences in the results might be due to reduced sensitivity to ABA suggested by Caspar et al 2013. This warrants further study since these results suggest that the role of RD20 in the regulation of stomatal density is complex and may differ between younger and older leaves.

The stomatal index was significantly lower in ABA treated WS and *gpa1* than in untreated plants while the stomatal index observed for *rd20* mutant line under ABA treatment was higher than that under control conditions. The fact that *rd20* mutant line had lower numbers of non-stomatal cells in the ABA treated plants and the density of stomata did not change after the treatment (Table 6) indicates that *rd20* mutant line had reduced division of non-stomatal cells and that RD20 promotes both the division of non-stomatal cells and partially inhibits the rate of stomatal cells differentiation.

Protein Expression and Purification

The changes to the methods of protein expression in *E. coli* that lead to the highest level of protein expression were the change of the codon bias of the cDNAs from the original Arabidopsis sequence to that for improved expression in *E. coli*, and the use of lactose auto-induction during the culture. The His-tagged proteins purified from a nickel column co-eluted with many contaminating proteins (Figure 5). Although expression of both GPA1 and RD20 was dominant compared to other proteins coming from *E. coli,* the level of purity is not high enough to assay GTPase activity.

In such an assay that measures phosphate released upon GTP hydrolysis, if there is any non-specific nuclease contamination in the sample, the level of phosphate can be augmented the ability of non-specific nucleases to hydrolyze GTP lead to incorrect measure of GTPase and GAP activity. To avoid false positive result coming from nuclease activity it is important to further purify the proteins to eliminate contaminant. It is also important to perform a control ATPase assay in parallel with GTPase assay to compare the level of released phosphate in both. The ATPase activity is an indication of contaminating non-specific nucleases in the proteins preparation.

The work on protein purification presented here is initial step to purify both RD20 and GPA1 protein at a high purity level to support and validate previously reported GAP activity of RD20 reported by Justin Wright MSc Thesis 2014 on proteins purified from Nickel column.

Future Work

To verify role of each domain of RD20 it is important to characterize the amino acids responsible for the activity of each domain. This can be done by screening candidate amino acids in each domain and by using site directed mutagenesis to identify amino acids that are critical for interaction and activity. It is also important to do experiments with truncated versions of GPA1 and the full length and truncated versions of RD20 to determine the sites on GPA1 that interact with RD20. By studying site of interaction in the two proteins it will be possible to further characterize the role of RD20 in the regulation of G protein complex. It is also important to assay competition and synergy between caleosins and other GPA1 interacting partners, such as Gβ (AGB1) and Gγ (AGG1) to investigate whether members of the gene family regulate GPA1 as a GAP or if they interfere with interaction between the three subunits of the G protein complex.

For stomatal density and index, it is necessary to increase the sample size for the preliminary assay to characterize the consistent region in terms of stomatal count for accurate calculation of stomatal density and index. It is important to repeat the experiments to compare leaves at different ages and sizes. Previous analysis indicated that *rd20* mutant plants had higher stomatal density than the WT plants when older leaves were assayed, (Justin Wright, Thesis 2014). It is also important to perform similar assay as described in the thesis for stomatal density and index for mutant lines for other members of the caleosin gene family and also Gβ, and Gγ to investigate possible components of the regulatory pathways that control stoma development and plants response to ABA.

After successfully purifying large amount of proteins for both GPA1 and RD20, The purified proteins can be used for second or third step purifications using HPLC. The proteins can

be subjected to anionic exchange chromatography and size exclusion chromatography. If high levels of proteins were obtained from each step it would be also possible to perform both chromatography techniques in tandem to increase purity level of the protein. Since it is expected to eliminate all the contaminated bands that co-purifies with the proteins, the level of purification will be very high. Those proteins can be used for assaying GAP activity of RD20 toward GPA1.

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