Heterotrimeric Gα subunit from wheat (*Triticum aestivum*), GA3, interacts with the calcium-binding protein, Clo3, and the phosphoinositide-specific phospholipase C, PI-PLC1

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ABSTRACT

The canonical Gα subunit of the heterotrimeric G protein complex from wheat (*Triticum aestivum*), GA3, and the calcium-binding protein, Clo3, were revealed to interact both in vivo and in vitro and Clo3 was shown to enhance the GTPase activity of GA3. Clo3 is a member of the caleosin gene family in wheat with a single EF-hand domain and is induced during cold acclimation. Bimolecular Fluorescent Complementation (BiFC) was used to localize the interaction between Clo3 and GA3 to the plasma membrane (PM). Even though heterotrimeric G-protein-signaling and Ca^{2+} signaling have both been shown to play a role in the response to environmental stresses in plants, little is known about the interaction between calcium-binding proteins and Gα. The GAP activity of Clo3 towards GA3 suggests it may play a role in the inactivation of GA3 as part of the stress response in plants. GA3 was also shown to interact with the phosphoinositide-specific phospholipase C, PI-PLC1, not only in the PM but also in the endoplasmic reticulum (ER). Surprisingly, Clo3 was also shown to interact with PI-PLC1 in the PM and ER. In vitro analysis of the protein-protein interaction showed that the interaction of Clo3 with GA3 and PI-PLC1 is enhanced by high Ca^{2+} levels. Three-way affinity characterizations with GA3, Clo3 and PI-PLC1 showed the interaction with Clo3 to be competitive, which suggests that Clo3 may play a role in the Ca^{2+}-triggered feedback regulation of both GA3 and PI-PLC1. This hypothesis was further supported by the demonstration that Clo3 has GAP activity with GA3.

INTRODUCTION

Heterotrimeric GTP-binding proteins (G proteins) are involved in multiple signaling pathways in plants and animals initiated through G-protein-coupled receptors (GPCR). The G protein complex is composed of three subunits, Gα, Gβ and Gγ. In animals the genes encoding these subunits comprises gene families; for example, the human genome has 23 Gα-, 6 Gβ-, and 12 Gγ-encoding genes. In contrast, plant genomes contain relatively few heterotrimeric G proteins, with the Arabidopsis genome encoding one Gα-, one Gβ- and two Gγ-subunits. In addition, there are 37 genes encoding regulator of G-protein-signaling (RGS) proteins and about 800 genes with significant sequence similarity to GPCRs (Jones and Assmann 2004). G-protein-signaling has been implicated in the plant response to pathogens (Blumward et al. 1998), light (Lapik and Kaufman 2003), high salinity, drought (Misra et al. 2007), hypoxia and ethylene signaling (Steffens and Sauter 2010), and in signaling pathways regulated by jasmonic acid (Okamoto et al. 2009), gibberellin (Ullah et al. 2003) and abscisic acid (ABA) (Ritche and Gilroy 2000; Wang et al. 2001; Pandey et al. 2006).

In the classical model of heterotrimeric G-protein-signaling, the receptor G-protein complex transmits signals through guanine nucleotide exchange and hydrolysis at the inside surface of the cell membrane. In the inactive state, Gα tightly binds the Gβγ subunits and a G-protein-coupled receptor (GPCR). When a ligand activates the GPCR, G proteins bound to the GPCR undergo conformational changes. The Gα subunit exchanges GDP for GTP, which results in the dissociation of the Gα subunit from the Gβγ dimer and GPCR. Gα-GTP and the Gβγ dimer can then activate downstream signaling cascades and effectors. The hydrolysis of the bound GTP to GDP by the GTPase activity of Gα allows the reconstitution of the inactive heterotrimeric complex (Sprang 1997; Hamm 1998). In plants, in contrast to animals, Gα was found to have a

high intrinsic GDP/GTP exchange rate that was predicted to favor a free GTP-bound resting state which suggests that GTPase activating proteins (GAPs) would play a critical role in the regulation of Gα-signaling (Johnston et al. 2007). The GAP activity of Arabidopsis RGS1 in association with GPA1 supports this notion, and is hypothesized to shift the G protein to a GDP-bound state (Chen 2008; Jones et al. 2011). The low number of genes encoding G proteins in plant genomes suggests that the multiplicity of signaling associated with G proteins is modulated through multiple interacting proteins, and it is likely that other GAPs exist in plants that are not part of the RGS-protein family. One such protein, PLDα1, in spite of its lack of an RGS-box, was shown to have GAP activity with the Arabidopsis GPA1 (Zhao and Wang 2004). The role of effector proteins associated with Gα is an important area of research and the number of identified interacting proteins is growing. To date, five upstream G-protein-coupled receptors have been shown to interact with the Gα subunit, AtGCR1 (Pandey and Assmann 2004), AtRGS1 (Chen et al. 2006), the pea GPCR (Misra et al. 2007), and two GPCR-type G proteins, GTG1 and GTG2 (Pandey et al. 2009). There are also few known downstream effectors of the plant Gα subunit. The Arabidopsis Gα (GPA1) interacts with Atpirin1, a cupin domain protein, which regulates seed germination and seedling development (Lapik and Kaufman 2003), phospholipase D (PLDα1) (Zhao and Wang 2004), prephenate dehydratase protein (PD1) (Warpeha et al. 2006), and the plastid protein thylakoid formation 1 (THF1) (Huang et al. 2006). Gα has also been shown to interact with phospholipase A_2 (PLA_2) in *Eschscholzia californica* (Heinze et al. 2007) and phospholipase C in *Pisum sativum* (Misra et al. 2007).

Bread wheat (*Triticum aestivum*) is one of the two major cereals worldwide and is the major crop that is most widely adapted to harsh environmental conditions (Tardif et al. 2007). It is among the few crops that are most tolerant to low temperatures, with the ability to survive at temperatures as low as -21ºC after a period of acclimation. Gene expression studies and microarray analyses of cold acclimation identified a large number of genes that were more strongly induced or repressed in cold tolerant winter wheat than in freezing sensitive spring wheat (Gulick et al. 2005; Monroy et al. 2007). A cold acclimation-induced calcium-binding protein, Clo3, formerly J900, was identified among these and subsequent yeast two-hybrid screening studies identified a protein-protein interaction between Clo3 and a protein with high sequence similarity to the Gα subunit, GA1 (Tardif et al. 2007). This suggested that the Gα protein may be involved in signaling pathways regulating cold acclimation. This was one of the first reports of a protein-protein interaction between a plant Gα and calcium-binding protein. Little is known about Clo3, and much less about its potential role in signaling. Its closest homologs in Arabidopsis are RD20/AtClo3 (At2g33380) and AtClo7 (At1G23240), which are members of a small gene family that contains single EF-hand and Ca^{2+}-binding domains. RD20 was first characterized as a drought-induced gene that was shown to bind Ca^{2+}. It was expressed in aerial tissues, mainly in the leaves and flowers. RD20 was also induced by dehydration, salt, ABA and cold treatments (Takahashi et al. 2000; Fujita et al. 2004).

In animal systems, activated Gα proteins are known to activate effector enzymes including phosphoinositide-specific phospholipase C (PI-PLC), which cleaves the phosphodiester bond of phosphatidylinositol 4,5-bisphosphate (PIP2) in the inner leaflet of the plasma membrane (PM), releasing inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 is a primary effector of calcium release, interacting with receptors on intracellular calcium storage sites to release

calcium into the cytoplasm (Berridge 1993). The existence of homologous genes for these proteins suggests that similar signaling also functions in plants. The PI-PLCs identified in plants are closely related to mammalian PLCδ (Munnik et al. 1998); they contain X and Y domains that are necessary for phosphoesterase activity and a calcium-binding C2 domain. PI-PLCs have been shown to play a role in signaling involved in disease resistance in tomato (Vossen et al. 2010). Misra et al. (2007) demonstrated that PLCδ functions as an intracellular signaling enzyme for the Gα-subunit of pea (*Pisum sativum*), and that Gα interacts with the calcium-binding C2 domain of the pea PLCδ.

Signaling proteins may have multiple functions that depend on their cellular context. The cell-type-specific and signaling-dependent functions of these proteins are often determined by their interaction partners (Hu and Kerppola 2005). Ca^{2+} plays a critical role in abiotic and biotic responses, and Ca^{2+}-binding proteins that function as signal sensor proteins may regulate specific protein-protein interactions, affect downstream phosphorylation cascades and regulate the response to environmental cues (Kudla et al. 2010).

The present study was conducted to characterize in vivo and in vitro protein-protein interactions between the wheat heterotrimeric Gα subunit, GA3, the calcium-binding protein, Clo3 and the phosphoinositide-specific phospholipase C, PI-PLC1.

MATERIALS AND METHODS

Plant materials and growth conditions

Tobacco seeds (*Nicotiana benthamiana*) were germinated in 10 cm pots in a potting mixture with equal volumes of peat moss, vermiculite and soil. The plants were grown for 2-4 weeks in the greenhouse with supplemental light to extend the day length to 16h light / 8h dark at 20°C.

Ga3, Clo3, Pi-Plc1 and _Pi-Plc2_ full length cDNA cloning and expression clones

The full length cDNA clone of *Ga3*-CS, from wheat cultivar Chinese Spring, encoding the Gα subunit of wheat was obtained from the Arizona Genomics Institute (AGI). This cDNA clone had a 17 bp duplication 91 nucleotides upstream of the normal stop codon. The intact version of the *Ga3* cDNA was generated by combining the 358 nucleotides of the 3`end of the cDNA PCR-amplified from a cDNA library of cultivar Norstar and 1027 nucleotides from the 5`end of the *Ga3*-CS cDNA. Gene-specific primers for the cloning are listed in Supplemental Table S1. A full-length *Clo3* cDNA, from the *T. aestivum* cultivar Norstar, was obtained from the Functional Genomics of Abiotic Stress (FGAS) cDNA clone collection (Houde et al. 2006). The partial cDNA clone of *Pi-Plc1* corresponding to GenBank EST accession GI: 55684870, also from the FGAS clone set, had a 1192 nucleotide ORF, but lacked its 5' end. The 5' end of the cDNA was cloned from a cDNA library by PCR amplification using gene-specific and vector-specific primers (Supplemental Table S1) and two rounds of cloning were carried out to obtain the missing portion of the cDNA. The full ORF of *Pi-Plc1* was subcloned into pDONR207 by Gateway ® BP clonase II Enzyme mix (Invitrogen) and subsequently transformed into the TOP10 *E. coli* strain.

The coding regions of *Ga3*, *Ga3*^{Q223L}, *Ga3*-CS, *Clo3*, *Pi-Plc1*, *Pi-Plc2*, *Pi-Plc*2ΔEF-hand, *Pi-Plc*2ΔC2 and C2 domain of *Pi-Plc*2 were cloned as fusions with fluorescent proteins in plant expression vectors using the Gateway ® cloning system (Invitrogen). Gateway ® LR reactions were used to transfer the inserts of the entry clones of *Ga3*, *Ga3*^{Q223L}, *Ga3*-CS, *Clo3*, *Pi-Plc* 1, *Pi-Plc* 2, *Pi-Plc*ΔEF-hand *Pi-Plc* 2ΔC2 and C2 domain to the plant destination binary vector, PK7FWG2, to generate enhanced Green Fluorescent Protein (eGFP) C-terminal fusions. They were also transferred to BiFC binary Yellow Fluorescent Protein (YFP) vectors, pBatL-B-sYFP-

N and pBatL-B-sYFP-C. For protein expression in *E. coli*, PI-PLC1 and Clo3 were cloned in pDEST17, a Gateway ® vector with a 6-His-tag N-terminal fusion (Invitrogen). *Pi-PLC1* was also cloned into pDEST15, a Gateway ® vector with a GST N-terminal fusion. *Ga3* was amplified using a pair of primers, digested with BamHI and SalI (Supplemental Table S1) and directionally cloned in to pGEX-2T, an N-terminal GST-fusion expression vector to which a SalI restriction site had been added.

Agrobacterium transformation and agroinfiltration

Electrocompetent *Agrobacterium* strain AGL1 was transformed with plant gene expression constructs; overnight cultures were centrifuged at 4000*g* for 15 min. at 4°C and resuspended in 10 mM $MgCl_2$ and 150 µM acetosyringone to an OD_{600} of 0.1 and incubated at room temperature for 2 hours (Walter et al., 2004). The leaf infiltration suspensions were a mixture of equal volumes of three or four *Agrobacterium* culture suspensions containing expression vectors for the experimental samples, the mCherry fluorescent cellular marker proteins (Nelson et al. 2007) (Supplemental Table S2) and a culture expressing P19 of tomato bushy stunt virus, to suppress gene silencing (Voinnet et al. 2003). The agroinfiltration solution was co-infiltrated into the leaf abaxial air space of two-four week old *N. benthamiana* plants. The plants were incubated in environmental growth chambers under long days (16h light / 8h dark) at 20ºC for two to four days and subsequently analyzed by microscopy.

Confocal laser scanning microscopy

The epidermal tissues of tobacco leaves were examined using the Spinning Disk2 (SD2) confocal microscope, at The Cell Imaging and Analysis Network (CIAN) laboratory, McGill University. GFP was excited at a wavelength of 491 nm by the diode laser and the emitted fluorescence was collected through a 520/535 nm band-pass. YFP was excited at the same-

wavelength laser, and the emitted fluorescence was collected through a 543 nm long-pass filter. The Cherry Red Fluorescent Protein (cRFP) was excited at a wavelength of 561 nm and the emitted fluorescence was collected through a 624/640 nm long-pass filter. The image of the interaction between GA3 and Clo3, as well as the supplemental movies, were taken with a Zeiss LSM 510 Meta confocal laser scanning microscope (Carl-Zeiss, Germany) with a C-Apochromat 63X/1.2 water objective with correction collar. Two laser sources were used for this work: 30mW Argon Ion laser (488/514 nm) and 1mW HeNe Green (543 nm). For multichannel GFP and mCherry Red, the electron was excited at 488 nm and 543 nm, respectively. Also, the HFT/UV 488/543/633 nm beam splitter and NFT 545 (secondary dichromic mirror) were used. NFT 545 was used to discriminate between GFP and RFP. In order to acquire an image in the multi-tracking mode, the band pass (BP) 505-530 nm filter and BP 560-615 nm filter were used for the GFP and RFP detection channels, respectively.

Expression and in vitro protein-protein interaction between PI-PLC1, GA3 and Clo3

For protein-protein interaction characterization and expression in *E. coli,* BL21 cells transformed with the recombinant plasmids were grown at 37^{o}C with 100 μg/ml ampicillin for 6 hours and then induced with 1 mM IPTG for 12 hours at room temperature. Cultures expressing GA3-GST were lysed in buffer: 50 mM Tris-HCl, pH 8.8, 100 mM NaCl, 2% Triton X-100, 5% Glycerol, 1 mM PMSF. The recombinant GA3-GST protein was purified with Glutathione Sepharose^{TM} (GE Healthcare Life Sciences), according to the manufacturer's protocol. Cultures expressing PI-PLC1-His or Clo3-His were lysed in the same buffer, centrifuged at 12,000*g* for 15 min. and protein was recovered from the pellet by solubilization in 50 mM Tris-HCl, pH 8.8, 0.5% SDS, 5% Glycerol, 1 mM PMSF. PI-PLC1 and Clo3 were purified using Ni-NTA Agarose (Qiagen) according to Todorova (2009) with some modifications: the Ni-NTA Agarose was

equilibrated with the wash buffer containing 50 mM Tris-HCl, pH 8.8, 0.05% SDS, and 1 mM PMSF. The protein sample was immobilized on the equilibrated beads, subsequently rinsed twice with wash buffer and then eluted with 2X-SDS-PAGE buffer (100 mM Tris-HCl, pH 7.0, 200 mM DTT, 4% (W/V) SDS, 0.2% (W/V) bromophenol blue, 30% glycerol and 100 mM imidazole).

The His-tag pull-down assay (Todorova, 2009) was also used to study the pairwise interaction among the three proteins, GA3, PI-PLC1 and Clo3. A mixture of 50 µl of crude extract of the Clo3 or PI-PLC1 culture and 50 µl of Ni-NTA Agarose beads, previously equilibrated with the wash buffer (50 mM Tris-HCl, pH 8.8, 0.05% SDS and 1 mM PMSF), were incubated for two hours at 4°C with gentle shaking. The mixture was rinsed with the wash buffer, centrifuged at 2000*g* for 1 min. and the supernatant was discarded. The Ni-NTA Agarose beads bound with PI-PLC1-His or Clo3-His proteins were incubated with 100 µl of GA3-GST crude *E. coli* lysate in 50 mM Tris-HCl, pH 8.8, 100 mM NaCl, 2% Triton X-100, 5% Glycerol, 1 mM PMSF and either 10 mM $CaCl_2$ or 1 mM EGTA for two hours at 4°C with gentle shaking. Samples were centrifuged at 2000*g*, washed twice with wash buffer, then eluted in 50 µl of 2X-SDS-PAGE buffer. To assay GTP/GDP bound forms of Gα, GA3 lysate was pre-treated with 5 mM EDTA and 5 mM of either GDP or GTP for 10 min. followed by the addition of 20 mM $MgCl_2$.

The three-way interaction between Clo3-His, GA3-GST, and PI-PLC1-GST was studied by first binding Clo3-His to Ni-NTA Agarose beads, then GTP-loaded GA3-GST in the buffer, 50 mM Tris-HCl, pH 8.8, 100 mM NaCl, 2% Triton X-100, 5% Glycerol, 1 mM PMSF was added at levels to achieve saturation of the binding to Clo3-His. Subsequently-increasing amounts of PI-PLC1-GST (in buffer: 50 mM Tris-HCl, pH 8.8, 0.5% SDS, 5% Glycerol, 1 mM

PMSF) were added to the interaction mixture. Bound protein was rinsed with wash buffer and eluted with 2X SDS-PAGE buffer and samples were analyzed by SDS-PAGE electrophoresis on 12% polyacrylamide gels subsequently stained with Coomassie Brilliant Blue R-250.

GTPase assay for GA3 and Clo3

GA3 and Clo3 proteins were expressed in *E. coli* as described above. GA3-GST was purified as described in Willard and Siderovski (2004), with modified lysis buffer: 25 mM Tris-HCl, pH 7.6, 100 mM NaCl, 5% Glycerol, 5 mM $MgCl_2$, 30 µM $AlCl_3$, 20 mM NaF, 5 mM PMSF, 50 µM GDP, 1 mM DTT, and 3x Complete EDTA-free protease inhibitor (Roche) at 3 tablets/l. Subsequently, 1 mg/ml lysozyme and 20 mM $MgCl_2$ were added and the solution was rocked for 30 min. and sonicated for two min. 200 mM NaCl was added, and the solution was rocked again for 30 min. The sample was centrifuged for 45 min. at 13,000*g* and the recombinant GA3-GST protein was purified from the supernatant with Glutathione Sepharose™ 4B beads (GE Healthcare Life Sciences) according to the manufacturer's protocol by incubation for 90 minutes at 4°C with rocking. The matrix was rinsed twice in a chromatography column (Bio-Rad) with 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.76 mM KH_2PO_4 and eluted with 25 mM glutathione, 50mM Tris-HCl, pH 8.0, and 150 mM NaCl. Clo3-His was isolated from a bacterial pellet from a 1-liter culture with 10 ml of B-PER lysis buffer (Pierce) and centrifuged at 13,000*g* for 30 min. Clo3 protein was purified from the supernatant using Ni-NTA Agarose (Qiagen) according to Pandey et al. (2009) and stored in aliquots of 100ul at -80°C.

Purified GA3 protein was incubated with 2mM EDTA for five min., and GTP was added to 5mM final concentration and incubated for 30 min. at 4°C. Both GA3 and Clo3 protein samples were buffer-exchanged three times in 50mM Tris-HCL, pH 7.5, and 100mM NaCl at 4°C, with VivaSpin 500 columns (Stedim) according to the manufacturer's protocol. GTPase

reactions were performed in the exchange buffer with GA3 at 49 μM and Clo3 at 48 μM with 10μM GTP and 10 mM $CaCl_2$; the reaction was activated with 10mM $MgCl_2$ and incubated for 30 min. at room temperature. Inorganic phosphate, the GTPase reaction product, was assayed using the P_iPer Phosphate assay kit (Invitrogen) according to the manufacturer's protocol, with samples being read over a 1 hour time course.

RESULTS

The heterotrimeric Gα subunit, *Ga3*, calcium-binding protein, *Clo3*, and phosphoinositide-specific phospholipase C, *Pi-Plc*, genes from wheat

The *Ga3* cDNA from the wheat cultivar Norstar has 98% nucleotide sequence identity with the coding regions of two previously reported wheat Gα genes from the cultivar S615, GA1 and GA2 (Hossain *et al.*, 2003). *Ga3* likely represents a homeologous copy of *GA1*, whereas *GA1* and *GA2* appear to be recent gene copies with small rearrangements. *GA1* is 99.4% identical to *GA2* but *GA2* has a 21 bp insertion. A second version of *Ga3* was found as a cDNA clone from the wheat cultivar Chinese Spring, *Ga3*-CS, which has a 17 bp insertion located 1038 bp downstream from the start codon; this causes a frame shift and results in an ORF encoding a protein with 367 aa instead of the 382 aa of the normal allele, *Ga3*. In addition, the last 20 aa of the shortened protein are completely changed relative to a similar clone identified in the cultivar Norstar. Except for the insertion, the nucleotide sequence is 100% identical to the *Ga3* clone from Norstar and thus it is likely an allele of *Ga3*; it is referred to as *Ga3*-CS. $GA3^{Q223L}$, a constitutive GTP-bound form of GA3, was also constructed by replacing A with T at position 668 of the *Ga3* coding region according to Ullah et al. (2003) by PCR-based site-directed mutagenesis to create a Q to L change.

A cDNA clone for *Clo3* (previously referred to as J900) (Tardif *et al.*, 2007) which belongs to a caleosin superfamily, has a high sequence similarity to caleosin-like genes identified in wheat and Arabidopsis. It has 63% aa sequence similarity with At *Clo3* of Arabidopsis (also known as *RD20)*, a calcium-binding protein gene family member that is strongly induced by drought and ABA treatments (Partridge and Murphy 2009). *Clo3* contains an EF-hand motif (Supplemental Fig. S1), has 76% aa sequence similarity with a Ca^{2+}-binding protein in rice (GB acc. BAD45228) and 69% aa sequence similarity with a barley Ca^{2+}-binding protein (GB acc. CAB71337).

Clones encoding phosphoinositide-specific phospholipase C genes, *Pi-Plc1* and *Pi-Plc2* cDNAs were identified among a FGAS wheat EST clone collection (Houde et al. 2006). The full-length cDNA clone of *Pi-Plc1* was derived from the combination of a partial-length cDNA and the 5’ end of the cDNA including the 5’ UTR and the 5' end of the ORF of *Pi-Plc1* obtained by PCR amplification from a wheat cDNA library. The 1860 nucleotide cDNA clone encodes a 506 aa protein with a molecular mass of 57 kDa that has 80% amino acid sequence identity with *Oryza sativa* PI-PLC1 (GB acc. AAK01711). PI-PLC1 contains X and Y domains that are necessary for the phosphoesterase activity as well as a C2 calcium-binding domain, but does not have an EF-hand calcium-binding domain in the N-terminal region of the protein that is found in many plant PI-PLCs. There are other examples of PI-PLC genes that lack an N-terminal EF-hand domain which have been identified in *Zea mays* (Zhai et al. 2005) and *Vigna unguiculata* (GB acc. AAB41107) (El-Maarouf et al. 2001). The full-length cDNA sequence of *Pi-Plc2* is 1761 nucleotides long and encodes a 586 aa protein. It has 59% amino acid identity to *Pi-Plc1* and contains an EF-hand in the N-terminal region, the X and Y phosphoesterase domains, as well as the C2 calcium-binding domain in the C-terminal region of the protein.

GA3 interacts with Clo3

The three-dimensional reconstruction of the confocal images of the BiFC interaction assay of Clo3 and GA3 clearly show a pattern of localization with the PM (Supplemental Movie S1, Fig. 1a) and not with any other structures. Though tonoplast-localized proteins would also be expected to localize to the periphery of the cell, they are also expected to be seen in internal membranes due to the presence of multiple adjacent vacuoles in a cell; however, this was not observed. The BiFC interaction assay of Clo3 with the constitutively GTP-bound form of GA3, $GA3^{Q223L}$, and the truncated version of GA3, GA3-CS, did not show significant fluorescence. The specificity of the BiFC assay was supported by positive and negative controls including the interaction of Arabidopsis Gα, At GPA1-C-terminal-YFP, and At RGS1-N-terminal-YFP (Grigston et al. 2008), which showed a clear interaction in the PM. No interaction was observed between negative controls, Arabidopsis Ptdlns synthase, At PIS-C-terminal-YFP, and an Arabidopsis ABA-responsive protein, At HVA22d-N-terminnal-YFP.

GA3-eGFP and GA3-CS-eGFP fusions to full length eGPF proteins were localized to the PM (Fig. 2a, 2b) and ER (Supplemental Fig. S2a, S2b) in tobacco epidermal cells. GA3-eGFP and GA3-CS-eGFP did not co-localize with any of the other 5 cellular makers (Supplemental Fig. S3, S2). In contrast to the PM localization of the interaction between Clo3 and GA3, Clo3-eGFP expressed in *N. benthamiana* epidermal cells as a fusion to the full-length eGFP was localized to the ER and tonoplast (Fig. 2c, 2d, Supplemental Fig. S4). The localization to the ER is seen by both co-localization with the ER marker and by the clear network structure that is seen in the upper focal planes of the cell. In mid-focal planes the labeling appears as a punctuate signal seen at the edges of the cells, which is expected in the cross-section of a network. The punctuate label co-localized with ER makers and was distinguishable from the PM and tonoplast makers. Gα in

Arabidopsis has previously been reported to localize to the PM and the ER (Weiss et al. 1997) and Clo3 homologs in Arabidopsis and *Brassica* have been localized to the ER (Hernandez-Pinzon et al. 2001). The difference between the localization of Clo3-eGFP and the localization of its interaction with GA3 suggests that there is a small subpopulation of Clo3 in the PM that interacts with GA3 that is not visible when the protein is expressed as a Clo3-eGFP construct.

To test the in vitro protein-protein interaction between GA3 and Clo3, Clo3 expressed as a His-tagged protein fusion in *E. coli* was immobilized on Ni-NTA Agarose beads and incubated with GA3 expressed as a GST-fusion. The interaction of GA3 and Clo3 was shown in vitro by a His-tag pull-down assay of Clo3, which resulted in the co-purification of GA3-GST (Fig. 3a). There was approximately a fivefold higher ratio of the GA3-GST/Clo3 recovery in the presence of 10 mM $CaCl_2$, than when proteins had been pretreated with 1 mM EGTA to remove bound calcium ions. GA3 interacted more strongly with Clo3 when it was preloaded with GTP than when it was preloaded with GDP in the absence of calcium ions, whereas no difference was found in the affinity between the GTP- or GDP-loaded forms of GA3 in its binding to Clo3 in the presence of 10 mM calcium ions (Fig. 3a).

The specificity of the interaction between GA3 and PI-PLCs

The interaction of GA3 and PI-PLC1 was observed by a BiFC assay as a reconstitution of the YFP, and was localized to the ER and PM (Fig. 1b, 1c). The subcellular localization of the fusion to the full-length eGFP, PI-PLC1-eGFP, was also seen on the PM and ER (Fig. 2e, 2f, Supplemental Fig. S5). In contrast, the second phospholipase C, PI-PLC2, did not show any interaction with the GA3 in the same assay. The PI-PLC2-eGFP fusion was localized predominantly to the PM (Fig. 2g, Supplemental Fig. S6). The localization of three truncations of PI-PLC2 was also carried out. PI-PLC2ΔEF-hand-eGFP, which lacked an EF-hand, was

detected on the PM. This was similar to the full-length construct; however, the level of expression appeared to be lower than the full-length version of PI-PLC2 (Supplemental Fig. S7a). Both PI-PLC2ΔC2 (which lacked the C2 domain) and C2 domain fused to eGFP, were not targeted to the PM but were found on the ER (Supplemental Fig. S7b). Though some deletions affected the localization of the PI-PLC2 protein to the PM, none of the truncated versions of PI-PLC2 showed interaction with GA3.

PI-PLC1 was also found to form homopolymeric complexes on the ER and PM when two fusion products, one as a fusion with the N-terminus of YFP and the other with the C-terminus of YFP were expressed together in the BiFC assay (Supplemental Fig. S8a). On the other hand, PI-PLC2 did not show evidence of dimerization in the same assay. The difference in the interaction and localization of PI-PLC1 and PI-PLC2 demonstrates the specificity of the interaction assay and suggests that the two proteins play different roles in plant metabolism and signaling.

In vitro binding experiments were also performed to confirm the interaction between GA3 and PI-PLC1. PI-PLC1 was expressed as a His-tagged protein fusion in *E. coli* and Ni-NTA Agarose-bound-PI-PLC1 protein was incubated with GA3 expressed as a GST-fusion. GA3 was co-purified with PI-PLC1-His when it was isolated in a His-tag pull-down assay using Ni-NTA Agarose beads (Fig. 3b). The recovery of GA3 from the in vitro interaction between GA3 and PI-PLC1 was approximately two times higher in the presence of 10 mM $CaCl_2$ than when calcium was sequestered by the addition of 1 mM EGTA to the interaction solution.

When the His-tag pull-down assay was employed after preloading GA3 with either GDP or GTP, the GTP-bound form of GA3 showed three times more binding with PI-PLC1 than the GDP-bound form of the protein in the absence of calcium ions. In the presence of calcium there was no difference between the interactions of PI-PLC1 with the GA3-GTP- and GA3-GDP-

bound forms (Fig. 3b). The BiFC in vivo interaction between a constitutive GTP-bound form of wheat Gα, $GA3^{Q223L}$, and PI-PLC1 also appeared to have higher levels of fluorescence than the interaction between the wild type GA3 and PI-PLC1 (Supplemental Fig. S8b, S8c).

PI-PLC1 also interacts with Clo3

PI-PLC1 was also found to interact with the calcium-binding protein, Clo3, on the ER and PM (Fig.1d, 1e). The localization of the interaction between the two proteins was seen by co-localization with PM and ER markers and by the labeling of the ER network structure in the upper focal planes of the cell (Supplemental Fig. S8d). A second phospholipase C, PI-PLC2, did not show any interaction with Clo3. The interaction between PI-PLC1 and Clo3 was verified by an in vitro pull-down assay; Clo3 expressed as a His-tagged protein was effective in co-purifying a PI-PLC1-GST-fusion protein in the presence of 10 mM $CaCl_2$ (Fig. 3c). The interaction between the two proteins was ten times higher in the presence of 10 mM calcium ions than when calcium ions were sequestered by the addition of EGTA.

The PI-PLC1 -GA3 - Clo3 interactions are competitive

Since Clo3 was found to interact with both GA3 and PI-PLC1, the dynamics of the three-way interaction were investigated by in vitro interaction studies to assess whether the interaction was synergistic or competitive. Clo3-His-tag-bound Ni-NTA Agarose beads were incubated with increasing amounts of GA3 preloaded with GTP in the presence of 10 mM $CaCl_2$, to reach saturation of GA3 binding to Clo3 (Fig. 4a). With GA3 at the saturation level, increasing amounts of PI-PLC1-GST were subsequently added to the interaction complex of Clo3 and GA3. The addition of PI-PLC1 resulted in decreased binding of GA3 to Clo3, while increasing amounts of PI-PLC1 were recovered as binding partners of Clo3 (Fig. 4b). This indicates competitive binding between the three proteins.

Clo3 has GAP activity

Calcium release is a downstream signaling consequence of PLC activation, and the hydrolysis of GTP is the critical regulatory step in inactivation of G proteins. Consequently, the role of the Clo3 interaction with GA3 was further investigated by characterizing the effect of protein-protein interactions on the GTPase activity of GA3. GA3-GST and Clo3-His-fusion proteins were purified from *E. coli* cultures and assayed for GTPase activity in the presence of calcium. GA3-GST alone showed intrinsic GTPase activity in vitro and the activity was stimulated 25% by the equimolar addition of Clo3-His in the presence of calcium as shown in Fig. 5. Clo3 alone showed no GTPase activity. This level of GAP activity was similar to that reported for the Arabidopsis PLDα1 (Zhao and Wang 2004).

DISCUSSION

Clo3 interaction with GA3

The low copy number of genes encoding Gα, Gβ and Gγ's in plants as compared to animals raises questions of how heterotrimeric G proteins might be involved in multiple signaling pathways in plants. The additional gene copies and allelic variations for Gα in wheat reported here indicate a greater complexity and divergence within this gene family than in Arabidopsis and rice, a phenomena reflected also in the recent analysis of the gene family in soybean (Bisht et al. 2011). However, the complexity of the family is modest relative to that in animal species. The complexity of signaling in plants may be derived from a variety of effector molecules that interact with the heterotrimeric G proteins (Assmann 2005; Panday et al. 2010); thus the analysis of the interaction between Gα, PI-PLC and Clo3 from wheat makes an important contribution towards understanding the complexity of protein-protein interactions involved in G-protein-signaling.

Studies in non-plant systems have shown that heterotrimeric G-protein-signaling is intrinsically linked to calcium signaling by activation of PI-PLCs, which in turn leads to calcium release into the cytoplasm. In spite of the complex array of signaling pathways in which both calcium and G proteins are implicated, the direct interaction between calcium-binding proteins and heterotrimeric G proteins has not been reported in plants. In non-plant systems, there are few examples of calcium-binding proteins known to interact with G proteins directly. The calcium-sensing receptor (CaR) in human pituitary cells is a G-protein receptor (Mamillapalli and Wysolmerski 2010), and the rat calcium-binding protein, calnuc, binds to Gα, though its function is not clear (Kanuru et al. 2009). More significantly, the avian regulator of G-protein-signaling, RGS3, which has the hallmark EF-hand domain of calcium-binding proteins, was shown to bind Gα and interrupt Gα-protein-mediated-signaling in neural cells in a Ca^{2+}-dependent manner (Tosetti et al. 2003). With so few calcium-binding proteins known to interact with Gα, the possible role of the calcium-binding protein Clo3 interacting with Gα is intriguing.

Ca^{2+} is a central regulator in cell physiology and plays an important role in the response to abiotic stress including low temperature, salt and water stress. Release of Ca^{2+} from the apoplast or intercellular stores to the cytoplasm gives rise to signature patterns of oscillations of cytosolic Ca^{2+} concentrations in response to different environmental cues (Dodd et al. 2007). Several classes of calcium-binding regulatory proteins including calcium-dependent protein kinases (CDPK), calcium-regulated transcription factors, calmodulins and calcineurins have been implicated in the response to abiotic stress (reviewed by Tuteja and Sopory 2008). Overexpression of the CDPK, At CPK6 (Xu et al. 2010), and of the calcineurin B-like protein, CLB5, (Cheong et al. 2010) have both been shown to enhance salt and drought tolerance in Arabidopsis.

RD20/Clo3, the ortholog of *Clo3* in Arabidopsis, is strongly induced by ABA treatment (Takahashi et al. 2000) and by overexpression of RD26, an ABA- and drought-inducible NAC transcription factor (Fujita et al. 2004). GPA1, the Arabidopsis ortholog of *Ga3*, is also implicated in ABA signaling; the *gpa1* mutant has enhanced ABA suppression of germination (Ullah et al. 2003), reduced stomatal closure in response to ABA treatment (Fan et al. 2008) and increased transpiration efficiency related to a reduced density of stomates (Nilson and Assmann 2010). Thus, the physical interaction of the ABA-induced Clo3 and GA3 present an important potential to decipher the role of Gα in ABA signaling.

The sequence of events in the well-known models of Gα activation of PI-PLC and subsequent IP3-trigged release of calcium suggests that the Clo3 interaction with GA3 could play a role in the feedback inactivation of Gα. This model is supported by the GTPase Activating Proteins (GAP) activity demonstrated by Clo3 in the present work.

GA3 interaction with Clo3 is enhanced by high Ca^{2+} concentrations, and at high Ca^{2+} concentrations the binding to Clo3 is similar to the GTP- and GDP-bound state of Gα (Fig. 3a). Though the competitive interaction between GA3, Clo3 and PI-PLC1 demonstrated by in vitro interactions alone does not predict the chronological order of binding events, existing models of PI-PLC activation by Gα suggest that PI-PLC binds to Gα and may be displaced by Clo3, since binding and displacement is enhanced by Ca^{2+}. GAP proteins promote the inactivation of G proteins by GTP hydrolysis, and the inactivation of GA3 by Clo3 is consistent with a number of stress-related responses in which Gα mutants have been shown to have phenotypes linked to increased stress tolerance, including enhanced ABA sensitivity and stomatal closure (Fan et al. 2008), increased transpiration efficiency (Nilson and Assmann 2010), the regulation of root proliferation in Arabidopsis (Chen et al., 2006) and adaptation to hypoxia in rice (Steffens and Sauter 2010). The fact that Clo3 also binds to PI-PLC1 suggests that it has functions other than

GTPase activation. If Clo3 is a negative regulator of GA3, the logical hypothesis is that it is also a negative regulator of PI-PLC.

GA3 and PI-PLC1

The activation of PI-PLC by Gα has long been known in animal systems, but the interaction of these two classes of proteins has only recently been reported in plants. The interaction of PI-PLC and Gα proteins from *Pisum* was characterized in vitro and by yeast two-hybrid analyses (Misra et al. 2007). Here we report the localization of the PI-PLC1 interaction with GA3 on the PM and ER, which raises the possibility that the two locations may relate to different functions. In addition to the classical model of G-protein-coupled receptor signaling that has been described for PM receptors, the localization of different Gα gene family members to endomembranes in animal systems has also been associated with the regulation of membrane trafficking (Marrari et al. 2007). The activity and localization of PI-PLCs and other phosphoinositide-metabolizing enzymes has also been shown to regulate membrane trafficking (Thole and Nielsen 2008), indicating that the localization of the PI-PLC interaction with GA3 to the ER and the PM may contribute to the multiplicity of signaling from G-protein receptors.

The interaction of GA3 with PI-PLC1 and its lack of interaction with PI-PLC2 demonstrates the specificity of the interaction. The C2 domain of PI-PLC is found in several classes of proteins involved in signaling and membrane trafficking and has been implicated in binding with phospholipids and other proteins (Nalefski and Falke 1996). The C2 domain of a PI-PLC from *Pisum* was shown to be sufficient for PI-PLC binding with Gα (Misra et al. 2007). The 131 aa C2 domain of the *Pisum sativum* PI-PLC has 74% amino acid sequence identity with the *T. aestivum* PI-PLC1 but only 68% identity with PI-PLC2 over the same region. PI-PLC1 and the *Pisum* PI-PLC share 13 aa within the sequence of their C2 domains that is~~are~~ not shared by

the two wheat PLCs; these are candidates for the critical amino acids mediating the interaction of PI-PLC1 and GA3. The same 13 aa are also conserved in two stress-induced PI-PLCs identified in other species, the systemic-acquired resistance induced *Oryza sativa* PI-PLC (Song and Goodman 2002) and the drought-, salinity- and ABA-induced *Vigna radiata* PI-PLC3 (Kim et al. 2004) (Fig. 6).

Clo3 and PI-PLC interaction

The significance of the interaction between Clo3 and PI-PLC is open to speculation as there have been relatively few reports of protein-protein interactions for PI-PLCs. The overexpression of a PI-PLC in maize enhanced drought tolerance (Wang et al. 2008) and the overexpression of NtC7, a protein that anchors PI-PLC to the plasma membrane in tobacco, was found to enhance salt tolerance (Nakamura and Sano 2009). PI-PLC was also reported to interact with actin in the cytoskeleton of oat roots (Huang and Crain 2009). In addition to their role in cleaving phosphoinositides from phospholipids, PI-PLCs have been studied for their role in releasing glycosylphosphatidylinositol (GPI)-anchored proteins from membranes (Coonrod et al. 1999). The role of the interaction with GPI proteins implies a large number of potential interaction partners; nevertheless there is little known about the interaction between a PI-PLC and a calcium-binding protein in plants. Clo3 is predicted by TopPred 5 (Claros and Von Heijne 1994) to have one membrane-spanning domain and was localized as a GFP-fusion protein to the ER and tonoplast; thus it may play a role in anchoring PI-PLC to the membrane.

Our results indicate a role for the calcium-binding protein Clo3 in the regulation of the signaling proteins Gα and PI-PLC. The GAP activity of Clo3 indicates that it plays a role in attenuating the activity of GA3. In addition, the phenotypes of Gα mutants in Arabidopsis and rice suggest that such attenuation could contribute to a number of physiological and

developmental adaptations to environmental stress. The localization of the interactions to both the PM and the ER suggest diverse roles for the interaction. The effect of the protein-protein interaction on the activity of GA3 and PI-PLC and the role of Clo3 is the subject of further investigation.

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FIGURE LEGENDS

Fig. 1 BiFC interactions of GA3, Clo3 and PI-PLC1 proteins expressed in tobacco epidermal leaf tissue.

A Co-localization of GA3-CYFP and Clo3-NYFP interaction to the PM; Scale bar = 20 µM; **B** co-localization of GA3-CYFP and PI-PLC1-NYFP interaction to the ER; **C** co-localization of GA3-CYFP and PI-PLC1-NYFP interaction to the PM; **D** co-localization of PI-PLC1-CYFP and Clo3-NYFP interaction to the ER; **E** co-localization of PI-PLC1-CYFP and Clo3-NYFP interaction to the PM; Scale bar = 24 µm.

Fig. 2 Subcellular localization of GA3-, Clo3-, PI-PLC1- and PI-PLC2-eGFP protein fusions expressed in tobacco epidermal leaf tissue.

A and **B** co-localization of GA3-eGFP to the PM and ER; **c** and **d** co-localization of Clo3-eGFP to the ER and tonoplast; **E** and **F** co-localization of PI-PLC1-eGFP to the PM and ER; **G** co-localization of PI-PLC2-eGFP to the PM; Scale bar = 24 µm.

Fig. 3 The pairwise in vitro protein-protein interactions of GA3, Clo3 and PI-PLC1 using a His-tag pull-down assay and SDS-PAGE.

A Clo3 and GA3. Clo3-His (26KD) was immobilized on Ni-NTA Agarose beads and incubated with GA3-GST (70KD) in the presence of $CaCl_2$, EGTA, GTP, or GDP. The Clo3-His bait and binding partner were eluted and separated by SDS-PAGE on a single gel. The portions of the gel for each of the two proteins are shown in separate panels as their molecular masses were substantially different.

B PI-PLC1 and GA3. PI-PLC1-His (59KD) was immobilized on Ni-NTA Agarose beads and incubated with GA3-GST (70KD) in the presence of $CaCl_2$, EGTA, GTP, or GDP. The proteins were eluted and separated on a single SDS-PAGE gel.

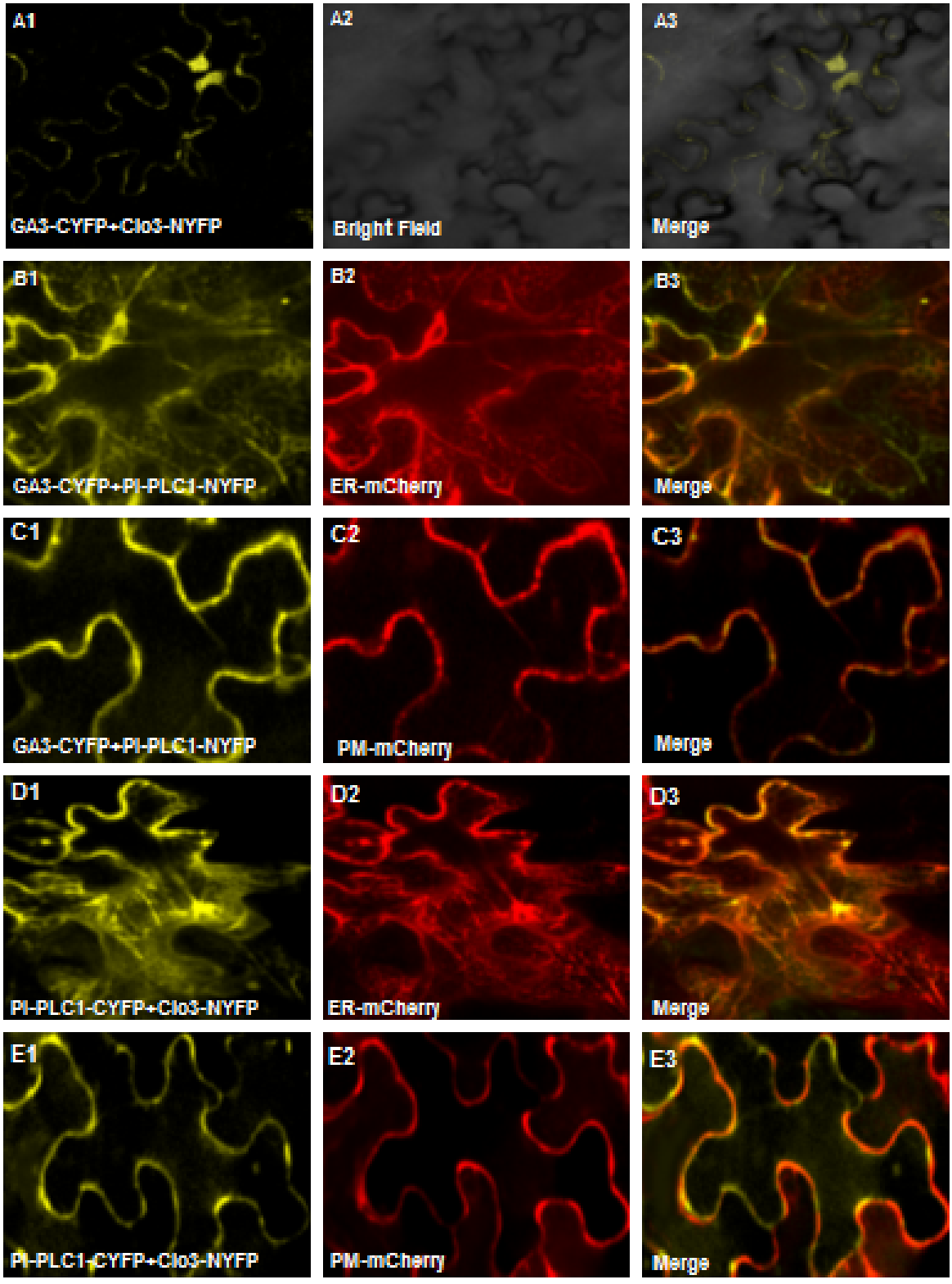
C Clo3 and PI-PLC1. Clo3-His (26K D) was immobilized on NiNTA Agarose beads and incubated with PI-PLC1-GST (83 KD) in the presence of either $CaCl_2$ or EGTA. The proteins were eluted and separated on a single SDS-PAGE gel. The portions of the gel for each of the two proteins are shown in separate panels.

Fig. 4 In vitro interaction of GA3, Clo3, and PI-PLC1 via a His-tag pull-down assay and SDS-PAGE. **A** Clo3-His immobilized on Ni-NTA Agarose beads was incubated for 2 hours at 4°C with 50, 100, 200, 300 and 400 µl of GA3-GST preloaded with GTP in lysate with 10 mM $CaCl_2$. Proteins were separated by SDS-PAGE. **B** The Clo3-His, bound with saturating levels of TaGA3-GST, preloaded GTP complex immobilized on Ni-NTA Agrose beads was incubated for 2 hours at 4°C with 50, 100, 200, 300 and 400 µl of PI-PLC1-GST in lysate with 10 mM $CaCl_2$. Proteins were separated by SDS-PAGE.

Fig.5 Clo3 stimulation of GA3 GTPase activity. GA3-GST and Clo3-His were expressed in *E. coli* and purified with affinity matrices for the GST and 6-His-tags, respectively. The proteins were incubated in equimolar amounts with 10 µM GTP in the presence of 10 mM $CaCl_2$ for 30 min. GTPase activity was assayed by measuring P_i released in the reaction mix and measured by a fluorescence emission assay. Clo3 showed no GTPase activity when it was assayed alone.

Fig. 6 Multiple sequence alignment of PI-PLC-C2 domains.

The alignment between 131 aa of the C2 domain of PI-PLCs from *Triticum aestivum, Pisum sativum, Oryza sativa* and *Vigna radita*. Thirteen amino acids are common between wheat PI-PLC1 and the Pisum PI-PLC, both of which interact with Gα, and are conserved in the other PI-PLCs known to be stress-induced, but are not common between PI-PLC1 and PI-PLC2; the latter does not interact with GA3.


A1
GA3-CYFP+Clo3-NYFP
A2
Bright Field
A3
Merge
B1
GA3-CYFP+PI-PLC1-NYFP
B2
ER-mCherry
B3
Merge
C1
GA3-CYFP+PI-PLC1-NYFP
C2
PM-mCherry
C3
Merge
D1
PI-PLC1-CYFP+Clo3-NYFP
D2
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E2
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E3
Merge

Figure 1

1

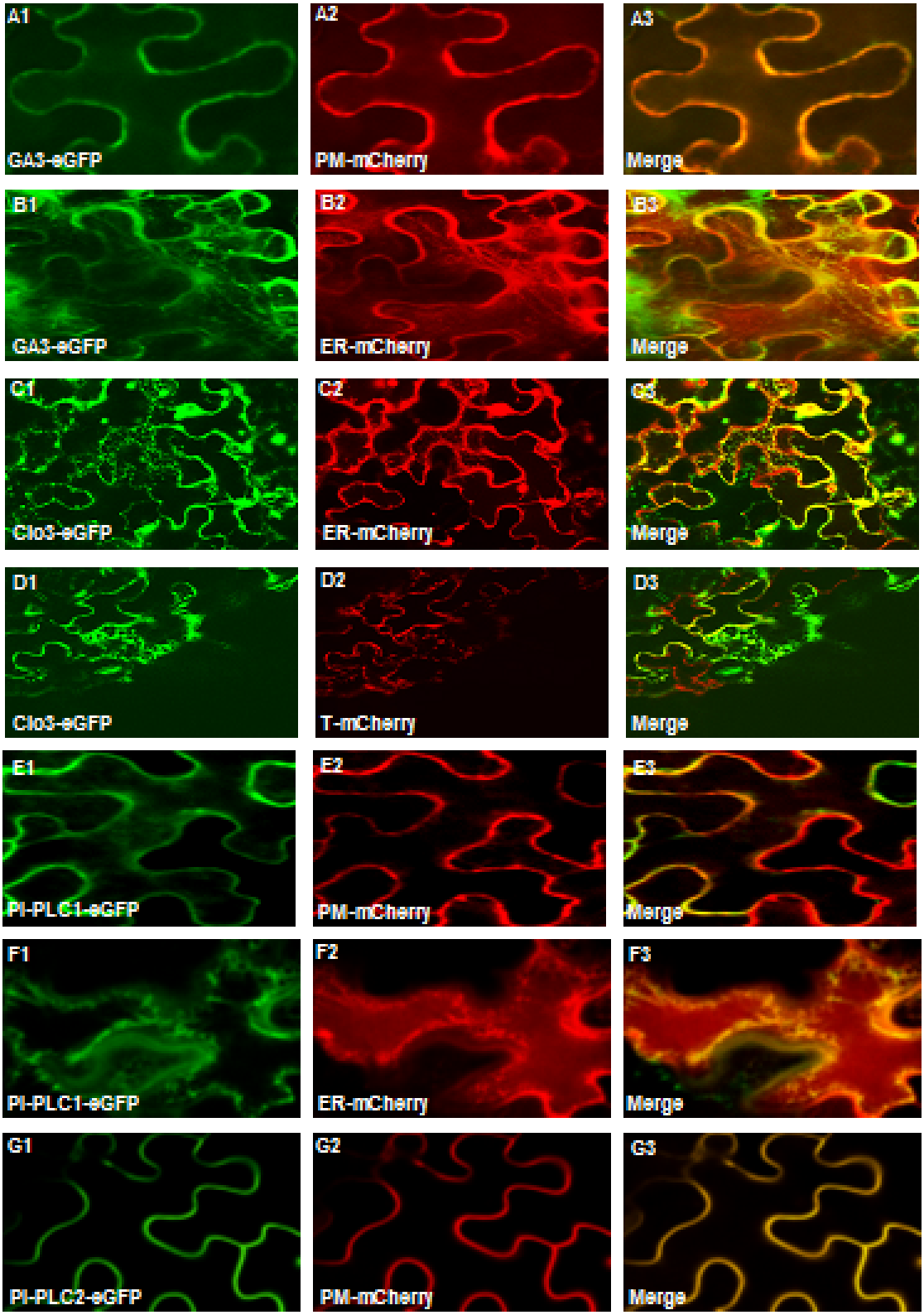

A1
GA3-sGFP
A2
PM-mCherry
A3
Merge
B1
GA3-sGFP
B2
ER-mCherry
B3
Merge
C1
Clo3-sGFP
C2
ER-mCherry
C3
Merge
D1
Clo3-sGFP
D2
T-mCherry
D3
Merge
E1
PI-PLC1-sGFP
E2
PM-mCherry
E3
Merge
F1
PI-PLC1-sGFP
F2
ER-mCherry
F3
Merge
G1
PI-PLC2-sGFP
G2
PM-mCherry
G3
Merge

Figure 2

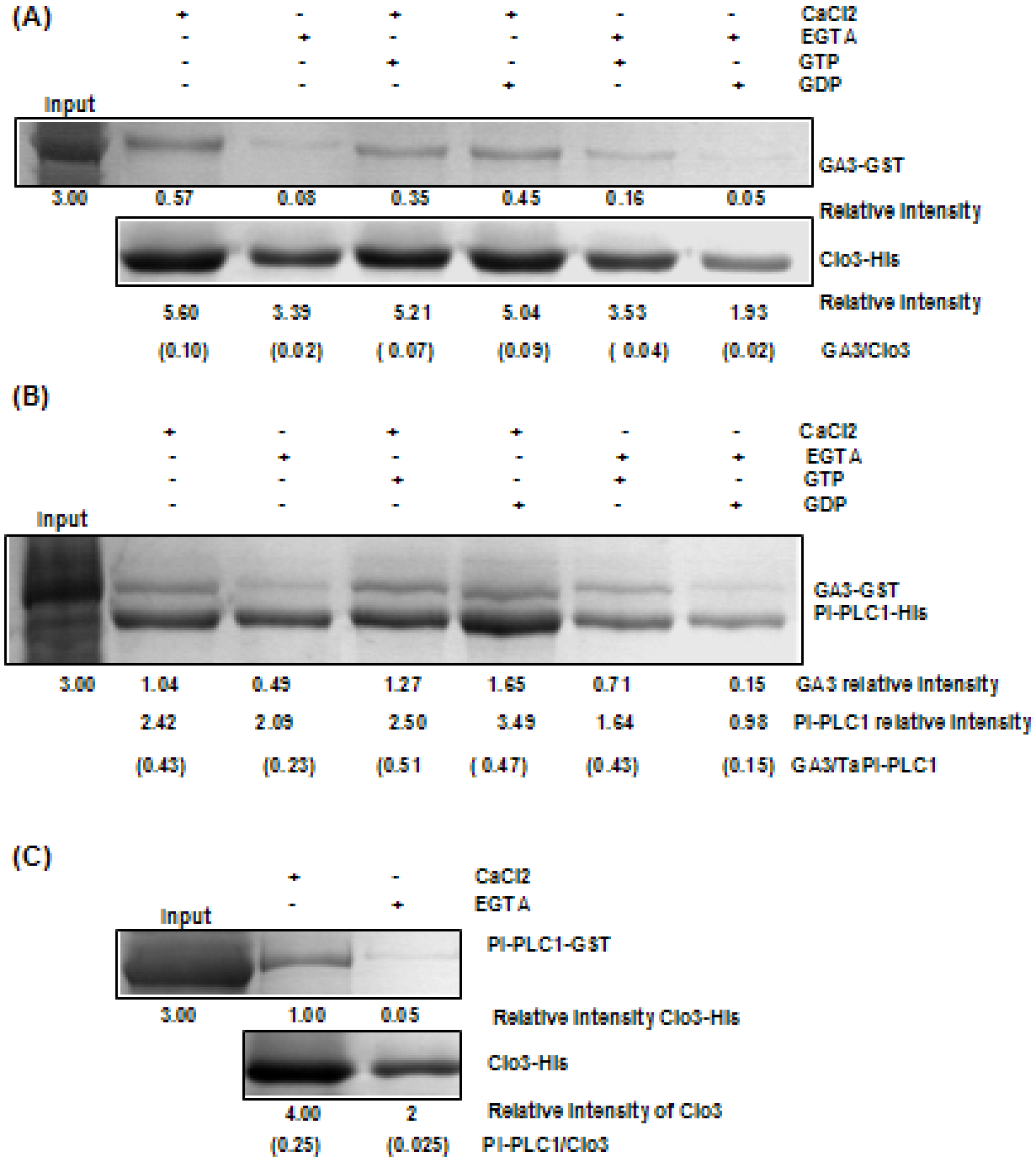

(A)
+ - + + - - CaCl2
- + - - + + EGTA
- - + - + - GTP
- - - + - + GDP
Input
GA3-GST
3.00 0.57 0.08 0.35 0.45 0.16 0.05 Relative Intensity
Clo3-His
5.60 3.39 5.21 5.04 3.53 1.93 Relative Intensity
(0.10) (0.02) (0.07) (0.09) (0.04) (0.02) GA3/Clo3
(B)
+ - + + - - CaCl2
- + - - + + EGTA
- - + - + - GTP
- - - + - + GDP
Input
GA3-GST
PI-PLC1-His
3.00 1.04 0.49 1.27 1.65 0.71 0.15 GA3 relative intensity
2.42 2.09 2.50 3.49 1.64 0.98 PI-PLC1 relative intensity
(0.43) (0.23) (0.51 (0.47) (0.43) (0.15) GA3/TaPI-PLC1
(C)
+ - CaCl2
- + EGTA
Input
PI-PLC1-GST
3.00 1.00 0.05 Relative Intensity Clo3-His
Clo3-His
4.00 2 Relative Intensity of Clo3
(0.25) (0.025) PI-PLC1/Clo3

Figure 3

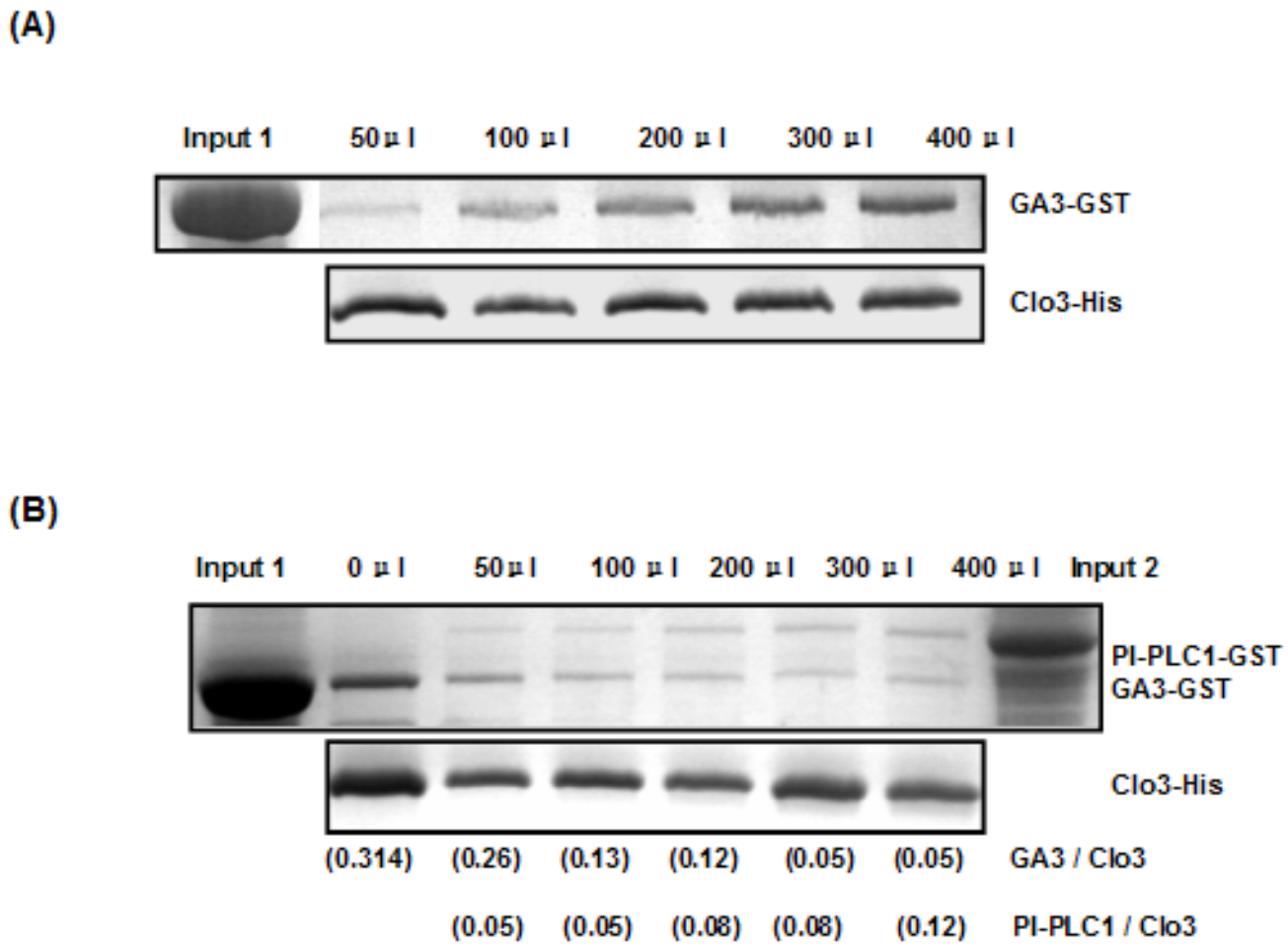
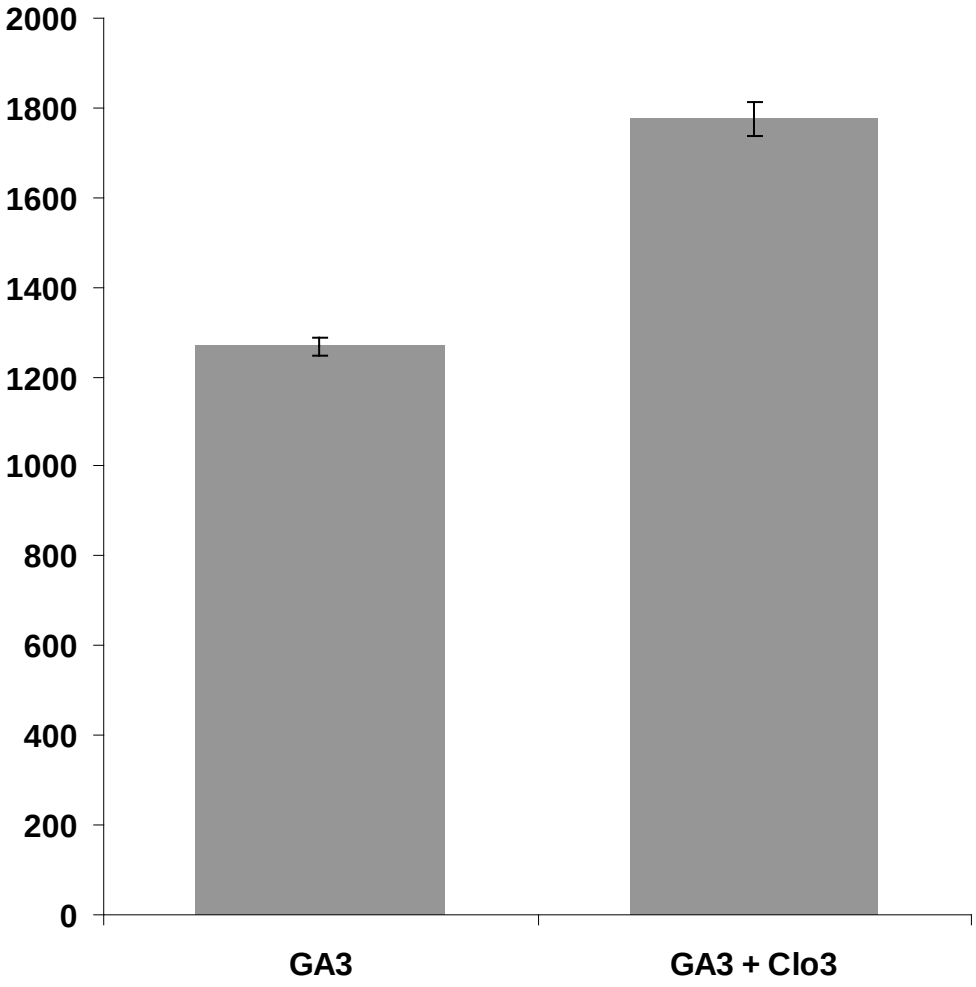

(A)
Input 1
50 µ l
100 µ l
200 µ l
300 µ l
400 µ l
GA3-GST
Clo3-His
(B)
Input 1
0 µ l
50 µ l
100 µ l
200 µ l
300 µ l
400 µ l
Input 2
PI-PLC1-GST
GA3-GST
Clo3-His
(0.314)
(0.26)
(0.13)
(0.12)
(0.05)
(0.05)
GA3 / Clo3
(0.05)
(0.05)
(0.08)
(0.08)
(0.12)
PI-PLC1 / Clo3

Figure 4

Clo3 interacts with Gα and PiPlc1

Figure 5



```
TaPI-PLC2          KTRLKVTVYMGDGWRFDFRKTHFDKCSPPDFYARVGIAGVVADTMMKETK 50
TaPI-PLC1          KKTLKVKVYMGDGWRMDFKQTHFDQYSPPDFYARVGIAGVPADSVMKKTK 50
PsPI-PLC           KTTLKVTVYMGEGWYYDFDHTHFDQFSPPDFYARVGIAGVPFDTIMKKTK 50
OsPI-PLC           KKTLKVKVYMGDGWRMDFTQTHFDQYSPPDFYARVGIAGVPADSVMKRTR 50
VrPI-PLC3          KKTLKVTIYMGEGWFHDFKHTHFDQYSPPDFYARVGIAGVPYDTVMKKTK 50
                   *. ***.:***:**  ** :****: **************  *::**.*:

TaPI-PLC2          VIMDNWIPTWDHEFEFPLSVPELALLRVEVHESDNHQKDDFAGQTCLPVW 100
TaPI-PLC1          AVEDNWVPVWGEEFSFDLTVPELALLRVEAHEYDMSEKDDFAGQTVLPVS 100
PsPI-PLC           TVEDSWLPSWNEVFEFPLSVPELALLRIEVHEYDMSEKDDFGGQTCLPVW 100
OsPI-PLC           AIEDNWVPVWEEDFTFKLTVPEIALLRVEVHEYDMSEKDDFGGQTVLPVS 100
VrPI-PLC3          SVEDNWSPSWNEEFKFPLSVPELALLRVEVHEYDMSEKDDFGGQTCLPVW 100
                    : *.* * * . * * *:***:****:*.** *  :****.*** ***

TaPI-PLC2          ELRSGIRSVRLYARDGEVLRSVKLLMRFEFS 131
TaPI-PLC1          ELQPGIRAVALFDRKGNKLPNVKLLMRFEFV 131
PsPI-PLC           ELRTGIRAVPLHSRKGDKYNNVKLLMRFEFI 131
OsPI-PLC           ELIPGIRAVALHDRKGIKLNNVKLLMRFEFE 131
VrPI-PLC3          ELRSGIRAVPLYSRKGEKYHNVKLLMRFEFI 131
                   ** .***:* *. *.*    .*********
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Figure 6

Supplemental Table 1: Oligo nucleotide PCR primers used in this study.

Primer Name	Sequence
M13 (For.)	5' GTAAAACGACGGCCAGT 3 '
***Ta*Clo3-internal (For.)**	5' GGTGGCTGCTCTTCTGTTTC 3'
Clo3 (For.)	5' TGCATTTGATGTTAAAGGAAGGT3'
PCMVSPORT6 (For.)	**5'**CCATAGAAGACACCGGGA **3'**
Partial-TaPI-PLC1(Rev.)	5' CAGGTGAAGGGAGCTCTT 3'
AttB1 pCMVSPORT6 (For.)	5'GGGGACAAGTTTGTACAAAAAAGCAGGCTCAGCCTCCGGACTCTAGC3'
AttB2 Partial PI-PLC1-1 (Rev.)	5' GGGGACCACTTTGTACAAGAAAGCTGGGTCTCGGGTGTAAGGTGGTCT 3'
AttB2 partial PI_PLC1-2 (Rev.)	5' GGGGACCACTTTGTACAAGAAAGCTGGGTCCGCTGCTCAGCTGGT 3'
AttB1-P1 GA3 (For.)	5' GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGGCTCCTCCTGCAGCA 3'
AttB1-P2 GA3 (For.)	5' GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGAAGGAGGATGTGCTCCATG 3'
AttB2-P3 GA3 (Rev.)	5' GGGGACCACTTTGTACAAGAAAGCTGGGTCCGTCCCCGTTCCCTCCCT 3'
***Ta*GA3-CS (For.)**	5' ATGGGCTCCTCCTGCAGCA 3'
***Ta*GA3-CS (Rev.)**	5' CCGGCTTGCTGCTCTGGA 3'
***Ta*GA3-internal (For.)**	5' GGAGACGAAGGAACTGTTCGACTG 3'
***Ta*GA3 (Rev.)**	5' CGTCCCCGTTCCCTCCCT 3'
Q223LTaGA3 (Rev.)	5' CCTCCTCTCATTCCTTAGACCTCCTAC 3'
Q223LTaGA3 (For.)	5' GTAGGAGGTCTAAGGAATGAGAGG 3'
AttB1 Clo3 (For.)	5' GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGCGATCCGGCGACAAT 3'
AttB1 Clo3 (Rev.)	5' GGGGACCACTTTGTACAAGAAAGCTGGGTCCATTGCACTATGATGAGAAAAGGCCC 3'
AttB1 PI_PLC1 (For.)	5' GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGGCACCTACAAGTGC 3'
AttB2 PI_PLC1 (Rev.)	5' GGGGACCACTTTGTACAAGAAAGCTGGGTCCACAAACTCAAAGCGCATG 3'
AttB1 PI_PLC2 (For.)	5' GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGACGACGTACAGGGTGTG 3'
AttB2 PI_PLC2 (Rev.)	5' GGGGACCACTTTGTACAAGAAAGCTGGGTCCGAAAACTCGAAGCGCAT 3'
AttB1 PI-PLC2ΔEF-hand (For.)	5' GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGGTTCACCATGACAT 3'
AttB2 PI-PLC2ΔC2 (Rev.)	5' GGGGACCACTTTGTACAAGAAAGCTGGGTCTGTCTTCACTGGTAGTTT 3'
AttB2 PI-PLC1-stop (Rev.)	5' GGGGACCACTTTGTACAAGAAAGCTGGGTCTCACACAAACTCAAAGCGCAT 3'
SeqL A (For.)	5' TCGCGTTAACGCTAGCATGGATCTC 3'
SeqL B (Rev.)	5' GTAACATCAGAGATTTTGAGACAC 3'
BamH1 GA3 (For.)	5' CGCGGATCCGCGATGGGCTCCTCCTGCAGCAGACCT 3'
Sal1TaGA3 (Rev.)	5' TTCCGCGGCCGCTATGGCCGACGTCCCCGTTCCCTCCCT 3'

Supplemental Table 2: Organelle markers as red fluorescent-protein fusions.

Organelle	Construct Name	Targeting Protein
Plasma Membrane	PM-rk CD3-1007	The full length of AtPIP2A, a plasma membrane aquaporin
Tonoplast	T-rk CD3-975	C-terminus of c-TIP, an aquaporin of the vacuolar membrane fused to the fluorescent protein
Endoplasmic Reticulum	ER-rk CD3-959	contains the signal peptide of AtWAK2, wall-associated kinase2, at the N-terminus of protein and an ER retention signal, His-Asp-Glu-Leu, at the C- terminus.
Plastids	Pt- rk CD3-999	The first 79 aa of the small subunit of tobacco rubisco.
Mitochondria	Mt-rk CD3-991	The first 29 aa of yeast cytochrome C oxidease IV.
Golgi	G-rk CD3-967	The first49 aa of GmMan1, soybean α-1,2 mannosidae 1.
Peroxisome	Px-rk CD3-983	Peroxisomal targeting signal1, Ser-Lys-Leu , at the C- terminus of the fluorescent protein.

PM, Plasma Membrane; **T,** Tonoplast; **ER**, Endoplasmic Reticulum; **Pt**, Plasitds; **Mt**, Mitochondria; **G**, Golgi; **PX**, Peroxisome; **r**, mCherry fluorescent protein; **k**, kanamycin resistance.