

**The interaction between the G α subunit of the heterotrimeric G protein complex
with calcium binding proteins, AtClo3/RD20, and AtClo7 from Arabidopsis and
TaClo3 from wheat, *Triticum aestivum***

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ABSTRACT

The interaction between the $G\alpha$ subunit of the heterotrimeric G protein complex with calcium binding proteins, AtClo3/RD20, and AtClo7 from Arabidopsis and TaClo3 from wheat, *Triticum aestivum*

ZHE JUN WANG

The alpha subunits of heterotrimeric G proteins and calcium binding proteins play important roles in signal transduction and cellular regulation in plants. However, the molecular function of $G\alpha$ proteins in plants is still poorly understood. In order to understand the role of $G\alpha$ proteins in signaling pathways, the downstream effectors and upstream regulators need to be investigated. Here we report the first study of interactions between a canonical $G\alpha$ subunit, GPA1, and calcium binding proteins in plants. Two of these proteins in Arabidopsis are caleosins, RD20/AtClo3 and AtClo7, which are small Ca^{2+} binding proteins with single EF hand domains. Interactions between the two calcium binding proteins and $G\alpha$ were studied using bimolecular fluorescence complementation (BiFC) *in vivo*. RD20/AtClo3 and AtClo7 localized to the ER and tonoplast, however interactions between RD20/Clo3 and Clo7 with $G\alpha$ were detected in the ER and PM respectively. A similar approach was used to determine the interaction between TaClo3, a homolog of the caleosins, and TaGA3, a $G\alpha$ from wheat. Similar to Arabidopsis caleosins, the TaClo3 protein also localized to the ER and tonoplast. Interactions between TaClo3 and TaGA3 were detected by BiFC in *Nicotiana benthamiana*, though their localization is not clear.

Wheat is hexaploid and is expected to contain more than one $G\alpha$. The detection of the five $G\alpha$ genes, some of which contain small insertions in the coding

region, suggests a diversity of functions among $G\alpha$ wheat homologs. The role of proteins is discussed as a basis for future studies.

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PART I. INTRODUCTION

1. Heterotrimeric G protein signaling in animals and plants

Heterotrimeric G proteins are involved in multiple cellular responses in animals and in a variety of signaling events in plants initiated by the G protein-coupled receptors (GPCR). The G protein complex is composed of three subunits, $G\alpha$, $G\beta$, and $G\gamma$ and in mammals each of the genes encoding the subunits comprises gene families. In the human genome there are 23 $G\alpha$ -, 6 $G\beta$ -, and 12 $G\gamma$ -encoding genes, as well as 37 genes encoding regulator of G-protein signaling (RGS) proteins and about 800 genes with significant sequence similarity to G protein-coupled receptors (GPCRs) (Jones and Assmann, 2004). Distinct G proteins can be involved in separate signaling pathways. For example, different heterotrimeric G proteins are involved in controlling cardiovascular activity, urine output and vasopressin secretion (Richard et al., 2008). In plants, G proteins are also involved in signaling pathways regulated by abiotic and biotic stresses such as pathogens responses (Beffa et al., 1995; Blumward et al., 1998), the light-signaled response (Assmann, 2002; Barnes et al., 1997; Lapik and Kaufman, 2003), high salinity and drought (Misra et al., 2007). In addition, G proteins are involved in signaling pathways regulated by a number of plant hormones including jasmonic acid (JA) (Okamoto et al., 2009), gibberellin (GA) (Ullah et al., 2003), and abscisic acid (ABA) (Pandey et al., 2006; Ritche and Gilroy, 2000; Wang et al., 2001). However, unlike mammals, plants have very few heterotrimeric G proteins; the model species *Arabidopsis* has genes encoding one $G\alpha$ -, one $G\beta$ -, and two $G\gamma$ -subunits (Jones and Assmann, 2004).

The heterotrimeric GTP-binding protein (G protein) complex transduces signals through guanine nucleotide exchange and hydrolysis at the inside surface of the cell membrane. In the inactive state, $G\alpha$ tightly binds with the $G\beta\gamma$ subunits and a G protein coupled receptor (GPCR). When a ligand activates the GPCR, G proteins bound to GPCR undergo a conformation change. The $G\alpha$ subunit releases GDP, and binds GTP, resulting in the dissociation of the $G\alpha$ subunit from the $G\beta\gamma$ subunit and GPCR. $G\alpha$ -GTP and the $G\beta\gamma$ can then activate downstream signaling cascades and effectors. The hydrolysis of the GTP bound by $G\alpha$ to GDP by the GTPase activity of $G\alpha$ itself allows the reconstitution of the inactive heterotrimeric complex (Hamm, 1998; Sprang, 1997). In plants, in contrast to animals, $G\alpha$ is hypothesized to be GTP-bound in its resting state and have GTP hydrolysis as a rate limiting step in its modulation (Johnston et al., 2007). The role of effector proteins associated with $G\alpha$ is an important area of inquiry as few proteins are known to interact with $G\alpha$ in plants.

2. The $G\alpha$ subunit in wheat interacts with a stress regulated protein

Bread wheat (*Triticum aestivum*) is one of the two major cereals world wide and also is the most widely adapted major crop with respect to harsh environmental conditions (Tardif et al., 2007). It is among the crops that are most tolerant to low temperatures, with the potential to survive temperatures as low as -21°C . However, tolerance to such extreme temperatures can only be achieved by a period of acclimation, in which plants are exposed to low, non-freezing temperatures. Full acclimation normally can be achieved by a period of 14 days of exposure to 4°C . Gene expression

studies and microarray analysis during the period of acclimation have identified many genes whose expression is changed by low temperature treatment. Several hundred genes are either up- or down-regulated in wheat in response to cold treatment and a significant fraction of these have more strongly induced or repressed in cold tolerant winter wheat than in freezing sensitive spring wheat (Gulick et al., 2005; Monroy et al., 2007). Microarray analysis showed that TaClo3 transcript was regulated differently during cold acclimation in spring and winter wheat (Gulick et al., 2005). Subsequent yeast two-hybrid screening studies identified a protein-protein interaction between TaClo3, the cold induced calcium binding EF-hand protein (Gulick et al., 2005), and the G protein alpha subunit, TaGA1 (Tardif et al., 2007). This protein-protein interaction suggests that the G α protein may be involved in signaling pathways regulating in response to cold in wheat. Single genes encoding G α s have been identified in the genomes of the model species Arabidopsis and rice, the first plants to have a completed genome sequence (Jones and Assmann, 2004). Two G α s have been identified in pea (Marsh and Kaufman, 1999), soybean (Gotor et al., 1996; Kim et al., 1995), and tobacco (Ando et al., 2000; Saalbach et al., 1999). Two G α cDNA clones, TaGA1 and TaGA2, were isolated from common wheat cultivar cv. S615 (Hossain et al., 2003). TaGA1 and TaGA2 differ from each other by the presence of a 21 bp insertion in the coding region of TaGA2. Aside from the insertion, they share 99.4% nucleotide identity in the coding region.

3. Heterotrimeric G-proteins signaling in Arabidopsis

Genetic studies in wheat are challenging due to difficulties in transformation and

mutagenesis and the hexaploid nature of wheat. The orthologous genes can be studied in the model species *Arabidopsis*, and mutants for such genes may be identified in existing T-DNA mutant collections. The homolog to wheat $G\alpha$ subunit of wheat in *Arabidopsis thaliana* is GPA1, which share 72% amino acid identity and 86% amino acid sequence similarity.

The *Arabidopsis* genome encodes one $G\alpha$, one $G\beta$, two $G\gamma$ subunits, one regulator of G-protein-signaling (RGS) protein, and 4 GPCRs (Jones and Assmann, 2004). Recently, Pandey et al. (2009) reported that two ABA receptors, GPCR-type G proteins, GTG1 and GTG2. ABA is a plant hormone that acts in the stress response pathways tied to osmotic stresses and low temperature responses. GTG1 and GTG2 bind to ABA and AtGPA1 and have GTPase activity like AtGPA1. Interestingly, AtGPA1 accelerated the GTP-binding of GTG1 and GTG2 but strongly inhibited their GTPase activity.

$G\alpha$ modulates cell division in the primary root meristem and free $G\beta\gamma$ is proposed to negatively regulate the formation of lateral roots (Chen et al., 2006; Perfus-Barbeoch et al., 2004). In addition, there is evidence that G proteins are involved in ABA signaling in barley (Ritche and Gilroy, 2000) and in *Arabidopsis* (Lapik and Kaufman, 2003; Pandey et al., 2006), light signaling pathways (Barnes et al., 1997), and in the regulation of ion channels in guard cells (Assmann, 1996). Lapik and Kaufman (2003) reported that seedlings of *gpa1* mutants had delayed expansion and greening in the cotyledons. Microarray analysis of gene expression comparison between $G\alpha$ loss- and gain-of-function *Arabidopsis* mutant lines showed that GPA1 is involved in the signaling

pathway of the defense and stress associated plant hormone, jasmonic acid (JA) response (Okamoto et al., 2009).

Protein-protein interaction approaches have accelerated the study of components of signaling and regulation of the stress responses at the cell and molecular level. To date, only four upstream G protein coupled receptors-like have been shown to interact with G α subunit, AtGCR1 (Pandey and Assmann, 2004), G protein signaling protein 1 (AtRGS1) (Chen et al., 2003), GTG1 and GTG2 (Pandey et al 2009). Previous studies showed that there are a few known downstream effectors of the plant G α subunit. GPA1 interacts with the Atpirin1, Arabidopsis cupin domain protein, which regulates seed germination and seedling development (Lapik and Kaufman, 2003), phospholipase A₂ (PLA₂) of *Eschscholzia californica* (Heinze et al., 2007), phospholipase D (PLD α 1) (Zhao and Wang, 2004), prephenate dehydratase protein (PD1) (Warpeha et al., 2006) and the plastid protein thylakoid formation 1 (THF1) (Huang et al., 2006).

4. The properties of Arabidopsis RD20 and Clo7

The calcium binding protein TaClo3 interacts with TaGal1 as determined by yeast 2-hybrid assay (Tardif et al., 2007). The closest homologs to TaClo3 in Arabidopsis are RD20/AtClo3 (At2g33380) and AtClo7 (At1G23240) which shared 57% and 61% amino acid sequence similarity to TaClo3 respectively. The RD20 gene and AtClo7 gene encode proteins of 236 and 174 amino acid residues with a single EF-hand Ca²⁺-binding domain. They belong to the caleosin superfamily with at least 7 family members in

Arabidopsis (Inmaculada et al., 2001). Caleosins were initially identified as a minor group of proteins abundant in oil bodies, with oleosins being the most abundant class of oil body proteins (Huang, 1992). All seven caleosins have an EF-hand, a hydrophobic transmembrane domain, a proline knot, tyrosine kinase binding site and casein kinase phosphorylation sites (Chen et al., 1999; Partridge and Murphy, 2009). Caleosin 1 (AtClo1) has peroxygenase activity and involved in lipid oxidation tied to the production of biologically active compounds referred to as oxylipins (Hanano et al., 2006). Based on NASCArrays microarray data RD20/AtClo3 mRNA is most abundant in sepals, senescing leaves and siliques. The members of the Clo gene family are grouped into two classes based on the position of their hydrophobic regions (Hanano et al., 2006). RD20 was grouped into class II in which the hydrophobic region is located centrally; AtClo7 (At1g23240) is in class I in which the hydrophobic region is located near the N terminus of the protein (Hanano et al., 2006). Several studies showed that caleosins are ER-bound, vacuole membrane-associated and lipid-associated proteins by immunofluorescence (Huang, 1992; Inmaculada et al., 2001; Partridge and Murphy, 2009).

RD20 was shown to bind Ca^{2+} by electrophoresis mobility shift and is expressed in aerial tissues, especially in leaves and flowers and under stress conditions such as dehydration, high salt, and ABA treatment (Partridge and Murphy, 2009; Takahashi et al., 2000). In addition, three ABA-responsive element (ABRE) sequences were found in the 1-kb 5'-flanking promoter region of RD20 (Takahashi et al., 2000). ABRE have previously been shown to regulate ABA responsive gene expression (Marcotte et al., 1989; Skriver and Mundy, 1990; Yamaguchi-Shinozaki et al., 1990). Microarray

analysis found that the RD20 gene is up regulated in lines overexpressing the NAC transcription factor RD26 (Fujita et al., 2004). RD26 is up regulated in response to dehydration, NaCl, and ABA treatment, and is involved in ABA-dependent signaling (Fujita et al., 2004). Together, these data suggested that RD20 may be involved in ABA-dependent signaling pathways. Other microarray studies showed that RD20 is up-regulated at an early stage after cold treatment and strongly down-regulated during cold deacclimation (Oono et al., 2006). There have been no reports describing the phenotype of RD20 mutants.

The AtClo7 (At1g23240) gene product is abundant as a pollen coat protein (Mayfield et al., 2001). The proposed function of AtClo7 is similar to RD20/AtClo3 since they share conserved domains and belong to a same caleosin family. However, there is no experimental evidence to demonstrate the function of Clo7. Microarray analysis showed the Clo7 was down regulated in the male sterility 1 (MS1) mutants (Takuya et al., 2007). MS1 belongs to the NAC transcription factor family and is important for transcriptional regulation for postmeiotic pollen maturation. The microarray data suggests that MS1 upregulated Clo7 expression indirectly (Takuya et al., 2007).

5. Bimolecular fluorescent complementation (BiFC)

Bimolecular fluorescent complementation (BiFC) is a technique for visualization of protein-protein interaction in living cells. This method has been successfully used in a

variety of expression systems in different organisms including yeast and plants. This approach relies on the formation of a fluorescent complex by two non-fluorescent fragments of the enhanced yellow fluorescent protein (eYFP) or its derivatives (Hu et al., 2002; Hu and Kerppola, 2003). The two non-fluorescent candidate proteins are engineered to be expressed as fusions to either the N-terminal or C-terminal portion of eYFP. The constructs are transformed into *Nicotiana benthamiana*, a wild tobacco species, by *Agrobacterium* infiltration and the yellow fluorescence is restored by the interaction of the two candidate proteins and the cellular location of the protein-protein interaction can be visualized microscopically (Hu et al., 2002; Hu and Kerppola, 2003). The two non-fluorescent fragments of YFP do not interact by themselves; if the two non-fluorescent candidate proteins do not interact, the full length eYFP protein is not reconstituted and no eYFP signal appears in the sample. *Agrobacterium* infiltration gives very high rates of transformation in which nearly every infiltration leads to expression in some cells. The BiFC technique allows the expression and visualization of multiple protein complexes in the same cell (Hu and Kerppola, 2003) and the localization of the BiFC can be combined with known markers.

6. Objectives

Yeast two hybrid data showing an interaction between TaClo3 protein and Ga subunit of wheat suggests that G proteins may play important roles in signaling pathways that respond to abiotic stress. This study conducted to characterize protein-protein interactions between the calcium EF-hand binding proteins. TaClo3 and the Ga subunit

of wheat by bimolecular fluorescent complementation *in vivo* and to similarly test if there are interactions between Arabidopsis Ga and RD20/AtClo3 protein and AtClo7 proteins. This study also proposed to confirm protein-protein interactions by GST-fusion protein pull down assays *in vitro*. Additional experiments were designed to characterize the phenotype of RD20/AtClo3 mutants in Arabidopsis in response to treatment with ABA hormones and abiotic stresses such as salt and drought.

PART II. MATERIALS AND METHODS

1 Plant Materials and Growth conditions

1.1 Plant materials

T-DNA insertion mutant information for gene RD20/AtClo3 was obtained from the Salk Institute's Genomic Analysis Laboratory's website (<http://signal.Salk.edu>). The T3 seeds of *A. thaliana* RD20/AtClo3 T-DNA insertion line (FLAG_237F07; Wassilewskija (WS) ecotype) and WS wild type seeds were obtained from the Institute National de la Recherche Agronomique (INRA), Versailles, France and the Arabidopsis Biological Resource Center (ABRC), Ames, USA respectively. *Nicotiana benthamiana* were obtained from Dr. Francois Labilerté, Institut national de la recherche scientifique (INRS) in Laval.

1.2 Growth conditions and soil preparation

Three volumes of black earth and one volume of vermiculite were mixed and baked in the oven at 100°C for one hour, to eliminate fungi and insects. Dry seeds of the wild type (WT) and mutant plants were stratified in the dark at 4°C for 2-3 days prior to sowing to ensure that the seeds would germinate uniformly. Wild type and mutant plant seeds were sown in the prepared soil and grown in a growth chamber (22°C, ~125 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ fluorescent light) with a light cycle of 16 hr light and 8 hr dark unless otherwise stated.

1.3 Identification of the *rd20/AtClo3* T-DNA insertion mutant

The *rd20/AtClo3* homozygous Arabidopsis mutants were identified by PCR. A pair of gene specific primers was designed using T-DNA primer design tool provided by Salk Institute Genomic Analysis Laboratory (SiGnAL, <http://signal.salk.edu/tdnaprimers.2.html>), and one T-DNA left border primer based on sequence information on the Institut national de la recherche agronomique (INRA) website (<http://www-ijpb.-versailles.inra.fr/en/sgap-/equipes/fichiers/FSTinformation.html>) were ordered from Biocorp, Montreal. The T-DNA insertion sites were confirmed using the T-DNA LB primer (FLAG_LB (Tag5)) and *RD20* specific right primer (Table 1). The *RD20* gene specific left primer (RD20_LP) and RD20_RP (Table 1) were used to detect *RD20* loci without a T-DNA insertion. Plant genomic DNA was isolated from a single leave of each plant using the Plant DNA extraction kit (Sigma). Homozygous mutant plants were further characterized by sequencing the PCR product with the pair of *RD20* gene specific primers. T4 seeds were collected from homozygous mutant plants.

Table 1. Primers used to identify RD20 homozygous mutant plants

Primer Name	Sequence (5'-3')	Application
M13 For	GTAAAACGACGGCCAGT	Vector primer reverse
WEF109_B14For	GGTGGCTGCTCTTCTGTTTC	TaClo3/J900 gene specific forward
WEF035_L22For	TGCATTTGATGTAAAGGAAGGT	TaClo3/J900 gene specific forward
FLAG_LB (Tag5)	CTACAAATTGCCTTTTCTTATCGAC	T-DNA left border
RD20_RP	AACAAGCGGGAAATTAAGTGG	RD20 gene specific forward
RD20_LP	ACTAACCATCCAAAAGGATCG	RD20 gene specific reverse

2. Identification and cloning of full length cDNAs

2.1 G protein alpha subunit TaGA3_CS cDNA sequence

The full length cDNA clone of TaGA3_CS encoding the G protein alpha subunit of wheat was obtained from in the Arizona Genomics Institute (AGI). This cDNA clone originated from the wheat cultivar Chinese Spring cultivar, and the clones is referred to as TaGA3_CS.

2.2 Construction of TaGA3_Nor full length entry clone

The sequences for two full length cDNAs TaGA1 and TaGA2 which encode G protein alpha subunit from wheat cultivar cv. S615 were available in GenBank with accession numbers AB090158 and AB090159 respectively. Three gene specific primers were designed based on the TaGA1 sequence to amplify and clone the G protein alpha subunit from a Norstar wheat cDNA library 4. All primers had Gateway *attB* recombination sites incorporated for subsequent cloning into Gateway donor vectors. Three gene specific primers include two gene specific forward primers and one gene reverse primer (Table 2). One forward primer (TaGA3_NorForP1) included the translational start ATG. A second forward primer was annealed 559bp downstream of the ATG, but introduced a new ATG translational start (TaGA3_NorForP2). Gene specific reverse primer (TaGA3_NorRevP3) began at original stop codon, but was designed to mutate and remove the stop codon. The *attB*-PCR products using TaGA3_NorForP2 and TaGA3_NorRevP3 were successfully amplified and recombined with pDonr207. The resulted entry clones named as PartialTaGA3_Nor entry clone was

sequenced using SeqL-A (proximal to attL1) and SeqL-B (proximal to attL2) primers (Table 2).

The cDNA clones for Ga for *T. aestivum* Ga obtained from the cultivar Norstar lacked the 5' end of the ORF (PartialTaGA3_Nor). The clone obtained from the University of Arizona stock centre the cultivar Chinese Spring had a 17 bp duplication leading to a frame shift in the 3' end of the ORF, 91 nt upstream of the stop codon. A full length intact cDNA was generated by combining parts of the two clones by PCR. Full length TaGA3_Nor was generated by fusing two PCR fragments. All primers used to clone full the length TaGA3_Nor are listed in Table 2. A pair of primers used to amplify the PCR fragment 1 which is the coding region from nucleotide 1 to 1027 based on TaGA3_CS cDNA clone, was TaGA3_CSFOR and TaGA3_CSREV. To amplify the last 358 nt corresponding to the 3'end of TaGA3_Nor gene, another pair of primers TaGA3_Nor interFor and TaGA3_Nor Rev was used to amplify the cDNA from Norstar wheat cDNA library 4 (PCR fragment 2). PCR fragments 1 and 2, which overlapped, were fused by additional PCR. The attB-PCR products with or without stop codon were amplified using the fused PCR products as templates and then cloned into pDonr207. Recombination of the attB-PCR products to pDonr207 is described in section 4.

Table 2. Primers used to construct TaGA3_Nor, TaGA3^{Q223L}, AtGPA1^{Q222L} and RD20 promoter::GUS expression clones

Primer Name	Sequence (5'-3')	Application
TaGA3_NorForP1	<u>GGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGGCT</u> CCTCCTGCAGCA	AttB-TaGA3_Nor forward P1
TaGA3_NorForP2	<u>GGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAAGG</u> AGGATGTGCTCCATG	AttB-TaGA3_Nor forward P2
TaGA3_NorRevP3	<u>GGGACCACCTTGTACAAAGAAAGCTGGGTACGTCCCCCGT</u> TCCCTCCCT	AttB-TaGA3_Nor reverse P3
SeqL-A	TCCGGTTAACGCTAGCATGGATCTC	Entry clone sequencing A
SeqL-B	GTAACATCAGAGATTTTGAGACAC	Entry clone sequencing B
TaGA3_CSFOR	ATGGGCTCCTCCTGCAGCA	TaGA3_CS forward
TaGA3_CSREV	CCGGCTTGCTGCTCTGGA	TaGA3_CS reverse
TaGA3_NorinterFor	GGAGACGAAGGAACTGTTCCGACTG	TaGA3_Nor internal forward
TaGA3_NorRev	CGTCCCCGTTCCCTCCCT	TaGA3_Nor reverse
P1For	<u>GGGACAACCTTGTACAAAAAAGCAGGCTTCATGGGCTT</u> ACTCTGCGTAGAAGTCG	AttB-AtGPA1 forward P1
MutaRev	CTCATTCTCAGTCCACCCACG	AtGPA1 GTPmuta reverse
MutaFor	CGTGGGTGGACTGAGAAATGAG	AtGPA1 GTPmuta forward

Table 2. Continued..

Primer Name	Sequence (5'-3')	Application
P2Rev	<u>GGGACCAC</u> CTTTGTACAAGAAAGCTGGGTATAAAAGGC CAGCCTCCAGTAAATTTC	AttB-AtGPA1 reverse P2
MutaTaGA3_NorRev	CCTCCTCTCAATTCCTTAGACCTCCTAC	TaGA3_Nor GTPmuta reverse
MutaTaGA3_NorFor	GTAGGAGGTCTAAGGAATGAGAGG	TaGA3_Nor GTPmuta forward
GWPrord20for	<u>GGGACAAC</u> CTTTGTACA AAAAAGCAGGCTTCCGTAA TGAATATCTTGACAACAA	AttB-RD20 promoter forward
GWPrord20rev	<u>GGGACCAC</u> CTTTGTACAAGAAAGCTGGGTATCTCTCTCT CTTACTCTTTTACAATC	AttB-RD20 promoter reverse

Note: Underlined sequences are the attB sites with extra 4Gs at 5' end

3. DNA sequencing

3.1 Wheat caleosin TaClo3/J900

The initial cDNA for TaClo3/J900 which was used for the yeast two hybrid screening (Tardif et al., 2007) was a partial length clone. Two independent full length cDNAs WEF035_L22 and WEF109_B14 from the *T. aestivum* cultivar Norstar, for TaClo3/J900 were obtained from the Functional Genome of Abiotic Stress (FGAS), Genome Canada Project. A gene specific and vector primers in Table 1 were used to sequence the clones at the Genome Quebec Innovation Centre (Montreal). The sequences were assembled with EST sequences from the FGAS data base. Blastx alignment for two sequences was performed on the NCBI web site (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome). The CAP3 Sequence Assembly Program was used to compile full length sequences (<http://pbil.univ-lyon1.fr/cap3.php>) (Huang and Madan, 1999). The quality of the contig was determined by the PHRED scores provided for the sequences. The cDNA sequences were compared with the best Blastx hit in the GenBank nr data base with rice and barley. Clustal W was used for multiple sequence alignment with related sequences from other species.

3.2 Generation of G α subunit TaGA4 and TaGA5 EST contigs

Blastn with the TaGA1 nucleotide sequence was used to identify twenty nine *T. aestivum* sequences in the GenBank EST database. The ESTs were compared by pair wise analysis by Blastn and grouped into sets with regions that shared at least 120 nt of 100% identity. These grouped ESTs in a group were assembled as contigs using CAP3 (<http://pbil.univ-lyon1.fr/cap3.php>) (Huang and Madan, 1999). Regions of the contigs with at least 2X sequence coverage were used for comparison between groups.

3.3 Multiple alignment and phylogenetic tree

All sequence alignments were performed using Cluatal W (version 2.0.11) with the BLOSUM62 scoring matrix. A phylogenetic bootstrapped tree was constructed using a neighbor-joining bootstrap analysis of 1000 replicates (Saitou and Nei, 1987) using software Clastal W version 2.0.11. The phylogenetic tree was viewed by Tree view software.

3.4 Protein topology prediction and Molecular weight calculation

TopPred version 5 (Claros and Von Heijne, 1994) was used to predict TaClo3/J900, RD20/AtClo3 and AtClo7 membrane topography and to generate hydrophobicity graphs. The default settings were used except for Kyte and Doolittle hydrophobicity scale and eukaryote for organism type (<http://mobyli.pasteur.fr/cgi-bin/portal.py?form=toppred>). Compute pI/Mw Tool was used to calculate the molecular mass of TaClo3/J900, RD20/AtClo3 and the AtClo7 proteins.

4. Gateway cloning

4.1 Bacterium strain

E. coli TOP10 (Invitrogen) was used as the host strain for bacterial transformation. Its genotype is F- mcrA Δ (mrr-hsdRMS-mcrBC) ϕ 80lacZ Δ M15 Δ lacX74 nupG recA1 araD139 Δ (ara-leu) 7697 galE15 galK16 rpsL (Str^R) endA1 λ .

The *E. coli* strain DB3.1 (Invitrogen) was used for transformation with Gateway plasmid vectors. Its genotype is: F- gyrA462 endA1 glnV44 Δ (sr1-recA) mcrB mrr hsdS20 (rB-, mB-) ara14 galK2 lacY1 proA2 rpsL20 (Smr) xyl5 Δ leu mtl1 (Miki et al., 1992). This strain permits the growth of Gateway destination vectors containing the ccdB gene. CcdB protein in wild type cells inactivates the DNA-topoisomerase II complexes and eventually kills the cells (Bernard and Couturier, 1992).

4.2 Gateway Cloning

Plasmids were constructed using the Gateway cloning system (Invitrogen, www.invitrogen.com). Two recombination steps are involved in Gateway cloning: BP recombination and LR recombination. *In vitro* recombination is facilitated between specific attachment sites (Hartley et al., 2000 ; Landy, 1989) in the following: attB x attP \rightarrow attL x attR (BP reaction), is used to introduce a DNA fragment into an entry vector. The attL x attR \rightarrow attB x attP (LR reaction) is used to transfer the DNA fragment from the entry vector to a destination vector, which is normally an expression vector.

4.3 BP Recombination

BP recombination was carried out between attB-PCR products and a donor vector (pDONR207) at 25°C for 2 hours or overnight according to the manufacturer's protocol (Table 3). The BP reaction was stopped by the addition of 0.5 µl of the proteinase K and incubation at 37°C for 10 min.

Table 3. BP Reaction

DNA Reagents	Volume
AttB-PCR product (15-150µl/µl)	1-3 µl
Donor vector pDonr207 (150ng/µl)	1µl
BP Clonase II enzyme mix	0.5µl
TE buffer, pH 8.0	0.5µl
Total 5µl	

In order to perform BP recombination, the attB-PCR primers for *A. thaliana* genes and *T. aestivum* genes were designed according to Gateway Technology manual (Invitrogen) and ordered from Biocop, Montreal (Table 4). The full length attB-PCR products without stop codon of *A. thaliana* genes were amplified using full length cDNA clone as a template (Table 5). The full length attB-PCR product of the TaClo3/J900 gene without stop codon was generated using wheat cDNA clone WEF035_L22 (Table 5). AttB-PCR products were amplified by the Touch down PCR program listed in Table 6.

Table 4. PCR Gateway primers used in this study

Primer Name	Sequence (5'-3')	Application
RD20GWFOR	<u>GGGGACAAC</u> TTTGTACAAAAAA GCAGGCTTCATGGCAGGAGAGG CAGAGGCTTT	AttB-RD20 forward primer
RD20GWREV	GGGGACCACTTTGTACAAGAAA <u>GCTGGGT</u> AGTCTTGTTTGCGAGA ATTGGCCCT	AttB-RD20 reverse primer
GPA1GWFOR	<u>GGGGACAAC</u> TTTGTACAAAAAA GCAGGCTTCATGGGCTTACTCTG CAGTAGAAGTCG	AttB-AtGPA1 forward primer
GPA1GWREV	GGGGACCACTTTGTACAAGAAA <u>GCTGGGT</u> TATAAAAGGCCAGCCT CCAGTAAATTC	AttB-AtGPA1 reverse primer
J900GWFOR	<u>GGGGACAAG</u> TTTGTACAAAAAA GCAGGCTTCATGGCGATCCGGC GACAAT	AttB-J900 forward primer
J900GWREV	GGGGACCACTTTGTACAAGAAA <u>GCTGGGT</u> CCATTGCACTATGATG AGAAAAGGCC	AttB-J900 reverse primer
At1g23240GWFOR	<u>GGGGACAAG</u> TTTGTACAAAAAA GCAGGCTTCATGTTTTTTTGT TTGTTT TTGTGAG	AttB-At1g23240 forward primer
At1g23240GWREV	GGGGACCACTTTGTACAAGAAA <u>GCTGGGT</u> AATTCGTTTCAAATA ATTCAGA AACAAC	AttB-At1g23240 reverse primer

Note: Underlined sequences are the attB sites with extra 4Gs at 5' end

Table 5. List of cDNA clones used in this study

Name	Description	GeneBank Accession #	Source and stock number
RD20 (At2g33380)	EF hand calcium binding protein of Arabidopsis	NM_128898	ABRC ^a ; U16599
AtClo7 (At1g23240)	Caleosin related protein	BX814132	INRA-CNRCV ^b ; GSLTFB
AtGPA1 (At2g26300)	G protein alpha subunit of Arabidopsis	NM_128187	ABRC ^a ; U12585
TaClo3 (J900)	EF hand calcium binding protein of wheat	DY742468	FGAS ^c ; WEF035_L22
TaGA3_CS	G protein alpha subunit of wheat (Chinese spring) and it has 17nt duplication at 3'end of the coding region	BG262238	AGI ^d ; Ta_CEA0081C15

a Arabidopsis Biological Resource Center (ABRC), Ames, USA

b Institut National de la Recherche Agronomique (INRA), France

c Functional Genome of Abiotic Stress (FGAS), Genome Canada

d Arizona Genomics Institute (AGI), NSF-USDA, The University of Arizona

Table 6. PCR Touch-down program

Steps	Temperature °C	Time	Cycles
1	95	2min	
2	94	30sec	3
3	50	30sec	
4	72	3min	3
5	94	30sec	
6	55	30sec	
7	72	3min	24
8	94	30sec	
9	68	30sec	
10	72	3min	
11	72	7min	
12	4		

Every 30 seconds the annealing temperature was decreased by 5 °C

4.4 *TOP10*–mediated chemical transformation

Top10 chemical competent cells were made as previously described (Dagert and Ehrlich, 1979). Transformation was performed using by mixing 2.5µl of BP reaction with 50µl of TOP10 competent cells and followed by incubation on ice for 30 min. The mixture was then heat shocked at 37°C for exactly 5 min. 100 µl of LB media was added and the cells were incubated at 37°C for 1 hour with shaking at 250 rpm. All of the transformed cells were spread on LB agar plates containing 7 µg/ml gentamycin. The plates were incubated overnight at 37°C.

4.5 Plasmid isolation and sequencing

A single colony of each entry clone was used to inoculate a 2 ml liquid culture with LB media with gentamycin which was incubated overnight with shaking at 37°C. The plasmid DNA was isolated using a Qiagen Plasmid Miniprep Kit (Qiagen) and quantified on a 1% Agarose gel. The entry clones were first confirmed to have inserts by PCR and then sent for sequencing. Sequencing primers (Table 7), SeqL-A and SeqL-B were designed based on information from the Gateway Technology manual (Qiagen) and used to sequence all the entry clones.

4.6 LR Recombination

LR recombination was carried out between entry clones and plant Gateway destination vectors to create plant expression constructs. Table 7 shows the LR reaction reaction. All of the plant expression constructs were confirmed by PCR using the pairs of gene specific primers flanked with attB sites.

Table 7. LR Reaction

DNA Reagents	Volume
Entry clone (150ng/ μ l)	1 μ l
Plant or E.coli Gateway Cloning vector (150ng/ μ l)	1 μ l
LR Clonase II enzyme mix	0.5 μ l
TE buffer, pH 8.0	2.5 μ l
	Total 5 μ l

5. Generation of plant gene expression constructs

5.1 Plant Gateway cloning vector and Plant expression vector

Plant Gateway expression vectors for subcellular localization and BiFC experiment are listed in Table 8. The plant cellular markers expressed as the fluorescent red mCherry fusion proteins have been established for plant transformation and immunofluorescence (Nelson et al., 2007).

Table 8. Gateway Plant expression vector list

	Binary plasmids	Description	Source
Plant Gateway Cloning Vector	pK7FWG2	35S: eGFP plant binary Gateway vector, for GFP fusion to the C terminus of experimental proteins. It has kanamycin selection in plants and spectinomycin in <i>E. coli</i> .	VIB-Ghent University ^a
	pBatTL-B-sYFP-N	Plant binary vector for protein fusion of the N terminal half of sYFP for BiFC to the C terminus of experimental proteins	Dr. AM.Jones ^b
	pBatTL-B-sYFP-C	Plant binary vector for protein fusion of the C terminal half of sYFP for BiFC to the C terminus of experimental proteins	Dr. AM.Jones ^b
	pKGWFS7	eGFP:gus plant binary Gateway vector for GFP fusion protein expression under the control of an experimental promoter. It has kanamycin selection in plants	VIB-Ghent University ^a
BiFC pairs of Positive And Negative Controls	sYFP-N-RGS	RGS1-N-sYFP binary vector for BiFC positive control	Dr. AM.Jones ^b
	sYFP-C-GPA1	GPA1-C-sYFP binary vector for BiFC positive control	Dr. AM.Jones ^b
	sYFP-C-PIP2A	PIP2A-C-sYFP binary vector for BiFC negative control	Dr. AM.Jones ^b
	HA V22D-nYFP	HA V22D-nYFP binary vector for BiFC negative control	Dr. Hugo Zheng ^c
	PIS2-cYFP	PIS2-nYFP binary vector for BiFC negative control	Dr. Hugo Zheng ^c
	P24β1-cYFP	P24β1-cYFP binary vector for BiFC negative control	Dr. Hugo Zheng ^c
	RD20-sYFP-N	RD20-sYFP-N binary vector for BiFC negative control	This work

	J900-sYFP-N	J900-sYFP-N binary vector for BiFC negative control	This work
	Binary plasmids	Cellular makers Description	Source
Plant	PM-rk	Plasma Membrane	ABRC ^d , CD3-1007
cellular markers as mCherry		The full length of AtPIP2A, a plasma membrane aquaporin	
fusion proteins	ER-rk	Endoplasmic Reticulum The combination between the signal peptide of AtWAK2, wall- associated kinase 2 at the N-terminus of Fluorescent protein and ER retention signal, His-Asp-Glu-leu, at the C-terminus	ABRC ^d , CD3-959
	Pt- rk	Plastid The first 79 aa of the small subunit of tobacco rubisco	ABRC ^d , CD3-999
	Mt-rk	Mitochondria The first 29 aa of yeast cytochrome C oxidase	ABRC ^d , CD3-991
	G-rk	Golgi The first 49 aa of GmMan1, soybean α -1,2 mannosidase I	ABRC ^d , CD3-967
	Px-rk ,	Peroxisome Peroxisomal targeting signal 1, Ser-Lys-Leu, at the C-terminus of fluorescent protein	ABRC ^d , CD3-983
	Vac-rk,	Tonoplast An aquaporin of the vacuolar membrane protein, γ -TIP	ABRC ^d , CD3-975

^a Ghent University, Belgium

^b University of North Carolina, North Carolina

^c McGill University, Montreal

^d Arabidopsis resource center

r, mCherry fluorescent protein; k, kanamycin resistance; aa, amino acid

5.2 Construction of AtGPA1^{Q222L} and TaGA3^{Q223L} full length expression clones

AtGPA1^{Q222L} was generated to produce a constitutive GTP bound form of Ga by replacing A with T at position 665 to create a Q to L of *AtGPA1* coding region (Ullah et al., 2003) using PCR-based site-directed mutagenesis. PCR-based site-directed mutagenesis was performed by three PCR reactions. Primers used to make AtGPA1^{Q222L} are listed in Table 2. In the first PCR reaction, primer P1For was paired with mutant primer (MutaRev) which is used to introduce a point mutation at position 665 of AtGPA1 coding region and amplify the N' terminal portion of the gene. In the second PCR reaction, mutant primer (MutaFor) which is reverse and complementary of MutaRev, was paired with primer 2 (P2Rev) were used to amplify the C terminal portion of the gene. In the third PCR reaction used the two PCR fragments as the template with P1For and P2Rev, end primers with *att* recombination sites. The attB-containing PCR product without stop codon was cloned into pDonr207 by *in vitro* recombination.

Constitutive GTP bound form of the other Ga, TaGA3_Nor, was generated. Therefore TaGA3_Nor^{Q223L} was generated by replacing A to T at N-terminal position 668 of TaGA3_Nor coding region using the same method as that used for the generation of AtGPA1^{Q222L}. The primers used to generate this clone are listed in Table 2. These two entry clones were recombined into the vector, pBatTL-B-sYFP-C, used for BiFC.

5.3 Generation of RD20/AtClo3 native promoter GUS expression clone

The RD20/AtClo3 promoter entry clone was constructed by amplifying a 1-kb DNA fragment upstream of the transcription start site using a pair of gene specific primers (Table 2) flanked with attB site by PCR (GWPrord20for and GWPrord20). The template DNA was extracted using a Plant DNA extraction kit (Sigma) from Arabidopsis WS ecotype wild type plants. The entry clone was confirmed by sequencing and recombined into the expression vector, pKGWFS7.

6 Transformation and expression in *N. benthamiana* and Arabidopsis

6.1 Agrobacterium strain

Agrobacterium tumefaciens strain AGL1 (Rifampicin, Gentamycin and Ampicillin resistant) was used for plant transient expression experiments. The *Agrobacterium* strain was obtained from Dr. Francois Labilerté, Institut national de la recherche scientifique (INRS) in Laval.

6.2 Electro-transformation of *A. tumefaciens*

Electroporation of *Agrobacterium* was carried out according to the instruction manual for MicroPulser electroporator (Bio-Rad) with some modifications. The electro-competent cells were prepared according to (Sharma and Schimk, 1996). About 30-50ng of plasmid DNA of interest was mixed with 50 µl of AGL1 electro-competent cells on ice. The mixture of AGL1 electro-competent cells and the plasmid DNA was transferred into a pre-chilled 0.1 cm electroporation cuvette (Fisher Scientific). The electroporation was

performed with the settings: 1.25KV, 25 μ FD, 200Ohms and about 5msec time constant. 200 μ l of LB medium was added to the cuvette immediately after electroporation, and transferred to a sterile test tube and incubated at 30°C for 2-4 hours with shaking of 250 rpm. The cells were spread on LB agar containing appropriate antibiotics, depending on the selectable marker of the plasmid DNA. The LB agar plates were incubated at 30°C for 2-3 days.

6.3 *Agrobacterium*-mediated infiltration of *N. benthamiana*

The agroinfiltration protocol was performed according to (Kapila et al., 1997). Separate 50 ml cultures of *A. tumefaciens* carrying plant gene expression experimental constructs and a strain carrying an expression vector with the p19 gene, which is a RNA silencing suppressor were incubated at 30°C with shaking at 250 rpm overnight. The initial OD₆₀₀ of the overnight culture was measured. Cultures were centrifuged and the cell pellets were resuspended in agroinfiltration solution [10Mm MgCl₂, 150 μ M actosyringone (3',5'-Dimethoxy-4'-hydroxyacetophenone)] to achieve a final OD₆₀₀=0.1. The *A. tumefaciens* suspensions with different expression plasmids were mixed in equal ratio and incubated at room temperature for at least 2 hours. The mixtures of one or more plasmid of interest and p19 vector were then infiltrated into the abaxial side of 4-5 weeks old *N. benthamiana* using a 3cc syringe without the needle. The infiltrated tobacco plants were then grown in the greenhouse for 3 days.

6.4 *Agrobacterium*-mediated stable transformation of *A. thaliana*

Agrobacterium harboring the RD20 native promoter::GUS;;eGFP construct was transformed into *A. thaliana*. The transformation was carried out according to “*Simplified Arabidopsis Transformation Protocol*” (Clough and Bent, 1998). Positive transformants were selected on half strength MS media with 100µg/ml kanamycin.

7. Microscopy analyses

Sections of *Agrobacterium* infiltrated leaf tissue approximately 2 mm x 2 mm were cut and placed on a microscope slide, covered with a drop of water and then with a cover slip. Samples were viewed with a Zeiss LSM 510 Meta confocal laser scanning microscope (Carl-Zeiss, Germany) at Cell Imaging and Analysis Network (CIAN) center in McGill University, Montreal (<http://biology.mcgill.ca/CIAN/microscopy.htm>). The objective was used a C-Apochromat 63X/1.2 water with correction collar. Two laser sources were used for this work: 30mW Ar Ion laser (488/514nm) and 1mW HeNe Green (543nm). Electron excitation was at 488nm and 543nm for GFP and mCherry Red respectively, also the HFT/UV 488/543/633 beam splitter and NFT 545(secondary dichroic mirror) were used. NFT545 was used to discriminate between GFP and RFP. In order to acquire an image in the multi-tracking mode, the band pass (BP) 505-530nm filter and BP 560-615nm filter were used for GFP detection channel and RFP detection channel respectively. An AxioCam HRm camera was used on this microscope. BiFC images and as well as some of images with GFP fusions were taken by the Leica DMI6000B Spinning Disc 2 (SD2) confocal microscope. The objective HCX PL APO CS 20X/1.25 oil pH 3 DIC was used. YFP fluorescence was viewed with excitation with

a 25mW diode laser at 491nm with BP 540-543 emission filter. The 25mW diode laser at 561nm with BP 624-640 filter was used to detect RFP signals. Samples with GFP construct were excited by 25mW diode laser at 491nm with BP 520-535 filter. An EM CCD 512x512 BT camera was used to record images.

PART III. RESULTS

1. Identification of two full length clones for TaClo3/J900

A cDNA clone for TaClo3, previously referred to J900, a Ca²⁺ binding protein was sequenced in its entirety from clone WEF035_L22 (Figure 1). A second clone WEF109_B14 (Appendix 1) had 100% identity with WEF035_L22 but also carried a likely chimeric cDNA fragment of 333 nt derived from another gene. The first 333 nucleotides have modest similarity to unrelated genes in *Vitis vinifera* and *Beta vulgaris*. The full length gene sequence will be referred to as TaClo3, due to its high sequence similarity to caleosin like genes identified in wheat, and Arabidopsis. TaClo3/J900 has 76% aa sequence similarity with a Ca²⁺ binding protein in rice, (GB acc. BAD45228) and 69% aa sequence similarity with Barley Ca²⁺ binding protein (GB acc. CAB71337).

GenBank database searches indicated that the TaClo3/J900 gene is a conserved protein belonging to the caleosin superfamily. The gene in Arabidopsis with the highest similarity to TaClo3/J900 is RD20/AtClo3, which has 40% amino acid identity and 63% amino acid sequence similarity. Other members of the Caleosin gene family, AtClo4 (At1g70670) and AtClo7 (At1g23240) were found to have 61% and 57%, amino acid similarity respectively, with TaClo3/J900. The RD20/AtClo3 has 43% identity and 61% similarity to AtClo 7. Two homologues of the TaClo3/J900 gene from the Arabidopsis genome, RD20/AtClo3 and AtClo7, were studied in this work.

(a)

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cggacagagaagaaggggttaccggcggcggcagcagcagcagcgatggcgatccggcgacaa 60
M A I R R Q
tcatcagcagcagcttctctactcctgcccgggtggctgctcttctgtttctatggatg 120
S S A A A S L L L P P V A A L L F L W M
tttggcggggggcatgtgatggcacatactgagattcgcaacatgacggcactccagaaa 180
F G G G H V M A H T E I R N M T A L Q K
catgtctcctttttcgaccgtaacaaggatggcatcattactccttcggaaacatttgaa 240
H V S F F D R N K D G I I T P S E T F E
gggtctgtcgcaattggttttaatgttacatagcagagagaatttgccaccttggtgcat 300
G S V A I G F N V T Y A R E F A T L V H
gctgctaattggcctataacaagccccgctgatgcaccattgcctcacttatcaatatac 360
A A N G P I T S P A D A P L P H L S I Y
atagagaatatgcagagaggaatgcatgggagtataccgggtgcatttgatgttaaagga 420
I E N M Q R G M H G S D T G A F D V K G
aggtttgttccacaaaagtttgaggaaatattcataaagcatgcaaaaactagaccagat 460
R F V P Q K F E E I F I K H A K T R P D
ggtttgacatatttgagggtggaggatatagatcctagcaaatcgagatccactggaccct 520
G L T Y L E V E D M I L A N R D P L D P
gcatcatgggagggacctcaaataagaatggggcggaaatatacaacgtcgcgagtgacaat 580
A S W E G P Q I E W G G I Y N V A S D N
gatggatttcttcataaggacgatgagagaggatatacgatggaagtgtgtttgtaaag 640
D G F L H K D D A R G I Y D G S V F V K
ctggaggaaaagagggccttttctcatcatagtgaatgtaatagagtgaacatgttgt 700
L E E K R A F S H H S A M
gggctgaaataattaggggaacacatagtgtgtgtactaagactggtatatatatttgttca 760
agtgtttgtttgcacataagtaaaatactgtgatgtttatcaagatatatatatgaactc 820
tgaaaaaaaaaaaaaaaaaaaaa 839
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Figure 1. TaClo3 calcium EF-hand binding protein full length sequence.
The start codon and stop codon were highlighted in grey.

2. Protein sequence analysis of Ca²⁺ binding proteins and Ga

2.1 EF-hand calcium binding sites

The deduced amino acid sequences of the TaClo3/J900, RD20/AtClo3 and AtClo7 indicate that they each contain a single EF-hand motif calcium-binding domain. The EF-hand motif of the RD20/AtClo3 and AtClo7 amino acid is between amino acid 60 and 90 (Hernandez-Pinzon et al., 2001; Takahashi et al., 2000). The Clastal W alignment of the protein amino acid sequence (Figure 2) shows that the proline knot sites are conserved, as well tyrosine kinase binding site. TaClo3/J900 has casein kinase phosphorylation sites I, II and III are which are also conserved in RD20/AtClo3, except that AtClo7 lacks the casein kinase target site III (Partridge and Murphy, 2009).

The molecular masses of protein TaClo3/J900, RD20/AtClo3 and AtClo7 proteins were predicted by the Compute pI/Mw Tool (Bjellqvist et al., 1994; Bjellqvist et al., 1993; Gasteiger et al., 2005) to be 24.2 kDa, 26.6 kDa and 20.1 kDa respectively. Their amino acids were analyzed using the TopPred program (Claros and Von Heijne, 1994; Heijne, 1992) with the Kyte-Doolittle hydrophobicity scale. The RD20/AtClo3, TaClo3/J900 and AtClo7 proteins are predicted to have one membrane-spanning domain with hydrophobicity values exceeding the upper (UC) and lower (LC) cutoff values (Figure 3).

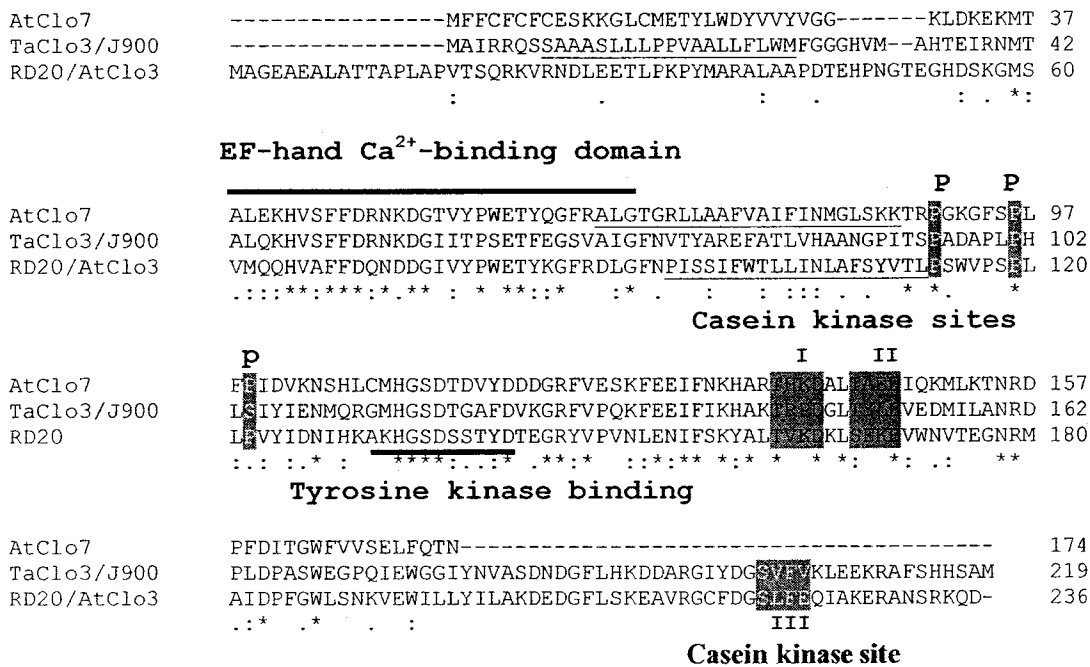
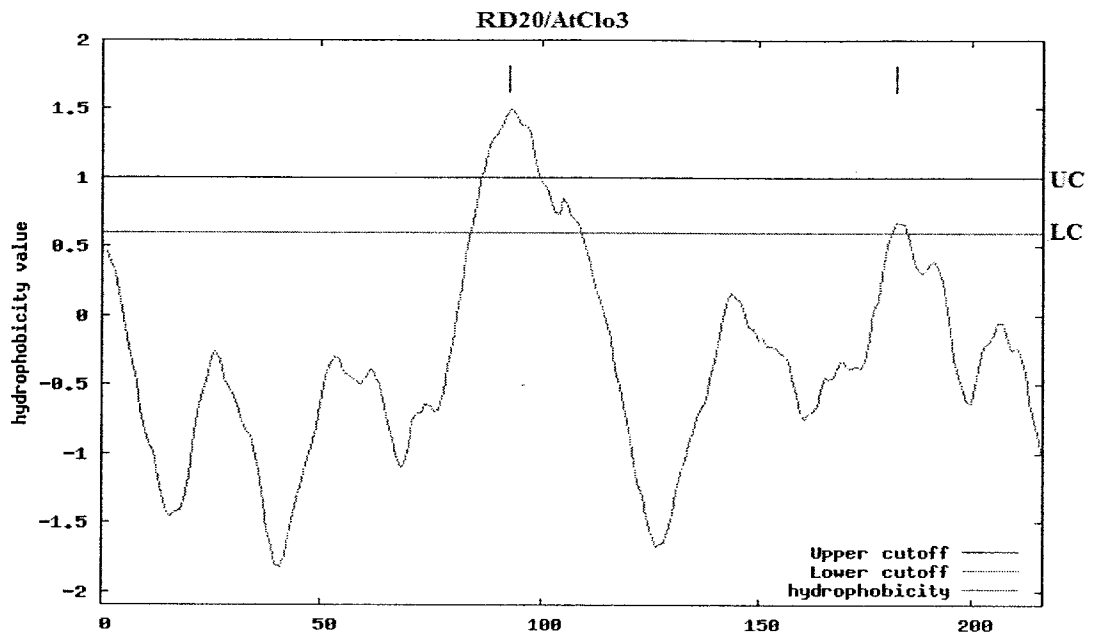


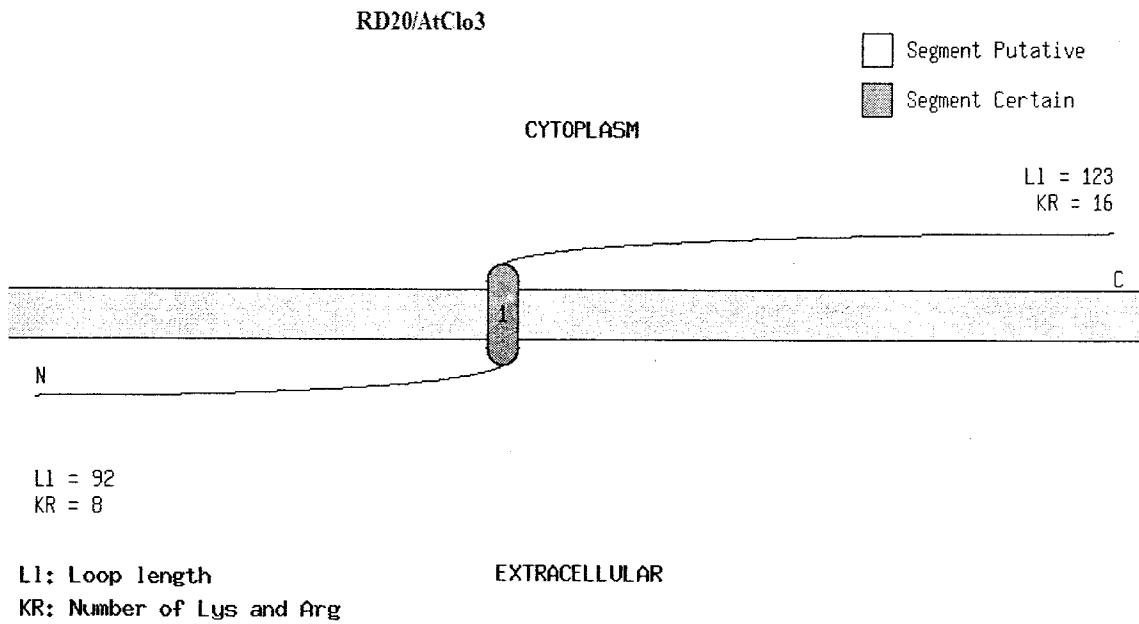
Figure 2. Alignment of the amino acid sequences of TaClo3/J900, RD20/AtClo3 and AtClo7.

The calcium binding EF hand domain was upperlined according to Shinozaki at al. (2000). Three proline knot sites are conserved and labeled by P, one conserved tyrosine kinase binding site is underlined and three casein kinase sites are highlighted with grey (Chen at al., 1999; MR Partridge and DJ Murphy, 2009, supplement). The predicted transmembrane domains were underlined in each protein.

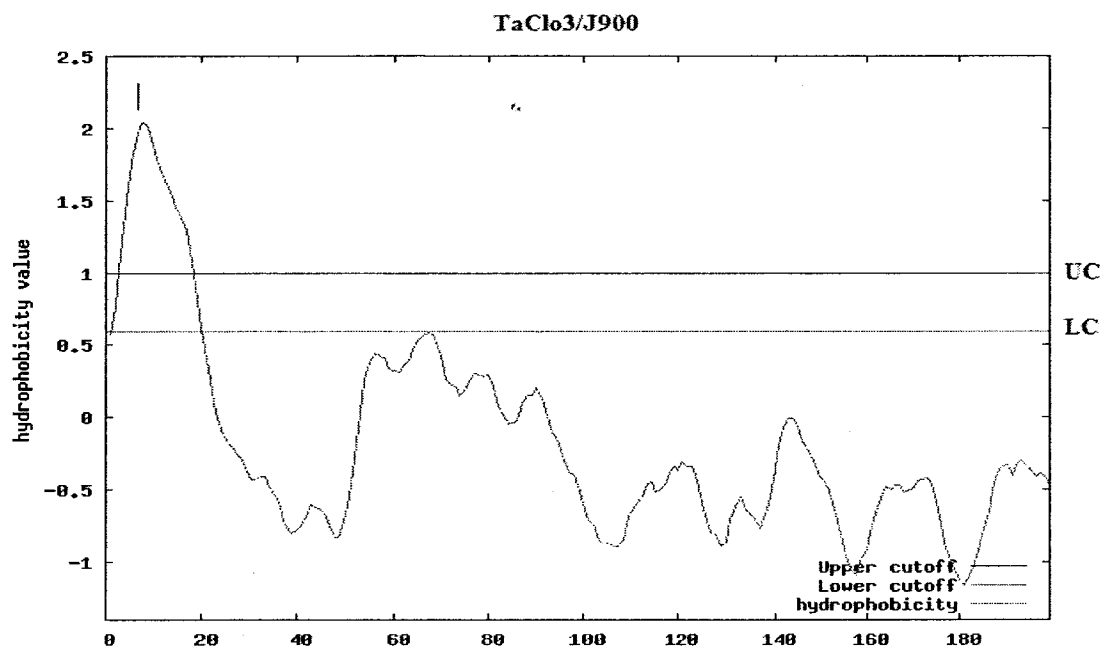
(a)



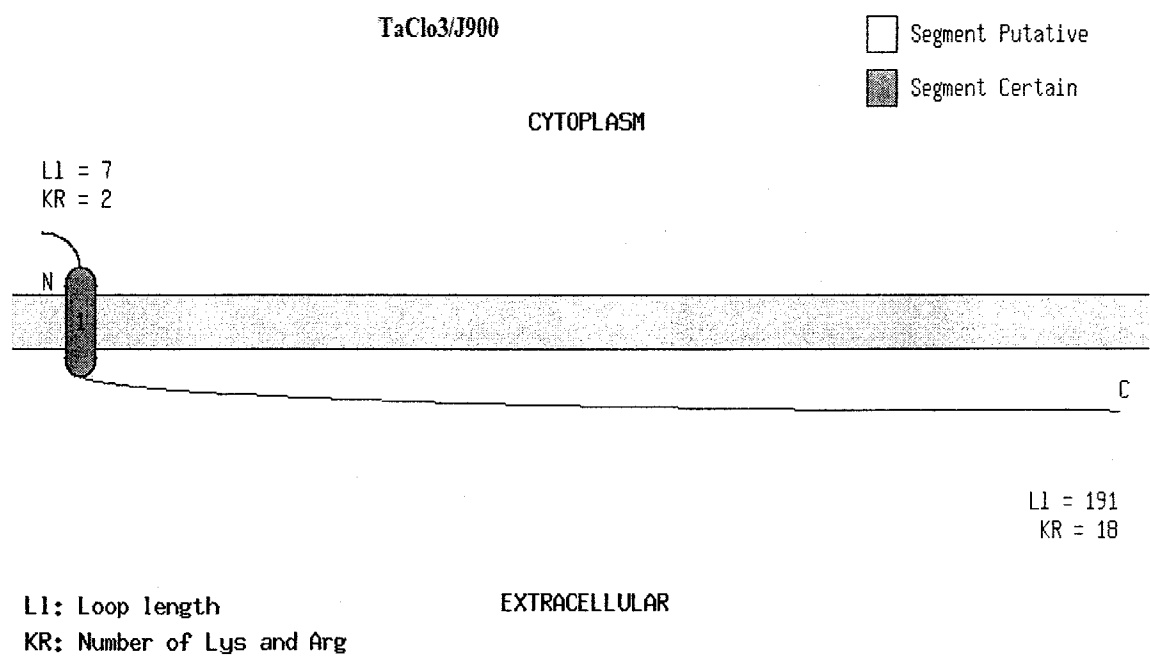
(b)



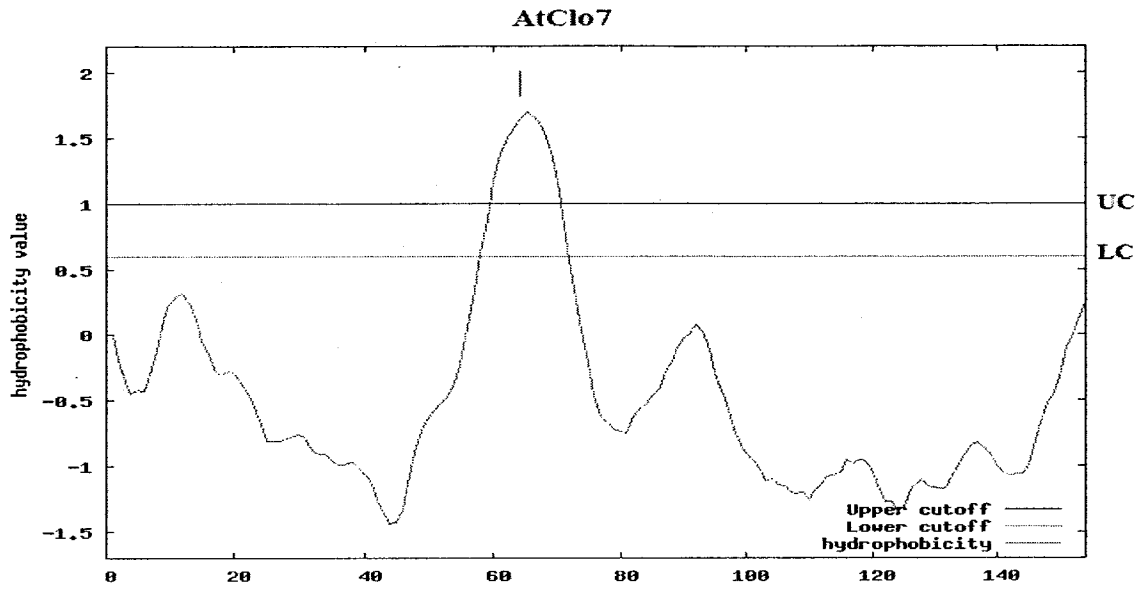
(c)



(d)



(e)



(f)

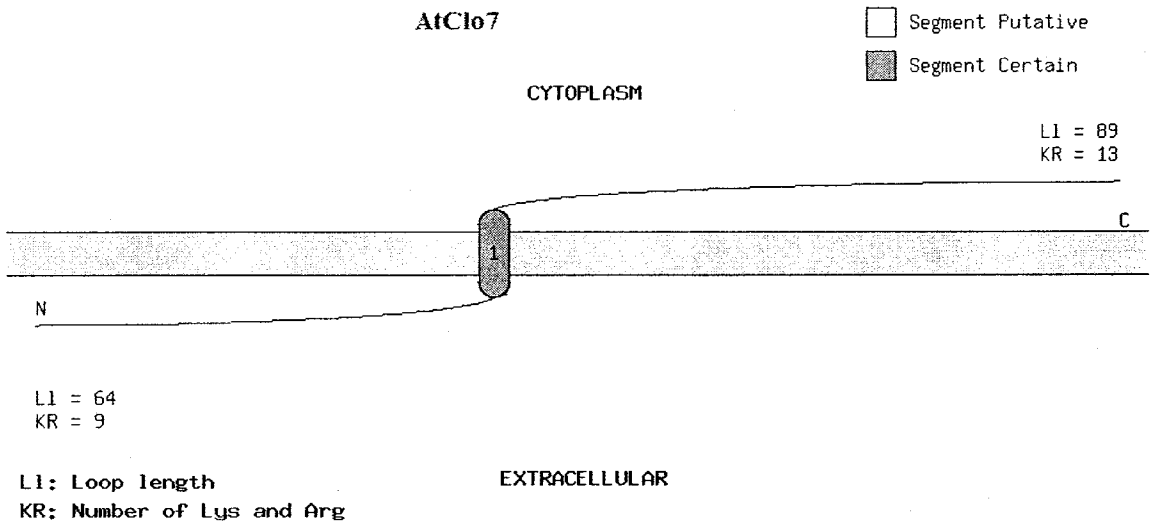


Figure 3. Transmembrane domain predictions for RD20/AtClo3, J900/TaClo3 and AtClo7.

The Hydrophobicity graph of and predicted transmembrane domains of (a, b) Arabidopsis RD20 protein (c, d) wheat TaClo3 (e, f) Arabidopsis Clo7. The transmembrane domain was predicted using TopPred 5 with the default settings except using Kyte and Doolittle hydrophobicity scale and eukaryote for organism type.

2.2 G α genes in wheat

The full length sequence of TaGA3_CS contains a 17 bp duplication at position 1038bp of the coding region (Figure 4). The 17 bp duplication is not present in TaGA1, TaGA2 nor in the homolog in rice. The 17 bp duplication causes a frame shift and a truncation of the ORF. The partial length TaGA3_Nor cDNA is amplified from a wheat Norstar library 4 had 100% nt sequence identity with TaGA3_CS over 600 nt of the TaGA3_Nor coding region except that it lacked the 17bp duplication found in TaGA3_CS. These cDNAs most likely represent different alleles of the same locus, and the full length cDNA TaGA3_Nor was constructed by combining the 5' end of the TaGA3_CS clone with the 3' end of the TaGA3_Nor clone. TaGA3_Nor has 98% nt sequence identity with the coding regions of two G α genes, TaGA1 and TaGA2 which were previously identified in wheat cultivar S615 (Hossain et al., 2003). TaGA2 has a 21-bp insertion and TaGA1 is 99.4% identical to TaGA2 if the 21-bp insertion is ignored.

ctcctcctgttccagccgccgggatcgacctcgccacatcagtcgctccgacgcccggac	60
cattgaagctgcctacgaggagcccatctcttcttccatcaccctcctactgcagat	120
agatctcaagtggacagctgctatctgcctcgtcatcaaccggaatgcactaggaggat	180
ctagggctctgtctgtctgtctttgcaaggaagcatgagaccacgcgcccgtctagaata	240
ata TTT tccatgctcgcgtgcgcttcaaaccatgggctcctcctgcagcagacctcac	300
TTT S M L A C A L Q T M G S S C S R P H	
tccgtaaacgaggccgaggcagccgacaacacaagatctgcagacatcgaccggcggatt	360
S V N E A E A A D N T R S A D I D R R I	
ctgcaggagacaaaggcggatcagcacgtccacaagctcttgcttctcggtgctggagaa	420
L Q E T K A D Q H V H K L L L L G A G E	
tcagaaagtccacgatatttaagcagattaagcttcttttcgaaccggcttcgacgag	480
S G K S T I F K Q I K L L F R T G F D E	
gcagaactcaagggtatatacgccggtcatccatgccaacgtgttccagacaatcaaaata	540
A E L K G Y M P V I H A N V F Q T I K I	
ctgtatgatggagctaaagagcttgcccaactggaaactgagcttcaaaacatggtata	600
L Y D G A K E L A Q L E T E S S K H V I	
tccccggataatcaggagattggagaaaaactatcagaaatcgaggcaggttggattac	660
S P D N Q E I G E K L S E I G G R L D Y	
ccactccttaacaaagaactcgtacaggatgtaagaaaattatgggaagattcagccatt	720
P L L N K E L V Q D V R K L W E D S A I	
caggaacttactcgtgtggaagtgtgctgcaagttcctgattgtgcacactacttcatg	780
Q E T Y S C G S V L Q V P D C A H Y F M	
gagaatctggaccgattagctgaaccagattatataccaacaaaggaggatgtgctccat	840
E N L D R L A E P D Y I P T K E D V L H	
gccagagtacggacaaatggggttgtggaaattcaatttagcccccttgagagagataaa	900
A R V R T N G V V E I Q F S P L G E S K	
agaggcggagaggtatacaggtgtacgtagtagggtcaaaggaatgagaggaggaag	960
R G G E V Y R L Y D V G G Q R N E R R K	
tggattcatcttttgaaggcgtcgcgatgccgtcatcttttgcgctgccattagcgagat	1020
W I H L F E G V D A V I F C A A I S E Y	
gatcagctgttgtttgaggacgagacacagaacagaatgatggagacgaaggaactgttc	1080
D Q L L F E D E T Q N R M M E T K E L F	
gactgggtactaaagcaaagatgttttgagaaaacatcgttcatgctgttccctcaacaaa	1140
D W V L K Q R C F E K T S F M L F L N K	
ttcgacatatttgagaggaaaatacaaaagggttcctttgaccgtgtgcgagtggtttaa	1200
F D I F E R K I Q K V P L T V C E W F K	
gattatgagccgatcgcgcctggcaaacaggatgtggaacatgcctatgagtttgtgaag	1260
D Y E P I A P G K Q D V E H A Y E F V K	
aagaaatttgaggaggtctacttccagagcagcaagccggaccggtgtggacc <u>gggtgttc</u>	1320
<u>aagatcta</u> cagaacgacggcgtggaccagaaacttgtaaagaagacgttcaagctgac	1380
K I Y R T T A L D Q K L V K K T F K L I	
gacgagagtatgagacgctccagggaggaacggggacg TTT tgaagactaggttgttgt	1440
D E S M R R S R E G T G T	
gactgagccacaggaggggggaaaacggcaattaggatgacacattaagtttacggtgtcg	1500
caactctgtgttgaattcattcttttagtgtagaagaggaagaagactgatttgatccct	1560
ttgtgcttgcctttcaaaaacaaagtccaaatttgatgaatccagtacagtattatgcaaa	1640
gtttggatctgtcattttgctttcaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa	1691

Figure 4. TaGA3_Nor full length sequence with amino acid.
The initial ATG start codon and TGA stop codon are highlighted. The cDNA clone from the cultivar Chinese Spring (TaGA3_CS) has a 17 bp tandem duplication for the region underlined.

In the *T. aestivum* EST database at GenBank there are 29 ESTs with high similarity to TaGA's. The ESTs were assembled into 5 contigs. Contigs were formed from sequences that had at least 120 nt of 100% sequence identity and are referred to as contigs 1 to 5. The accession numbers of all ESTs for each gene are reported in Table 9. Contig 3 has is 100% identical to TaGA3_CS which is 100% identical to TaGA3_Nor except for the 17 bp duplication. Contigs 4 and 5 represent independent genes and are referred to TaGA4 and TaGA5 respectively, though they are likely partial length contigs. The nucleic acid sequence identity within each groups and between groups are shown in Table 10. There seems to be five genes that encode G α in wheat genome (Figure 5). TaGA3_CS and TaGA3_Nor have 98% nt sequence identity with TaGA1 and TaGA2 within the coding region which suggests that TaGA3 is homeologous to TaGA1 or 2. TaGA4 is 97% identical TaGA5 which indicate that they are a homeologous pair. TaGA4 is 93% identical with TaGA1 and TaGA2 (without the 21 nt insertion) and to TaGA3_CS (without the 17 nt duplication). TaGA5 is 91%-92% identical to TaGA1, 2 and 3. These data suggest that TaGA4 and TaGA5 are paralogs to the other three genes. There are 3 single ESTs that did not contig with the other 5 genes (Table 9). CJ899506, BE415109 and BQ237279 do not have any overlap with TaGA4 or TaGA5. They may be from additional genes or may represent missing ends of the partial length gene sequences in the TaGA4 and TaGA5 contigs. Although, CJ899506, BE415109 and BQ237279 have 86%-91% identity with the TaGA1 to 3, the 1X coverage of these sequences are not sufficiently reliable to define their relationship to the other TaGA sequences.

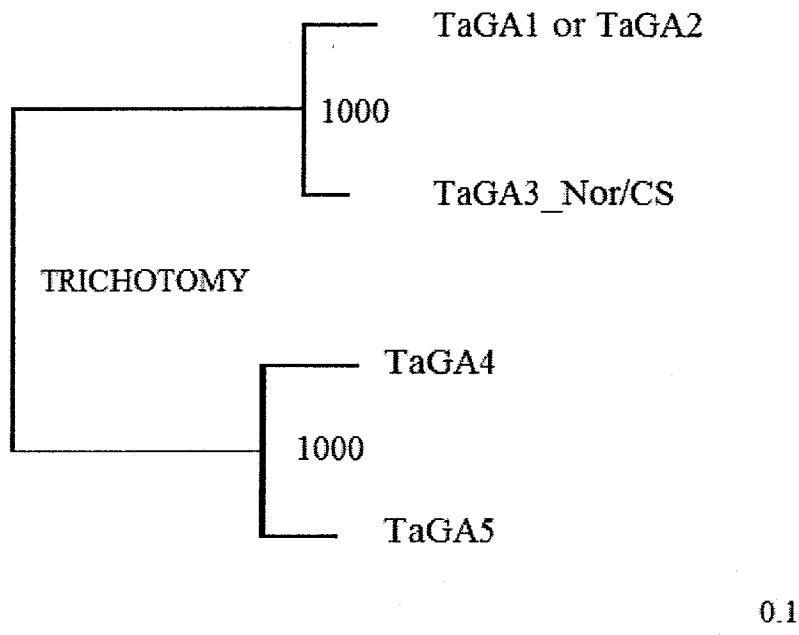


Figure 5. Phylogenetic tree of G protein alpha subunits from wheat.

The nucleotide sequences of G alpha subunit were aligned using the Clustal W 2.011 with the following parameter sets: gap open penalty=15, gap extension penalty=6.66, delay divergent sequences (%)=30, DNA transition weight=0.5, DNA weight matrix=IUB. A phylogenetic tree was constructed by neighbor-joining method and TreeView v1.6.6 software (Roderic D. M. Page, 2000). The confidence level of each group was analyzed by bootstrap analysis of 1000 replicates.

TaGA1	TaGA2	TaGA 3	TaGA 4	TaGA5	3 single ESTs
AB090158	AB090159	CJ568330	CJ623850	CJ808857	CJ899506
		BJ265521	CJ631665	CJ809236	BE415109
		BJ216880	CJ933261	CJ822043	BQ237279
		CJ675275	CJ523102	CJ821861	
		BG262238	CJ921243		
		BJ209444	CJ727658		
		BQ166408	BJ275749		
		CJ773864	BJ270754		
		BJ259799	CJ523102		
		CJ785517	BQ240307		
		CJ829028			

Table 9. GenBank accession IDs for full length *T. aestivum* TaGA1 and TaGA2 and EST accessions encoding Ga subunit genes identified in the GenBank EST database

Table 10. Nucleic acid sequence identity for coding region among *T. aestivum* Ga genes

	TaGA1	TaGA2	TaGA3_Nor	TaGA3_CS	TaGA4	TaGA5
TaGA1	100	97.7	98	97	93	91
TaGA2	99.4	100	97	95	93	91
TaGA3_Nor	98	98	100	98	93	92
TaGA3_CS	98	98	100	100	91	89
TaGA4	93	93	93	93	100	97
TaGA5	91	91	92	92	97	100

2.3 Protein sequence analysis of TaGA3_CS and TaGA3_Nor in *T. aestivum*

The structural domains of TaGA3_CS and TaGA3_Nor amino acid sequences were predicted based on the known TaGA1, TaGA2 (Hossain et al., 2003) and AtGPA1 amino sequences (Ma et al., 1990). The G α subunits have one $\beta\gamma$ binding region and six regions involved in GTP-binding (Figure 6). The GTP-binding regions are conserved among all G α subunits shown in Figure 6 except GTP-binding region 6 and GTP-binding site IV of TaGA3_CS, which were lost due to the frame shift introduced by the duplication in this allele. The binding regions for all three effectors binding regions are conserved among all of G α subunits listed in Figure 6. However, the amino acid sequence in the receptor binding region of TaGA3_CS is changed due to the 17nt duplication at the 3'end. In addition, the myristoylation sites for posttranslational modification and the cholera toxin (CTX) site for ADP-ribosylation are conserved in all TaGA sequences.

***thaliana*) G alpha subunit.**

There are 4 GTP binding sites and one myristoylation site shown by dark lines and the cholera toxin (CTX) site were highlighted in grey respectively (Simon et al., 1991). The 6 GTP binding regions are underlined; three effectors binding regions (EBR) were underlined by a solid line and the receptor binding region was underlined with a dashed line (Lambright et al., 1994; Noel et al., 1993). The conserved regions are indicated by asterisks below the sequences.

3. RD20/AtClo3 and AtClo7 interact with AtGPA1 in vivo

Previous studies found interaction between TaClo3 protein and the G α subunit through yeast analysis. This study was conducted to characterize protein-protein interactions between the Arabidopsis homolog of TaClo3, RD20/AtClo3 or AtClo7 and the G α subunit using transient expression assay *in vivo*.

EGFP tagged RD20/AtClo3 (Figure 7a and 7b) and AtClo7 (Figure 8a and 8b) were localized in the ER and tonoplast membrane in the epidermal cells of *Nicotiana benthamiana*. The localization to the ER is seen by co-localization with the ER marker and by the clear network structure that is seen in the upper focal plains of the cell. In mid-focal planes the labeling appears to be punctuated seen at the edges of the cells which would be expected for the cross section of a network. The punctuate label co-localized with ER makers and were distinguishable from the PM and tonoplast makers. RD20/AtClo3::eGFP and AtClo7::eGFP did not co-localize with any other 5 cellular makers listed in Table 1. AtGPA1::eGFP was only localized in the plasma membrane (Figure 9a).

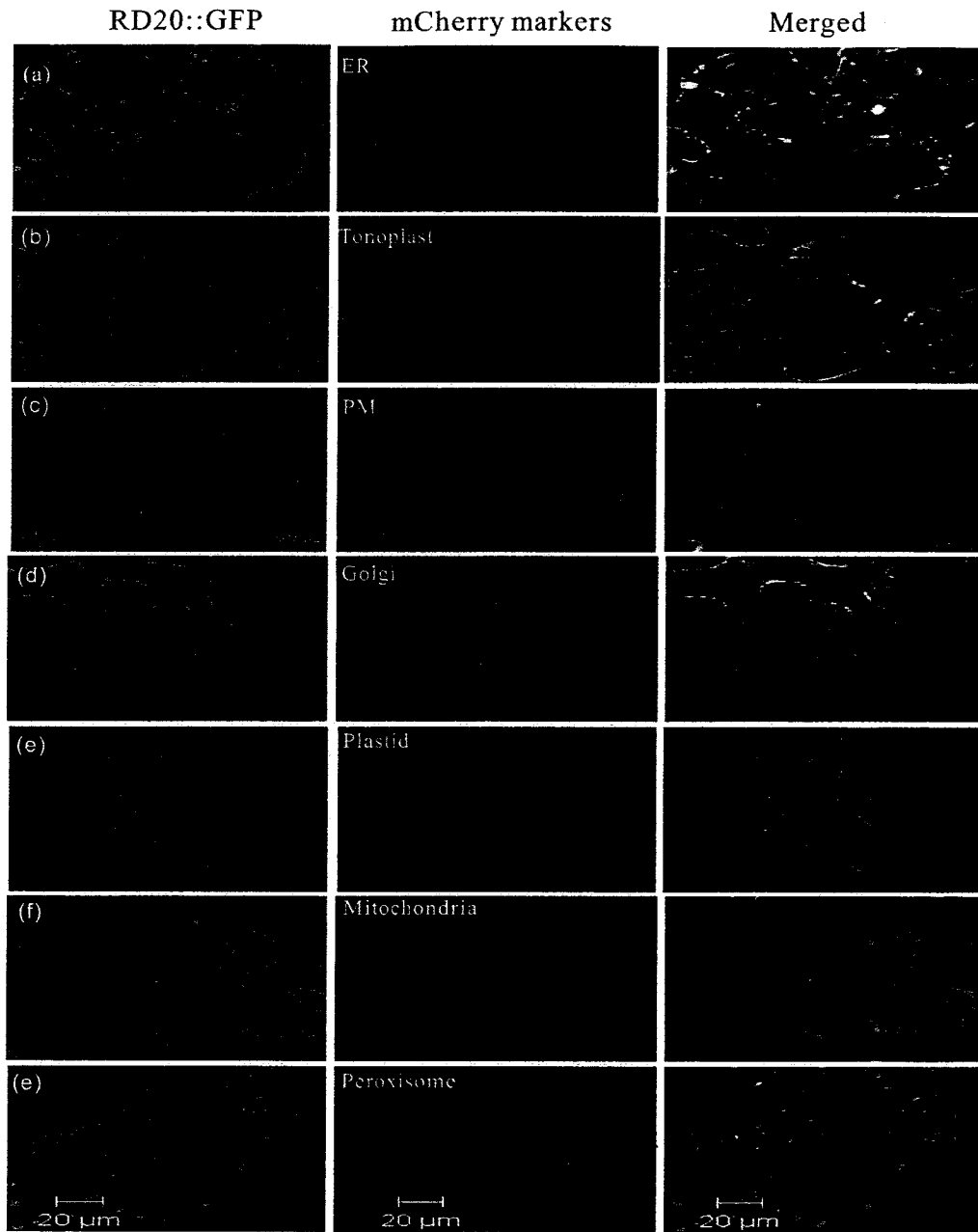


Figure 7. Cellular localization of RD20::GFP fusion protein co-expressed transiently with seven cellular mCherry markers in the tobacco leaf epidermal cells.

RD20::GFP was co-expressed with markers for (a) ER, (b) Tonoplast membrane, (c) PM, (d) Golgi, (e) Plastid, (f) Mitochondria, (g) Peroxisome. Scale bars indicate 20 μm.

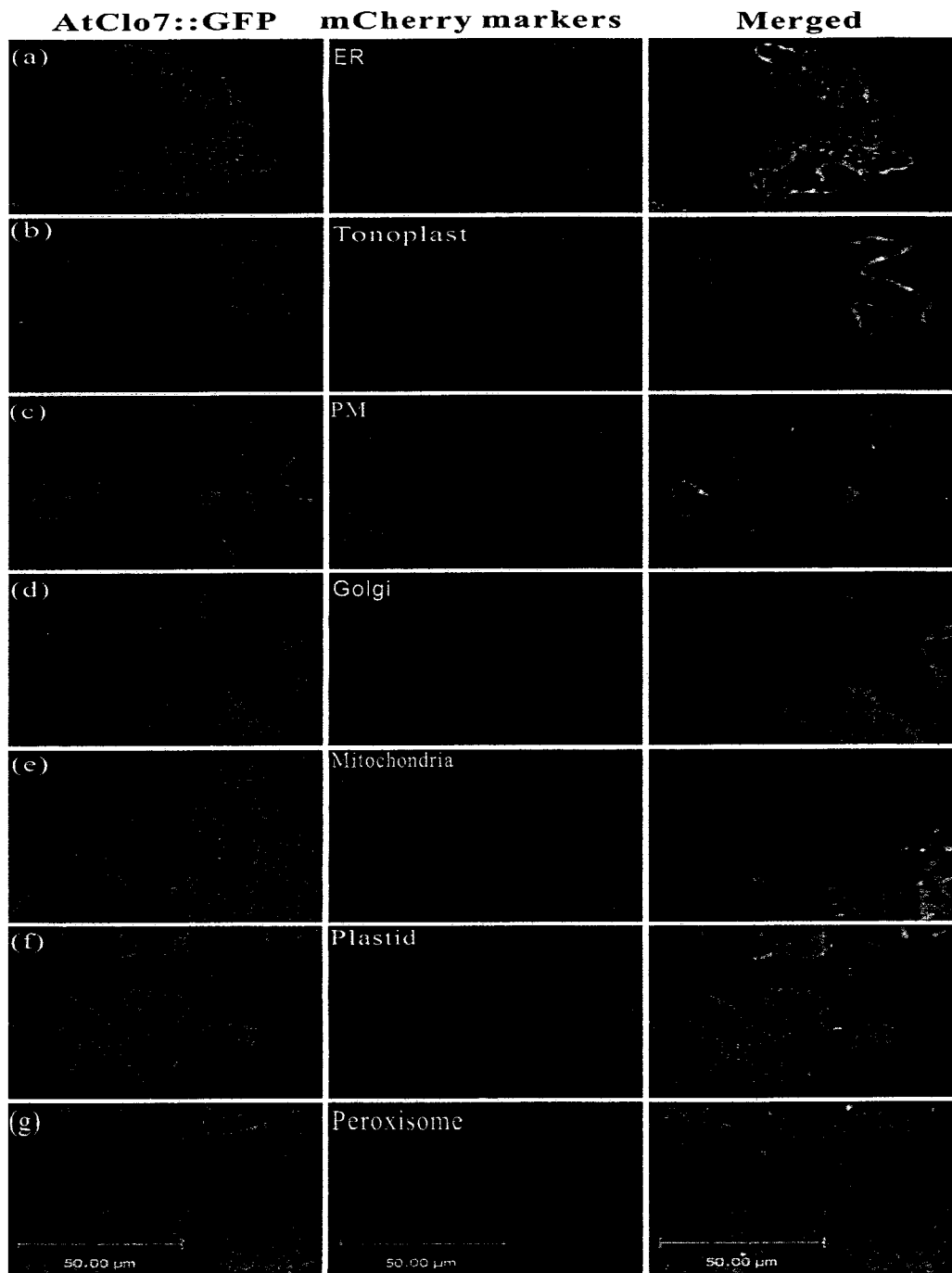


Figure 8. Cellular localization of AtClo7::GFP fusion protein co-expressed transiently with seven cellular mCherry markers in the tobacco leaf epidermal cells.

AtClo7::GFP was co-expressed with markers for (a) ER, (b) Tonoplast membrane, (c) PM, (d) Golgi, (e) Mitochondria, (f) Plastid, (g) Peroxisome. Scale bars indicate 50μM.

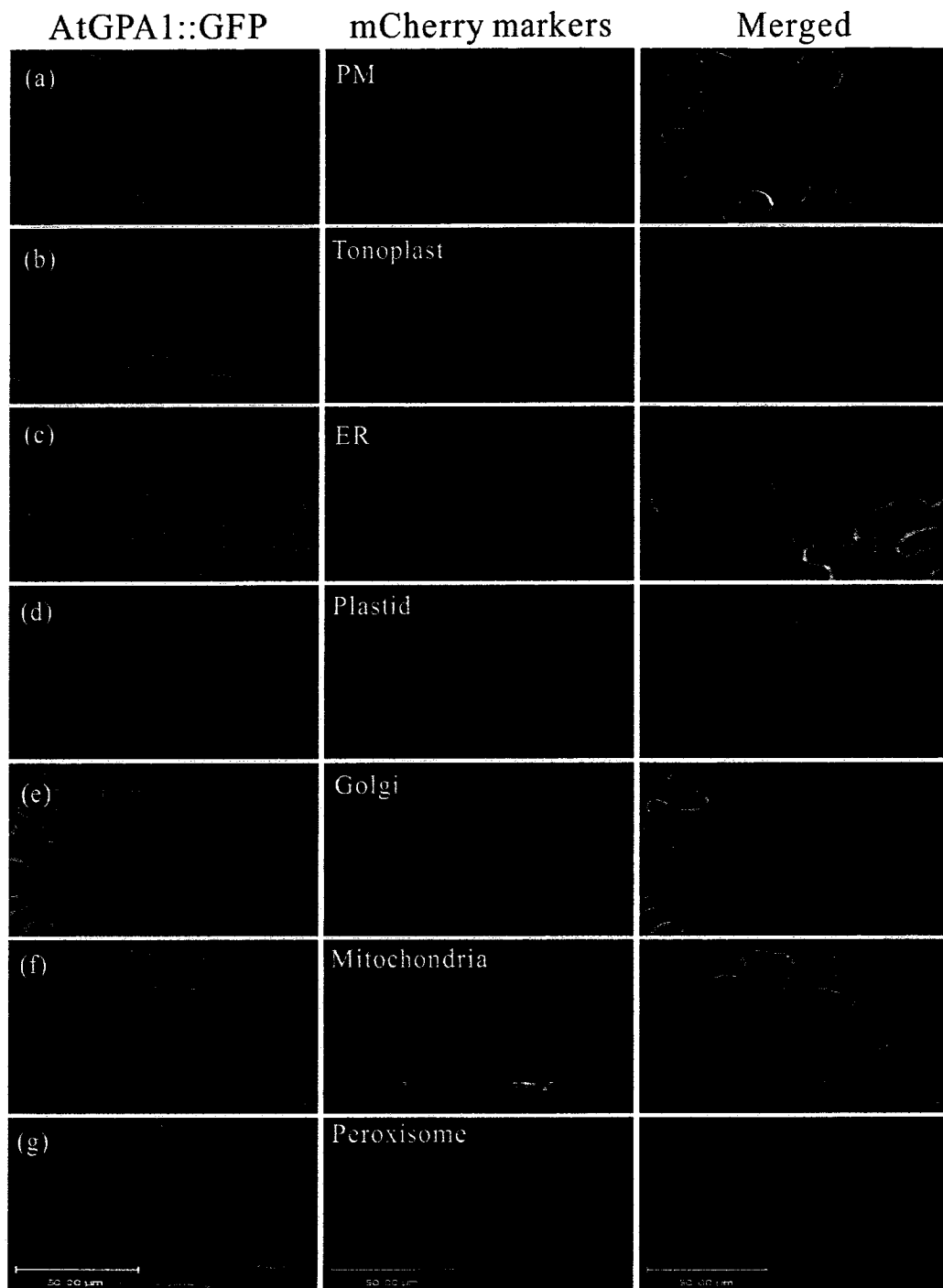


Figure 9. Cellular localization of AtGPA1::GFP fusions protein co-expressed transiently with seven cellular mCherry makers in the tobacco leaf epidermal cells.

AtGPA1::GFP was co-expressed with markers for (a) PM, (b) Tonoplast membrane, (c) ER, (d) Plastid, (e) Golgi, (f) Mitochondria, (g) Peroxisome. Scale bars indicate 50 μ M.

Protein-protein interaction between candidate proteins monitored by the BiFC assay showed RD20/AtClo3 interaction with AtGPA1 in the ER. This localization was shown by both the ER network structure and by co-localization with the ER marker (Figure 10a). This localization was somewhat surprising since the bulk of AtGPA1::eGFP was localized in the PM. It is clear that the YFP signal did not co-localize with other 6 mCherry markers (Table 1). Though the full length GFP fusion indicated that the bulk of the AtGPA1 was localized in the PM, there was no evidence for interaction with RD20 in the PM (data not shown). Similarly, RD20/AtClo3 interacts with AtGPA1^{Q222L} only in ER (Figure 11a). The Q222L mutation inactivates the GTPase activity of G α , and results in a constitutive GTP bound form of the protein.

AtClo7::eGFP had similar localizations as RD20/AtClo3::eGFP, in the ER and tonoplast but AtClo7 interacts with AtGPA1 in the plasma membrane and the tonoplast (Figure 12a and 12b). Similar results showed that AtClo7 interacts with AtGPA1^{Q222L} in the plasma membrane and the tonoplast (Figure 13a and 13b).

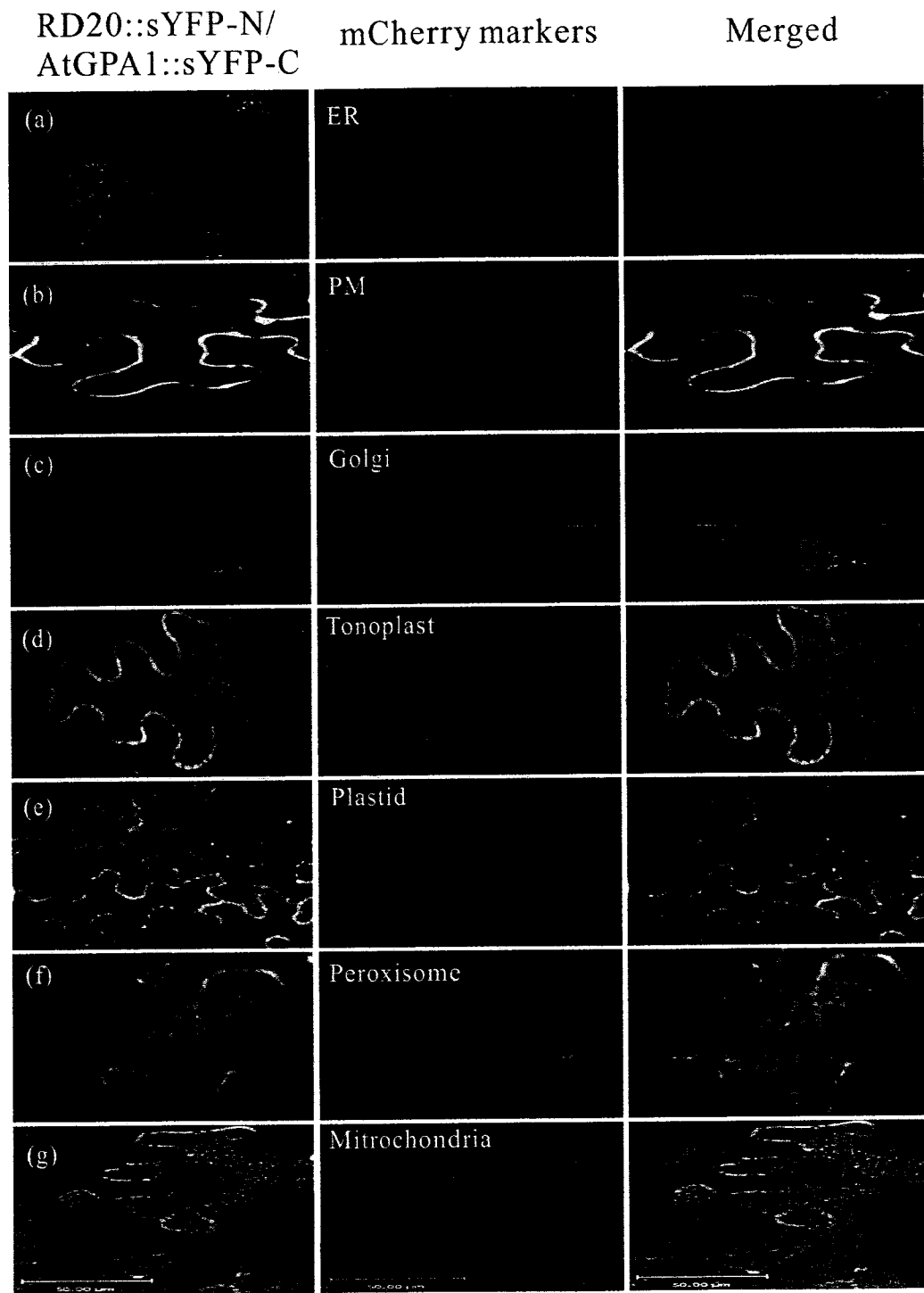


Figure 10. Interaction of AtGPA1::*sYFP-C* and RD20::*sYFP-N* fusions protein co-expressed transiently with seven cellular mCherry makers in the tobacco leaf epidermal cells.

The interaction fusion proteins was co-expressed with markers for (a) ER, (b) PM, (c) Golgi, (d) Tonoplast membrane, (e) Plastid, (f) Peroxisome, (g) Mitochondria. Scale bars indicate 50 μ M.

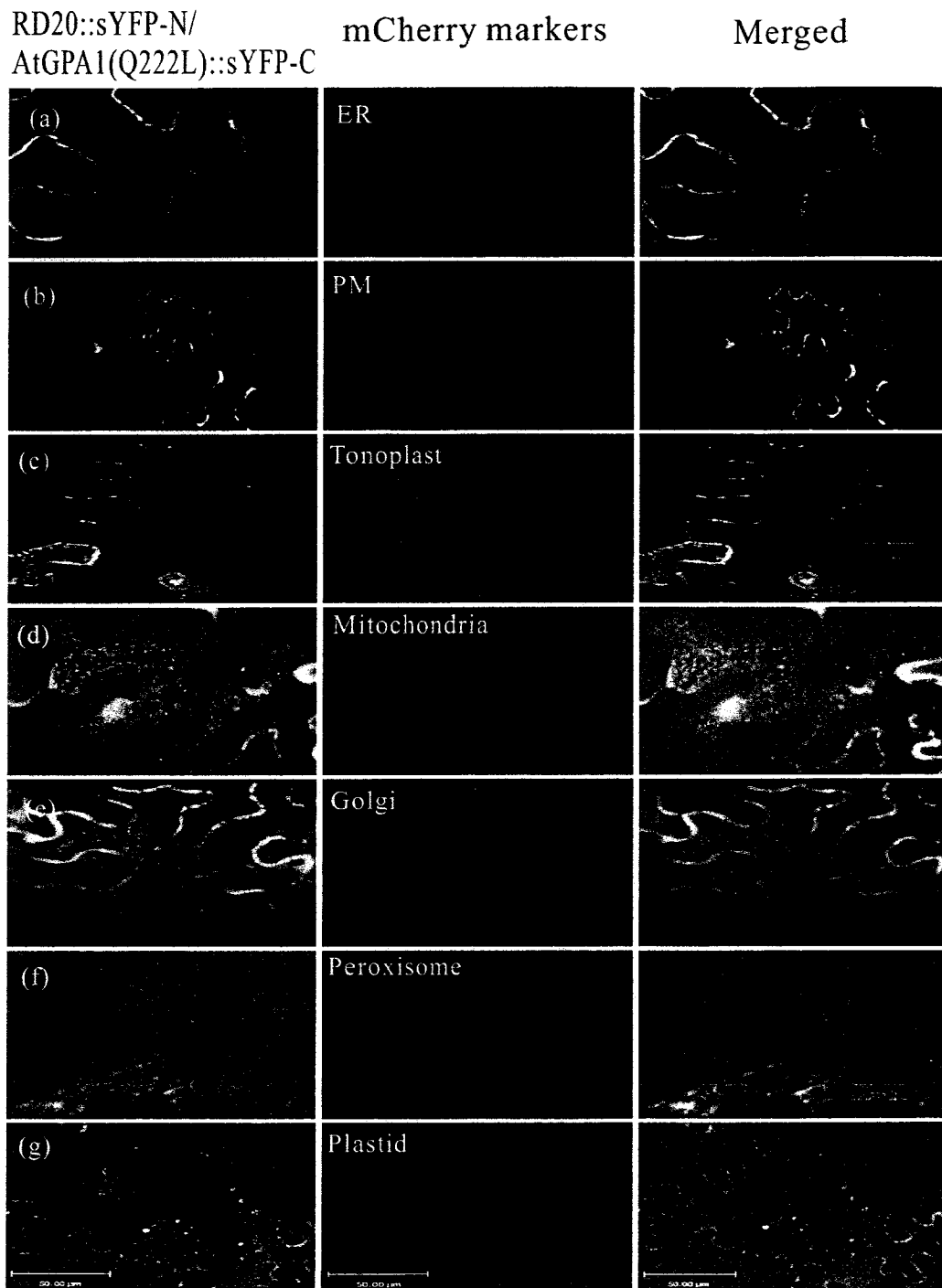


Figure 11. Interaction of AtGPA1(Q222L)::sYFP-C and RD20::sYFP-N fusions protein co-expressed transiently with seven cellular mCherry makers in the tobacco leaf epidermal cells.

The interaction fusion proteins was co-expressed with markers for (a) ER, (b) Tonoplast membrane, (c) PM, (d) Mitochondria, (e) Golgi, (f) Peroxisome, (g) Plastid. Scale bars indicate 50 μ M.

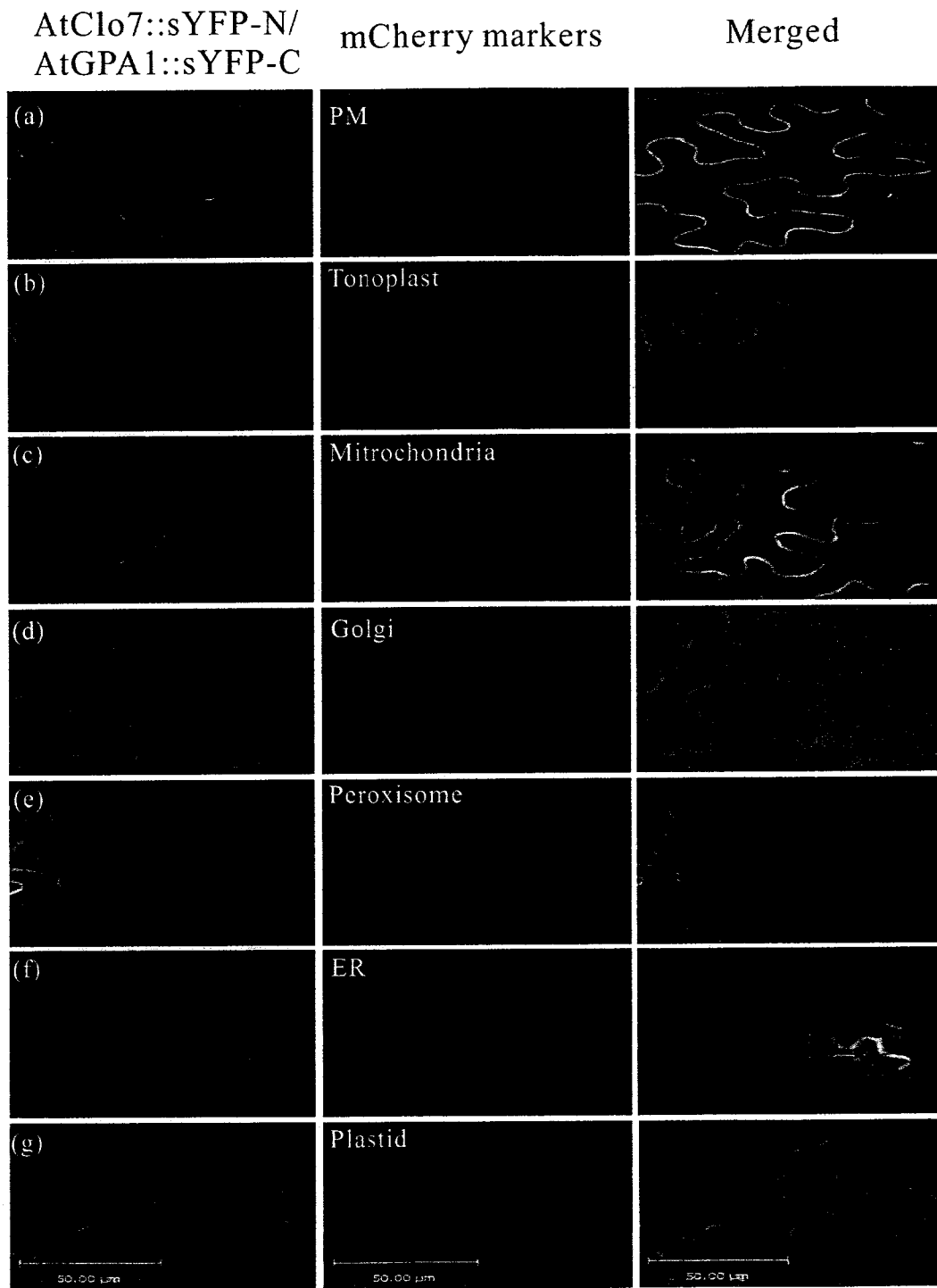


Figure 12. Interaction of AtGPA1::sYFP-C and AtClo7::sYFP-N fusions protein co-expressed transiently with seven cellular mCherry makers in the tobacco leaf epidermal cells.

The interaction fusion proteins was co-expressed with markers for (a) PM, (b) Tonoplast membrane, (c) Mitochondria, (d) Golgi, (e) Peroxisome, (f) ER, (g) Plastid. Scale bars indicate 50 μ M.

AtClo7::sYFP-N/
AtGPA1(Q222L)::sYFP-C

mCherry markers

Merged

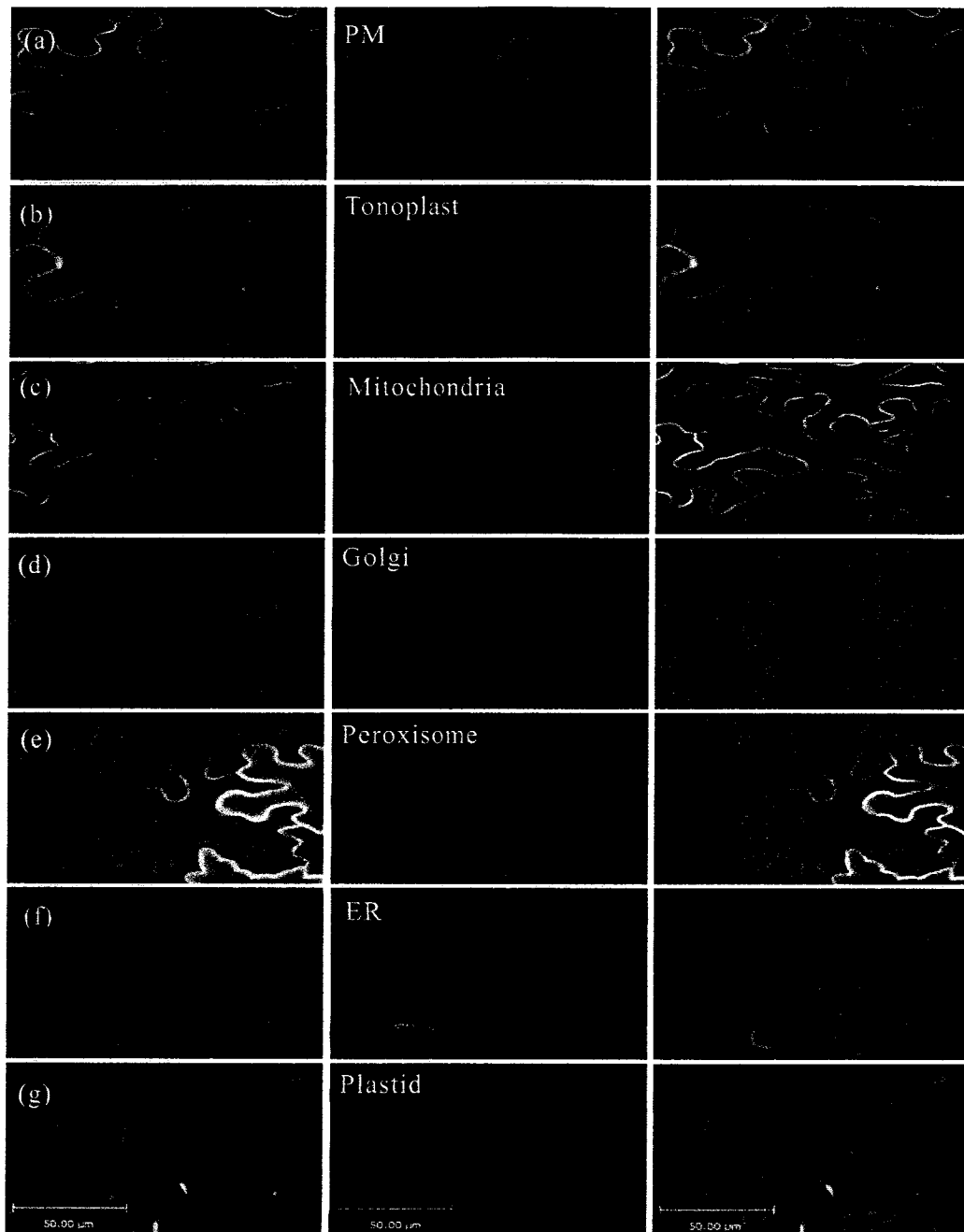


Figure 13. Interaction of AtGPA1(Q222L)::sYFP-C and AtClo7::sYFP-N fusions protein co-expressed transiently with seven cellular mCherry makers in the tobacco leaf epidermal cells.

The interaction fusion proteins was co-expressed with markers for (a) PM, (b) Tonoplast membrane, (c) Mitochondria, (d) Golgi, (e) Peroxisome, (f) ER, (g) Plastid. Scale bars indicate 50μM.

Both positive and negative controls for BiFC localized as expected. The Arabidopsis G α subunit (AtGPA1) and the Arabidopsis regulator of G-protein signaling protein 1 (AtRGS1) clearly interacted in the PM (Figure 14e). The interaction of these proteins was previously shown by BiFC assay, yeast split-ubiquitin assay and by co-immunoprecipitation in vitro (Grigston et al., 2008). Four pairs of protein-YFP fusions that are localized in similar cellular compartments but are known to not interact were used as negative controls. The first two pairs, Hva22d-nYFP and P24B1-cYFP, and Hva22d-nYFP and PIS2-cYFP (Table 8), were shown not to interact by BiFC assay (unpublished data, Hugo Zheng) (Figure 14a and 14b). The third pair and fourth pair were J900-sYFP-N with PIP2A-sYFP-C and RD20-sYFP-N with P24B1-cYFP. The last two pairs were shown not to interact in the BiFC assay in this work (Figure 14c and 14d) and indicate that the YFP fusion proteins will not interact based solely on the interaction potential of the YFP fragments.

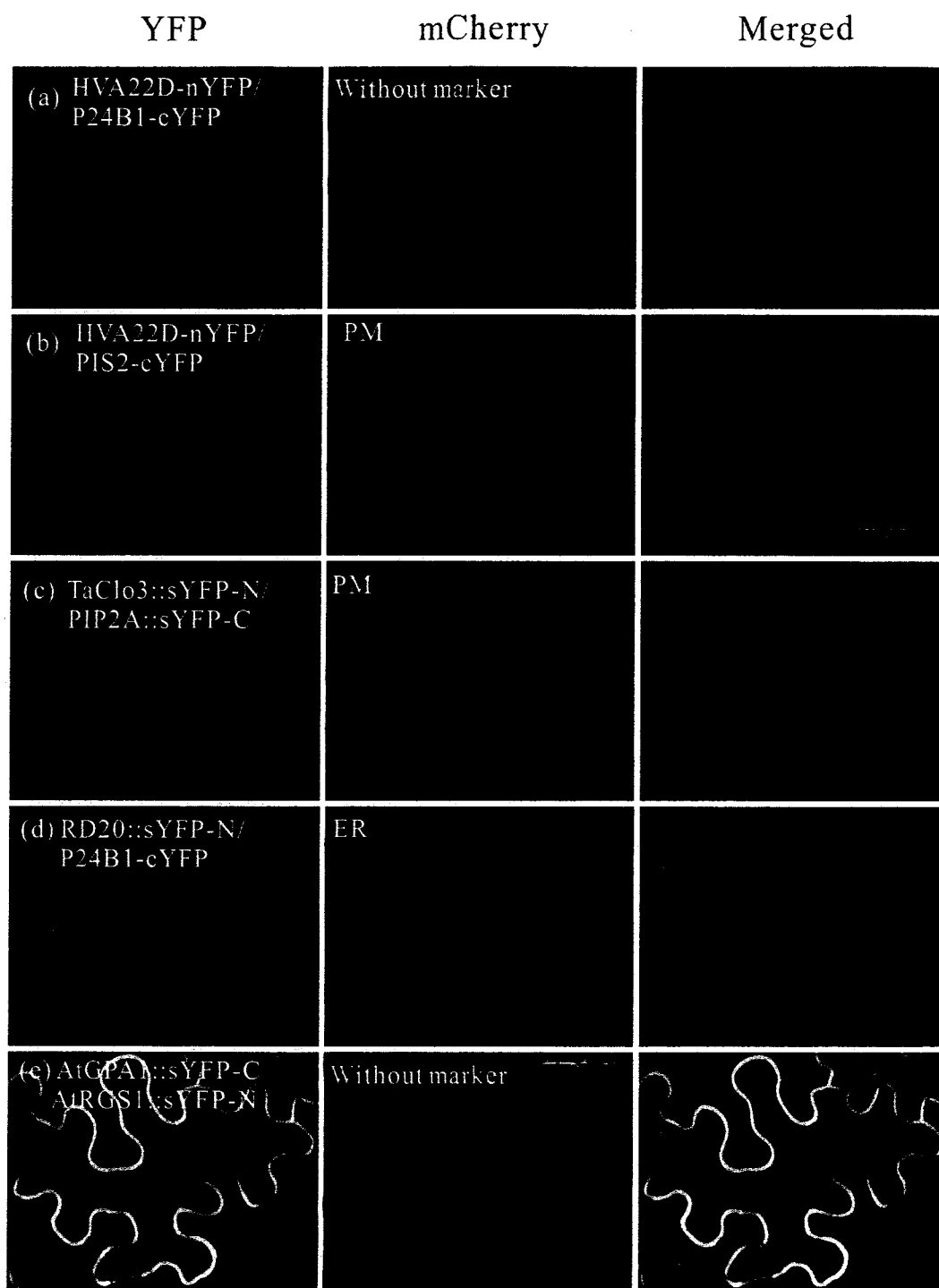


Figure 14. Negative and positive controls used in BiFC experiment.

Negative control pairs (a) HVA22D-nYFP and P24 β 1-cYFP, (b) HVA22D-nYFP and PIS2-cYFP, (c) TaClo3::sYFP-N and PIP2A::sYFP-C, (d) RD20::sYFP-N and P24 β 1-cYFP, (e) Positive control pairs (scale, 100 μ M) : AtGPA1::sYFP-C and AtRGS1::sYFP-N. Scales for (a) to (d) is 50 μ M. Scales for (e) is 100 μ M.

4. TaClo3/J900 protein interacts with TaGA3_Nor, but not TaGA3_Nor^{Q223L} and TaGA3_CS

TaClo3/J900::eGFP fusion localized in the ER as indicated by ER network structure and co-localization with ER markers, as well as in the tonoplast (Figure 15a and b). TaGA3_Nor::eGFP and TaGA3_CS::eGFP localized in both ER and PM (Figure 16a and 17a). TaClo3::eGFP, TaGA3_Nor::eGFP and TaGA3_CS::eGFP did not localize with other cellular makers (Figure 16 and 17).

The interaction between TaClo3/J900 and TaGA3_Nor; TaClo3/J900 and TaGA3_Nor^{Q223L}; TaClo3/J900 and TaGA3_CS was also studied. Approximately 20% of epidermal cells in leaves infiltrated with *Agrobacterium tumefaciens* harboring TaClo3/J900-sYFP-N and TaGA3_Nor were shown to have YFP fluorescence. The localization of the interaction between TaClo3 and TaGA3_Nor is difficult to analyze, because the level of expression for this interaction was quite low. It was not possible to unambiguously determine the location of the fluorescent signal (Figure 18a). The BiFC interaction assay between TaClo3/J900 and TaGA3_Nor^{Q223L} and between TaGA3_CS and TaGA3_Nor^{Q223L} did not show significant fluorescence (Figure 18b and 18c).

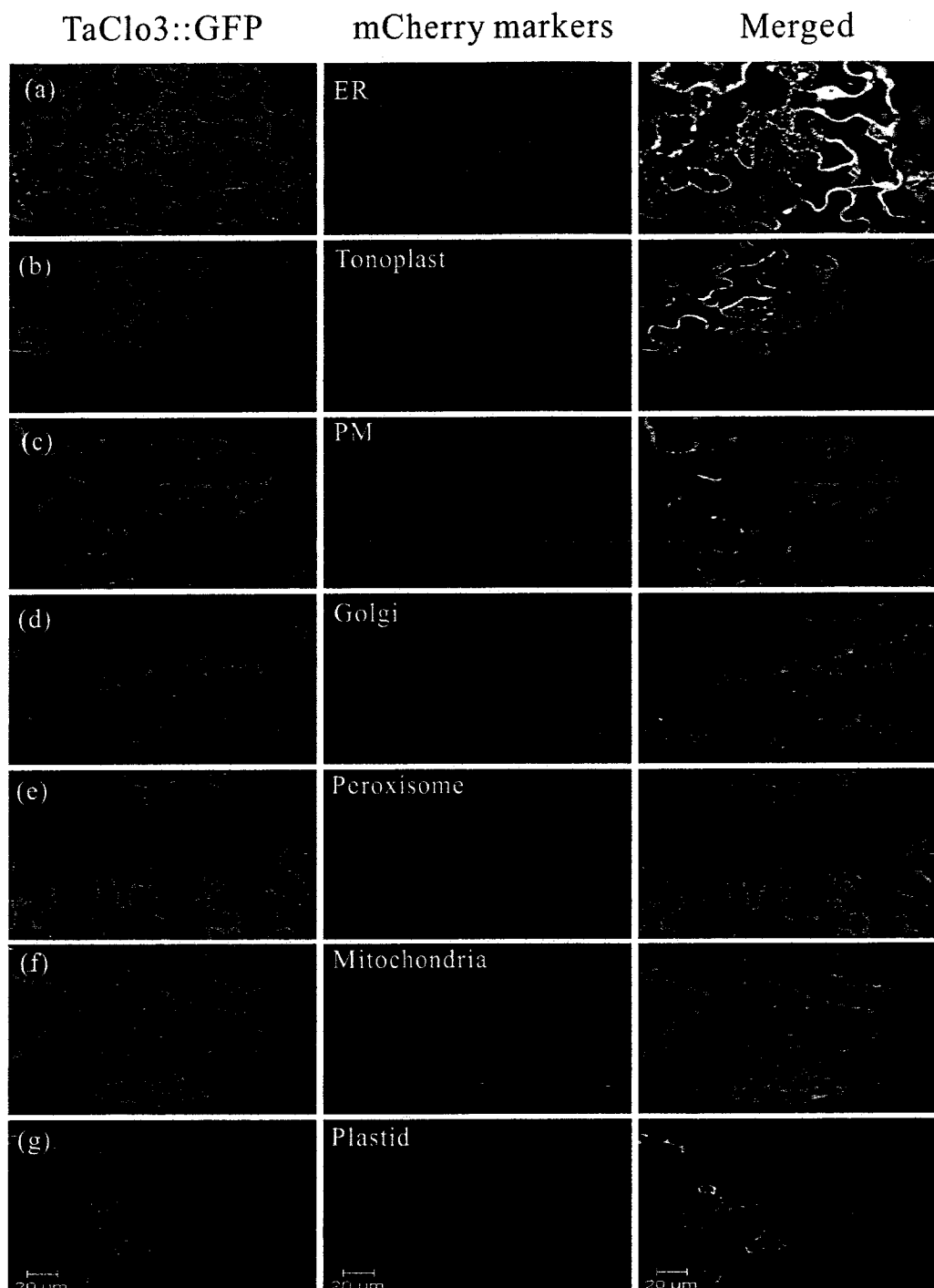


Figure 15. Cellular localization of TaClo3::GFP fusions protein co-expressed transiently with seven cellular mCherry makers in the tobacco leaf epidermal cells.

TaClo3::GFP was co-expressed with markers for (a) ER, (b) Tonoplast membrane, (c) PM, (d) Golgi, (e) Peroxisome, (f) Mitochondria, (g) Plastid. Scale bars indicate 20 μ M.

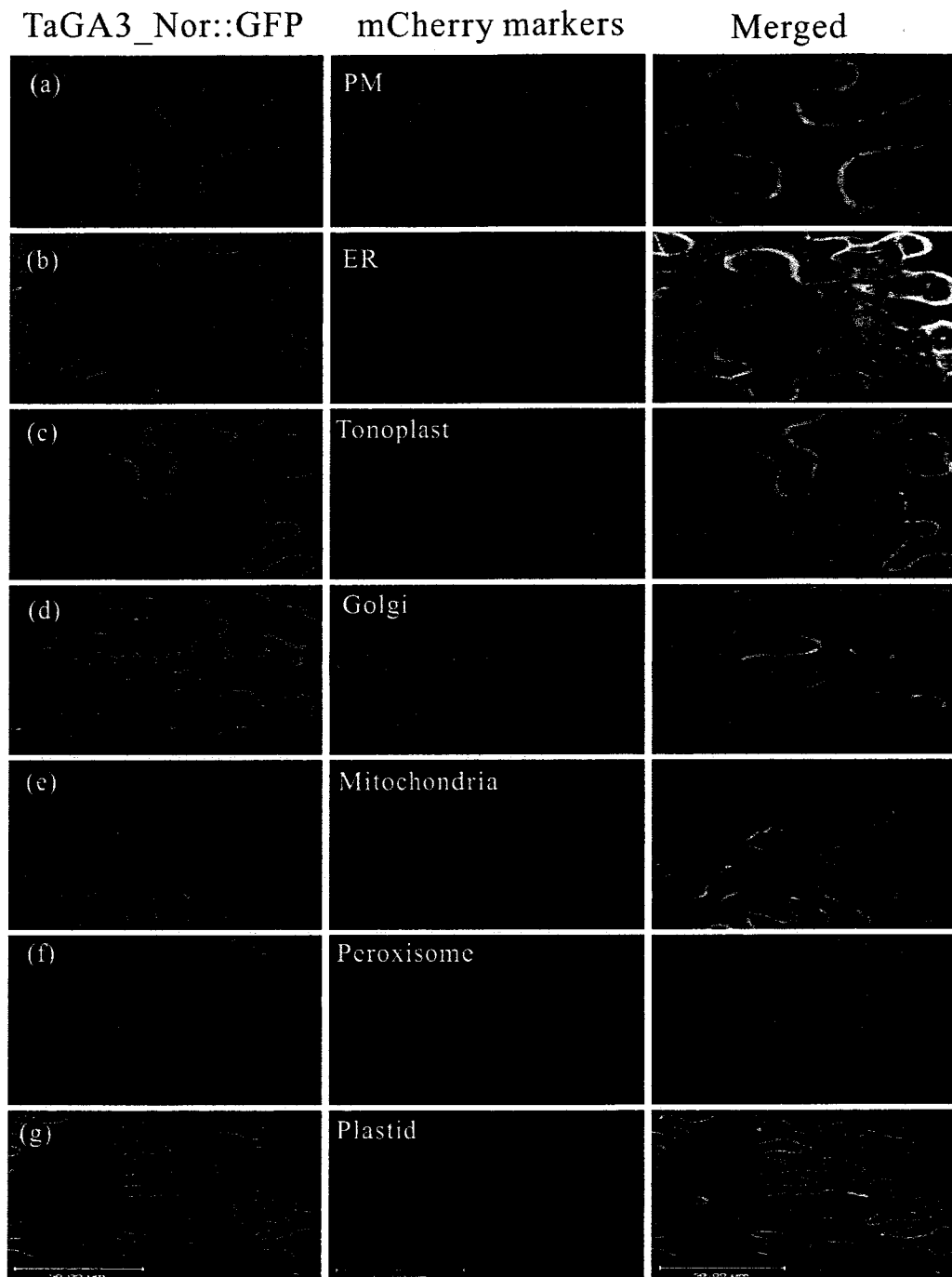


Figure 16. Cellular localization of TaGA3_Nor::GFP fusions protein co-expressed transiently with seven cellular mCherry makers in the tobacco leaf epidermal cells.

TaGA3_Nor::GFP was co-expressed with markers for (a) PM, (b) ER, (c) Tonoplast membrane, (d) Golgi, (e) Mitochondria, (f) Peroxisome, (g) Plastid. Scale bars indicate 50 μ M.

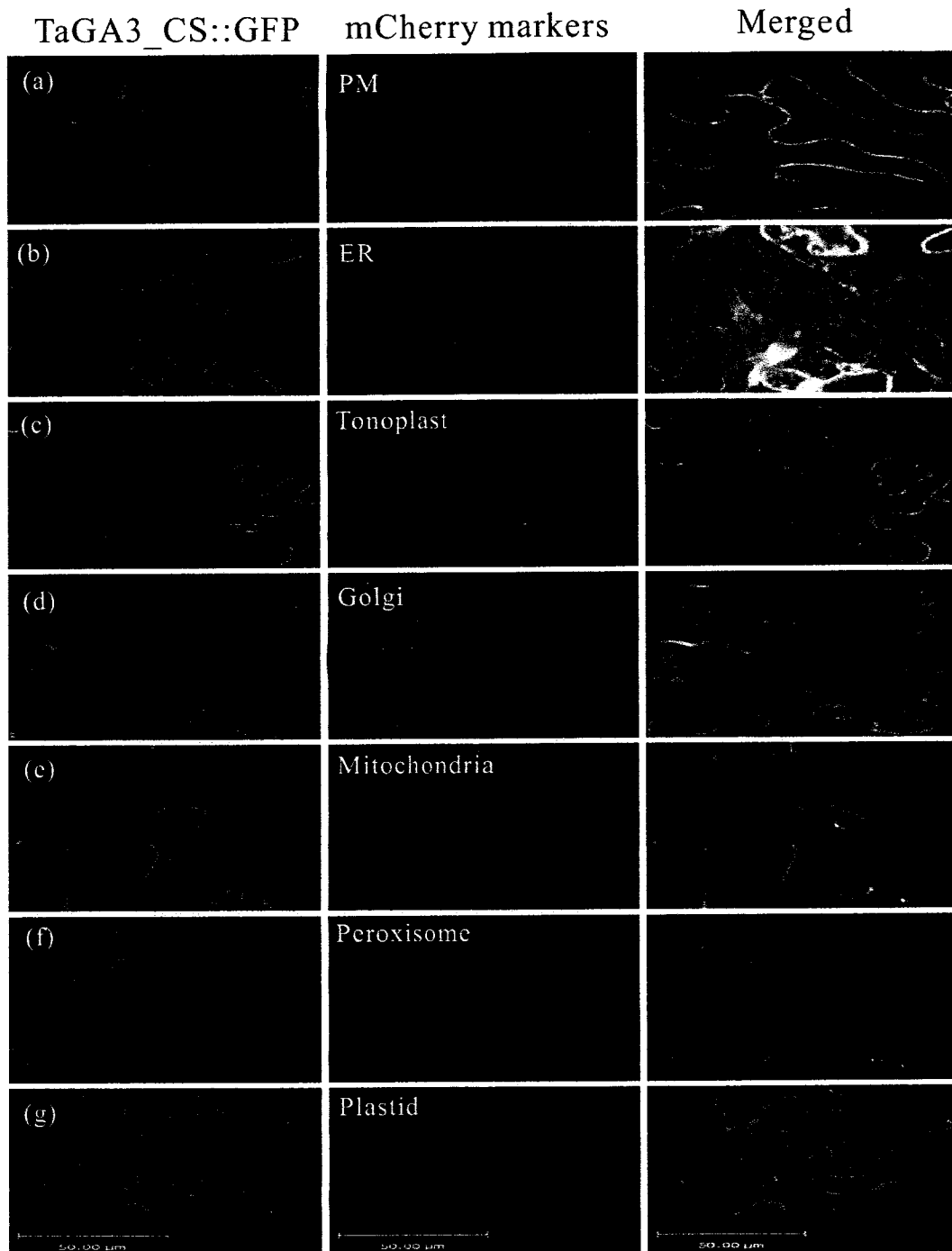


Figure 17. Cellular localization of TaGA3_CS::GFP fusions protein co-expressed transiently with seven cellular mCherry makers in the tobacco leaf epidermal cells.

TaGA3_CS::GFP was co-expressed with markers for (a) PM, (b) ER, (c) Tonoplast membrane, (d) Golgi, (e) Mitochondria, (f) Peroxisome, (g) Plastid. Scale bars indicate 50 μ M.

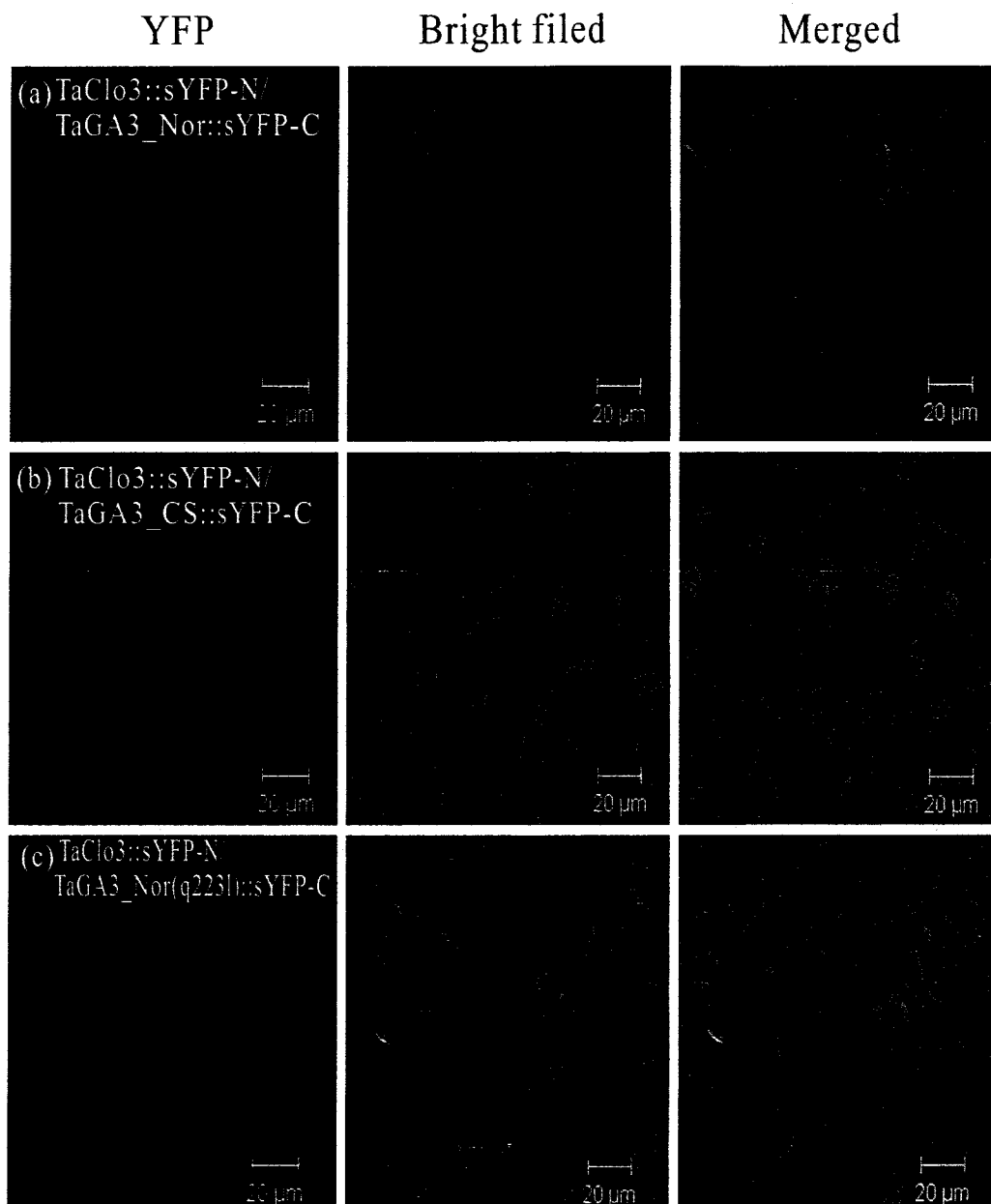


Figure 18. Interaction of TaClo3::sYFP-C and TaGA3::sYFP-N fusions protein expressed transiently in the tobacco leaf epidermal cells.

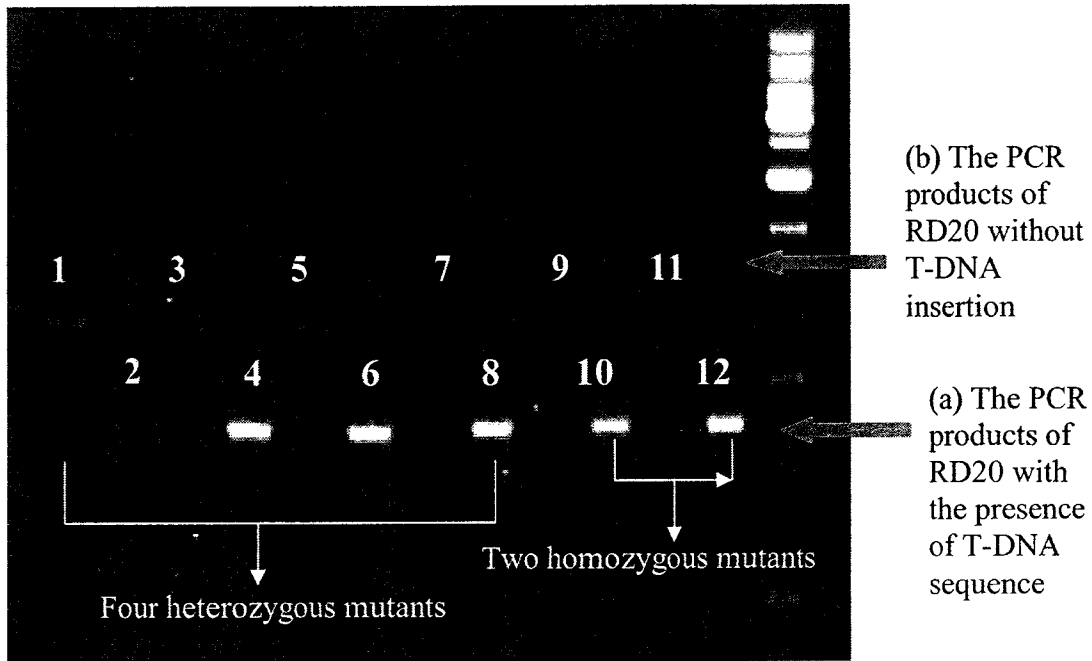
(a) TaClo3::sYFP-N and TaGA3_Nor::sYFP-C, (b) TaClo3::sYFP-N and TaGA3_CS, (c) TaClo3::sYFP-N and TaGA3_Nor (Q223L)::sYFP-C. Scale bars indicate 20 μ M.

5. Identification of *rd20* T-DNA Insertion Mutants

To examine the role of RD20 in plants, two homozygous mutant plants which carry a T-DNA insertion in the RD20 gene were identified from a mutant seed stock (Figure 19a). The amplification of single product using a RD20 gene specific primer and a T-DNA insertion specific primer indicated the presence of a T-DNA insertion in the coding region of RD20. There was no amplification product from two RD20 gene specific primers designed to span the insertion site, which indicated the plants were homozygous for the T-DNA insertion. Amplification from both LB and gene specific primer and the two gene specific primers would indicate that the plants were heterozygous for the mutation. The amplification products from the mutant plants were further confirmed by sequencing and the results show that the homozygous RD20 mutant harbors a T-DNA insertion in exon 2, is 490 bases from 5' to the start of transcription (Figure 19b).

Further studies are needed to characterize the phenotype of *rd20* mutants in *Arabidopsis* in response to treatment with ABA, D-glucose and abiotic stresses including high salt, and osmotic stress.

(a)



(b)

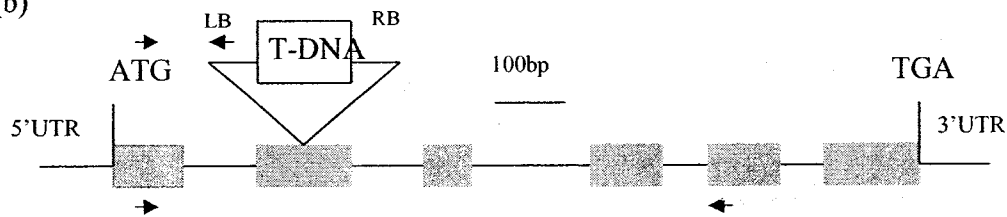


Figure 19. RD20 T-DNA homozygous mutant identification and T-DNA location.

(a) RD20 homozygous mutant plants identified by the PCR. Each two lanes have the PCR products of a single plant. The odd numbered lanes are the PCR products of two gene specific primers for RD20 designed to span the T-DNA insertion site; a PCR product is only generated in the absence of the T-DNA insertion. The even numbered lanes are the PCR products from amplification with one gene specific primer and a left border T-DNA primer used to detect the presence of T-DNA insertion. Homozygous mutants have only PCR product generated the second pair of primers.

(b) Map showing the relative position of the rd20 mutant T-DNA insertion and the primers used in (a).

PART IV. DISCUSSION

1. AtGPA1 interacts with RD20/AtClo3 and AtClo7

Heterotrimeric G-protein proteins play an important role in signal transduction pathways in plants and animals. The presence of one or two copies of $G\alpha$ genes in well characterized plant species as well as low numbers of genes encoding $G\beta$ and $G\gamma$ raises questions about whether or not they might be involved in multiple signaling pathways in plants. It has been hypothesized that complexity of signaling may be derived from a variety of effector molecules that interact with the heterotrimeric G proteins (Assmann, 2005). The diverse localization of $G\alpha$ and interacting calcium binding proteins investigated in this study suggests additional mechanisms for the diversity of signaling derived from G protein signaling. Two proteins, RD20/AtClo3 and AtClo7 from the same gene family interact with AtGPA1 in two different locations. RD20/AtClo3 interacts with AtGPA1 in the ER (Figure 10a); while AtClo7 interacts with AtGPA1 at the PM (Figure 11a). In addition there are differential localization patterns between GFP tagged RD20, AtClo7, and AtGPA1 compared with the location of the interaction. AtGPA1 localizes to the PM (Figure 9a) but AtGPA1 interacts with RD20 in the ER; AtClo7::eGFP localizes in the ER and the tonoplast (Figure 8a and 8b) but interacts with AtGPA1 at the PM and the tonoplast. The different locations of these interactions indicate that the single conical $G\alpha$ in Arabidopsis is involved in different signaling pathways in these cellular locations.

Clo3/RD20 and Clo7 are the first calcium binding proteins in Arabidopsis reported to interact with AtGPA1. Calcium signaling is a well established component of molecular signaling (Tuteja and Mahajan, 2007). It would be important to further investigate the role of AtGPA1 interaction in calcium initiated stress response signaling in plants. This study shows that both of the GFP tagged RD20/AtClo3 and AtClo7 proteins are in the ER and tonoplast (Figure 7a and 7b; Figure 8a and 8b). The orientation of EF hand calcium binding domain in the lumen or cytosol is important for Ca^{2+} signaling. The TopPred program predicted that RD20/AtClo3 contains one hydrophobic transmembrane domain on the C terminal side of the calcium EF hand binding domain (Figure 2 and Figure 4a and 4b), but it did not predict whether the C-terminal side or N-terminal side is facing to the cytosol. AtGPA1 localization is dynamic from the ER to the Golgi to the PM indicating that AtGPA1 is on the cytosolic side of endomembrane and the PM. In the BiFC assay the C-terminal end of RD20/AtClo3 fused with sYFP-N half interacted with AtGPA1 in the ER; this indicates that the C-terminus of RD20/AtClo3 is on the cytosolic side of the ER membrane. This is consistent with previous evidence that the C-terminal end of RD20/AtClo3 is lost after protease treatment of microsomes and subsequent detection of intact N-terminal end of RD20/AtClo3 by western blot analysis (Partridge and Murphy, 2009). This orientation would enable the calcium EF hand binding domain to interact with pools of calcium within the ER lumen and this might regulate the interaction between RD20/AtClo3 and AtGPA1, possibly by affecting its GTPase activity on the cytosolic side of the ER membrane. Similarly, the results of the transmembrane prediction of AtClo7 (Figure 2 and Figure 4e and 4f) show that the calcium binding domain is on the C-terminal side of

the transmembrane domain. The interaction of AtClo7 and AtGPA1 at the PM (Figure 12a) and the tonoplast (Figure 12b) indicate that the C-terminal side of AtClo7 is facing the cytosolic side of the tonoplast membrane and that the N-terminal side including the calcium binding domain is facing the vacuole lumen. The possibility that AtClo7 may activate or inactivate AtGPA1 in response to calcium signalling could be investigated with *in vitro* interaction studies using proteins purified from *E. coli* expression systems. The interaction and activity of the proteins could be quantified with different Ca^{2+} , GTP/GDP levels as well as different states of protein phosphorylation. This approach is currently in progress in our laboratory.

$\text{G}\alpha$ might be regulated in different ways for its signaling: release from the heterotrimeric complex, interaction with proteins affecting its GTPase activity, GDP/GTP exchange, and interaction with downstream proteins such as phospholipases C and D. RD20 and AtClo7 could be GTPase accelerating proteins (GAPs) or could regulate GDP/GTP exchange. Our data show that RD20 and AtClo7 interact with GTP-bound form of AtGPA1 (Q222L) (Figure 11a and Figure 13a and 13b). Johnston et al. (2007) reported that *in vitro* characterization of GTP hydrolysis and GDP release of AtGPA1 suggest that under steady state conditions, the protein is predominately in the GTP-bound form. This is in contrast to other $\text{G}\alpha$ s described in animals, where show the $\text{G}\alpha$ ground state is in the GDP bound form. Further studies should be done to investigate the GTPase activity of AtGPA1 after interaction with AtClo7 or RD20/AtClo3.

The interaction of AtGPA1 with RD20 in the ER suggests that G α may play a role in membrane trafficking. The ER is where the heterotrimeric complex forms and G α is palmitoylated before moving to the plasma membrane (Marrari et al., 2007). BiFC results showed that AtGPA1 interacts with RD20 in the ER suggesting that RD20 might enhance the accumulation or retention of AtGPA1 on the surface of the ER. Therefore, we co-expressed full length AtGPA1::eGFP fusion with RD20/AtClo3::mCherry to test if high levels of RD20/AtClo3 expression would cause AtGPA1 to accumulate in the ER rather than the PM, but there was no evidence for this (data not shown). This might be because of agroinfiltration approach is not very sensitive for detecting the small pool of the AtGPA1 in the ER. Evidence from humans G α s showed the fast trafficking of the activated G protein subunits between the plasma membrane and intracellular membranes with a $t_{1/2}$ less than 1 minute (Chisari et al., 2007). Interaction of AtGPA1 with RD20/AtClo3 in the ER suggests that there is a minor portion of AtGPA1 in the ER. Studies of human G α indicate that a number of G α subunits play a role in membrane trafficking (Marrari et al., 2007). This raises the possibility that RD20/AtClo3 might be involved in the trafficking of AtGPA1 from the ER to the PM. This interaction may be important before AtGPA1 progresses to the PM or for shuttling activated G α from the PM to the ER. In contrast AtClo7 protein interacts with AtGPA1 at the PM. AtClo7 might mediate the movement of heterotrimeric complex movement from the ER to the PM. This hypothesis can be further test using fluorescent protein-tagged AtClo7 with photoswitchable Dronpa (Chisari et al., 2007) and FRAP (Chisari et al., 2007) on living cells to observe the dynamic movement of the proteins.

Though AtClo3/RD20 and AtClo7 belong to the same gene family, their distinct localizations indicate that they likely have different functions. The percentage similarity between AtClo7 and RD20/AtClo3 is not high; they share only 61% aa sequence similarity. In addition, the location of the interaction of AtGPA1 with AtClo7 in the tonoplast and the PM, is different from RD20/AtClo3. This also indicates that their interaction with AtGPA1 is very specific. Microarray data from NASCarray, showed that the most abundant expression of AtClo7 is in the stamen and does not change during ABA stress. RD20/AtClo3 is most highly expressed in petals and sepals and is significantly induced in mesophyll cells with ABA treatment. RD20/AtClo3 expression is also highly induced by salt and drought and the consensus sequence for the ABA-responsive element (ABRE) is found in the promoter region of the gene (Takahashi et al., 2000). AtGPA1 is involved in ABA, drought, and NaCl signaling pathways based on studies with the loss-of-function mutant *gpa1* (Lapik and Kaufman, 2003). However, there are no previous studies to report that AtClo7 is in the ABA, drought or salt stress responses and microarray data indicate that the level of AtClo7 gene expression is not changed under drought conditions.

2. TaClo3 and TaGA3 in wheat

Analysis of Ga and TaClo3/J900 from wheat showed important commonalities and differences with studies on the Arabidopsis homologs. The structural predictions by TopPred for TaClo3/J900 in wheat surprisingly indicate that the EF-hand calcium binding domain is on the cytosolic side of the protein (Figure 2 and 3f), unlike the prediction for RD20 and AtClo7 which indicates the EF-hand domain would be on the

luminal side of the ER membrane. Though the location of the interaction of TaGA3 with TaClo3 is unclear (Figure 18a) TaClo3/J900::eGFP was shown to localize in the ER and the tonoplast (Figure 15a and 15b). TaClo3 might play different roles in calcium signaling than AtClo3.

The fact that G α (TaGA3) in wheat was observed in the ER indicates that there may be different localizations of other members of the gene family in wheat. GFP tagged TaGA3_Nor and TaGA3_CS localize in the ER and the plasma membrane (Figure 16a and 17a). G α is a well known plasma membrane protein; post translational modifications in the ER or a role in organelle trafficking might explain its presence in the ER (Marrari et al., 2007). The localization of G α in Arabidopsis is primarily in the PM, and its apparent transit through the ER is sufficiently fast for it not to be detected as a GFP fusion by microscopy. Thus the relatively high signal for the wheat TaGA3::eGFP fusion in the ER is surprising.

3. TaClo3 interacts with TaGA3_Nor, but not TaGA3_CS and GTP-bound form G α

Wheat G α proteins may have more diverse roles in signaling than Arabidopsis. There is an interaction between TaClo3/J900 and full length allele TaGA3_Nor allele, the G α allele from the cultivar Chinese Spring TaGA3_CS, which is shortened by 31 amino acids showed no interaction with TaClo3/J900 (Figure 18b). Its truncation results in the loss of the GTP binding region 6 and part of the G protein receptor region due to an internal duplication in the coding region. Interactions between AtClo7 and RD20/AtClo3

with TaGA3_CS were tested. TaGA3_CS did not interact with RD20/AtClo3, however, TaGA3_CS did interact with AtClo7 (data not shown). These data suggest that TaGA3_CS might have different functions from TaGA3_Nor and the terminal 31 amino acid are critical for binding to RD20/AtClo3 and TaClo3/J900 but not for AtClo7.

The G α gene family in rice, and by extension, in monocots, might be in the GDP bound form in their ground state (Seo et al., 1997). Further investigation is needed to assess the regulation of G α GTPase activity or GTP/GDP exchange by AtClo3 and AtClo7 and TaClo3. We used the mutant form of TaGA3_Nor (TaGA3_Nor^{Q223L}) which binds to GTP constitutively and has no GTPase activity to determine interaction with TaClo3 (Oki et al., 2005; Ullah et al., 2003). The Q223L mutant did not interact with TaClo3/J900 by BiFC (Figure 18c). TaClo3/J900 has preferential interactions with the wild type, presumably GDP bound state. The different forms of G α in wheat ought to be studied *in vitro* to characterize their affinity for these calcium binding proteins and the effect of Ca²⁺ on their interaction and their effect on GTPase activity and GTP/GDP exchange.

The diversity of the G α gene family in wheat is remarkable. Since rice is the most closely related species to wheat among species with fully sequenced genomes and because wheat is a hexaploid species, wheat might be predicted to have three homeologous gene copies. This study identified five genes encoding G α , including the two genes that were previously described and those identified in EST databases in this study. These can be classified into two groups that share less than 92% nucleotide

sequence identity, indicating two non-homeologous clades for Ga in wheat. Two full length Ga cDNA clones, TaGA1 and TaGA2, were isolated from the same hexaploid common wheat cultivar, cv. S615 by Hossain et al. (2003). They reported a 21nt insertion in the coding region of TaGA2 cDNA sequence relative to TaGA1. TaGA1 and TaGA2 have 99.4% nt sequence identity in their coding regions if the 21 nt insertion in TaGA2 is ignored (Table 10). Homeologous gene copies, i.e. orthologous genes derived from the different diploid ancestors of polyploid wheat; typically have 95% to 98% identity within the coding region (Gulick et al., 2005; Ridha Farajalla and Gulick, 2007). The 99.4% sequence identity between TaGA1 and TaGA2 is higher than that of homeologs and suggests that TaGA1 and TaGA2 represent a recent gene duplication. In addition, TaGA2 has a 21bp insertion relative to all other gene copies identified in the *T. aestivum* EST database in GenBank. There are 7 single nucleotide differences within the 1152 ORF of TaGA2 compared to TaGA1 and 11 single nucleotide insertion and deletions and one mismatch in the 3'UTR region. The latter is a much higher difference than one could expect for sequencing errors or allelic differences. The TaGA3_CS cDNA clone from the cultivar Chinese Spring has a 17 bp duplication at position 1038bp of the coding region. The TaGA3_Nor cDNA clone, from the wheat cultivar Norstar has 100% identity (Table 10) to the TaGA3_CS clone except it lacks the 17 bp duplication; this suggests that TaGA3_CS and TaGA3_Nor represent alleles of the same gene. TaGA3 has 98% identity to TaGA1 and TaGA2 in gene coding region, indicating that TaGA3 is a homeolog of TaGA1. The TaGA4 contig is 97% identical to TaGA5 in the coding region, but has 91% identity with TaGA1 and TaGA2, 92% identity with TaGA3. This suggests that TaGA4 is a homeolog of TaGA5, and is paralogous to TaGA1, TaGA2

and TaGA3. Any sequence divergence between genes and alleles could cause differences functional differences. The large degree of gene and genome duplication is apparent from extensive genome sequencing of several species and the multiple gene copies are hypothesized to be redundant. Many examples of genetic redundancy have been described in which no phenotype is apparent until multiple members of gene families have been mutated. In one example, single ethylene DNA binding factors (EDF) knock outs did not show any change in ethylene response phenotype but the quadruple mutant (*edf1/2/3/4*) did show significant phenotypes which indicates that EDF gene family members have functional overlap (Alonso et al., 2003). Single mutants for the abscisic acid receptors GTG1 or GTG2 are not affected by ABA treatment, however, the double mutant *gtg1gtg2* show significant hyposensitivity to 1 μ M ABA during germination (Pandey et al., 2009). The sequence diversity among the Gas in wheat raises the question as to whether the sequence differences have functional significance; the most striking features of this diversity are the apparent two non-homeologous groups and the insertional events that gave rise to TaGA2 and the CS allele of TaGA3.

4. Future perspective

Based on the BiFC interaction studies *in vivo* on G alpha proteins and calcosin proteins in wheat and Arabidopsis, a possible working model is proposed for the role and further investigation of the RD20/AtClo3-AtGPA1, AtClo7-AtGPA1 and TaClo3/J900-TaGA3 interaction (Figure 20). AtClo3 and AtClo7 were observed to bind to the constitutive GTP bound form of AtGPA1 and the wild type form, thus is may bind to either GDP or both form of AtGPA1. In order investigate this model, further studies are needed to examine the effect of GTP/GDP binding to AtGPA1 on the interaction of AtClo3 and AtClo7 as well as the effect of this interaction on the GTPase activity of GPA1. These studies could be carried out as *in vitro* studies with proteins produced in an *E. coli* expression system. The RD20/AtClo3-AtGPA1, AtClo7-AtGPA1 interactions study using a GST/His-tagged pull down assay is in progress in our lab. This system could also be used to investigate Ca²⁺ binding of RD20 and Clo7 and its effect of Ca²⁺ binding on protein interaction and the regulation of GTPase activity. In addition, it would be important to study the possible role of these calcium binding proteins with Gα and Gβγ reassociation and on the interaction of Gα and the heterotrimeric complex with the G protein coupled receptors from Arabidopsis, RGS1 and the ABA receptors GTG1 and GTG2. These interactions could be studied both *in vitro* with GTS or His tag pull down experiments or by co-immunoprecipitation with the complex of RD20/AtClo3- or AtClo7-AtGPA1 *in vivo*.

The investigation of the GTP or GDP bound forms of Gα warrant further study, especially in light of somewhat different reports of the kinetic properties of rice Gα and the Gα variants that described in this work in wheat. GDP release and the turnover GTP

hydrolysis rate of TaGA3 assay can be further studied with TaGA3 and TaClo3 purified from an *E.coli* expression system. In addition, the GDP bound form of the enzyme can be produced by site directed mutation (Lane et al., 2008). Another important line of investigation is whether the RD20/AtClo3 plays a role in retention of AtGPA1 in ER or the relocalization of AtGPA1 from the PM to the ER. It is important to investigate if the localization and interaction of RD20 and Ga may play a role in membrane trafficking.

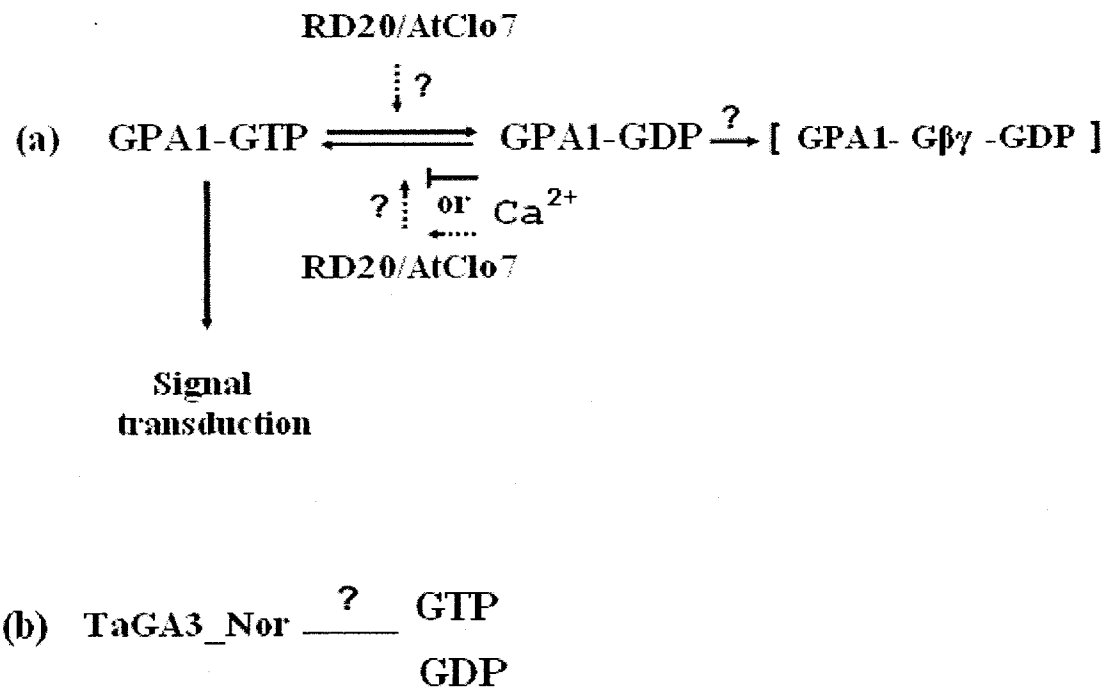


Figure 20. Proposed work model.

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APENDIX 1

1. WEF109_B14, calcium EF-hand binding protein full length sequence

CCACGCGTCCGCTGTCTTTTCTGTATACTTTCCCCGTTCTATTGCTACCGCGGGTCTTATGCAATCGA
TCGGATCATATAGATATCCCTTCAACACAACATAGGTCATCGAAAAGATCTCGGACGACTCACCAAAG
CACGAAAGCCAGTTAGAAAATGGATTCCATTTTGAAGAGTGCCTAACCGCATGGATAAGCTCACATTA
ACCCGTCAATTTTGGATCCAATTCGGGATTTTTCTTGGGAAGTTTCGGGAAGAAATTGGAATGGAATA
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