AtCLO4, a stress responsive calcium binding protein, interacts with the heterotrimeric Gα subunit in *Arabidopsis thaliana.*

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complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Abstract

Heterotrimeric G-proteins have been shown to be involved in plant responses to abiotic stress. Recent evidence has shown that a caleosin, RD20/AtCLO3, is involved in stomate and root responses to ABA treatment in addition to interacting with the G alpha subunit (GPA1) in *Arabidopsis thaliana.* The role of the other caleosin gene family members and their interaction with G-proteins still remains largely unknown. This work demonstrated the interaction of another caleosin, AtCLO4, with GPA1 by Bimolecular Fluorescence Complementation (BiFC). Phenotypic analysis of *clo4* mutants showed significantly less inhibition of lateral root formation during salt and mannitol stress compared to wild type; while CLO4 gene expression was characterized in the roots by GUS staining during periods of stress. These results present a novel interaction between CLO4 and GPA1 and indicate a role for CLO4 in regulating GPA1 during periods of stress that culminate in inhibition of lateral root formation.

Acknowledgments

I would first like to thank my supervisor, Dr. Patrick Gulick, whose door (and email) was always open for guidance throughout my research work and writing. I would also like to thank my committee members, Dr. Reginald Storms and Dr. Vladimir Titorenko, for their time in examining this work.

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Introduction

Plant growth and reproduction rates are highest within optimal ranges of temperature, water availability and nutrient ion concentration in their growth environment. However, a remarkable feat of plants is their ability to withstand non-optimal conditions and some species are able to survive and grow through times of abiotic stress such as high salinity, drought and extreme high and low temperatures. Within species, there is wide genetic variation for tolerating environmental stress and numerous molecular mechanisms that have been associated with the adaption to stress conditions (Liu et al., 2000, Bright et al., 2006, Xiong *et al.,* 2006). The molecular mechanisms associated with these have been studied for years with many overlapping triggers and switches. To date, hundreds of genes have been shown via cDNA microarray technology to be induced in plants by cold, heat, drought as well as high salinity (Seki et al., 2002, Shinozaki et al., 2003 , Maruyama et al., 2004, Atkinson et al., 2013, Cheng et al., 2013, Leviatan et al., 2013).

Plants undergoing abiotic stress have been shown to increase their cytosolic calcium levels in response to salt stress, drought, abscisic acid (ABA) treatment and, to a lesser extent, cold stress (Nambara and Marion-Poll, 2005, Hepler 2005). Increases in ABA levels have been shown to cause stomatal closure (Bright et al., 2006) and induce expression of stress-related transcription factors that aid in drought and high salinity responses. The ABSCISIC ACID–RESPONSIVE ELEMENT BINDING PROTEIN1 (AREB1) is upregulated by ABA treatment and has been shown to activate downstream genes such as RD29B, KIN2, AIL1 and RAB18 (Fujita et al., 2005) during periods of drought. There are also several transcription factors such as

Dehydration Responsive Element Binding Proteins DREB1/CBF and DREB2 that activate the expression of downstream RD29A during periods of cold and drought respectively (Liu et al., 2000); however, this has been shown to be unaffected by exogenous ABA which demonstrate both ABA dependent and independent regulatory systems for stress induced gene expression (Maruyama et al., 2004, Nakashima et al., 2006). Plant physiological changes under abiotic stress manifest themselves in the forms of root morphology changes (Xiong *et al.,* 2006), stomatal closure (Pospíšilová et al., 2009) aperture and density.

One member of the caleosin gene family, At*Clo3/RD20* has been shown to be markedly induced by drought, salt and ABA (Takahashi. 2000). Members of this gene family are calcium binding proteins with a single EF-hand motif and have been identified in many plant species including *Arabidopsis thaliana* (Arabidopsis), rice and wheat and other members of the Triticeae tribe (Frandsen et al., 1996, Naested et al., 2000, Takahashi, 2000, Khalil et al., 2011). The caleosin gene family in Arabidopsis consists of seven members (Partridge & Murphy 2009). Several of these caleosins have been implicated in controlling a wide array of plant responses that includes the response to abiotic stresses as well as lipid peroxidation (Hanano et al., 2006, Aubert et al., 2011, Kim et al., 2011). At*Clo3/RD20* is a non-seed caleosin, which when mutated has been shown to impair the regulation of stomatal aperature in response to drought (Aubert et al., 2010). The involvement of *RD20* as a mediator of stress phenotypes has been shown to inhibit seed germination under ABA treatments as well (Aubert et al., 2011). AtCLO4 has been shown to be a stress responsive calcium binding protein that acts as a negative regulator in ABA and salt stress signaling. Loss of function mutants of *AtCLO4* have been shown to decrease germination rates under stress while being hypersensitive to salt and mannitol (Kim et al.,

2011). It is also worth noting that caleosin 1, *AtClo1* ,has been shown to affect oil bodies (OB) of the seed by inducing proliferation of lipid droplets and influencing the accumulation and storage of specific lipids (Froissard et al., 2009). AtCLO1 has also been shown to have a Ca^{2+}-dependent peroxygenase activity which may be involved in the oxylipin signaling pathways and plant defense responses (Hanano et al., 2006).

Interactions between caleosins and heterotrimeric G proteins have been proposed as a novel mechanism for mediating cell responses to abiotic stress. Heterotrimeric G protein complexes are involved in multiple regulatory pathways in both plants and animals, and are known to receive extracellular signals to relay to downstream target proteins known as effectors (Neubig & Siderovski, 2002). The standard model of G protein signaling is one in which the complex is comprised of Gα, Gβ and Gγ subunits and has GDP bound to the Gα subunit in its resting state. The Gulick lab has shown that Ta-Clo3 interacts with GA3, the Gα subunit of the heterotrimeric G protein complex in *Triticum aestivum (*Khalil et al., 2011). This was elaborated through work on AtClo3/RD20, which was found to interact with the G alpha subunit (GPA1) of *Arabidopsis thaliana* (unpublished, MSc thesis of Zhejun Wang). The heterotrimeric G proteins in animals and yeast are associated with 7 transmembrane (7TM) G protein coupled receptors (GPCR) that bind to a ligand, causing a conformational change in the protein on the cytoplasmic side of the cell membrane at the Gα subunit. This change causes the Gα subunit to release its GDP nucleotide in exchange for GTP. The binding of GTP causes conformational changes which separates the Gβγ dimer from the Gα subunit, and changing the subunits into their activated states. The activated subunits are free to interact with and activate downstream effectors (Gilman 1987).

The model organism *A. thaliana* G protein complex differs from that of animals. The genome encodes a single alpha subunit (GPA1) (Ma et al., 1990), one beta subunit (AGB1) (Weiss et al., 1994) and three gamma subunits (AGG1, AGG2, AGG3) (Mason, M. G., & Botella, J. R. 2000). The heterotrimeric G proteins, act cyclically as molecular switches between activated and inactivated states with GTP binding causing a conformational change in the Gα subunit which thereby disrupts interaction with the Gβγ dimer and separates the dimer from the Gα subunit. The Gα subunits in animal G proteins are affected by GTPase accelerating proteins (GAPs) and guanine nucleotide exchange factors (GEFs), with GEFs acting to accelerate exchange of bound GDP for GTP. In animal systems GEFs play key regulatory roles in the activation of Gα subunits, however, unlike mammalian heterotrimeric G protein complexes, the guanine nucleotide exchange steps seemingly take place in the absence of G-protein coupled receptors (GPCRs). The only known GTPase accelerating protein (GAP) shown in Arabidopsis is the regulator of G protein signaling 1 (AtRGS1), a protein with a 7TM domain that has been shown to inhibit the activity of GPA1 by uncoupling the Gα subunit via endocytosis brought on by sugar dependent relocalization. The Gβγ dimer and GαGTP subunits are then able to interact with other unknown presumptive downstream effectors (Urano & Jones 2013).

G proteins have been found to be involved in plant stress signaling pathways. It has been shown that G proteins are expressed in roots and affect root morphology. Mutations in *GPA1* reduced lateral root formation and increased primary root length which would allow plants to better survive droughts by promoting deeper penetration into soil for water while *agb1* mutants have increased lateral root formation (Ullah et al., 2003, Zhang et al., 2006). *GPA1* mutants have been shown to affect aerial tissue by playing a role in controlling stomatal

density which manifested in lower stomatal density and higher transpiration efficiency (Nilson & Assmann 2010). Meanwhile, *AGB1* mutants have also been shown to be required for stomatal density with increased stomatal density (Zhang et al., 2008). *GPA1* and *AGB1* mutants have also been shown to be less and more sensitive, respectively, to oxidative burst damage caused by O_3 which manifests itself with an increase in reactive oxygen species (ROS) and hydrogen peroxide (Joo & Fedoroff 2005).

Research on the interactions between caleosins and the G protein complex presents a novel approach to understanding and studying not only the effects that the caleosin family has on the G protein complex but also on downstream effectors that can contribute to the abiotic stress resistance. One such downstream effector is phosphoinositide specific phospholipase C (PI- PLC). PI- PLC is known to aid in calcium release into the cytoplasm by hydrolyzing phosphatidylinositol 4,5-bisphosphate (PIP2) to produce inositol triphosphate (IP3) which triggers calcium release from the vacuole or endoplasmic reticulum, and is also known to be activated by Gα in animals (Berridge 1993). Overexpression of PI –PLC (*Zm*PLC1) in maize has been shown to improve the plants response to drought (Wang et al., 2008). PLCδ was shown to interact with Gα in *Pisum* as (Misra et al., 2007) and PI-PLC1 was shown to interact with Gα from *Triticum aestivum* (Khalil et al., 2011). This raises the hypothesis that caleosins may regulate G proteins by affecting their interaction with PLCs, indeed Khalil (2011) demonstrated competitive binding between Pi-PLC1, GPA1 and CLO3, though its effect on PI-PLC activation has not been shown. The Gulick lab has shown that CLO3 as well as CLO7 interact with Gα in Arabidopsis *in vivo* (unpublished). Mutants of *rd20/clo3* have root phenotypes similar to *rgs1*

mutants in *rd20/clo3* mutants; they have decreased inhibition the formation of lateral roots and develop a longer primary root in response to ABA treatment (Chen et al., 2006).

The first objective of this thesis was to investigate other members of the caleosin gene family besides *CLO3* and *CLO7* to determine if they interact with the G protein complex subunits. Bimolecular Fluoresence Complementation method (BIFC) was used to test potential interactions of other members of the caleosin gene family, *CLO2, CLO4* and *CLO5* with the Gα (GPA1) protein subunit and to determine the localization of such interactions within the cell. The second objective was to conduct phenotypic analysis of caleosin mutant lines of *Arabidopsis*. The analysis measured changes in stomatal density and root morphology in response to ABA treatment, cold treatment, as well as mannitol and salt stress. Finally, transgenic Arabidopsis plants containing the promoter of *CLO1* and *CLO4* fused to the β-glucuronidase (GUS) gene were used to localize gene expression in different plant tissues in response to abiotic stress.

Materials and Methods

Plant growth conditions and imaging protocols:

Soil used for all experiments was made specifically by mixing three parts of black earth, one part peat moss and one part vermiculite. The mixture was then heat treated at 130^{O}C for one hour. After the soil cooled, seeds were sown in pots and cold treated in the dark at 4°C for three days. Pots were then moved the greenhouse with temperature at 22^{o}C with a light cycle extended to 16 h per day by supplementary lighting.

Mutant seed lines of *clo4* and the wild type (WT) Columbia ecotype were used to study stomatal density changes caused by ABA treatment. The plants were sprayed three times per week with a solution of 25 µM ABA in 0.05% ethanol/water mixture starting at the time of emergence and continued for fifteen-days, while control plants were sprayed three times per week with 0.05% ethanol. To determine stomatal density, leaf samples from leaves one and two were taken from six to eight plants and placed in 9:1 ethanol and acetic acid overnight to remove the green colouration caused by chlorophyll. These leaves were subsequently rinsed in 70%, 50% and 20% ethanol for 30 minutes at each ethanol level. Samples were placed in deionized water for at least 30 minutes or until staining. Each leaf was punctured twice with a needle next to the sites of interest at a point midway between the ends of the leaf and midway between the central vein and the edge of the leaf. The leaves are then placed in 0.5% toluidine blue (TBO) for 5-10 minutes depending on leaf size. Once staining was complete the leaves were rinsed with water. Samples were then photographed on a Zeiss Axioplan fluorescence

microscope using a Lumenera Infinity 3-1C 1.4 megapixel colour cooled CCD camera after which stomatal density was counted and tabulated.

Seeds to be grown on agar plates were sterilized prior to sowing. WT, *clo1 and clo4* seeds were placed in ependorf tubes containing 70% ethanol and vortexed. The ethanol is then decanted and the seeds soaked in a sterilization solution containing (3.09% sodium hyperchlorate, 1% Triton X-100) for five minutes. The seeds were rinsed with four to five washes of sterile distilled water and then left in the dark at 4°C for three days. Seeds were plated under sterile conditions on petri dishes with semi-solid MS media containing 0.5x Murashige and Skoog basal salt mixture, 1% (w/v) sucrose, 0.05% 4-Morpholineethanessulfonic acid (MES hydrate), 0.4% Gelzan^{Tm} CM agar substitute gelling agent adjusted to pH 5.7.

Seedlings were transferred to 150 mm treatment petri plates 48 h after germination. Seedlings were placed on media with 2 µM ABA, or mannitol treatment with either 250 or 300 mM of mannitol, or salt treatment plates which contained either 85 or 100 mM of NaCl; control seedlings were transferred onto new control plates which only contain MS and sucrose. All plates were sealed and placed vertically in growth chambers set to 22°C, 43.21 $\mu mol \cdot m^{-2} \cdot s^{-1}$ fluorescent light with a light cycle of 16 h light and 8 h dark. After eight days of growth on the ABA plates, they were photographed and the root lengths were measured and lateral root numbers counted. Similarly, plants were left for six days after germination on plates with different treatment conditions including 250 and 300 mM mannitol and 85 and 100 mM NaCl and then photographed. For cold treatments, plants were grown on MS and 1 % sucrose plates for four and six days after which they were transferred to growth chambers at 4°C for four days

and then photographed. Images taken for all experiments were analyzed with *imageJ* software to measure primary root length and count the number of lateral roots formed.

Statistical Analysis:

Data for stomata index, primary root lengths and lateral root count was analyzed by Student's *t*-test and 2 way ANOVA. Results with statistical significance of at least 0.05 were further analyzed by Duncan's multiple range test to detect significant differences between genotypes under different treatment conditions.

Construction of expression vectors:

The coding regions of *CLO1, CLO2, and CLO4* were amplified by PCR with Gateway® ends and cloned into pDonr207 vectors using the Gateway® BP Clonase II Enzyme system (Invitrogen). Constructs developed are listed in Table 1 and oligonucleotide primers used for cloning are listed in Table 2. The recombinant plasmids were transformed into electrocompetent TOP10 *E. coli* strain and selected on LB media containing 50 mg/ml of gentamycin antibiotic. Colonies grown were picked and plasmid purified, with positive constructs being confirmed via PCR with gene specific primers, by restriction digest as well as DNA sequencing. Confirmed *CLO1, CLO2, and CLO4* genes within the pDONR207 vectors were transferred to destination vectors using the Gateway® LR Clonase II Enzyme system. These vectors were PK7FWG2, a GFP fusion destination vector with full length eGFP as a C-terminal

fusion and to the Bimolecular fluorescence Complementation (BIFC) vectors, pBatTL-B-sYFP-C and pBatTL-B-sYFP-N. With the latter two vectors contain the C terminal and N terminal halves of the Yellow Fluorescent Protein (YFP). The promoter sequences of *CLO1 and CLO4,* taken 1 kb upstream of the start of transcription were also cloned into pDONR207 by Gateway cloning and then transferred to pFAST-G04 a plant expression vector which expresses β-glucuronidase (GUS) under the control of the selected gene promoter inserted into the vector (Shimada et al., 2010). The vector contains a visible marker which expresses GFP only in the transgenic seed coat which allows for selection of transgenic plants by screening seed for GPF expression. The full list of expression vectors and primers used are provided in **Table 1** and **Table 2** respectively.

Table 1. Expression vectors used in this study

Construct	Description
CLO1 /PBATTL-B-SYFP-N	Contains full length coding sequence of *CLO1* fused with N-terminal half of YFP.
CLO2 / PBATTL-B-SYFP-N	Contains full length coding sequence of *CLO2* fused with N-terminal half of YFP.
CLO4 / PBATTL-B-SYFP-N	Contains full length coding sequence of *CLO4* fused with N-terminal half of YFP.
GPA1/ PBATTL-B-SYFP-C	Contains full length coding sequence of *GPA1* fused with C-terminal half of YFP.
AGB1/ PBATTL-B-SYFP-C	Contains full length coding sequence of *AGB1* fused with C-terminal half of YFP.
AGG1/ PBATTL-B-SYFP-C	Contains full length coding sequence of *AGG1* fused with C-terminal half of YFP.
CLO1 /PK7FWG2	Contains full length coding sequence of *CLO1* fused with eGFP.
CLO4 / PK7FWG2	Contains full length coding sequence of *CLO4* fused with eGFP.

Tonoplast Marker; T-rk CD3-975	C-terminus of c-TIP, an aquaporin of the vacuolar membrane fused to the fluorescent protein
Endoplasmic Reticulum Marker; ER-rk CD3-959	Expresses the signal peptide of wall-associated kinase2, fluorescent at the N-terminus of protein and ER retention signal, His- Asp -Glu-leu, at the C- terminus.
Mitochondria Marker; Mt-rk CD3-991	The first 29 amino acids of yeast cytochrome C oxidase IV.
Plasma membrane Marker; PM-rk CD3-1007	Full length of AtPIP2A, a plasma membrane aquaporin.
P19	Protein of Tomato bushy stunt virus

Table 2. Primers used in this study

Clo4, AT1G70670, Forward Primer – 5' GGGGACAAGTTTGTACAAAAAAGCAGGCTT CATGGCTTCCTCTATTTCC 3' tm = 51.59
Clo4, AT1G70670, Reverse Primer – 5' GGGGACCACTTTGTACAAGAAAGCTGGGT CTGGATGTTTCTTAGAAGTTTTAG 3' tm = 52.61
Promoter Clo1 for GUS in Pfastg04, Forward Primer – 5' GGGGACAAGTTTGTACAAAAAAGCAGGCTT C ATGAAGTCTCGACTGCTGTA 3' tm = 53.42
Promoter Clo1 for GUS in Pfastg04, Reverse Primer – 5' GGGGACCACTTTGTACAAGAAAGCTGGGT C CATCTCTCTCTCACTTTGTCTC 3' tm = 53.28
Promoter Clo4 for GUS in Pfastg04, Forward Primer – 5' GGGGACAAGTTTGTACAAAAAAGCAGGCTT C ATGGGTCTACCGTCTACTACA 3' Tm = 53.86
Promoter Clo4 for GUS in Pfastg04, Reverse Primer – 5' GGGGACCACTTTGTACAAGAAAGCTGGGT CGAAGCCATGATCAGAATGA 3' Tm = 54.38

***Underlined sequences are Gateway ends**

Localization of gene expression with β-glucuronidase (GUS) reporter assay:

Transgenic Arabidopsis plants containing *CLO1* and *CLO4* promoter:GUS constructs, in the pfastg04 plasmid vector, were made using the floral dip method (Clough & Bent, 1998). T1 seed from the dipped plant was collected and screened for GFP expression. Putative transgenic seed was planted to screen its T2 seed to identify T1 lines with single T-DNA insertions. GFP expressing T2 seed were planted to identify homozygous lines with a single transgene insertion. Transgenic homozygous T2 seed were germinated on control MS media with 1% sucrose. After 8 days, plants were transferred to MS plates with 1% sucrose supplemented with either 150 mM NaCl, 300 mM mannitol, or to control media. The plants were treated for 6, 12 and 24 h with their respective stresses and then transferred to an X-Gluc solution overnight at 37°C according to the protocol described in Jefferson et al., (1987). Samples are then destained in 70% ethanol for at least 16 hours after which the ethanol was replaced.

The samples were removed from the ethanol and placed on a clear surface and observed under the Nikon SMZ1500 Stereomicroscope with a Leica DFC420 5 megapixel colour digital camera. Samples were illuminated from below with white light. Gene expression was scored by the appearance of blue stain in the treated tissues. Samples were photographed to show differences in tissue specific expression.

Protein-protein interaction and localization by Bimolecular Fluorescence Complementation :

Nicotiana benthamiana plants were grown similarly to the *Arabidopsis* as described above, in soil under the same conditions except that the plants were grown for two to four weeks after germination. Plasmid vectors containing the full length *CLO1, CLO2, CLO4 or CLO5* coding regions expressed in the BiFC vector pBatTL-B-sYFP-N were transformed into electrocompetent *Agrobacterium tumafaciens* strain AGL1 by electroporation transformation. Full length coding regions of *GPA1, AGB1 and AGG1-2* were expressed in C/YFP vector pBatTL-B-sYFP-C for BiFC, and were transformed in the same strain. *Agrobacterium* cultures transformed with the expression constructs were grown overnight in LB media at 30°C. Subsequently, cultures of *Agrobacterium* at OD 600 0.5 harboring the BiFC constructs and OD 0.3 for markers were centrifuged and resuspended in agroinfiltration solution of 10mM $MgCl_2$ and 150 µM of acetosyringone. The suspensions were incubated at room temperature for four hours and infiltrated with syringes without needles into on the lower mid part the abaxial side of the leaf of *N. benthamiana* plants. After two to three days growth in the greenhouse, tissue samples 5 X 5 mm were cut from the leaves and observed on the Olympus Fluoview FV10i Laser scanning microscope with a 60x oil immersion lens. The pre-set dye GFP/Mcherry/FRET; excitation peak 489 nm, emission 510 – 610 nm, was used to observe BIFC interactions and localizations shone from laser diodes. A photomultiplier tube based spectral system is used for detection and imaging.

Results

CLO4 interacts with GPA1 (Gα):

Since the caleosin CLO3/RD20 was previously found to interact with GPA1, the heterotrimeric G protein α subunit and AGG2, a γ subunit, (Zhejun Wang, MSc thesis) the current objective was to determine if any other caleosins have interactions with heterotrimeric G proteins. Potential protein-protein interactions between different members of the caleosin gene family and different heterotrimeric G protein subunits were examined by BIFC.

CLO4 was found to interact with GPA1 by BiFC assays (Figure 1). The CLO4-N-YFP construct gave positive fluorescent signals when they were co-expressed with GPA1- C-YFP. CLO4 was not observed to have interactions with the Gγ subunit AGG1. CLO4 interactions with GPA1 were localized to the PM (Figure2). CLO1 - N-YFP, CLO2 - N-YFP and CLO5- N- YFP were not found to produce any fluorescent signal with the C-YFP fusion constructs with GPA1, AGB1, AGG1 nor AGG2. These results indicate that there are no interactions between these caleosins and any of the heterotrimeric G protein subunits that were tested. These were tested multiple times and there was no indication of interaction whereas the positive controls, RD20 -N-YFP with GPA1-C-YFP consistently produced a fluorescent signal in these assays. The differences among the four caleosins, as shown by the BIFC assays, underscore the specificity of the protein-protein interaction detected for CLO4 with the G-protein subunits. This indicates that CLO4 could directly regulate G protein signalling by the physical interaction between CLO4 and the GPA1 subunit and singles it out for more extensive experimentation. Further characterization of

caleosins was focused to *CLO4* and *CLO1* to investigate the relationship between caleosins and the response to environmental stress conditions and to expand on the possible roles of caleosins in plant development.

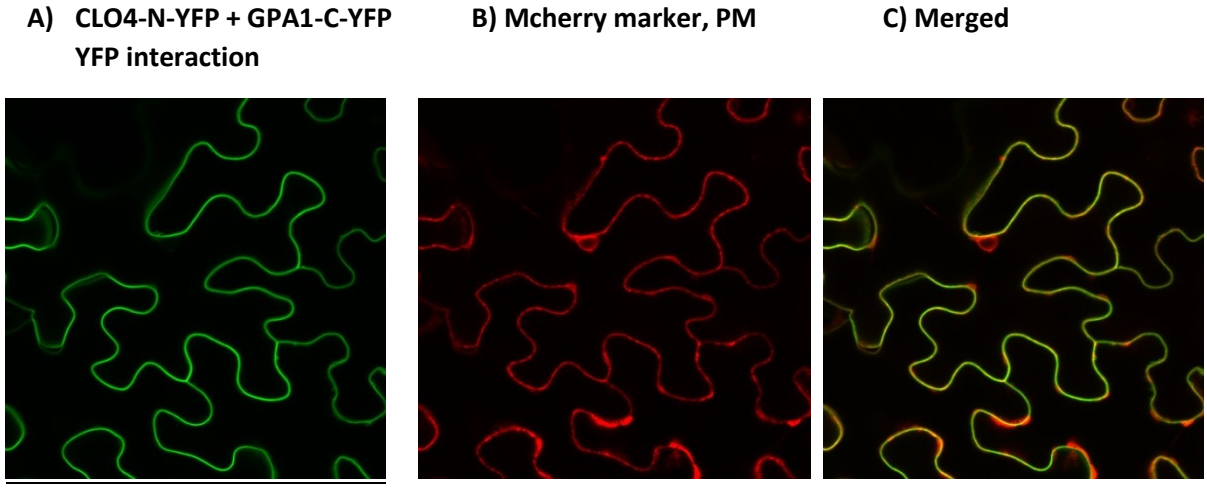


Figure 1. Interaction between CLO4 and GPA1 (Gα). **(A)** Shows BIFC results for assaying the interaction between gene fusion construct CLO4-NYFP interacting with GPA1-CYFP expressed in the leaf epidermal cells of *N. benthamiana* by transient transformation. **(B)** Plasma membrane marker, mCherry fusion with C-terminus of AtPIP2A. **(C)** Merged images of the overlap shows that the CLO4-GPA1 interaction takes place in the plasma membrane.

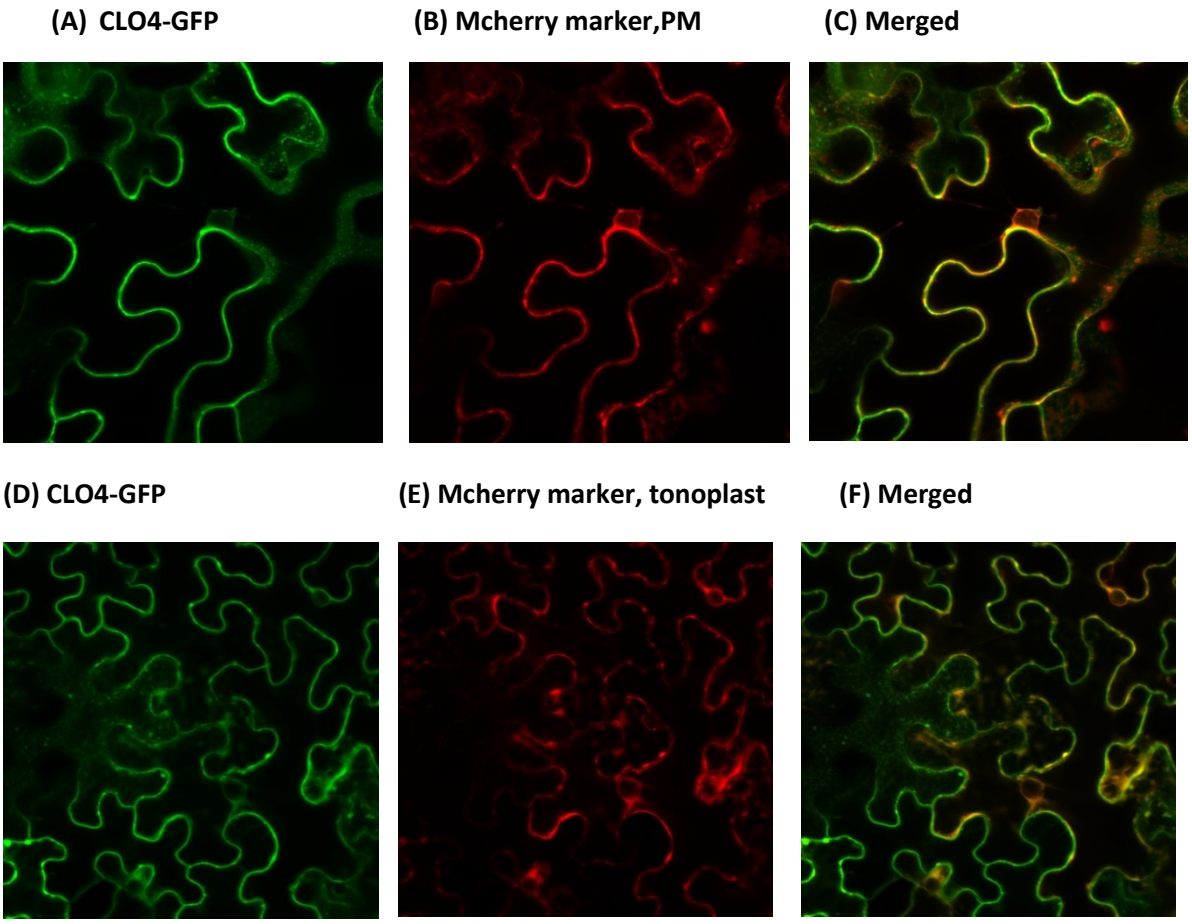


Figure 2. Localization of CLO4 within organelles. **(A and D)** Shows the localization of CLO4 fused with full length Green fluorescent protein expressed in the epidermal leaf cell of *N. benthamiana* by transient transformation. **(B)** Mcherry fusion with C-terminus of AtPIP2A, marker for plasma membrane. **(E)** Mcherry fusion with C-terminus of c-TIP, a marker for vacuole. **(C and F)** Show that CLO4 is localized both in the plasma membrane and tonoplast.

ABA treatment effects on stomatal density and root morphology in the *clo4* mutant do not differ from those of WT:

Mutant rd20/clo3 lines were previously shown to have reduced primary root growth and enhanced lateral root growth and changes in stomatal density in response to ABA

treatment (unpublished Gulick lab data). The *clo4* mutant lines were treated with ABA; this was to test if CLO4 plays a role in ABA signalling that affects the change in stomatal density in and root architecture in response to ABA treatment. In contrast to *RD20*, the root growth characteristics in response to ABA treatment for *clo4* were not found to differ significantly from wild type plants **(Figure 3)**. The number of lateral roots and primary root length both decreased in response to ABA treatment; however this was not significantly different from wild type. Effect of ABA on stomatal density in *clo4* mutants was negligible (**Figure 4**), however the results do not agree with previous results from the Gulick lab and the experiments warrant replication. The *clo4* mutants sprayed with ABA for 2 weeks displayed increased stomatal density but it was not significantly different from the untreated controls and there were no significant differences between the WT and *clo4*. These results do not agree with similar experiments conducted in our lab and the response to ABA warrants further study.

The mutant clo4 plants had a significantly lower stomatal index than the WT and ABA treated plants had a significantly reduced stomatal index but the response of the two genotypes to ABA treatment was not significantly different as seen by the non-significant P value for genotype x treatment interaction effect.

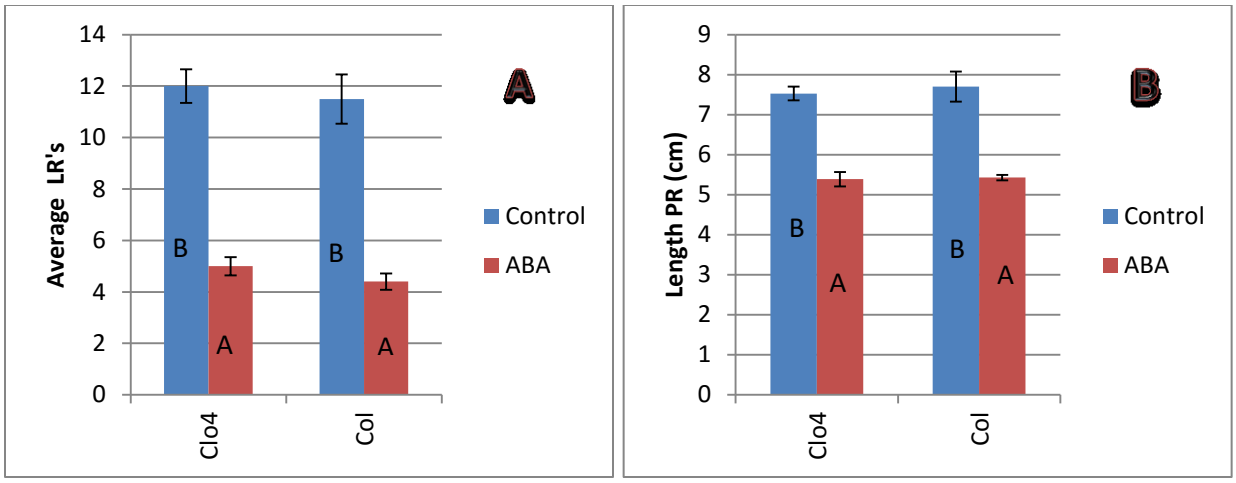
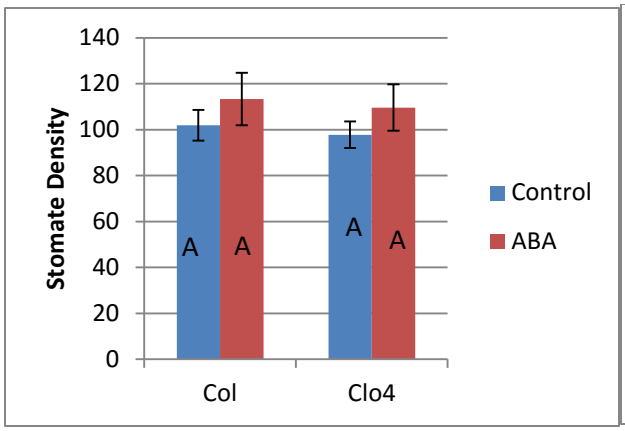
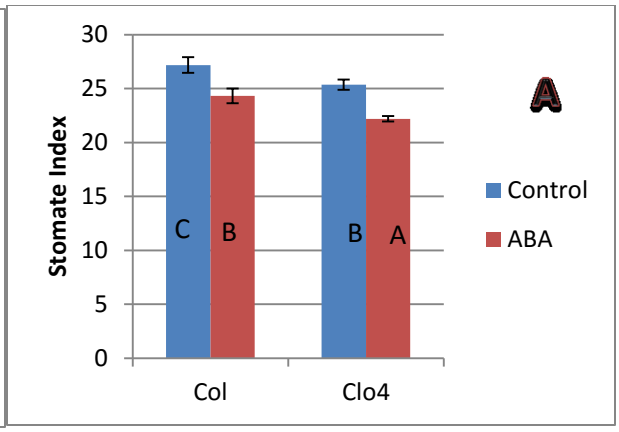


Figure 3. Root morphology changes after abscisic acid (ABA) treatment. **(A)** The number of lateral roots counted on wild type Colombia and mutant *clo4* plants after being grown on MS control plates (blue) and ABA treated plates (red) for 8 days. **(B)** The primary root lengths of WT and *clo4* mutant plants after being grown on MS control plates and ABA treated plates. 18 plants were used and were grown under similar conditions in growth chambers with no significant difference between *clo4* mutant plants and Colombia wild-type plants. Error bars represent standard errors.





2-way Analysis of Variance

Dependent Variable: Stomate_Index

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	189.501[a]	3	63.167	11.459	.000
Intercept	39368.054	1	39368.054	7141.775	.000
Treatment(ABA)	145.437	1	145.437	26.384	.000
Genotype	62.417	1	62.417	11.323	.001
Treatment (ABA) * Genotype	.344	1	.344	.062	.804

2-way Analysis of Variance

Dependent Variable: Stomate Density

B

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	875.535[a]	3	291.845	.668	.583
Intercept	243508.267	1	243508.000	557.416	.000
Treatment	739.472	1	739.472	1.693	.210
Genotype	83.480	1	83.480	.191	.667
Treatment * Genotype	.175	1	.175	.000	.984

Figure 4 (A) Comparison of the stomatal density and stomatal indices of Colombia wild type and clo4 mutants. Plants were grown in soil under control and ABA sprayed conditions. Six plants were examined per treatment with three microscopic images taken at similar location on the leaf. Error bars represent one standard error. **(B)** 2 way ANOVA test showed a significant effect of the genotype indices but no significant interaction effect. Responses of different genotypes to treatments were not significantly different for indices and density.

clo4 mutants have less inhibition of lateral root formation under salt and osmotic stress conditions relative to WT:

It has been shown that seed germination in *clo4* mutants was hypersensitive to salt and mannitol treatment (Kim et al., 2011). Here *clo4* as well as *clo1* mutants were tested for the sensitivity of root growth to salt and mannitol stress (**Figure 5, 6**). The *clo1* and *clo4* mutants were sown on MS plates containing 85 and 100 mM NaCl to test for salt stress. While all the plants showed reduced lateral root formation as well as shorter primary root growths, the salt treated *clo4* mutants had significantly less inhibition of lateral root formations compared to *clo1* and the wild type (**Figure 5, A-D**). Additionally, the *clo1* and *clo4* mutants were sown on MS plates containing 250 mM and 300 mM mannitol to test for root growth under osmotic stress conditions. Similar to the salt stress, plants showered reduced lateral root formations and shorter primary root growths compared to the WT control under control conditions. However, *clo4* mutants grown on 300 mM mannitol plates showed significantly greater lateral root formation than the wild type. The clo1 mutants also had significantly more lateral roots than WT with 300 mM mannitol, but significantly fewer lateral roots than the clo4 mutant (**Figure 6, A-D)**.

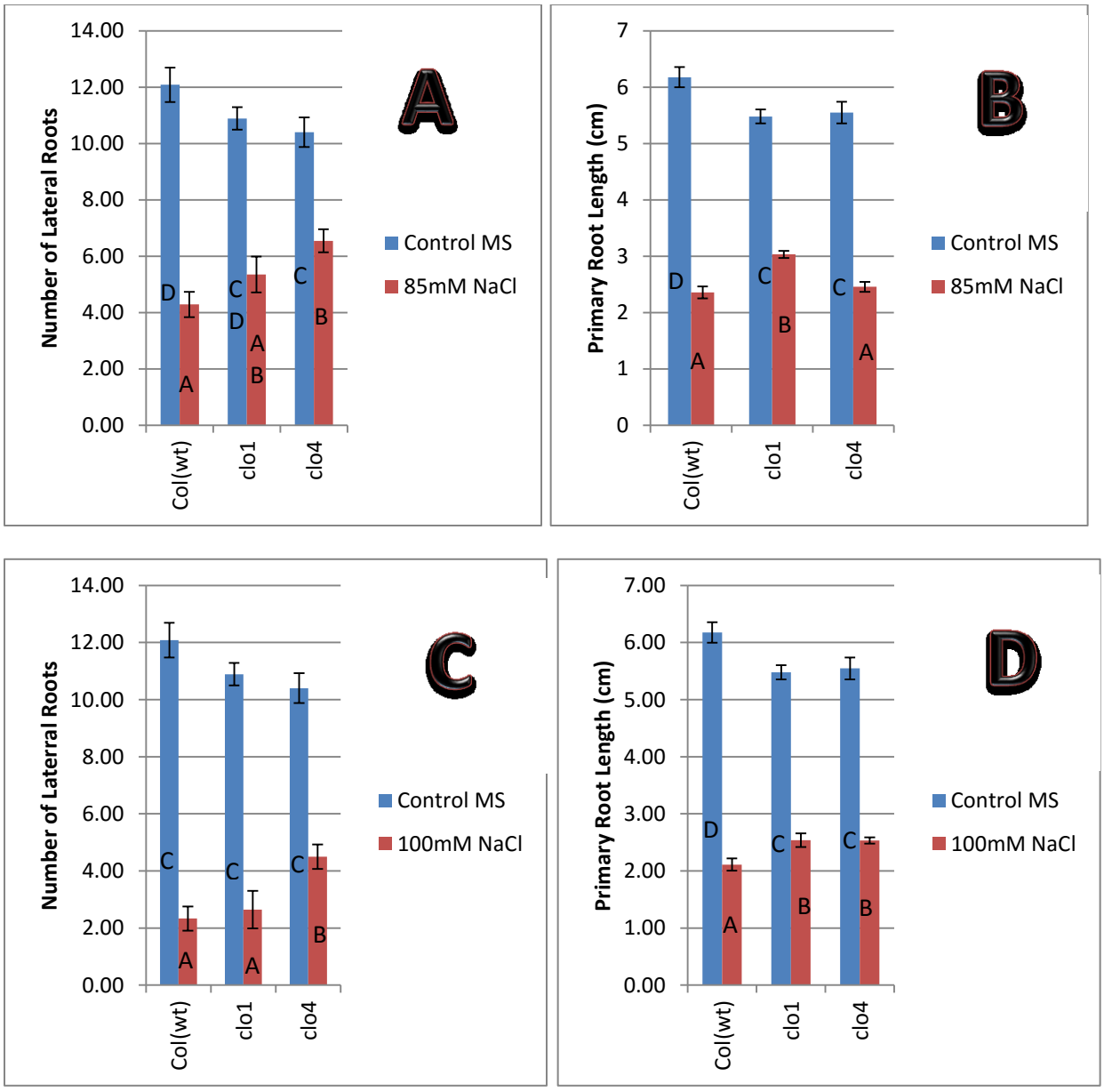


Figure 5. The number of lateral roots and primary root length for Colombia (WT), clo1 and clo4 mutants under control and salt stress. Graphs show primary root lengths and lateral root numbers measured/ counted respectively from wild type, *clo1 and clo4* mutants after growth on MS control plates compared to containing 85 mM and 100 mM of NaCl. Error bars represent standard error; letters on bar graphs indicate significant differences determined by Duncan's multiple range test. The results of statistical testing are given in supplementary data tables 1 and 2.

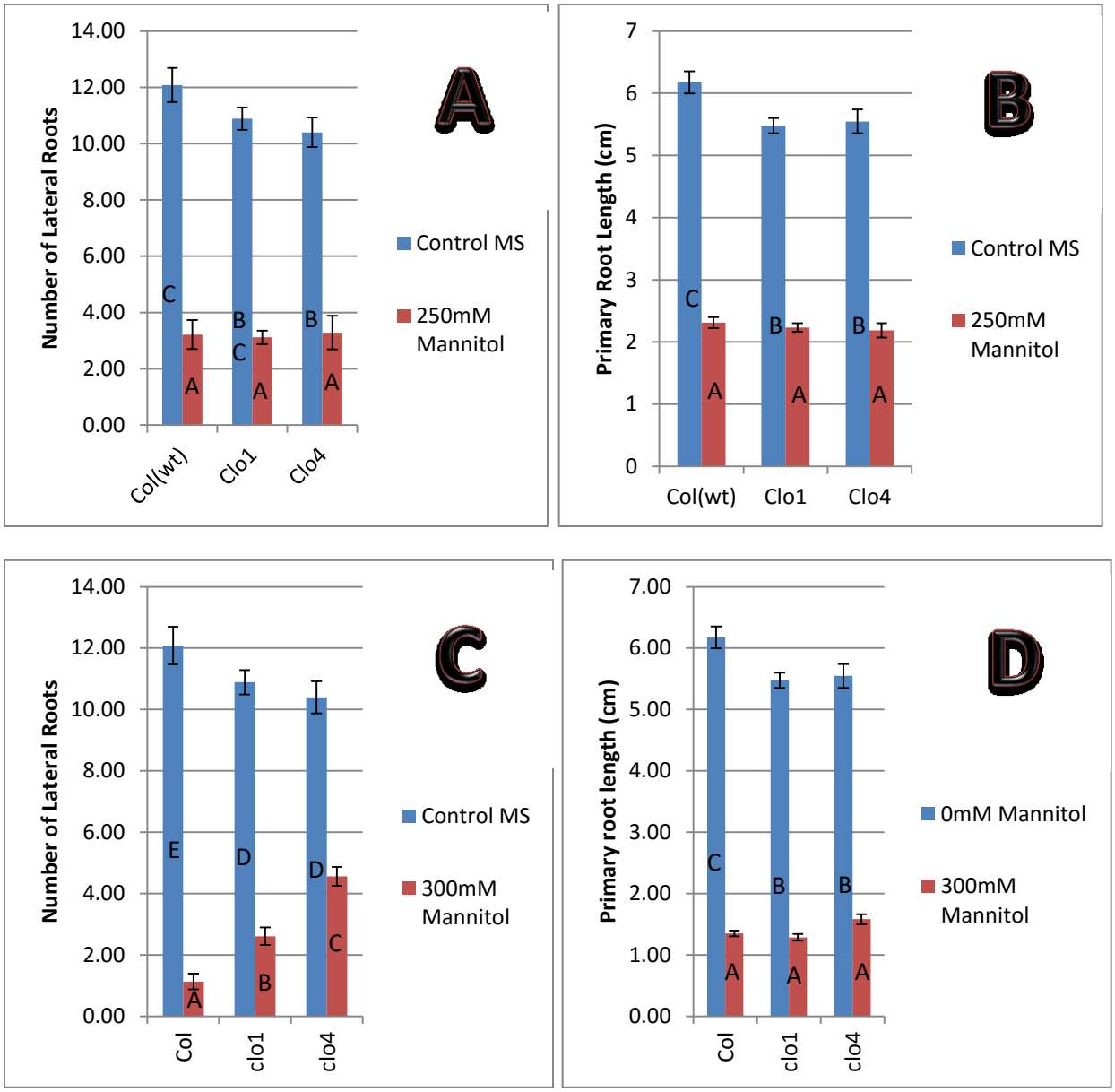


Figure 6. The number of lateral roots and primary root length for Colombia (WT), clo1 and clo4 mutants under control and mannitol stress. Graphs show primary root lengths and lateral root numbers measured/ counted respectively from wild type, *clo1 and clo4* mutants grown on MS control plates compared to plates containing 250 mM and 300 mM mannitol. Error bars represent standard error; letters on bar graphs indicate significant differences determined by Duncan's multiple range test. The results of statistical testing are given in supplementary data Tables 1 and 2.

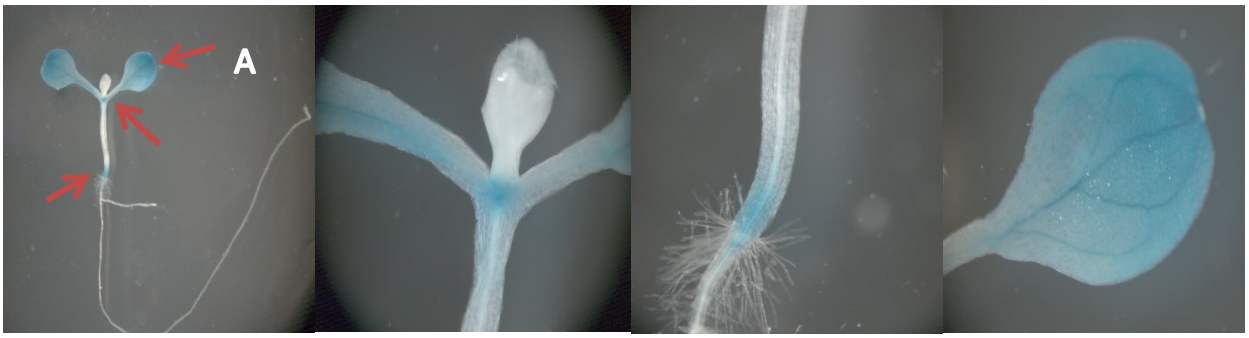
The *CLO4* promoter was characterized in the roots of Arabidopsis in response to cold, salt and mannitol stress:

Since phenotypic analysis of the *Clo4* mutants showed a significance difference in root growth in response to salt and mannitol treatment, the tissue specific pattern of promoter activity was characterized for *CLO4*. *CLO4* gene expression was monitored in response to salt and osmotic stress in the plant tissues. Transgenic plants expressing the β-glucuronidase (GUS) gene under the control of the *CLO4* promoter were used to visualize the localization of gene expression *in vivo*. Plants grown on regular MS plates for 6 days were transferred to different stress conditions including cold, 150 mM NaCl and 300 mM mannitol and assayed after different growth times.

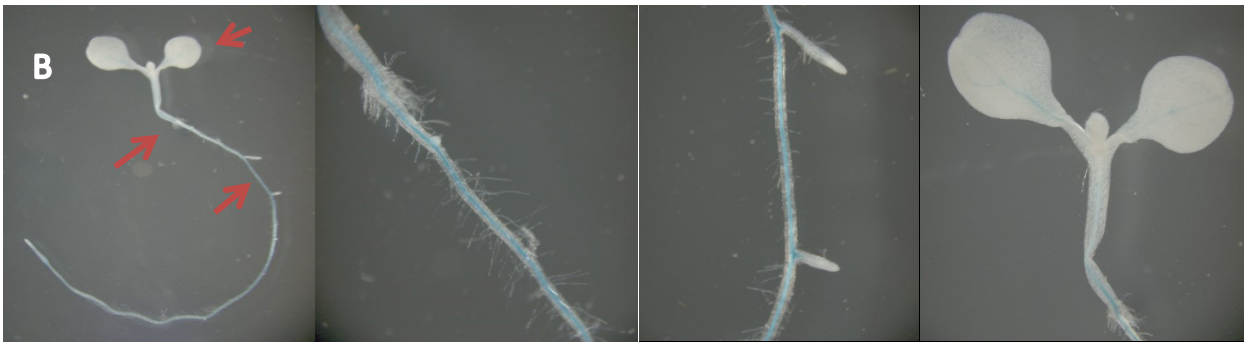
Untreated CLO4-promoter:GUS control plants did not exhibit any observable GUS expression **(Figure 7, C, control).** CLO4-promoter:GUS plants subjected to 150 mM NaCl treatment for 1 hour and 24 hours **(Figure 7)** had *CLO4* promoter driven expression of GUS along the length of the root, but not in the root tip region or budding lateral roots. CLO4-promoter:GUSplants were also tested with 300 mM mannitol for 1 hour and 24 hours **(Figure 8);** the *CLO4* promoter showed the a similar pattern of expression as plants treated with NaCl. CLO4-promoter:GUS plants at ages 2, 4 ,8 and 10 days were also subjected to cold stress. *CLO4* promoter activity after cold treatment for 1 hour showed a similar localization as that for salt and mannitol treatment. Transgenic CLO4-promoter:GUS plants tested at different growth stages showed expression in all root tissues (including lateral roots) except for the root tip. CLO4-promoter:GUS plants from control plates , did not show GUS staining in any parts of the plants up to the age of 8 days, however control 10 day old plants exhibited the same pattern as the

cold treated plants **(Figure 9)**. It should be noted that CLO4-promoter:GUS seeds used were taken from the T2 generation which has a 1 in 4 chance of having a non-transgenic plant; however 2-3 plants per treatment were set aside for control conditions and apart from any transgenic CLO4-promoter:GUS plants over the age of 8 days, none of the controls showed any GUS staining when subjected to treatment conditions. Transgenic plants expressing GUS under the control of the *CLO1* promoter were tested under similar conditions. Figures 7 (A) and 8 (A) show CLO1-promoter:GUS plants subjected to salt and mannitol treatments and showed promoter activity only in the aerial tissue and root tip . However, when CLO1-promoter:GUS plants at ages 2, 4 and 6 days were subjected to cold treatment for 1 hour **(Figure 10);** localization of CLO1 promoter activity was initially found to be in the leaves and aerial tissues of young shoots but decreased as the plant aged until only a small area around the meristematic region is observed in plants cold treated 6 days after germination **(Figure 10, C)**.

Clo1 24 hour 150mM NaCl Treatment



Clo4 24 hour 150mM NaCl Treatment



Clo4 1hour 150mM NaCl Treatment

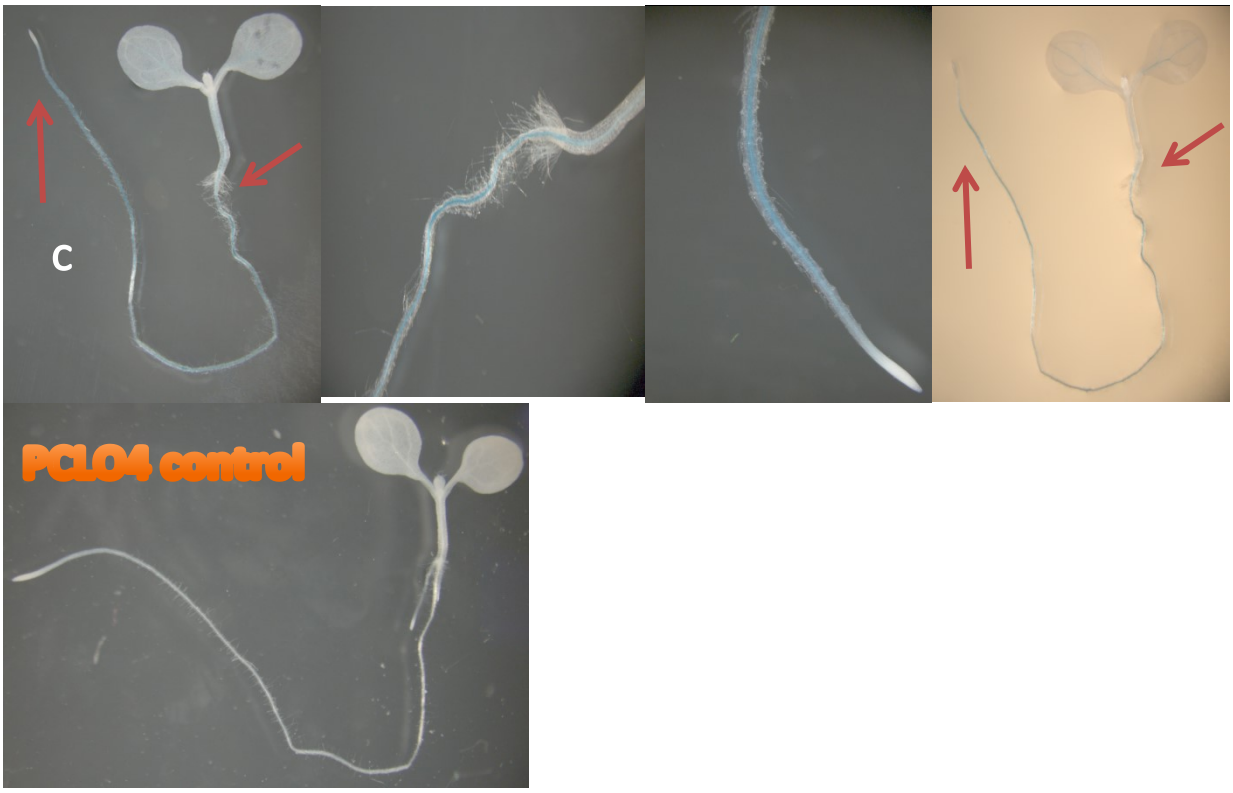


Figure 7: Salt Treatment; Histochemical localization of CLO1 and CLO4 promoter activity in transgenic *Arabidopsis* containing the CLO1- promoter:GUS and CLO4- promoter:GUS gene fusions after being subjected to 150 mM NaCl. **(A)** GUS staining appears in plant aerial tissue after CLO1-Promoter:GUS plants were placed on 150 mM NaCl treatment plates for 24 hours. **(B –C)** GUS staining appears in root tissue except in root tips after transgenic Promoter Clo4:GUS seeds were plated similarly to CLO1-promoter:GUS seeds and were subjected to 150 mM NaCl treatments for 24 hours **(B)** and 1 hour **(C).** Control, untreated plants .

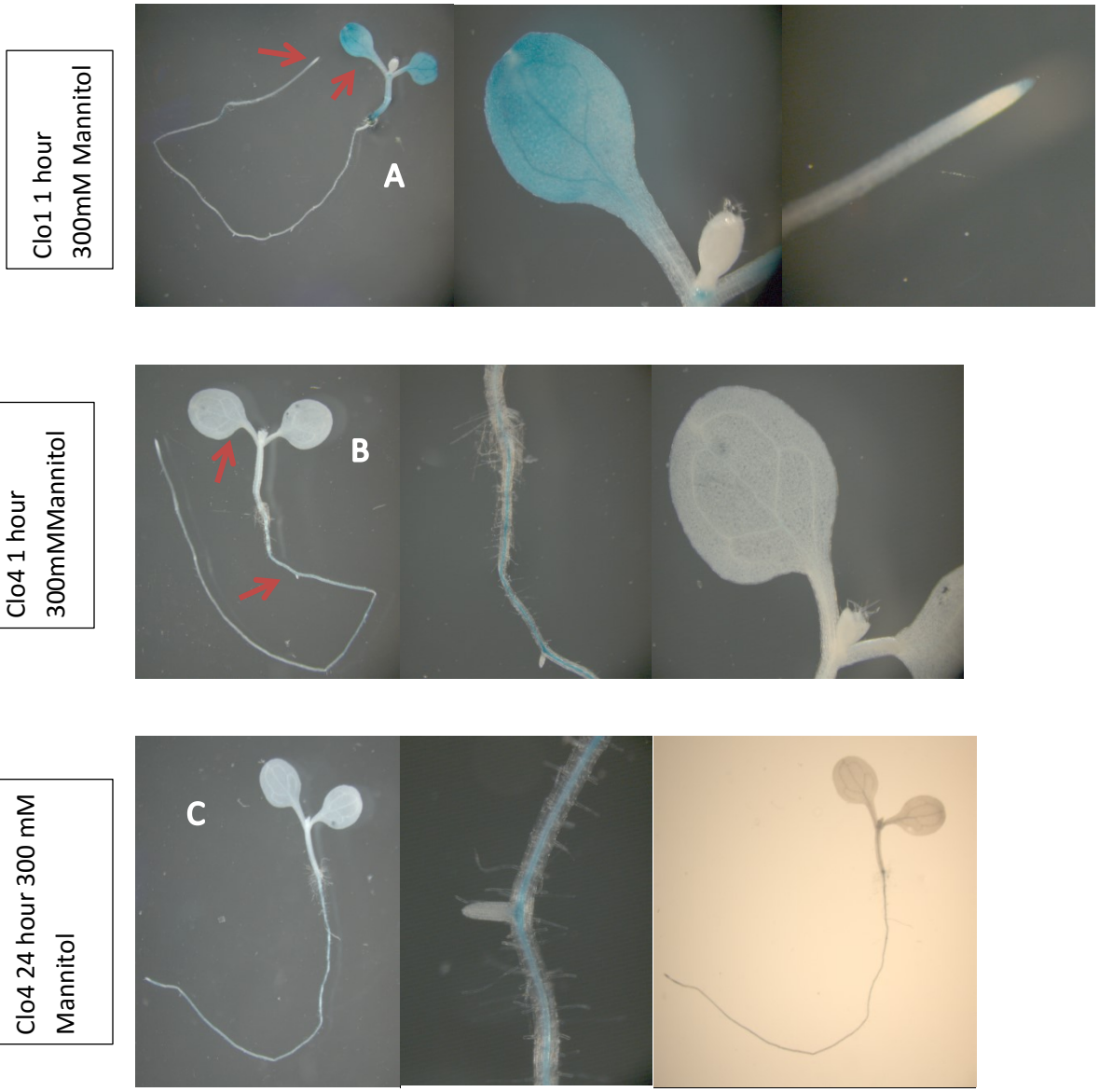


Figure 8: Mannitol Treatment; Histochemical localization of CLO1 and CLO4 promoter activity in transgenic *Arabidopsis* containing the CLO1- promoter:GUS and CLO4- promoter:GUS gene fusions after being subjected to 300 mM Mannitol treatment for 24 hours. **(A)** GUS staining appears in plant aerial tissue after CLO1-Promoter:GUS:GUS plants were placed on 300 mM mannitol treatment plates for 1 hour then removed and placed in GUS staining solution. **(B –C)** GUS staining appears in root tissue except in root tips after transgenic Promoter Clo4:GUS seeds were germinated and transferred to 300 mM mannitol treatments for 1 hour **(B)** and 24 hours **(C).**

2 days old: 1 hour cold treatment

4 days old: 1 hour cold treatment

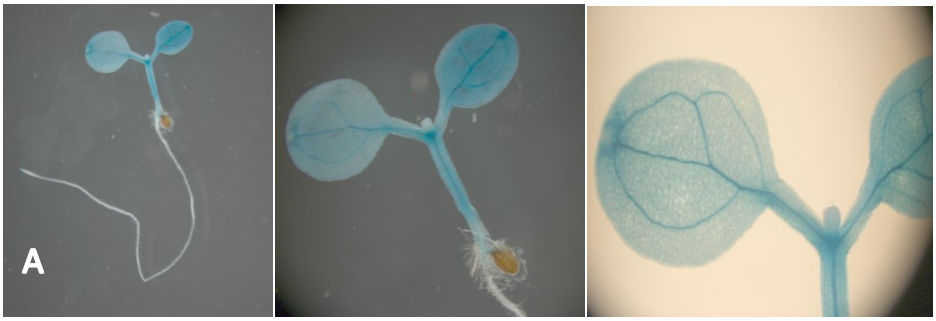
8 days: 1 hour cold treatment

10 days: 1 hour cold treatment

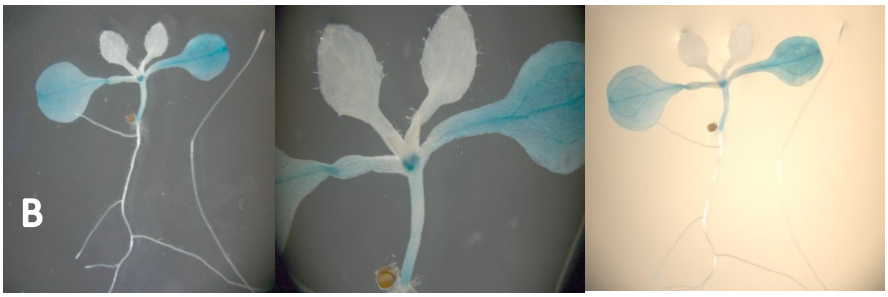
10 day Control

Figure 9: Cold Treatment; Histochemical GUS staining of transgenic plants in which GUS was expressed under the regulation of the CLO4 promoter region. **(A)** Shows CLO4 expression in plant's roots by the blue colouration. Transgenic Promoter Clo4:GUS seeds were sterilized and plated on MS plates. 2 days after germination plate is placed on ice in a 4°C cold room for 1 hour. **(B)** Plants were cold treated for 1 hour 4 days after germination. **(C)** Same as above except plants were cold treated for 1 hour 8 days after germination. **(D)** Same as above except plants were cold treated for 1 hour 10 days after germination.

2 day old: 1 hour cold treatment



4 day old: 1 hour cold treatment



6 day old: 1 hour cold treatment

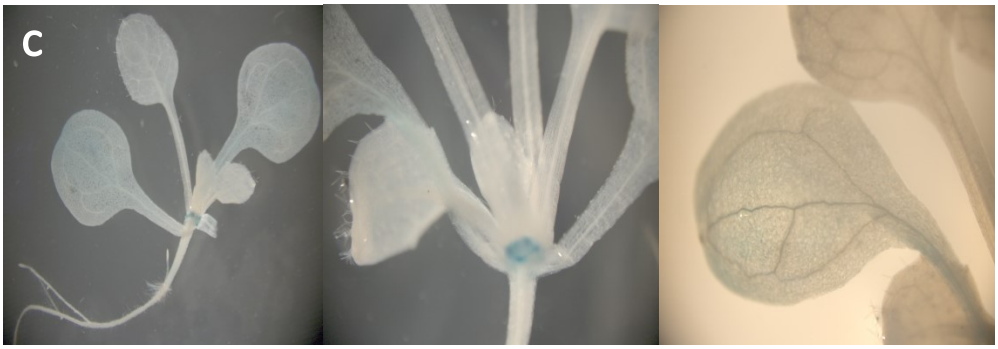


Figure 10: Cold Treatment; Histochemical GUS staining of transgenic plants in which GUS was expressed under the regulation of the Clo1 promoter region. **(A)** Shows *CLO1* expression in plant's cotyledons by the blue colouration. Transgenic Promoter Clo1:GUS seeds were sterilized and plated on MS plates. 2 days after germination plate is placed on ice in a 4°C cold room for 1 hour. **(B)** Plants were cold treated for 1 hour 4 days after germination. **(C)** Same as above except plants were cold treated for 1 hour 6 days after germination. As promoter-Clo1:GUS plants get older, expression decreases and is found only in the meristematic region.

Discussion

Clo4 could be a negative regulator of GPA1:

Mannitol and NaCl treatments significantly decreased primary root lengths and lateral root numbers in *clo1, clo4* and Colombia wild type plants. Although the number of lateral roots on *clo4* mutants decreased under stress compared to untreated *clo4* mutants, the number of lateral roots was significantly higher than those of stress-treated wild type and *clo1* mutants. These results suggest that *CLO4* is required for proper inhibition of lateral root formation during periods of stress; with inhibition of lateral root formation having been shown to be an important factor in plant stress response (Xiong et al., 2006). Loss of function of *CLO4* lessens the inhibition of lateral root formation which would cause a plant to be more sensitive to drought and salt conditions. This agrees with what has been shown previously that *clo4* mutants had decreased germination rates compared to wild type when plated on media with mannitol or NaCl (Kim et al., 2011).

It has been shown that GPA1 is required for normal lateral root formation since *gpa1* mutants had less lateral roots than wild type plants under control conditions (Chen et al., 2006). The response of *clo4* mutants to mannitol and salt stress suggest a mechanism by which CLO4 and GPA1 may be interrelated. CLO4 enhances the suppression of lateral root formation in response to salt and mannitol treatments. This is consistent with CLO4 acting as a negative regulator of GPA1 under stress conditions. Calcium is known as a secondary messenger that

works downstream of heterotrimeric g proteins (Hepler, 2005) and since CLO4 is a calcium binding protein; it is a possible that CLO4 is a negative downstream regulator of GPA1.

CLO4 interaction with GPA1 shown by BIFC is a novel interactions that indicates diversity among different members of the caleosin family and G proteins:

Three caleosins were tested for interactions with G proteins. These were CLO1, CLO2 and CLO4, and RD20/CLO3 was used as a control since this had already been shown to interact with GPA1 *via* BIFC (unpublished Gulick lab). Surprisingly, CLO1 and CLO2 showed no interactions with GPA1, whereas CLO4 had a very strong interaction localized at the plasma membrane. CLO4 was also shown to be naturally localized in the tonoplast as well as the plasma membrane by imaging a CLO4 fusion with an eGFP; helping to shed some light on the movements of transgenic CLO4 expressed in tobacco leaf cells without the presence of an Arabidopsis G protein counterpart. This interaction suggests that CLO4 can bind to GPA1 which suggests that CLO4 is capable of regulating GPA1.

CLO4 impacts lateral root numbers under stress and displays gene expression in roots under similar stress conditions:

The phenotypic analysis of root morphology under different levels of mannitol and salt stress levels showed a decrease in lateral root formation in *clo4* mutants and wild type but with significantly more lateral roots in *clo4* mutants. However, phenotypic analyses of *clo4* mutants under ABA treatment were found to have no significant differences from the wild type plants in aerial and root tissues. The lack of significant effects of ABA treatment on *clo4* mutants

suggests that CLO4 is not involved in the ABA pathway and prompts us to think of possible alternatives to *CLO4*'s role in plant stress. Plants under stress suppress lateral root formation in favor of deeper primary roots (Xiong et al., 2006); loss of function of *CLO4* seems to lead to plants partially losing that response. This coupled with the lack of ABA effects on *CLO4* could mean that *CLO4* is involved an ABA independent pathway for osmotic and salt stress signaling that is similar to the SOS (Salt Overtly Sensitive) pathway. SOS3 belongs to a subfamily of EF-hand-type calcium binding proteins and are involved in the activation of osmotic stress protein kinases (Guo et al., 2002). CLO4 could also be involved in other ABA independent signaling pathways. The Dehydration Responsive Element Binding Proteins (DREB) are transcription factors which are involved in responses to cold (DREB1) and drought (DREB2) that act in ABA independent pathways with a corresponding cis-acting element DRE (Shinozaki et al., 2000, Agarwal & Jha 2010, Yoshida et al., 2014). The Arabidopsis Gene Regulatory Information Server (http://arabidopsis.med.ohio-state.edu/) analysis of *cis* – regulatory elements in the CLO4 promoter region showed the presence of ten RAV1 binding sites while known downstream effector genes in the ABA pathway such as RAB18 and KIN2 (Fujita et al., 2005) had only five RAV1 binding sites. RAV1 is part of a subfamily of cold transcription factors which possesses significant sequence similarity to N-terminal AP2/ERF binding domains (Kagaya et al., 1999). This AP2/ ERF motif is also found in DREBs and both subfamilies have been shown to be a part of 5 subfamilies of transcription factors involved in dehydration and cold inducible gene expression (Sakuma et al., 2002, Fujita et al., 2005). This further suggests that CLO4 might be involved in ABA independent pathways for plant stress responses.

It is important to monitor gene expression of CLO4; especially in response to abiotic stress. GUS staining done on 6 day old plants treated with 300 mM mannitol, 150 mM salt or 4 °C for 1 or 24 hours, showed that CLO4 was expressed strongly in the primary and lateral roots but not in the lateral root tips. The seeds for the transgenic CLO4-Promoter:GUS plants were from the T2 generation which were segregating 1:2:1 with 25% of the plants not expected to carry the CLO4-Promoter:GUSreporter. The non-stressed controls included 2-3 plants per treatment which totals approximately 25 plants; none of the control non-stressed plants, except those above 8 days old, exhibited GUS staining. This degree of replication confirms that the lack of expression was not due to the absence of the transgene. CLO1-Promoter:GUS was also shown to be heavily expressed in the entirety of the aerial tissues and only the root tip of the primary root. This displays diversity in expression of different caleosins which could allow for their having different roles in responding to environmental stresses.

Future work:

This work highlights two major points. CLO4 has a direct interaction with the Gα subunit, GPA1 and CLO4 could play a key role in ABA independent regulatory systems for stress gene expression. Due to the importance of G-proteins and calcium in signal transduction, an interaction between Gα and a calcium binding protein such as CLO4 could signify a new horizon of experimental possibilities and understanding.

Future work could include more biochemical evidence of CLO4 and GPA1 interactions such as *in vitro* experiments to confirm and characterize protein-protein interaction by affinity purification and Western Blot or *via* Yeast Two Hyrbid analysis. In addition, GUS staining experiments can be repeated on the homozygous T3 generation of CLO4-PROMOTER:GUS plants for a more definitive outcome and with more frequent sampling times to monitor more closely monitor the changes in gene expression. Since GPA1 is important for root morphology and CLO4 could inhibit GPA1 under stress conditions, double mutants can be created to test if there any additive effects on root morphology under different conditions.

The different localization of CLO1 is interesting since there are seven known caleosins in Arabidopsis with different tissue specific patterns of expression (Aubert et al., 2011, Unpublished Gulick lab), which could mean that different caleosins act downstream of heterotrimeric G proteins in different plant tissues and in response to different stress factors such as water deficiency, cold, salt etc. Finally, it is important to identify which of the many existing plant stress response pathways CLO4 can fit into by testing of interactions with proteins such as DREB or AREB1 and to determine if CLO4 induces any changes in expression of known stress induced genes by microarray assays or by second generation mRNA sequencing.

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Supplementary Information

Genotypes tested	Control	ABA treatment
Col vs Clo4 Primary Root Length	.640	.819
Col vs Clo4 Lateral Root Number	.683	.248

Conditions tested	Colombia vs Clo1	Colombia vs Clo4	Clo1 vs Clo4
85mM NaCl Primary Root Length	.000	.510	.000
100mM NaCl Primary Root Length	.021	.001	.978
85mM NaCl Lateral Root Number	.199	.045	.001
100mM NaCl Lateral Root Number	.773	.008	.066
250mM Mannitol Primary Root Length	.481	.403	.725
300mM Mannitol Primary Root Length	.379	.024	.005
250mM Mannitol Lateral Root Number	.858	.929	.782
300mM Mannitol Lateral Root Number	.001	.000	.000

Supplementary table 1: The tables represents Student's T test done to test significant differences between genotypes and conditions tested including Salt, ABA and Mannitol stress.

Conditions tested	Genotype	Treatment * Genotype
85mM NaCl Primary Root Length	.000	.000
100mM NaCl Primary Root Length	.000	.000
85mM NaCl Lateral Root Number	.000	.03
100mM NaCl Lateral Root Number	.000	.012
250mM Mannitol Primary Root Length	.000	.059
300mM Mannitol Primary Root Length	.000	.003
250mM Mannitol Lateral Root Number	.000	.212
300mM Mannitol Lateral Root Number	.000	.000

Supplementary table 2: The table represents 2 way ANOVAs tests to check for significant effects from each independent variable as well as any interaction between them.