Differential Roles of At-*CLO4* in Regulation of Heterotrimeric G Protein Complex in *Arabidopsis thaliana* and Characterization of Members of the Extra-Large G Protein Family in *Triticeae*

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Abstract

Differential Roles of At-*CLO4* in Regulation of Heterotrimeric G Protein Complex in *Arabidopsis thaliana* and Characterization of Members of the Extra-Large G Protein Family in *Triticeae*

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Concordia University, 2020

Caleosins, calcium binding proteins characterized by a single EF hand domain, are known to be involved in ABA signaling and the response to abiotic stress in plants. They are encoded by gene family with seven members in the *Arabidopsis thaliana* genome. Caleosins have been shown to physically interact with the alpha subunit of the heterotrimeric G proteins *in vitro* and *in vivo*. The heterotrimeric G protein complex in Arabidopsis consists of three subunits; the α subunit, GPA1, the β subunit, AGB1, and one of three γ subunits named AGG1, AGG2 and AGG3 which are known to be involved in signalling pathways associated with development and the response to abiotic stresses. Caleosins and G protein subunits are known to physically interact, however the genetic interaction of these two classes of proteins and their combined roles in the stress response of plants is poorly understood. This study confirmed *in planta* interaction of GPA1 and CLO4, a member of the caleosin gene family in Arabidopsis and addressed questions arising from role of the interaction in plant signaling and stress responses. The *in vivo* promoter activity of *CLO4* showed that the gene is negatively regulated in both primary and lateral roots by abscisic acid. CLO4 was found to positively regulate stomatal development, through GPA1 related signalling and both genes were found not to be individually sufficient to control lateral root elongation in response to ABA treatment. Arabidopsis plants mutated in both *clo4* and *gpa1* genes showed significant reduction of total lateral root length. Gain of function of the *CLO4* gene through over-expression suggests that caleosins play a role in the ABA induced stomatal closure and ABA induced inhibition of primary root elongation. In addition to Arabidopsis CLO4, this study investigated the possible GTPase activity of another member of the caleosins gene family, RD20/CLO3, toward GPA1 and showed that the GAP activity of the RD20/CLO3 protein toward GPA1 is negligible compared to the other known plant GTPase accelerating protein, RGS1, which suggests different mechanism of regulation of the

heterotrimeric G protein Complex through caleosins. This study also characterized the Extra-Large G protein gene family in *Triticum aestivum*. Total of three *Xlgs* per haploid geneome, each with three homeologous copies for the total of 9 genes in the hexaploid genome were identified and characterized for expression patterns. Differential tissue specific gene expression of the members of *Xlg* gene family showed that the genes are expressed in seed, root, inflorescence, leaf and stem. Bioinformatics analysis on the transcript level abundance showed that, under stress conditions such as drought, heat, cold and *Fusarium graminearum* infection, members of the gene family altered expression which suggests transcriptional regulation of certain members of *Xlg* gene family in response to biotic and abiotic stress conditions.

To my sweet lovely daughters, Eliana and Selena

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Recognition of the shared work

Sabrina Brunetti and I have equally contributed to the protein purification and enzymatic assay of CLO3 and GPA1. Purification of GPA1 and the enzymatic assay was performed in Dr. Jean Rivoal's lab. Michael J. Lowden contributed to FPLC protein purification of the proteins. Zeynab Hamidi Tabar performed the BiFC assay as shown in figures 3 and repeated the Y2H assay as shown in Figure 10.

Jacqueline Siqueira Glasenapp repeated the GUS experiment as shown in figure 5.

Sabrina Brunetti and Zeynab Hamidi Tabar Ran CD-Hit algorithms for expression analysis of *Triticum aestivum.*

Chapter 1

Introduction

1.1 Heterotrimeric G protein complex, caleosin gene family and plant stress responses

In dept study of plant signalling pathways could be the fundamental step for better understanding of the relevance of the gene regulatory systems and challenges a plant needs to overcome to grow. This study is focused on Arabidopsis stress response, through the heterotrimeric G protein complex, caleosins gene family and the plant stress hormone Abscisic acid (ABA). Advancement in plant science through better understanding of underlying molecular mechanisms that affect plants under stress conditions could be the fundamental step to help humanity in the face of environmental changes that affect plant growth, to protect our environment and to advance a better understanding of safe and sustainable plant production.

Caleosins and members of the heterotrimeric G protein complex have been previously reported to be involved in plant stress response that is regulated by Abscisic acid (ABA) (Kim et al. 2011; Aubert et al. 2011; Jeon et al. 2019; Xu et al. 2015; Alvarez et al. 2011. Pandey et al. 2006). The plant hormone ABA is known to be elevated under environmental stress conditions and can control multiple traits such as seed germination, stomatal development and aperture and root architecture (Sah et al. 2016; Finkelstein 2013). Although members of the caleosins gene family and the G protein complex have been independently studied, little is known about regulation of the G protein complex through caleosin gene family and the effect of caleosins on certain traits that are affected by ABA, such as stomatal development and root architecture, are not well understood. This study will investigate the role of a member of the caleosins gene family, *At-CLO4,* in regulation of the G protein complex through its interaction with GPA1, the α subunit of the heterotrimeric G protein complex, to expand the current knowledge in interacting partners of the G protein complex in Arabidopsis. This study also investigates possible role of Arabidopsis CLO3 in regulation of GPA1 protein through *in vitro* enzymatic assay. In addition, this study investigates regulation of the Extra-Large G proteins in response to different stress conditions in *Triticum aestivum* with a bioinformatics analysis. Extra-Large G proteins have been reported to interact with certain members of the heterotrimeric G protein complex in Arabidopsis (Chakravorty et al 2015) and studying the composition of the gene

family and the study of gene expression of the members of the gene family expands the current knowledge in signaling through the G protein complex and their interacting proteins in plants.

The heterotrimeric G protein complex in Arabidopsis has been shown to be involved in most stages of plant's development and differential roles of the G protein complex in regulation of plant morphology and stress responses have been subject of research for many years among plant researchers, while still many unanswered questions remaine to be addressed to better understand plant stress response mechanisms (Pandey 2019). The signaling through the heterotrimeric G protein complex in plants is unique and does not follow the classical model of the heterotrimeric G protein complex (Urano et al. 2012).

The calcium ion Ca^{2+} and the plant hormone abscisic acid (ABA) play important roles in known plant stress response mechanisms (White et. al. 2003; Sah et al. 2016). Members of the caleosin gene family are calcium binding proteins that bind the Ca^{2+} ion and are transcriptionally regulated by the plant stress hormone, abscisic acid (Kim et al. 2011; Aubert et al. 2011). Multiple publications have shown the role of the heterotrimeric G protein complex in abscisic acid signaling pathways (Jeon et al. 2019; Xu et al. 2015; Alvarez et al. 2011. Pandey et al. 2006). Although both heterotrimeric G protein complex signaling and the caleosins have been independently studied, little is known about the connection between G protein complex and the calcium binding proteins. Previous studies had shown that the α subunit of the heterotrimeric G protein complex, GA3, in wheat (*Triticum aestivum*), interacts with the calcium binding protein Clo3 (Tardif et al. 2007) and that Clo3 competes with a phospholipase C 1 (PLC1) in binding to GA3 while interaction between Gα and Clo3 was enhanced in presence of the calcium ion Ca^{+2} (Khalil et al. 2011). Our lab previously showed that, the Arabidopsis Gα, GPA1, also interacts with members of the caleosin gene family in Arabidopsis; RD20/CLO3, CLO7 (Wang 2009) and CLO4 (Rafeh 2016) using Bimolecular Fluorescent Complementation assay (BiFC) in *Nicotiana benthamiana* leaves.

This study will start with validating previously reported interaction and localization of CLO4 and GPA1 using more stringent conditions to avoid possible over expression of the two genes caused by the 35S promoter. The main aim of the study is to explore the plant stress response and morphological changes through heterotrimeric G protein complex, members of the caleosin gene family and their relationship to the plants response to the phytohormone abscisic acid (ABA). Another objective of the study is to assay the potential GTPase accelerating protein

(GAP) activity of Arabidopsis CLO3/RD20, another member of the caleosins gene family, toward GPA1 based on the preliminary phenotypic analysis that suggests possible negative regulation of the Arabidopsis α subunit with CLO3 (Gulick lab, unpublished data). This study is investigating the hypothesis that the calcium binding protein *Arabidopsis thaliana* caleosin 4 (At-*CLO4*) , another member of the caleosin gene family, may have a role in regulation of signaling through the heterotrimeric G protein complex in Arabidopsis by physically interacting with the α subunit, GPA1, and can affect plant's morphological responses under abiotic stresses in different plant organs. This study will explore plant's stress responses by evaluating expression of *CLO4* gene under different stress conditions and the effect of gene expression on Arabidopsis morphological changes under stress conditions.

By generation of double mutant Arabidopsis plant by crossing single mutant lines *clo4* and *gpa1*, regulation of the G protein complex during stress response that affects Arabidopsis root morphology and stomatal regulation and development will be assayed. The double mutant analysis will determine if the genes are in the same or different pathways regulating stomatal development and aperture during plant stress response in addition to plant root responses. The interaction of different domains of both CLO4 and GPA1 proteins will be also assayed to obtain primary results on the interacting domain(s) or motif(s) of the proteins. Finding the interacting domains of the two proteins could be a fundamental step for better understanding of signaling through G protein complex and possible competition of the members of the caleosin gene family with the βγ dimer for binding to the α subunit, GPA1. Another objective of this study is to characterise members of the Extra-Large G protein complex in *Triticum aestivum* through bioinformatics. The study describes the gene family structure in this hexaploid species and will investigate tissue specific expression of the members of the *Xlg* gene family and the levels of expression of each gene and their homeologous copies under abiotic and biotic conditions i.e., temperature changes, drought and Fusarium infection.

1.2 Heterotrimeric G protein Signaling

Research that led to discovery of heterotrimeric G protein complex started in the early 1960's and despite the fact that the exact mechanism of G-protein signaling and the receptors were not known, research on hormone signaling and the secondary messenger cAMP and stimulation of adenylyl cyclase led to the discovery of the G protein complex signal transduction

in 1994 by the Nobel prize winners Alfred Gilman and Martin Rodbell (Birnbaumer 2007 ; Milligan & Kostenis 2006 ; Nobelprize.org). Further findings and publications on the G-protein complex and the mode of action of receptor activated internal signals, led to the discovery of more than 800 G Protein Coupled Receptors (GPCRs) in human and the development of medications which target these receptors. Such medications include about thirty percent of the medications that are currently available in the market (Hauser et al. 2017; Oldham & Hamm 2008).

The heterotrimeric G protein complex consists of three main subunits α, β and γ that bind together in inactive form that is activated once a ligand binds to the GPCR. G protein signalling is regulated by the phosphorylation state of guanine bound by the Gα protein; proteins bound to GTP disassociate from GβGγ duplex and are active; those bound to GDP are inactive, associate with GβGγ and form the trimeric complex. In the classical mammalian model for G protein complex, Gα is activated by interaction with nucleotide exchange factors (GEFs) which promote GTP binding through GTP/GDP exchange. G protein–coupled receptors (GPCRs) which act as GEFs are activated by specific ligands bound to the GPCR (Liu et al. 2016). The inactivation of Gα's is facilitated by GTPase activating or accelerating proteins (GAPs), and guanine nucleotide dissociation inhibitors (GDIs) (Loirand et al. 2013) and in most cases GEFs are considered the key regulatory protein in the GTP/GDP cycling with heterotrimeric G proteins (Figure 1) (Siderovski & Willard 2005).

The heterotrimeric G protein complex is involved in many pathways and human diseases including, asthma and allergic inflammation (Johnson et al. 2002), heart disease (Zolk et al. 2000), thyroid hormone regulation (Kleinau, et al. 2013), vision, taste and the sense of smell (McCudden et al. 2005). The human genome encodes 23 different alpha subunits of the heterotrimeric G protein complex (McCudden et al. 2005) that are divided to four main families based on the protein sequence similarity between the subtypes; G_i, G_q, G_s and G_{12}. The names of the families correspond to their α subunits which are respectively $G\alpha_i$, $G\alpha_q$, $G\alpha_s$ and $G\alpha_{12/13}$. The $G\alpha_q$ family consists of $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, and $G\alpha_{15/16}$ (Kamato et al. 2015; Oldham & Hamm 2008). The $G\alpha_i$ family consists of $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{t\text{-}rod}$, $G\alpha_o$, $G\alpha_{t\text{-}cone}$, $G\alpha_z$ and $G\alpha_{gust}$ and the $G\alpha_s$ family consists of $G\alpha_s$ and $G\alpha_{olf}$ and G_{12} family has two members; $G\alpha_{12}$ and $G\alpha_{13}$ (Oka et al. 2009; McCudden et al. 2005). The subtypes of the Gα subunits are regulators of many mechanisms in cells, from activation of adenylate cyclase and regulation of ion channels (Simon

et al. 1991) to regulation of β catenin signaling pathway (Banu et al. 2019). It has been reported that, the G_i and G_s families are regulators of adenylyl cyclase, the $G_{12/13}$ family are involved in cytoskeleton formation and cell proliferation and the G_q family are involved in phospholipase Cβ activation and downstream cascades (Juneja and Casey 2009; Kamato et al. 2017; Bryan and Del Poeta, 2018). In contrast to mammalian Gα's, most fungi including *Candida albican*s and *Saccharomyces cerevisiae*, have two Gα subunits and one Gβ and one Gγ (Dignard et al. 2008).

In addition to the 23 Gα subunits, human genome contains five Gβs and 12 Gγs which are involved in numerous signaling pathways such as K^+ channel activation (Smrcka 2008) and regulation of a low voltage activated calcium channels known as the T-type calcium channels (Wolfe et al. 2003). The Gβγ dimers are also important for proper folding of Gα and its association with GPCR and termination of Gα signaling (Oldham & Hamm 2006).

In addition to the well-known classical model of heterotrimeric G protein signaling activated by the guanine nucleotide exchange factors (GEFs), receptor independent activation of the α subunit of the G protein complex has been also reported. This pathway is regulated indirectly by nucleoside diphosphate kinases (NDPKs) which control the amount of GTP that is present, which in turn affects the level of GTP bound of Gα (Abu-Taha et al. 2018). Another mechanism of Gα activation, is GPCR independent Gα activation by the guanine exchange factor proteins, with a conserved GBA motif, which are not necessarily GPCRs. The GBA motif or Gα binding and activating motif are reported in proteins that showed *in vitro* GEF activity toward Gα i.e., "Gα-interacting vesicle-associated protein" (GIV) and "Dishevelled-associating protein with high frequency of leucines" (DAPLE) involved in cell proliferation and cell movement (Leyme et al. 2017). These proteins bind to GDP bound Gαi and not the GTP bound form and act as GEFs (De Opakua, et al. 2017; Leyme et al. 2017).

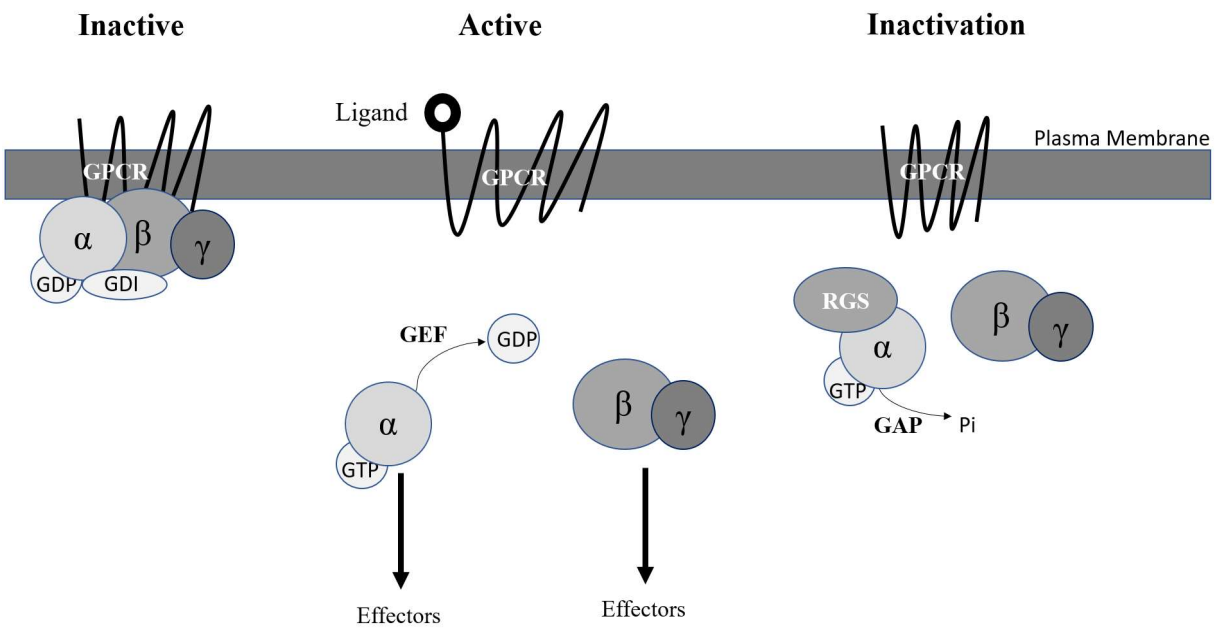


Figure 1. Classical model of signaling through Heterotrimeric G protein complex. In the inactive state, the trimer of α, β and γ is bound to a G protein coupled receptor (GPCR) while the α subunit is bound to GDP. The G protein dissociation inhibitor proteins bind to the trimer to assure the inactive state of the complex. The active state initiates upon binding of ligand to GPCR that acts as a GEF or Guanin Exchange Factor. The conformational changes in the receptor activates Gα to exchange its GDP with GTP. The activated Gα and dissociated βγ dimer will initiate the signaling through binding to the downstream effectors and activate a downstream signaling cascade. Inactivation of signaling will be facilitated by GAPs or GTPase accelerating proteins that promote GTP hydrolysis. The GTPase accelerating proteins increase the intrinsic GTP hydrolysis rate of Gα and the GDP bound Gα re-associates with the βγ dimer and form the inactive state in plasma membrane (McCudden et al. 2005; Liu 2016; Loinard 2013; Siderovski & Willard 2005).

1.3 Heterotrimeric G protein Complex in plant

The genome of rice, *Oryza sativa*, encodes one Gα, *RGA1*, one Gβ, *RGB1*, and four Gγ's (Ferrero-Serrano & Assmann 2016). In 1999, it was reported that the dwarf phenotype seen in rice is caused by mutation in *dwarf1* gene on chromosome 5 which encodes the α subunit of the heterotrimeric G protein complex (Ashikari et al. 1999). This gene is of world-wide significance

in rice cultivation, since the development of dwarf rice cultivars was a major advance in rice breeding in the 1970's and played a central role in crop improvement (Peng et al. 1999).

The heterotrimeric G protein complex in *Arabidopsis thaliana* is composed of G proteins encoded by one Gα gene, *GPA1*, one Gβ gene, *AGB1*, and three genes encoding Gγ's: *AGG1*, *AGG2* and *AGG3* (Urano et al. 2013). The classical heterotrimeric G protein signaling starts with activation of the α subunit upon recognition of external stimuli via GPCR. In contrast, Arabidopsis Gα, GPA1, has a fast-inherent nucleotide exchange rate which is 100 times higher than its intrinsic GTP hydrolysis rate which indicates that it does not rely on GEFs for activation (Jones et al. 2011). The intrinsic slow GTPase activity of GPA1 is accelerated by the Regulator of G protein Signaling 1, RGS1, which promotes the formation of the trimer (Figure 2) and re-association of the αγβ complex (Stateczny et al. 2016; Urano & Jones 2013). It has been shown that endocytosis of the transmembrane RGS1 protein dissociates it from GPA1 and activates signaling through heterotrimeric G protein complex (Urano et al. 2012). RGS1 is a unique protein containing both transmembrane domains and RGS box and its structure suggested that it could be served as both GAP and GEF however the protein was found to only have GAP activity toward GPA1 (Chen et al. 2006). In addition to RGS1, the phospholipase D alpha 1 (PLDα1) has been reported to have GTPase accelerating activity toward GPA1 (Roy Choudhury & Pandey 2016).

In contrast to human with 23 alpha subunits of the heterotrimeric G protein complex, most plants only have one Gα per their haploid genome (Chakravorty et al 2015). The Arabidopsis Gα, *GPA1*, was first identified and cloned in 1990 (Ma et al. 1990) and the last Gγ subunit (AGG3) was identified in 2011 (Chakravorty et al. 2011). After more than 20 years of research on the role of the heterotrimeric G protein complex in plants the fundamental key players in the heterotrimeric complex have been established and subsequent publications have shown a significant role of the G protein complex in plant's development and stress responses. The subunits of the heterotrimeric G protein signaling are known to be involved in plant root development (Chen et al. 2006), stomatal development (Nilson & Assmann 2010), cell proliferation (Ullah et al. 2001), regulation of ion channels in stomatal guard cells (Wang et al. 2001), defence signaling (Liu et al. 2013), salinity response (Yu & Assmann 2018) and brassinosteroid signaling (Zhang et al. 2018). The extensive role of plant heterotrimeric G protein complex and few subunits involved in the core trimer and GPCR independent activation

of plant G protein highlights the potential importance of other proteins in regulating the heterotrimeric G protein complex itself, or act as downstream effectors.

Arabidopsis plant subunits of the heterotrimeric G protein complex have been reported to alter phenotype under elevated concentration of glucose or in response to certain plant hormones such as ABA and auxin (Chen et al. 2006; Urano et al. 2013).

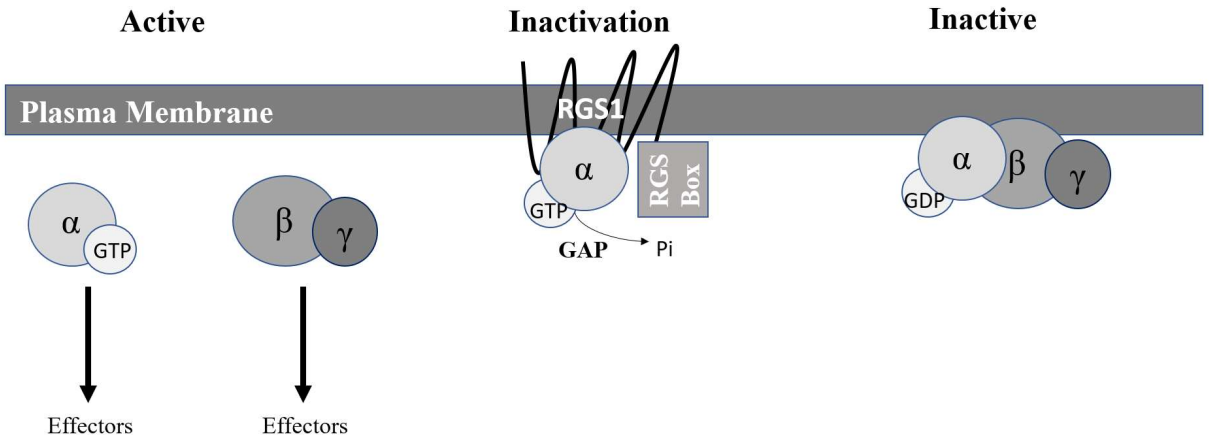


Figure 2. Model for Arabidopsis heterotrimeric G protein signaling. The Gα subunit of the heterotrimeric G protein complex, GPA1, is constitutively active and does not need Guanine Exchange Factor (GEF) for activation. The GPA1 protein has slow rate of GTP hydrolysis. The intrinsic GTPase activity of GPA1 is accelerated by RGS1, a GTPase accelerating protein (GAP). Since plant Gα is constitutively active, the inactivation of the signaling plays an important role in signaling through heterotrimeric G protein complex (Urano & Jones 2013; Chen et al. 2006; Pandey 2019).

The Arabidopsis *gpa1* mutant line has been reported to have lower stomatal density (Nilson and Assmann 2010) and less cell proliferation in Arabidopsis leaf causing the round leaf phenotype (Ullah et al. 2001). ABA is known to be a negative regulator of root growth and an inhibitor of seed germination in Arabidopsis (Sah et al. 2016). The *gpa1* mutant in Arabidopsis has been reported to be hyper-sensitive to exogenous ABA which leads to delayed germination and greater reduction of primary root length in response to ABA treatment than the WT (Pandey et al. 2006). Although it has not been reported that *gpa1* mutant alters the primary root length under normal growth condition, there are two different phenotypes observed for *gpa1* mutant lines in Arabidopsis. The *gpa1* mutant line was shown to have a lower number of lateral roots

compared to wild type plants (Chen et al. 2006) and also to have a lower overall root mass in mature plants (Ullah et al. 2003). Other work reported that Arabidopsis *gpa1* mutant plants not to be different from wild type in number of lateral roots (Pandey et al. 2006).

The Gβ mutant, *agb1,* has more lateral roots and longer primary root compared to wild type plant (Chen et al. 2006). The *agb1* mutant showed similarities in shoot morphology with *gpa1* such as shorter and wider leaves than the WT (Urano et al. 2016). The *agb1* mutant line also showed higher level of primary root and lateral root sensitivity to ABA treatment (Pandey et al. 2006). In addition to being a negative regulator of auxin induced cell division in Arabidopsis roots, the *agb1* mutant plants are distinguished by a higher number of lateral roots and development of double lateral root primordia, with two primordia developing adjacent to each other, rather than the more widely spaced primordia which are seen in WT plants under control condition (Ullah et al. 2003). The single mutants of either $G\gamma_1$, $G\gamma_2$ or $G\gamma_3$ have more lateral roots compared to wild type Arabidopsis seedlings, however mature plants of the single mutants of *agg1* and *agg2* have wild type phenotypes. The *agg3* mutant line showed a phenotype similar to *agb1* mutant line with shorter siliques and flower length than the WT and the $G\gamma_1$ and $G\gamma_2$ mutants (Urano et al. 2013). The triple knock-out of the three Gγ subunits, *agg1/agg2/agg3*, showed a mutant phenotype similar to that of *agb1* by having short flowers measured from the base of the sepal to the tip of the petals, shorter siliques and shorter rosette leaves (Thung et al. 2012).

The Arabidopsis G protein interactome study showed that heterotrimeric G protein complex interacts with several other proteins including BINDING PARTNER OF ACD11 1, (*BPA1*), N-MYC Downregulated-like 1 (*NDL1)*, Thylakoid Formation1 (*THF1*) and Vascular Plant One Zinc Finger (*VOZ1)* (Klopffleisch et al. 2011). The BPA1 protein is involved in plant immune response to *Phytophthora. capsici,* a plant pathogen which targets binding partners of ACD11 to destabilize the protein and promote cell death (Li et al. 2019), NDL1 is known to be involved in the regulation of auxin transport (Khatri et al. 2017). THF1 is known to be involved in regulation of light dependent cell death and maintaining chloroplast function (Hamel et al. 2016) and VOZ1, a member of Vascular Plant One-Zinc-Finger (VOZ) Transcription Factors, is involved in salt stress response and salt tolerance; The VOZ family contains two members, VOZ1 and VOZ2 that are reported to be expressed in vascular bundles and are mainly found in cytoplasm (Prasad et al. 2018).

In addition to the classical subunits of the heterotrimeric G protein complex in Arabidopsis, the Extra Large GTP binding proteins, named XLG1, XLG2 and XLG3, are also reported to compete with GPA1 in binding to the Gβγ dimmer (Chakravorty et al 2015) and it has been reported that XLG2 is involved in Arabidopsis immunity and disease resistance (Liang et al. 2016; Zhu et al. 2009). Findings on the extra-large G proteins increases the number of potentially different three-protein G protein complexes that can be formed in Arabidopsis (Chakravorty et al 2015).

1.4 Arabidopsis stomatal development and aperture

Plant stomata are small pores on leaf epidermis regulating plant transpiration and gas exchange (Lee et al. 2015; Jangra et al. 2019). Carbon dioxide that enters plant cells through these pores is used as the carbon source in photosynthesis (Gray & Hetherington 2004). Stomatal cell development is initiated by asymmetric cell divisions in meristemoid mother cells that eventually gives rises to the stomatal guard cells (Abrash & Bergmann 2010). Between stoma on leaf epidermis there is at least one pavement cell which separate stomata from each other; the one cell spacing pattern plays important role in stomatal function (Papanatsiou et al. 2016). The formation and development of stomata are not only controlled by plants but also by environmental factors; i.e., high CO_2 concentration around older leaves of Arabidopsis activates signaling pathways that reduces the number of stomata on younger leaves (Nadeau & Sack 2002). Abscisic acid (ABA) inhibits development of stomata in Arabidopsis cotyledons (Tanaka et al. 2013). ABA is also known to regulate stomatal conductance and elevated levels of ABA in guard cells causes closing of the stomata to reduce water loss (Wang et al. 2008). Interestingly stomatal guard cells are able to synthesize their own ABA to be able to have a fast response to drought without relying on ABA transporters (Bauer et al 2013).

The identity of the meristemoid mother cells are determined by the transcription factor SPEECHLESS (SPCH) which is negatively regulated by a Mitogen Activated Protein Kinase (MAP) cascade, which phosphorylates the basic helix-loop-helix in the SPEECHLESS transcription factor that eventually leads to a reduced number of stomata (Lampard et al. 2008; Kumari et al. 2014; Jewaria et al. 2013). There are three important key players in stomatal development; SPEECHLESS, MUTE and FAMA proteins, which respectively control initial step to asymmetric cell division, the differentiation to Guard Mother Cells (GMC) and the transition

from GMC to Guard Cells (GC). Arabidopsis mutants which lack expression of any of these three genes are impaired in the development of stoma in the different stages that these three transcription factors regulate (Lampard 2007).

Certain MAP kinases are reported to be involved in regulating Arabidopsis stomatal development (Lee et al. 2016). MAP kinases make up plant phosphorylation cascades that translate external stimuli into an internal cellular response and they are known to be involved in plant hormone signaling (Jagodzik et al. 2018). The main components and mechanism of the MAP Kinase cascade are the MAP Kinase Kinase Kinase (MAPKKK) that phosphorylates MAP Kinase Kinase (MAPKK) which in turn phosphorylates MAP Kinase (MAPK) (Gray & Hetherington 2004). In *Arabidopsis thaliana* there are about 80 MAPKKKs including three sub-classes; MEKK like, Raf like and Zik like kinases, in contrast to the large number of MAPKKK's there are ten MAPKK's and 20 MAP Kinases (Jagodzik et al. 2018). For regulation of the stomatal development, the MAPKKK, YDA, phosphorylates MAPKK4 and MAPKK5 which phosphorylate the downstream MAP kinases; MAP MAPK3 and MAPK6 which are the negative regulators of SPEECHLESS the latter being a critical transcription factor to initiate stomatal development (Wang et al. 2007). It has been shown that MAP Kinase Phosphatase 1 (MKP1) is also important for stomatal development and *mpk1* mutant Arabidopsis has been reported to decrease stomatal numbers showing that MKP1 is the positive regulator of stomatal development in *Arabidopsis thaliana* (Jangra et al. 2019). The MAPK cascade itself is negatively regulated by ERECTA, ERECTA LIKE1, ERECTA LIKE2 and TOO MANY MOUTH receptors which are in turn positively regulated by Stomagen and negatively regulated by Epidermal Patterning Factors; EPF2 and EPF1 (Kumari et al. 2014). An interesting finding by Lee et al. (2015), showed that, EPF2, the negative regulator of stomatal initiation, and Stomagen, the positive regulator of stomatal initiation, compete in binding to Erecta family to determine stomatal patterning.

Stomatal development is the key factor and determinant of the number of stomatal cells on leaf epidermis. This phenotype is usually as either stomatal density (which refers to number of stomata per mm^2 on leaf epidermis) or stomatal index which is the ratio of stomatal cells to pavement cells. In addition to Arabidopsis stomatal development and the number of stomata on leaf epidermis, stomatal opening and closing or stomatal aperture plays an important role in plant stress response, water loss and survival. Stomatal aperture is known to be mostly controlled by

the plant hormone abscisic acid (ABA), calcium ions (Ca^{2+}) and the concentration of CO_2. By the elevation in these three main regulators, stomatal closing and prevention of stomatal opening occurs (Kim et al. 2010). Opening of stomatal guard cells is positively regulated by blue light and the phytohormone auxin by activation of the H^+- ATPase pump which regulates H^+ efflux from the guard cells and K^+ uptake (Daszkowska-Golec et al. 2013). Under elevated abscisic acid concentrations, the Ca^{2+} permeable channels open which leads to increased cytosolic Ca^{2+} concentration in guard cells. The elevated Ca^{2+} will cause activation of both Rapid transient (R-type) and the Slow sustained (S-type) anion channels which will lead to depolarization of the guard cell plasma membrane and K^+ efflux which will result in closing of the stoma (Munemasa et al. 2015) while the increased Ca^{2+} inhibits K^+ influx channels (Melotto et al. 2017). In contrast, K^+ pumps in plasma membrane such as Arabidopsis K^+ transporter 1, AKT1, K^+ channel in Arabidopsis thaliana 1 and 2, KAT1 and KAT2 are inward potassium channels that promote turgid and open state of stoma (Daszkowska-Golec et al. 2013). In addition to reduction of stomatal density (Xu et al. 2016), increased CO_2-also causes stomatal closure. This process can be through elevation of ABA or ABA dependent pathway or as an ABA independent pathway co-regulating stomatal closure with ABA signal; certain mutants of ABA signaling pathway showed stomatal closing under elevated CO_2 confirming presence of ABA independent pathway that regulate stomatal closing under elevated CO_2 and activating S-type anion channel (Hsu et al. 2018).

The α subunit of the heterotrimeric G protein complex, GPA1, has been reported to be a positive regulator of stomatal development (Nilson & Assmann 2010) and it has been reported that the *gpa1* mutant plants are insensitive to ABA induced closing of stomata (Wang et al. 2001). GPA1 has been also shown to be inhibitor of K^+ influx (Wang et al. 2001; Perfus-Barbeoch et al. 2004).

1.5 Plant development and stress responses

Plants cannot change their location under stress conditions. The stress conditions can affect plants in any stage of their life cycle. Regardless if they are mature plants or seeds, external factors have a huge impact on plants at molecular level from gene regulation to post transcriptional and post translational regulations (Haak et al. 2017). Plant organs are developed from two main cell types; Shoot apical meristem (SAM) and Root Apical Meristem (RAM)

which are respectively initiation points of aboveground tissues and roots respectively (Olatunji et al. 2017). Morphogenesis, development of plant organs, from the meristematic cells is not only controlled by plant's genome but it is also influenced by external factors and stresses such as abiotic stresses i.e., high or low temperatures, salinity, drought, nutrient availability and light intensity, or biotic stresses such as certain pathogenic microorganisms and insects (Suzuki et al. 2014 ; Verma et al. 2016). Regardless of origin of external stimuli whether it is biotic or abiotic stress, it will cause cellular responses in plant which in most cases turns on certain cellular pathways that consequently can affect plant's morphology. Among well known cellular responses under stress conditions are elevation of the secondary messenger calcium ion (Ca^{2+}) in cytosol (Hepler 2005; Aldon et al. 2018; Liu et al. 2018) and synthesis of the plant hormone abscisic acid (ABA) which can regulate different signalling pathways in plants and affect plant morphology to increase tolerance to the stress situation and increase the chance of the plant's survival under the stress conditions (Sah et al. 2016; Finkelstein 2013).

1.5.1 Role of calcium ion (Ca^{2+}) in plant stress response

The calcium ion, Ca^{2+}, is an essential divalent cation that plays important roles in plant's cell wall and vacuole's membrane by neutralizing ionic charges as a counter ion. Concentration of the calcium ion in cytosol is among the main determinants of activation of many pathways that are involved in plant's development and stress responses (White et al. 2003). Plant's cytosolic Ca^{2+} concentration is lower than other organelles and it is usually between 100-200 nanomolar (Yang et al. 2017) while in the other organelles the concentration is between 1-10 millimolar (Moreau et al. 1987). Depending on the stimuli, the stored Ca^{2+} ions in organelles with higher concentrations will be released with different signatures into the cytosol to regulate plant stress response (Whalley et al. 2011). The increased Ca^{2+} concentration can regulate gene expression (Whalley et al. 2011) and regulation of ion channels to determine calcium influx and efflux (Edel et al. 2017).

External stimulus such as abiotic stresses; drought and salinity or even mechanical stresses are known to elevate cytosolic Ca^{2+} concentration and trigger stress responses (White et al. 2003). Most proteins involved in plant stress response through elevated cytosolic calcium ion concentration, have a helix-loop-helix domain known as EF hand that has the ability to bind to the calcium ion and stimulate certain signalling pathways in plants (Bender et al. 2013 & White

et al. 2003). The Arabidopsis genome contains 250 EF hand containing proteins (Bender et al. 2013) and among these proteins are caleosins (Chen et al. 1999; Kim et al. 2011; Aubert et al. 2011), calcium dependent protein kinases (CDPKs), calmodulins (CaM) and calmodulin like proteins (CMLs) (Bender et al. 2013 & White et al. 2003) and calcineurin B-like (CBL) protein kinases (CIPKs) (Edel et al. 2017).

1.5.2 Role of abscisic acid (ABA) in plant stress response

Plants are controlled by phytohormones which can regulate many plants traits and affect their morphology. Abscisic acid (ABA) is among phytohormones known to be involved in plant stress responses. Under certain stress circumstances, this phytohormone that results the elevated level of ABA, alters plant development at different stages by generally acting as a negative regulator of growth and development; root elongation and branching, seed germination, stomatal development and aperture and even plants aging and overall biomass (Sah et al. 2016). The Biosynthesis of ABA starts in plastids from beta carotene and by the aid of the enzymes Beta Carotenoid Hydroxylase 1 (BCH1) and Beta Carotenoid Hydroxylase 2 (BCH2), ABA Deficient 1 (ABA1), ABA Deficient 4 (ABA4) and Nine-Cis-Epoxy carotenoid Dioxygenase 3 (NCED3), the molecule known as Xanthoxin is secreted into the cytoplasm which in turn will be used as precursor to make abscisic aldehyde by the aid of the enzyme ABA Deficient 2 and finally abscisic acid (ABA) by the aid of two proteins; ABA Deficient 3 (ABA3) and Abscisic Aldehyde Oxidase (AAO) enzymes (Finkelstein 2013 & Bauer et al. 2013).

In the absence of ABA, the negative regulator of ABA signaling; protein phosphatase 2C (PP2C) Binds to the Sucrose Non-Fermenting SNF1 related protein kinase 2 (SnRK2) and by binding to the activation loop of this protein, inhibits phosphorylation and activation of SnRK2 and consequently blocks ABA signaling. In presence of ABA, this phytohormone binds to its receptor PYR/PYL/RCAR; Pyrabactin resistance (PYR), Pyrabactin Resistanc Like (PYL) and the Regulatory Component of ABA Receptors (RCAR) which will allow the ABA bound receptor to bind to PP2C protein and inhibits interaction of PP2C with SnRK2 which will lead to activation of SnRK2 (Sah et al. 2016). In presence of ABA the free and activated SnRK2 will be phosphorylated and subsequently phosphorylate downstream proteins such as KAT1 and SLAC1 in stomatal guard cells to close the stomata (Ng et al. 2014), ABI5 to regulate seed germination and post germination developments (Sánchez-Vicente 2019), ABA responsive element binding

protein (AREB) and ABA responsive element binding protein (ABF) transcription factors to regulate stress response genes (Sah et al. 2016).

Interestingly some elements of the ABA signaling pathway have been also shown to be regulated by another plant hormone auxin which positively regulates plant root growth and development. The phytohormone auxin is involved in most plant growth and developmental stages by promoting cell proliferation and expansion and the crosstalk between auxin and other plant hormones such as abscisic acid (ABA), ethylene and jasmonic acid can determine root architecture and decisions for primary root development and lateral root formation (Rock et al.2005; Olatunji et al. 2017; Verma et al. 2016). As an illustration: the transcription factor, *Abscisic acid Insensitive 3 (ABI3)* gene is expressed in primordia and has been shown to be induced by auxin and mutations in the *ABI3* gene has been shown to reduce sensitivity to auxin stimulation of lateral roots development in Arabidopsis (Brady et al. 2003).

1.6 Arabidopsis root development

Roots are vital organs to absorb nutrients and water. Although they are not easily visible and mostly located in soil, they play an important role in plant survival, nutrient uptake and symbiosis with soil microorganisms. After over 100 years of research, many questions remain to be answered to understand the complexity of regulation of root architecture and the different factors that are determinants of the shape, development and roles of this plant organ (Lux et al. 2012; Petricka et al. 2012). The Arabidopsis root consists of a primary root and its branches, referred to as lateral roots. The primary root is such an important organ that basically the entire post embryonic development of the plant is based on the single primary root (Delay et al. 2013). The Primary root consists of three main zones; differentiation, elongation and meristematic. As their names suggests, the meristematic zone is the origin of cell division, the elongation zone is where cell expansion takes place and the differentiation zone is the base for the development of lateral root formation (Petricka et al. 2012). Mature meristematic root cells sense their size and stop their growth; brasinisteroid signaling in meristematic cells play an important role in determination of their size by inhibition of root cells growth and allowing cell to differentiate (Pavelescue al. 2018).

Multiple genes and pathways have been shown to affect plant root development from small peptides, such as the root growth factor peptides Gloven 1 and 3 , GLV1 and GLV3, which

are positive regulators of gravitropism response (Delay et al. 2013) to the main determinants of both primary root and the lateral roots; the transcription factor, Wuschel-related homeobox 5 (WOX5), Scarecrow (SCR) and Short Root (SHR) which are involved in stem cell identity, maintenance and root cell differentiation (Delay et al. 2013; Tian et al. 2014). Other than the well-known regulators of root development, other genes have been reported to affect Arabidopsis root morphology. Among them are the heterotrimeric G protein complex; the β subunit of the heterotrimeric G protein complex, AGB1, is known to be negative regulator of lateral roots and Arabidopsis plants that have mutation in the *agb1* gene will develop more lateral roots and greater root mass (Ullah et al. 2003). The GβGγ dimer negatively regulates lateral roots initiation while the α subunit, GPA1, is positive regulator of primary root elongation (Chen et al. 2006).

The formation of lateral roots starts from cell division in root pericycle to produce four layers of cells near outer surface of the primary root. After additional cell divisions and elongation, the primordia push through the root cortex to form lateral root with mature root tip (Petricka et al. 2012). The plant's lateral roots play an important role in root architecture and stress response. Initiation and development of both primary and lateral roots are controlled by multiple factors and there are extensive publications on the role of the hormones and the genes and environmental factors that are the determinants of root morphology. Plant root development is known to be highly regulated by phytohormones such as abscisic acid (ABA) which inhibits primary root growth by downregulation of genes that are involved in cell cycle, interference with the effect of the positive regulators of root elongation such as auxin or ethylene and elevation of cytosolic Ca^{2+} (Sun et al. 2018; Perfus-Barbeoch et al. 2004).

Based on the concentration of ABA, decision of root elongation will be made by plant; under high concentration of ABA, plant root growth stops while lower concentration of ABA can promote root elongation (Rowe et al. 2016). It has been also shown that ABA signaling in root endodermis regulates plant salt-stress response by inhibiting development of lateral roots (Duan et al. 2013). Certain ABA responsive genes can have both positive and negative effect on lateral root development; the ABA response genes, *ABA1* and *ABA3* can act as positive regulators of lateral root initiation under low concentration of ABA while they are among negative regulators of lateral root initiation under higher concentrations of ABA (Harris 2015).

ABA and salt stress are inhibitors of lateral roots in Arabidopsis (Galvan et al. 2011), while auxin is the main positive regulator of lateral root formation and elongation (De Smet et al.

2003). Under abiotic stresses such as drought and reduced photosynthesis, plants reduce the number of lateral roots and total root mass (Xiong et al 2006) and can also increase the primary root length (Smith & De Smet 2012) to survive with lower amount of nutrients and increase the chance of finding a water source by deeper soil penetration (Rowe et al. 2016). However, soil penetration is also dependent on soil strength. The strength of different soils is quite variable, but strength above 1 megapascal is generally inhibitory for plant roots to be able to penetrate soil. The primary root cap shape and size are also among determinants of root growth which highlights the genes that are involved in this process such as the members of the NAC transcription factor family member; *FEZ-2*, the determinants of cell layers on root cap, and *SMB-3* which regulates root cap shape. The *fez2* mutant with narrowed root cap due to reduced cell layers showed lower level of penetration in MS media while the *smb-3* mutant with rectangular shape root cap showed higher levels of penetration (Roué et al. 2020).

1.7 Salt stress

Increased salt concentration is among abiotic stresses that affects plant growth and development. More than 20 percent of agricultural lands are affected by salinity (Yu & Assmann 2015). Increased salt concentrations affect plants growth by osmotic stress, similar to the stress caused by drought and ionic stresses from increased concentrations of Na^+ or Cl^- ions which cause ionic toxicity (Galvan et al. 2011). During salt stress, plant changes the root architecture to avoid salt toxicity or osmotic stress by inhibition of lateral root formation or by bending and re-directing the orientation of growth of their primary root (Galvan et al. 2011). The effect of salt on lateral root growth is more severe than that of the primary root. In the presence of 100mM NaCl, primary root length is reduced by approximately 50 percent while the number of lateral roots is diminished by about 80%, however in higher concentrations of salt, such as 140mM, the effect is severe on both primary and lateral roots (Duan et al. 2013). It has been reported that the heterotrimeric G protein complex regulates plant growth under salt stress by activating the α subunit; *agb1*Arabidopsis are more sensitive to salt stress than wildtype while the mutant of Regulator of G protein Signaling, *rgs1,* have higher salt tolerance (Colaneri et al. 2014). The salt hypersensitivity of *agb1* mutant mainly affects above-ground tissues rather than root architecture and the negative effect of NaCl on the mutant line is highly reduced by including calcium in the

form of $CaCl_2$ in the plant growth media (Yu & Assmann 2015). These findings can highlight the role of calcium binding proteins in signaling through G protein complex.

1.8 Purpose of the study

Calcium and abscisic acid are known internal plant signals that are elevated once plants are subjected to stress situations. The heterotrimeric G protein complex and some members of the caleosin gene family have been reported to be key elements in plants stress response. Although caleosins and the G protein complex have been extensively assayed for their roles in plant response during stress conditions, the role of caleosins in signaling in relation to the hetrotrimeric G protein complex is still unknown. The second chapter of this study investigates the role of a member of the caleosin gene family, *CLO4,* in signaling and its interaction with G protein complex during plants stress response by evaluating plants stomatal density, index and aperture in Arabidopsis plants with mutations in *clo4 and gpa1.* In addition to the above ground tissue, the study will also characterize role of the gene in root development through GUS analysis and morphologocial chamges in Arabidopsis plants lacking both genes, *clo4* and *gpa1*. Studying single mutant lines of *gpa1* and *clo4* in paralell with the double mutant *gpa1 clo4* and their effect on plant morphology will give isigths to wether caleosins act through heterotrimeric G protein complex under stress condition or through a paralel pathway.

This study also focuses on possible GTPase accelerating activity of another member of the caleosin gene family, RD20/CLO3, toward the α subunit of the heterotrimeric G protein complex, Arabidopsis GPA1, using *in vitro* enzymatic assay. Chapter 3 of this study is focused on annotation and characterization of the members of the Extra-Large G protein gene family in *Triticum aestivum* and evaluation of possible transcriptional regulations of these genes under biotic and abiotic stress responses by the aid of bioinformatics approaches.

Chapter 2

Differential Roles of At-*CLO4* in Regulation of Heterotrimeric G Protein Complex in *Arabidopsis thaliana*

2.1 Abstract

Regardless of origin of the stimuli whether it is abiotic or biotic stress, elevated levels of Ca^{2+} and the phytohormone abscisic acid (ABA) are among well studied plant stress responses to regulate gene expression and molecular responses to increase plant's survival rate. Heterotrimeric G protein complex is known to be involved in ABA signaling pathway and caleosin gene family are known Ca^{2+} ion binding proteins. Although the G protein complex and the members of the caleosin gene family have been studied separately, little is known about interaction effect of caleosins and the G protein complex. In this study, CLO4 and GPA1 were shown to physically interact at the plasma membrane. It was observed that the Arabidopsis *CLO4* gene is downregulated in plant roots under elevated ABA concentrations. Regarding the effects of CLO4 on plant morphology, the gene was shown to be positive regulator of stomatal development acting in the same pathway as GPA1. Although single mutants of *clo4* and *gpa1* did not alter plant's phenotype, both genes were found to be involved in Arabidopsis lateral root development by showing significant reduction of total lateral roots in *clo4 gpa1* double mutant Arabidopsis. Phenotypic analysis of the *CLO4* over expressing line in Arabidopsis suggests that, ectopic expression of the gene could mimic other members of the gene family that affect root morphology and responses in addition to the stomatal aperture.

In addition to the Arabidopsis CLO4, role of another member of the caleosins gene family, RD20/CLO3, was investigated using *in vitro* assay to measure GTP hydrolysis rate of Gα in presence of the protein. This study showed that CLO3/RD20, does not accelerate the intrinsic GTPase activity of GPA1 which suggests regulation of the heterotrimeric G protein complex by CLO3 is not mediated through the acceleration of the GTP hydrolysis rate.

2.2 Introduction

2.2.1 Caleosin gene family

The caleosin gene family is comprised of a group of calcium binding proteins characterized by a single EF hand Ca^{2+} binding motif in their N-Terminal domain, an amphipathic α helix and proline knot in their central domain and phosphorylation sites in their C-Terminal domain (Chen et al. 1999). Structure and function of caleosins relies on presence of calcium. The presence of calcium is shown to decrease solubility of a member of the caleosins gene family, Arabidopsis CLO1, and induce aggregation state of the protein (Purkrtova et al. 2008). Caleosin 1-7 define the caleosins gene family in *Arabidopsis thaliana*. Caleosins are detected in endoplasmic reticulum, chloroplast envelope, vacuole (Blee et al. 2014) and oil bodies in Arabidopsis (Shimada et al 2018). *Brachypodium dictachyon* and wheat, *Triticum aestivum,* respectively have 11 caleosin genes in the diploid genome, and 34 gene copies in the hexaploid genome (Khalil et al. 2014). Members of the gene family have been identified in a wide range of different plant lineages including algae, mosses, lycophytes, monocots and dicot plants (Song et al. 2014). Members of the caleosins gene family have been reported to have peroxygenase activity (Partridge et al. 2009; Blée et al. 2012). It has been shown that *RD20/CLO3* have specific peroxygenase activity toward unsaturated fatty acids (Blée et al. 2014) that could be involved in both biotic and abiotic stress responses in Arabidopsis and may be involved in certain antifungal compound production (Partridge et al. 2009). Their function has been shown to be different than the other two well known oil body membrane localized proteins such as oleosins that prevent fusion of oil bodies or steroleosins with specific reductase activity (Shimada et al 2018).

Shen et al, (2014) proposed two caleosins isoforms in Arabidopsis named “H”, and “L” according to their relative molecular mass. The H group contains a motif of approximately 29 amino acids in the N-terminal region of the protein that is not present in the “L” group and the N terminal extension is variable in length in the L group. Arabidopsis CLO1, 2, 3 and 8 are among H-caleosins while the other caleosins CLO4, 5, 6 and 7 are in the L group. Sequence comparison and phylogenetic analysis suggests that the “L” caleosins are evolved from the “H” group (Shen et al. 2014). Caleosins are known to be among oil body associated proteins together with the other two well-known oil body membrane localized proteins; oleosins and steroleosins (Shimada et. Al. 2010). Oil bodies are plant cell organelles that store triacylglycerides (TAGs) which is the

main energy source in seeds during germination (Poxleitner et al. 2006). Other than seeds, oil bodies in leaf have been shown to produce anti-fungal compounds known as phytoalexins and up-regulation of *CLO3/RD20* transcript in leaf during fungal infection and senescence has been reported (Shimada et al. 2014). Leaf oil bodies have been suggested to be involved in plant stress response. Very high or very low temperature increases number of oil bodies in leaves and the role and content of leaf oil body is different than that in seeds; oil bodies are normally in very low abundance in leaves compared to seeds since oleosin biosynthesis genes are not expressed in leaves (Shimada et al. 2018).

Previous findings related to *CLO4,* a member of caleosin gene family, showed that *CLO4* transcript is detectable in Arabidopsis roots, stem, and leaves and is highly expressed in the flower and the promoter activity of the gene was observed in most plant organs except dry seed (Kim et al. 2011). The *clo4* mutant line, SALK_090861, did not show any obvious morphological difference compared to WT under control conditions while the mutant line showed higher drought tolerance compared to the wild type plant Columbia and *CLO4* overexpressing lines were reported to have more sensitivity to drought conditions (Kim et al. 2011). Germination of *clo4* mutant seeds was more sensitive to salt and drought stresses and ABA treatment and expression of the two leucine zipper transcription factors *ABF3*, *ABF4* and *ABA insensitive 1, ABI1*, genes involved in ABA signalling pathway, have been shown to be increased in the *clo4* mutant line under ABA treatment conditions (Kim et al. 2011).

Another member of the caleosin gene family, *RD20/CLO3,* has been also reported to be involved in abiotic stress responses, and ABA signalling (Aubert et al. 2011). It has been reported that *RD20* transcript is elevated under drought, salinity and increased ABA concentrations (Takahashi et al. 2000). The *rd20* mutant plants have a lower germination rate when plated on media with ABA and plants over-expressing *RD20* have higher germination rate with ABA treatment compared to wild type Arabidopsis (Auber et al. 2011). The *rd20* mutant plants also showed higher transpiration and increased stomatal opening (Aubert et al. 2010).

It has been reported that, expression of plant At-*CLO1* in yeast increases total lipid content (Froissard et al. 2009) and the N-terminal domain of the protein is essential for protein sorting in yeast cytoplasmic lipid droplets (Purkrtova et al 2015) and this domain on another member of the caleosins gene family, CLO3/RD20, was shown to be sufficient for interaction with the α subunit of the heterotrimeric G protein complex (GPA1) using BiFC in *Nicotiana*

benthamiana leaves (Ehdaeivand 2014). The exact role of proline knot is not characterized in caleosins; however, it has been reported that this motif plays important role in oil body targeting (Abell et al. 1997).

2.2.2. Regulation of Arabidopsis Root Morphology and stomatal development through CLO4, GPA1- the α subunit of the heterotrimeric G protein complex, and the phytohormone abscisic acid (ABA).

Expression of *GPA1* and *CLO4* transcripts or promoter activity of these genes using GUS expression system in Arabidopsis showed that, *GPA1* is expressed in stomatal precursor cells (Nilson and Assmann 2010) as well as root and above ground tissues with higher expression in Arabidopsis roots (Chen et al. 2006). The study on *CLO4* showed that the gene is also expressed in root and Arabidopsis guard cells as well as other plant organs (Kim et al. 2011). Although downregulation of *CLO4* in Arabidopsis leaves had been reported in response to ABA treatment (Kim et al. 2011), the expression of the gene in Arabidopsis root system using homozygous *pCLO4:GUS* construct in response to ABA treatment has not been studied.

Formation of the heterotrimeric G protein αβγ trimer in Arabidopsis slows cell proliferation in primary root while the GTP bound Gα and the free βγ dimer are respectively positive regulators of primary root elongation and negative regulators of lateral root cell division (Chen et al. 2006). However, there are conflicting phenotypes observed for *gpa1* mutant line. The *gpa1* mutant line was reported to have a lower number of lateral roots compared to WT by Urano et al. (2003) and Chen et al. (2006) and was reported to have the same number of lateral roots as WT by Pandey et al. (2006). The differences may be due to different experimental conditions or ecotypes. The *gpa1* mutant line showed to be hypersensitive in its primary root response to ABA by showing higher percent reduction of primary root under elevated ABA concentrations (Pandey et al. 2006). Under control condition *clo4* mutant plants did not show any distinct morphology compared to the wild type Arabidopsis (Kim et al. 2011). It has been reported that the *gpa1* mutant Arabidopsis has between 1-2-fold, increased in expression of ABA regulators *ABI3* and *ABI5* genes (Pandey et al. 2006). Higher expression of the ABA response genes, *ABF3*, *ABF4* and *ABI1* have been reported by Kim et al. (2011) in *clo4* mutant line under ABA. This can suggest that both GPA1 and CLO4 may act through ABA stress responses in Arabidopsis under stress condition.

Kim et al. (2011), showed that, *clo4* mutant line has a greater degree of stomatal closing in response to ABA while overexpressing the gene showed insensitivity to ABA (Kim et al. 2011). The *gpa1* mutant in Arabidopsis has been reported to be insensitive to ABA inhibition of stomatal opening which highlights the role of GPA1 as positive regulator of ABA response in inhibition of K^+ inward channel and consequently stomatal closure (Wang et al. 2001; Perfus-Barbeoch et al. 2004). The opposite phenotypes observed for *clo4* and *gpa1* mutant plants will be investigated in this study through double mutant analysis of *clo4 gpa1* Arabidopsis plants to analyse whether the genes act in the same or parallel pathways. A summary of known roles of GPA1 and CLO4 in plant morphology is shown in Table 1.

In addition to the effect of CLO4 on GPA1 during Arabidopsis root development and response to ABA, this study will also investigate role of Arabidopsis CLO4 on regulation of G protein complex and its relationship to stomatal development and ABA responses. The *gpa1* mutant in Arabidopsis showed reduced stomatal density and index proposing positive role of this gene in stomatal and pavement cell development (Nilson and Assmann 2010). This study will investigate role of Arabidopsis *CLO4* in plant stomatal development with the analyses of single and double mutant lines of *clo4* and *gpa1* in addition to the *CLO4* overexpression in Arabidopsis.

Table 1. Role of GPA1 and CLO4 in plant morphology. Summary of the known roles of both genes, *GPA1* and *CLO4*, in regulation of stomata and root development in Arabidopsis are listed.

	***gpa1* mutants**	***clo4 mutant* *SALK_090861* Homozygous**
General Morphological observation of mutation under control condition	Reduction of cell division and proliferation in areal tissues and round leaves (Ullah et al. 2001)	No difference was observed between *clo4* mutant, *SALK_090861,* and WT under control condition (Kim et al. 2011)
Stomatal Development	Lower stomatal density and index compared to WT (Nilson & Assmann 2010)	No data available on stomatal development and stress response of the *clo4* mutant line used in this study
Development of Lateral Roots	**1.** Lower number of lateral roots (Urano et al. 2003; Chen et al. 2006) **2.** Same number of lateral roots as WT (Pandey et al. 2006)	No data available on lateral root morphology and their role in ABA stress response in the mutant line used in this study
Development of Primary Root	Higher percent reduction of primary root in presence of ABA (Pandey et al. 2006)	No data available on primary root morphology and stress response of the mutant line used in this study
Stomatal aperture	insensitive to ABA inhibition of stomatal opening (Wang et al. 2001; Perfus-Barbeoch et al. 2004)	Higher stomatal closing in response to ABA (Kim et al. 2011)
Regulation of ABA responsive genes	Elevation in *ABI3* and *ABI5* transcripts (Pandey et al. 2006)	Higher expression of *ABF3, ABF4* and *ABI1* (Kim et al. 2011) under ABA

2.3 Materials and Methods

2.3.1 Plant growth conditions

For all experiments with plants grown in soil, seeds were sown on soil mixture of 2:1:1 ratio of black earth, peat moss and vermiculite. The soil was heated for 90 minutes at 130°C and cooled to room temperature. Seeds were sown on moist soil and cold treated at 4°C for 48 hours

prior transferring to either the greenhouse or growth chambers. Plants were grown with a light cycle of 16 hours of light, 8 hours dark and culture temperature between 22-24°C.

2.3.2. Screening of *Arabidopsis thaliana* mutant lines

Homozygous mutant lines harboring the T-DNA insert in either *gpa1* or *clo4* gene and *CLO4* over-expressing (OE) line, and the homozygous double mutant line null in both *gpa1* and *clo4* genes were used in multiple assays. All mutant lines were in Columbia ecotype background. The *clo4* homozygous line mutant from the *SALK_090861* and *CLO4* over expressing line *4-3* used in the study were kindly provided from the lab of Dr. Jeong Sheop Shin, School of Life Sciences and Biotechnology, Korea University, Seoul. The *gpa1-3* in Columbia background was kindly provided by Dr. Alan Jones (North Carolina State University) The double mutant line was generated by crossing *gpa1-3 in* Columbia background with *clo4 SALK_090861*. The *CLO4* promoter:GUS reporter construct was developed in the Gulick lab using 1000 bp upstream of the transcription initiation site and cloned into the pFAST-G04 vector (Rafeh, 2016) and transgenic Arabidopsis lines were developed by using the floral dip method (Clough & Bent 1998). The line was re-screened to select a homozygous transformed line according to Shimada et al. (2010) prior to the assays.

2.3.3. Growth condition of *Nicotiana benthamiana* plants

Nicotiana benthamiana seeds were sown on the soil mixture of 2:1:1 ratio of black earth, peat moss and vermiculite and plants were grown in greenhouse with supplemental lighting to maintain a light cycle of 16 hours of light, 8 hours dark and maintained at a temperature between 22-24°C. Plants 2-3 weeks old were used for transient transformation by *Agrobacterium* infiltration.

2.3.4. Bimolecular Fluorescent Complementation Assay (BiFC)

The *in-planta* protein-protein interaction of the two proteins was examined using Bimolecular Fluorescent Complementation Assay (BiFC) as described by (Walter et al 2004) with slight modifications. The experiment was carried out using the full-length coding sequences for both *CLO4* and *GPA1* genes fused to two halves of a split Yellow Fluorescent Protein in the pBATLE-B-sYFP-C and pBATLE-B-sYFP-N- plasmid vectors respectively (Grigston et al

2008) provided by the Gulick lab. The abaxial side of leaves for *N. benthamiana* plants were Agro-infiltrated as previously described by (Kapila et al. 1997) using different concentrations of the *Agrobacterium tumefaciens* cultures carrying the constructs along with cultures carrying the expression vectors with organellar markers, fused to Red Fluorescent protein and P19 as suppressor of transgene expression (Walter et al. 2004). The infiltration mixtures were prepared from overnight cultures of the *Agrobacterium tumefaciens* strain AGL1 carrying the expression vectors. All cultures grown to an OD of 0.5 or diluted to that OD for the interaction and OD of 0.1 for the organellar markers before centrifugation and the pellets were resuspended in the agroinfiltration solution, incubated at room temperature for four hours and then used to infiltrate the plant leaves. The leaves were monitored at different time points starting 12 hours post injection up to 52 hours to select the optimal incubation time to detect interaction of the two proteins and to localize the interaction. The final images were taken using Olympus FV10i laser scanning microscope. The interaction of the two proteins was captured between 42-45 hours post-infiltration using eGFp filter, 473 nm, excitation at 489 nm and emission at 510 nm. Full length AtPIP2A protein fused to red fluorescent protein used as plasma membrane marker. The full length At-DMP1 protein fused to red fluorescent protein was used as tonoplast marker and full length At-WAK2 protein fused to red fluorescent protein was used as endoplasmic reticulum marker. All mcherry markers were detected with a filter at 559 nm with excitation at 580 nm and emission at 610 nm.

2.3.5. Promoter activity and tissue specific expression of *CLO4* gene

Promoter activity was assayed using plants transformed with plasmid constructs with the gene promoter fused to the glucuronidase gene (GUS) as described by (Jefferson et al 1987). The homozygous *pCLO4:GUS* carrying Arabidopsis seeds were sterilized in 70% ethanol for one minute followed by 5 minutes of incubation in sterilizing solution (4% sodium hypochlorite, 1% Triton X-100). Seeds were washed 5 times with distilled water and stratified in dark at 4°C for 2-4 days and then plated on plant growth semi-solid media containing ½ Murashige and Skoog (MS), 0.05% MES hydrate, 1% sucrose, 0.4% Gelzan with the pH adjusted to 5.7 with KOH for 10 days and then transferred to treatment plates for 6 hours complemented with either 10 µM ABA, 150 mM NaCl or 300 mM mannitol. For cold treatment, 10 day old plants were incubated in 4°C for 6 hours prior staining. For long term evaluation of effect of ABA on the promoter

activity of the *CLO4* gene, seeds were sterilized as mentioned, germinated on control plates, ½ Murashige and Skoog (MS), 0.05% MES hydrate, 1% sucrose, 0.4% Gelzan with the pH adjusted to 5.7 with KOH, and then transferred to new plates complemented with either 1 µM or 2 µM ABA and imaged several times during an 8 days span of growth to visualize GUS expression. The treated and the control plants were then incubated in GUS staining solution (50 mM NaHPO4-pH 7.2, 0.5% Triton X-100, 1 mM X-Glue at 37°C overnight and de-stained with 70% ethanol. The plants were imaged on Nikon SMZ1500 stereomicroscope using Leica DFC420, 5-megapixel color digital camera.

2.3.6. Screening for *clo4 gpa1* double mutant

The single mutant lines *gpa1-3* and *clo4* SALK_090861 were crossed. The F1 heterozygous plant was found via a PCR screen and the seed was planted and plants from the F2 generation were screened to identify the double mutant homozygous for both the *clo4 and gpa1* loci. Arabidopsis genomic DNA was extracted as previously described by Edwards et al. (1991), using extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA, pH 8.0, 0.5% SDS) and precipitated using isopropanol, washed with 70% ethanol and re-suspended in TE pH 8.0. PCR was performed using 50-70ng DNA in presence of gene specific and T-DNA targeting primers suggested by SALK institute [http://signal.salk.edu/cgi-bin/tdnaexpress] for each line. The list of primers for screening is shown in Table 2.

Table 2. Primers for screening the mutant *gpa1* and *clo4* lines.

Primer Name	Sequence	Tm
SALK_106193LP	CTCGACATTTTTCTTTGTCGG	59.73
SALK_106193RP	ATATTCACCTTGCCAAACACG	59.87
SALK_090861LP	GCGAAACGATCAAAATTATGC	59.58
SALK_090861RP	TTTGTCGTAAACGCCTGAATC	60.12
LBb1.3	ATTTTGCCGATTTCGGAAC	59.89
CLO4RTPCRF	ATGGCTTCCTCTATTTCCACTGG	62.79
CLO4RTPCRR	TTATGGATGTTTCTTAGAAGTTTTAGAAGATC	61.07

2.3.7. Root phenotyping

Root phenotyping was performed for the mutant and over-expressing lines and the control wild type plants. The seeds were sterilized in 70% ethanol for one minute followed by 5

minutes of incubation in sterilizing solution (4% sodium hypochlorite, 1% Triton X-100). The seeds were washed 5 times with sterilized distilled water and stratified in dark at 4°C. The stratified seeds were plated on plant growth semi-solid media containing ½ Murashige and Skoog (MS), 0.05% MES hydrate, 1% sucrose, 0.4% Gelzan with the pH adjusted to 5.7 with KOH. All plants were stratified for 48 hours and germinated on MS media and transferred 36 hours post germination to either control plates or treatment plants complemented with 2 µM ABA and grown for 10 days. Plants were imaged and measured for primary root length and the number and lengths of lateral roots were evaluated 10 days after transferring using ImageJ software (Rasband, 1997-2018; https://imagej.nih.gov/ij/). Total of three or four Arabidopsis plants were characterized per plate. Two plates were assayed for each genotype under each condition.

2.3.8. Stomatal density and index analysis

Stomatal analysis was performed on 15-day old soil grown plants grown in growth chamber complimented wirh16h light, 8h dark at 23°C. The plants were treated with either ABA solution (0.05% ethanol and 25 µM ABA) or control solution (0.05% ethanol) three times per week after germination as described in Nilson and Assmann (2010). Detached first and second leaves were incubated in 1ml of 9:1 ethanol: acetic acid overnight which was changed to 70%, 50% and 20% ethanol at 30 minutes intervals. The samples were placed in water prior to staining and were stained using 0.5% Toluidine blue TBO and mounted in 15% glycerol. All samples were imaged using ZEISS Axioplan microscope. Three plants per each genotype under either control condition or ABA treatment was imaged and four images per each leaf was captured.

2.3.9. Stomatal aperture experiment

The Stomatal aperture experiment was performed as previously described by Kim et al, 2011, with some modifications. Leaves from 3-4 weeks old plants grown in greenhouse were detached and incubated in the opening solution containing 5mM MES-KOH, 20 mM KCl, pH 6.15 for one hour. ABA was added to the final concentration of 5µM and the leaves were incubated for 2 hours. The leaves were gently scratched on the slide using blade to remove a thin layer of leaf epidermis to visualize the stomata and to obtain images with the ZEISS Axioplan microscope. Stomatal aperture was measured using ImageJ software (Rasband, 1997-2018;

https://imagej.nih.gov/ij/). Two leaves per plant for two plants per genotypes under each treatment were imaged and stomatal aperture was measured for each stoma.

2.3.10 Statistical analysis

All statistical analysis in this study were performed using IBM SPSS statistics and *p value* of ≤ 0.05 was considered significant. For all phenotypic analysis, two-way ANOVA was performed to test interaction effect between genotypes and treatments. One-way ANOVA was performed between treatments and control experiments followed by Duncan multiple range test. The mutants or treatments that are shown with different letters on each graph show statistically different responses or phenotypes.

2.3.11 Protein-protein interaction between truncated versions of CLO4 and GPA1 using Yeast Two Hybrid (Y2H) assay.

Constructs containing the full-length coding sequences of CLO4 as well as C-terminal and N-terminal domains of CLO4 were cloned in both pGADT7 and pGBKT7 Gateway compatible vectors kindly provided by Dr. Sacher lab, Concordia University, Biology Department. AGB1 Gamma3 (γ) was used as positive control (Chakravorty et al 2015) for interaction with GPA1-AD (Brunetti et al. Gulick lab unpublished data). The targeted sequences were cloned using Gateway cloning protocol according to manufacturer's guideline. Four different truncated versions of the CLO4 protein were cloned in both pGBKT7 and pGADT7 vectors as shown in Table 3 using primers shown in Table 4. Two versions of the N-terminal domain of the protein were used containing the calcium binding motif with either amino acids 1-44 or 1-46 included. The other N-terminal domain truncation version had amino acids 1-79 which contained calcium binding motif, amphipathic alpha helix and the proline knot. The C-terminal domain construct included amino acids 80 to 195. The full length CLO4 construct, amino acid 1-195, was also assayed in parallel with the truncated versions. The constructs were used to test for the interaction in configurations with the GAL4 fusions, one with GPA1 bound to the Gal4 activation domain (AD) the other Gal4 binding domain (BD), and were tested for interaction with the CLO4 constructs in complimentary configurations, as fusions to the BD or AD domains, respectively. The Y2H experiment was performed as previously described by Ito et al (1983) with minor modifications. An overnight culture of yeast cells strain AH109 was diluted

to OD 0.4 in 10ml YPD and then grown until OD 1.0. The cultures were pelleted and re-suspended and re-centrifuged three times with 5 ml of 1xTE (10 mM Tris-pH-7.5 and 1 mM EDTA). The pellet was re-suspended in 1 ml of 0.1 M LiOAc containing 30µl of carrier DNA stock (10 mg/ml). Aliquots of 150 µl of the cells were added to 1 µg of each construct along with 350 µl of 50% PEG. After one hour of incubation each reaction was placed in 42°C water bath for 5 minutes after addition of 50 µl DMSO, then plated on selective medium and incubated at 30°C.

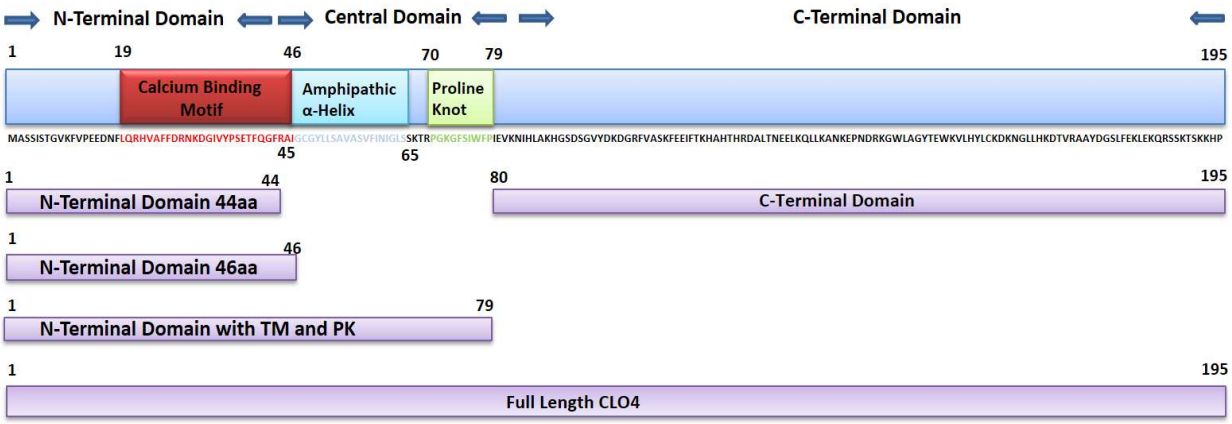


Figure 1. Full length CLO4 protein and the four truncated versions used for the Y2H experiment. Different truncated versions of the protein were cloned for yeast two hybrid assay. three clones targeting the N-terminal domain of the protein from either amino acids 1-44 and 1-46 including the calcium binding motif and a clone including calcium binding motif, the transmembrane domain and the proline knot from amino acids 1-79. C-terminal domain of the protein from amino acids 80-195 and a full length CLO4 protein amino acid 1-195.

Table 3 Lists of Constructs made for Yeast Two Hybrid (Y2H) experiment

Construct name	Description
CLO4 NTer 44aa pGADT7	CLO4 N-terminal first 44 amino acid fused to Gal4 activation domain
CLO4 NTer 44aa pGBKT7	CLO4 N-terminal first 44 amino acid fused to Gal4 binding domain
CLO4 NTer TMPK-pGADT7	CLO4 N-terminal domain containing both transmembrane and proline knot domains bound to Gal4 activation domain aa 1 to aa 79
CLO4 NTer TMPK-pGBKT7	CLO4 N-terminal domain containing both transmembrane and proline knot domains bound to Gal4 binding domain aa 1 to aa79
CLO4-N-Ter PGADT7	CLO4 N-Terminal first 46 amino acids fused to Gal4 activation domain
CLO4-N-Ter PGBKT7	CLO4 N-Terminal first 46 amino acids fused to Gal4 binding domain
CLO4-C-Ter PGADT7	CLO4 C-terminal aa80 to 195aa fused to Gal4 activation domain
CLO4-C-Ter PGBKT7	CLO4 C-terminal 80aa to 195aa fused to Gal4 binding domain
CLO4-Full Length PGADT7	CLO4 Full length 1aa-195aa fused to Gal4 activation domain
CLO4-Full Length PGBKT7	CLO4 Full length1aa-195aa fused to Gal4 binding domain

Table 4 List of the primers used to make Y2H constructs

Primer Name	Sequence
CLO4_N_Ter_REV_TMPK	ggggaccactttgtacaagaaagctgggtcTTAAGGAAACCAGATAGAGAATCCTTTACCC
CLO4_N_Ter_REV_44aa	ggggaccactttgtacaagaaagctgggtcTTATCTAAATCCTTGAAATGTCTCCGAGG
CLO4 N_TerF	ggggacaagtttgtacaaaaaagcaggcttcATGGCTTCCTCTATTTCCACTGGAG
CLO4 N_TerR	ggggaccactttgtacaagaaagctgggtcTTAAATTGCTCTAAATCCTTGAAATGTCTCC
CLO4 C_TerF	ggggacaagtttgtacaaaaaagcaggcttcATGGGAATAGAGGTTAAGAATATTCACCTTGC
CLO4 C_TerR	ggggaccactttgtacaagaaagctgggtcTTATGGATGTTTCTTAGAAGTTTTAGAAG
CLO4_Full_Y2H_F	ggggacaagtttgtacaaaaaagcaggcttcATGGCTTCCTCTATTTCCACTGG
CLO4_Full_Y2H_R	ggggaccactttgtacaagaaagctgggtcTTATGGATGTTTCTTAGAAGTTTTAGAAGATC

2.3.12 Truncated versions of GPA1 protein tested for interaction with CLO4 protein.

Cloning of the expression vectors carried out using Gateway technology method by PCR amplification of either full length or truncated versions of the targeted sequences of *GPA1* using cDNA clone as the template. Three different truncated version of the protein were cloned; The N-terminal domain of the protein, amino acids 1-100, containing Myriostoylated glycin and the p-loop for nucleotide recognition. The C-terminal domain of the protein, amino acids 101-204, containing critical threonine residues for Gα and the RGS box interaction. The C-terminal domain of the protein, amino acids 205-383, containing residues that interact with the Gβγ dimer. The primers used in the study are listed in Table 5. The amplified DNA products were transferred to pDONR207 using BP Clonase (Invitrogen), transferred to *E. coli* strain *TOP10*

using electroporation and selected on the appropriate media. The entry clones were then purified and used to transfer the insert into the destination vector pBATLE-B-sYFP-C using LR Clonase, and then used in Bimolecular Fluorescent Complementation Assay as described by (Walter et al 2004) with some modification.

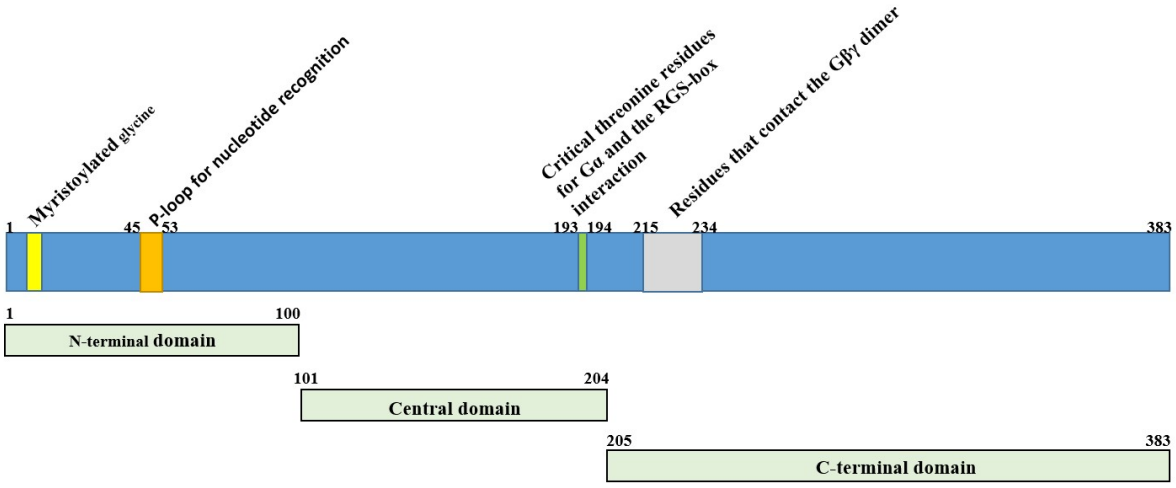


Figure 2 Truncated versions of GPA1 protein tested for interaction with CLO4 protein. The predictions are based on Temple and Jones 2007. The protein-protein interaction assay between truncated versions of GPA1 and CLO4 was performed using Bimolecular Fluorescent Complementation Assay (BiFC). Three different truncated versions of the GPA1 protein were assayed for their interaction with CLO4. The interaction was screened using ZEISS Axioplan microscope.

Table 5. Primers used to make constructs for interaction between truncated versions of GPA1 with full length CLO4 protein

Primer Name	Sequence
GPA1 N-Terminamal Forward	ggggacaagtttgtacaaaaaagcaggcttCATGGGCTTACTCTGCAGTAGAAGTC
GPA1 N-Terminal Reverse	ggggaccactttgtacaagaaagctgggtCTTCATTTTGAGCAAACTCCTTTG
GPA1 Central domain Forward	ggggacaagtttgtacaaaaaagcaggcttCATGGGCACAGATTCTGCTAAATATATGTTATC
GPA1 Central domain Reverse	ggggaccactttgtacaagaaagctgggtCCACAGGGCTGAACTGTATTTC
GPA1 C-Terminal Forward	ggggacaagtttgtacaaaaaagcaggcttCATGGGCGGAGAGAATAAAAAAAGTGGTGAAGTG
GPA1 C-Terminal Reverse	ggggaccactttgtacaagaaagctgggtCTAAAAGGCCAGCCTCCAGTAAATTTC

2.3.13 Arabidopsis CLO3/RD20 protein expression and purification

A DNA strand encoding the Arabidopsis CLO3/RD20 coding sequence was synthesized with codons optimized for expression in *E. coli* and cloned into the pRsetA-His tagged vector (Life Technologies). The plasmid was transformed into *E. coli* strain BL21-DE3 by electroporation. After testing different induction protocols and culture conditions for high level expression of soluble protein and facilitation of the purification in *E. coli,* an auto induction protocol developed by Studier (2005) was used to induce expression of CLO3/RD20 in *E. coli* without the use of Isopropyl β-d-1-thiogalactopyranoside. The culture was grown in LB media containing 100µg/ml of ampicillin at 37°C overnight. The growth media (ZY media) was made using 4g of Yeast extract and 8g Bio-Tryptone dissolved in water and brought to 800 ml volume and divided into two 400ml portions in two 2L flasks. A 2.5 ml aliquot of the overnight culture was added to each flask. Each culture was complemented with 8 ml of 50x5052 induction solution (25% (w/v) glycerol, 2.5% (w/v) glucose and 10% (w/v) α-lactose) and supplemented with 8 ml of 50xM solution (1.25M Na2HPO$_4$, 1.25M KH_2PO_4, 2.5M NH_4Cl and 0.25M Na_2S0_4), 0.8 ml 1M $MgSO_4$ and ampicillin at 100 µg/ml as previously described by Studier (2005). The culture was incubated at 37°C with shaking for 8 h at 200 rpm and then the temperature was reduced to 18°C and the culture was grown overnight. Content of each culture flask was centrifuged in two 250 ml centrifuge bottles for 25 min at 10,000 x g and the pellets were frozen at -80°C until they were used for protein purification. The freezing step was shown to improve lysis of the *E. coli* cells to obtain higher CLO3/RD20 protein yield. Each frozen bacterial cell pellet was lysed using the B-PER lysis buffer (Thermo Scientific, Pierce, USA) as per manufacturers protocol with addition of 0.25% (v/v) nonyl phenoxypolyethoxylethanol (NP-40), 2500 U of deoxyribonuclease (DNase) (Sigma-Aldrich) and an EDTA-free protease inhibitor tablet (Sigma-Aldrich). After centrifugation of the lysate at 10,000 x g for 30 minutes the protein was purified from the supernatant by affinity chromatography using nickel agarose beads (GoldBio) as previously described by Pandey et al. (2009) with slight modification. The lysate was incubated with the equilibrated nickel agarose beads (GoldBio) and washed with buffer 1 (50mM Tris-HCl pH 7.5, 200mM NaCl, 15mM imidazole, 10% glycerol, 0.25% Tween 20) with three column volumes five times, followed by three washes with three column volumes of wash buffer 2 (50mM Tris-HCl (pH 7.0), 300mM NaCl, 30mM imidazole, 12% glycerol, 0.25% Tween 20) and eluted using 5ml of elution buffer (50mM Tris-HCl pH 7.0, 300mM NaCl,

300mM imidazole, 12% glycerol, 0.1% Tween 20, EDTA-free protease inhibitor tablet). Since presence of bacterial nucleases co-purified with CLO3-His tagged protein can interfere with a GTPase assay, the CLO3/RD20 was further purified using ion exchange chromatography. To achieve higher purity level of the protein, the CLO3-His protein was desalted in 20mM Tris-HCl pH 8, 0.1% TWEEN-20 and applied to 1mL HiTrap Q FF Sepharose (GE Healthcare) column equilibrated with the same buffer. Protein bound to the column was eluted using linear gradient of 0-50% between buffer A (20mM Tris-HCl pH 8, 0.1% TWEEN-20) and buffer B (20mM Tris-HCl pH 8, 0.1% (v/v) TWEEN-20, 1M NaCl). The elution was collected in 5ml fractions and aliquots of each fraction were run on SDS-PAGE. The CLO3 protein was found to be eluted in the gradient around 300mM NaCl. The protein was concentrated using a 10 kDa MWCO spin filter (Pierce concentrator, Thermo Scientific) and applied to a Superdex 75 26/60 gel filtration column with 50 mM NaCl. The purified protein was acid precipitated and analyzed by SDS-PAGE and the protein sample was frozen at -80°C prior to GTPase assay.

2.3.14 Arabidopsis GPA1 protein expression and purification

The codon optimized GPA1 coding sequence was synthesised by Life Technologies and subsequently cloned into the pDonor 207 plasmid and then transferred to pGex6p1 GST expression vector using Gateway cloning method. The GST tagged GPA1 was co-expressed with the chaperones to increase the solubility of the protein. For this purpose, the GroES-GroEL chaperones encoded by the pGro7 plasmid in *E. coli* strains HB101 (Takara Bio, Mountain View, CA) was used. The protein was induced as previously described by Auslender et al. 2015 and the total culture of 175 ml was centrifuged at 10,000 x g for 30 minutes and the pellet was frozen at -80°C prior to the purification. The pellet was re-suspended in 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl, 140 mM NaCl, 30 µM $AlCl_3$, 20 mM NaF, 1 mM DTT, 0.1 % (v/v) Triton X-100, EDTA-free protease inhibitor (Roche) and 1 mM PMSF, pH 7.3. Cells were lysed twice at 4°C using a French Press (18,000 psi). The lysate was centrifuged at 10,000 x g for 30 minutes and the supernatant was filtered through a 0.22µm cellulose acetate filter and the GPA1 protein was purified batchwise using GoldBio glutathione agarose resin as per manufacturer's protocol using 0.5ml settled bed volume equilibrated in the extraction buffer. Since GPA1 was co expressed with GroES-GroEL chaperones, an additional wash step was performed with 15ml of the extraction buffer plus 5mM ATP and 10mM $MgCl_2$ to increase solubility and yield of

GPA1 as previously explained by Auslender et al. (2015). GPA1-GST was eluted after the wash step with 5 mL of the elution buffer (50 mM Tris-HCl pH 8.0, 0.1 % (v/v) Triton X-100, 30 µM $AlCl_3$, 20 mM NaF, 1 mM DTT, complete EDTA-free protease inhibitor (Roche), 1 mM PMSF and 10 mM reduced glutathione) and the protein in the elution was verified by SDS-PAGE. The protein sample was buffer exchanged into 20 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol (DTT), 25µM GDP and 5% (v/v) glycerol by diafiltration using an Amicon Ultra-4 centrifugal filter and further purified by negative chromatography using MonoQ HR5/5 column in the same buffer containing 20 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol (DTT), 25µM GDP and 5% (v/v) glycerol. The GPA1-GST was incubated with 5mM GTP for 30 minutes after incubation with 2mM EDTA for 5 minutes. The purified protein was buffer exchanged into the following buffer containing; 20mM Tris-HCl pH 8.0, 10µM CaCl2 and 25 mM KCl prior to the assay.

2.3.15 GTPase activity assays

Spectrophotometric coupled enzyme assay was used to test potential GTPase activity of CLO3 toward GPA1 as explained previously for the nucleoside diphosphate kinase by Dorion and Rivoal (2003) and the reaction was monitored for disappearance of NADH at 340nm. GTP hydrolysis rate of GPA1 was monitored in presence of CLO3 to assay CLO3's potential as a GTPase accelerating protein (GAP). Higher GTPase activity was expected to show more disappearance of NADH. The reaction contained 10 mM Tris–HCl, pH 7.5, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM $MgCl_2$, 10 µM GTP, 10 µM $CaCl_2$, 50 mM KCl, 0.32 mM nicotinamide adenine dinucleotide, 4 mM phosphoenolpyruvate, 0.4 unit rabbit muscle pyruvate kinase, and one unit rabbit muscle lactate dehydrogenase in a final volume of 200µl. The assay was performed at 25°C on a VERSA max microplate reader (Molecular Devices, Sunnyvale, CA) for 40 minutes; absorbance at 340 nm was used to measure the disappearance of NADH.

2.4 Results

2.4.1 *In Planta* interaction assay with GPA1 *and* CLO4

Using Bimolecular Fluorescent Complementation assay (BiFC) in *N. benthamiana* leaf, the CLO4 and GPA1 proteins were shown to physically interact and the interaction was located

at plasma membrane (Figure 3). The interaction between the two proteins was observed between 42-45 hours post infiltration. The interaction fluorescent signal co-localized with the plasma membrane marker, the full length AtPIP2A protein fused to red fluorescent protein. Since the plasma membrane and the vacuole membrane, tonoplast, are adjacent to each other in most cell periphery, Arabidopsis DMP1 protein was used as tonoplast marker which showed that the protein-protein interaction does not localize with the tonoplast marker and confirmed that the interaction takes place in the plasma membrane. In addition to the tonoplast marker, the endoplasmic reticulum marker also did not show overlapping signals with the GPA1-CLO4 interaction (Figure 3).

Although full length proteins of CLO4 and GPA1 showed interaction, the truncated versions of GPA1 did not show interaction with full length CLO4 using Bimolecular Fluorescent complementation assay (Figure 2) and the truncated and the full-length versions of the CLO4 protein did not show interaction with GPA1 using yeast two hybrid assay (Figure 10).

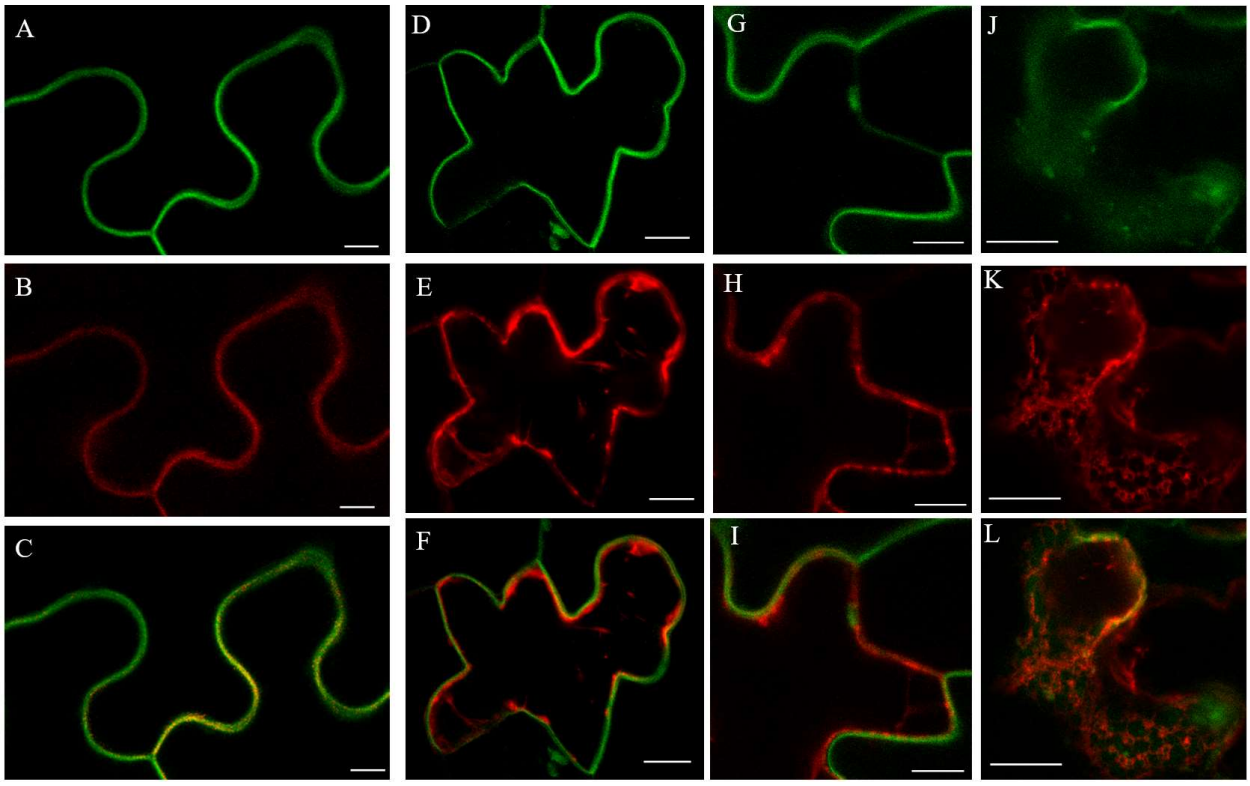


Figure 3. *In vivo* interaction of CLO4 and GPA1 in *Nicotiana benthamiana* leaf using Bimolecular Fluorescent Complementation assay. A) Physical interaction between the two proteins GPA1 and CLO4. **B)** Full length AtPIP2A protein fused to red fluorescent protein used as plasma membrane marker. **C)** Overlap of the two images showing the interaction is located in

the plasma membrane. **D & G)** Interaction between the two proteins GPA1 and CLO4. **E & H)** Full length At-DMP1 protein fused to red fluorescent protein used as tonoplast marker. **F & I)** Overlap of the two images showing the interaction is not located in the tonoplast. **J)** Interaction between the two proteins GPA1 and CLO4. **K)** Full length At-WAK2 protein fused to red fluorescent protein used as endoplasmic reticulum marker. **L)** Overlap of the two images showing the interaction is not located in the ER. Scale bar indicates 10µm.

2.4.2 Histochemical GUS analysis of promoter of the At-*CLO4* gene

The localization of the *CLO4* gene expression in transgenic plants with the *pCLO4:GUS* reporter construct was initially assayed using ten day old Arabidopsis plants after 6 hours of mannitol, NaCl, cold or ABA stress treatments. In the untreated control plants the beta-glucuronidase reporter gene expression was detected in both lateral roots and the primary root (Figure 4. A & F). To mimic drought and salt conditions the plants were subjected to 300 mM mannitol and 150 mM NaCl, respectively. There were no significant changes in the expression level of the beta-glucuronidase reporter gene in the primary and the lateral roots under mannitol treatment (Figure 4. B & G) compared to the untreated control plants. Under salt stress, the primary root showed higher promoter activity compared to the control conditions and no significant difference was observed for the lateral roots (Figure 4 C & H). Under cold treatment at 4°C both the primary and the lateral root showed reduction in the promoter activity (Figure 4 D & I). Under ABA treatment the relative promoter activity and expression of the beta-glucuronidase gene was reduced compared to the control conditions (Figure 4. E & J).

Enlarged images of the primary and the lateral roots comparing the promoter activity under ABA to the control condition showed that the expression level of the beta-glucuronidase gene is significantly reduced in the lateral root (Figure 4 M & N). ABA treatment also reduced the promoter activity of the *CLO4* gene in the primary root (Figure 4 K & L). The result showed that although the promoter of the gene remains active, the activity is reduced under ABA treatments which needed more detailed analysis on when and how the expression is reduced in Arabidopsis roots when they are subjected to longer period of exogenous ABA. For this purpose, Arabidopsis transgenic seedlings, carrying *CLO4* promoter fused to GUS reporter gene, 36 hours after germination were transferred to either control or ABA containing plates and monitored to evaluate long term effects of ABA on expression of *CLO4* gene. Expression of the GUS reporter

gene was evidently reduced 4 days post transfer in media complemented with 2µM ABA and at day 7 post transfer to 2µM ABA plates, the expression was reduced in Arabidopsis root system (Figure 5).

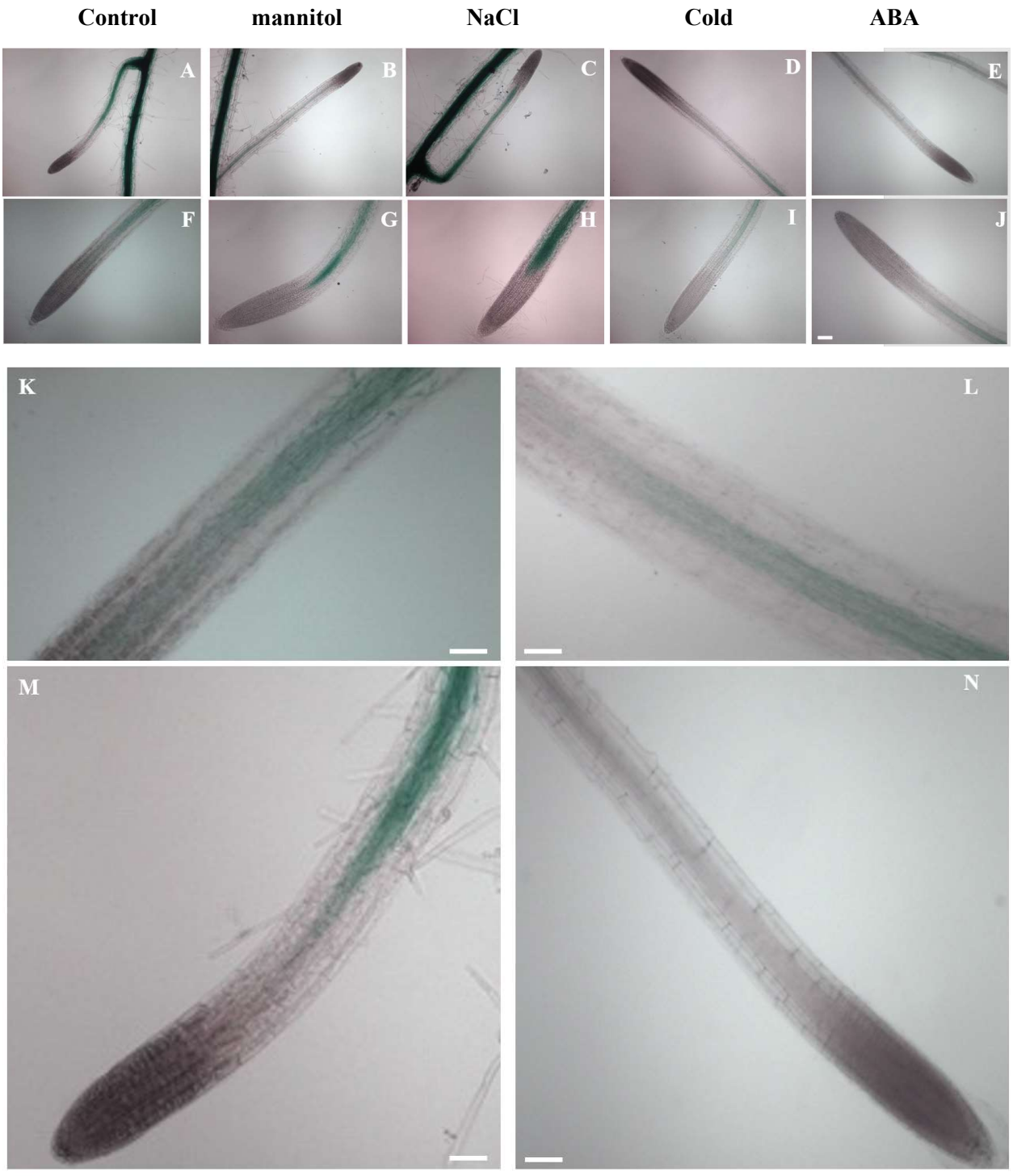
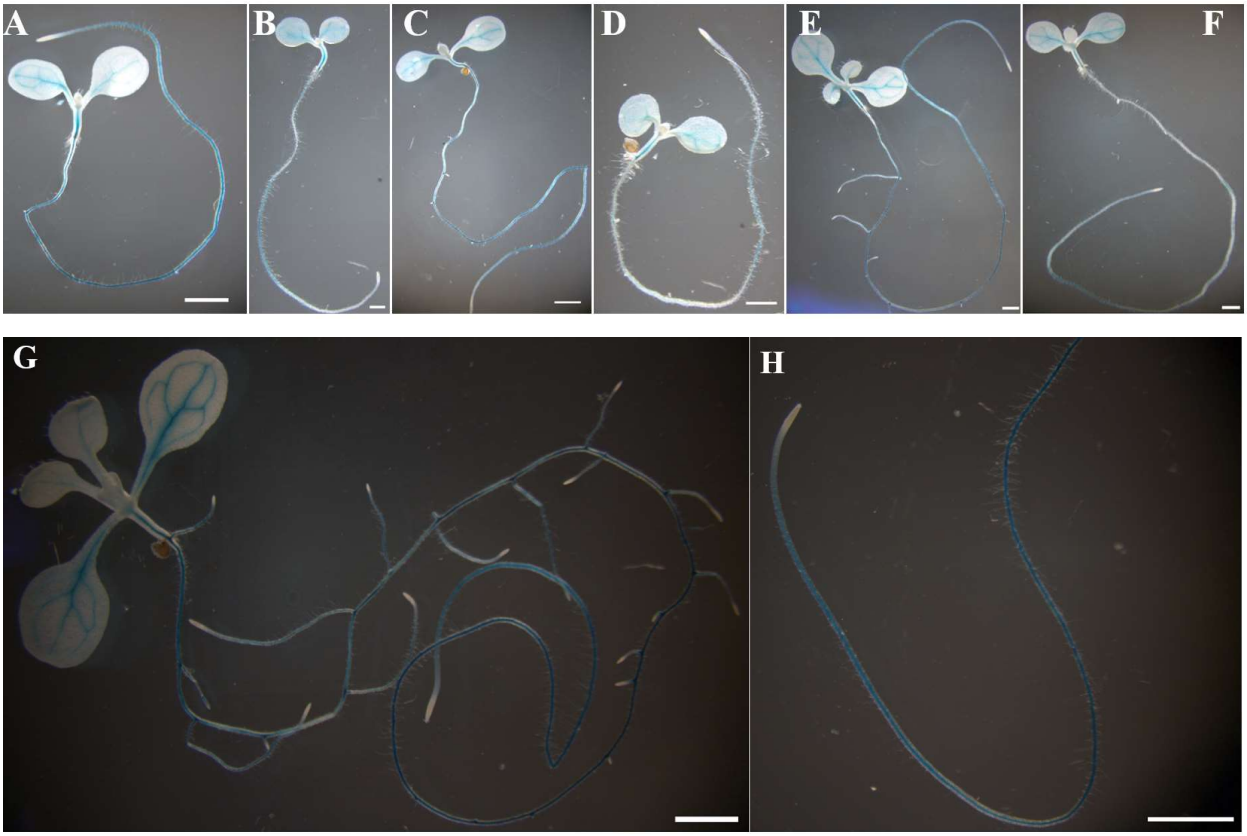


Figure 4. *CLO4* promoter activity of 10 day old Arabidopsis under different stress conditions after 6 hours of treatment. Tissue was stained to reveal GUS activity. **A.** Lateral Root under control condition **B.** Lateral Root under 300 mM mannitol after six hours of treatment **C.** Lateral Root under 150 mM NaCl after six hours of treatment **D.** Lateral Root after six hours of cold stress **E.** Lateral Root treated for six hours under 10 µM ABA **F.** Primary Root Tip Control. **G.** Primary Root Tip after six hours of 300 mM mannitol **H.** Primary Root Tip after six hours of 150 mM NaCl treatment **I.** Primary Root Tip under cold stress after 6 hours **J.** Primary Root Tip under 10 µM ABA for six hours. **K.** Enlarged image of the primary root under control condition. **L.** Enlarged image of the primary root under 10 µM ABA after six hours. **M.** Enlarged image of the lateral root under control conditions. **N.** Enlarged image of the lateral root after 10 µM ABA treatment, for six hours. Scale bar is 100µM.



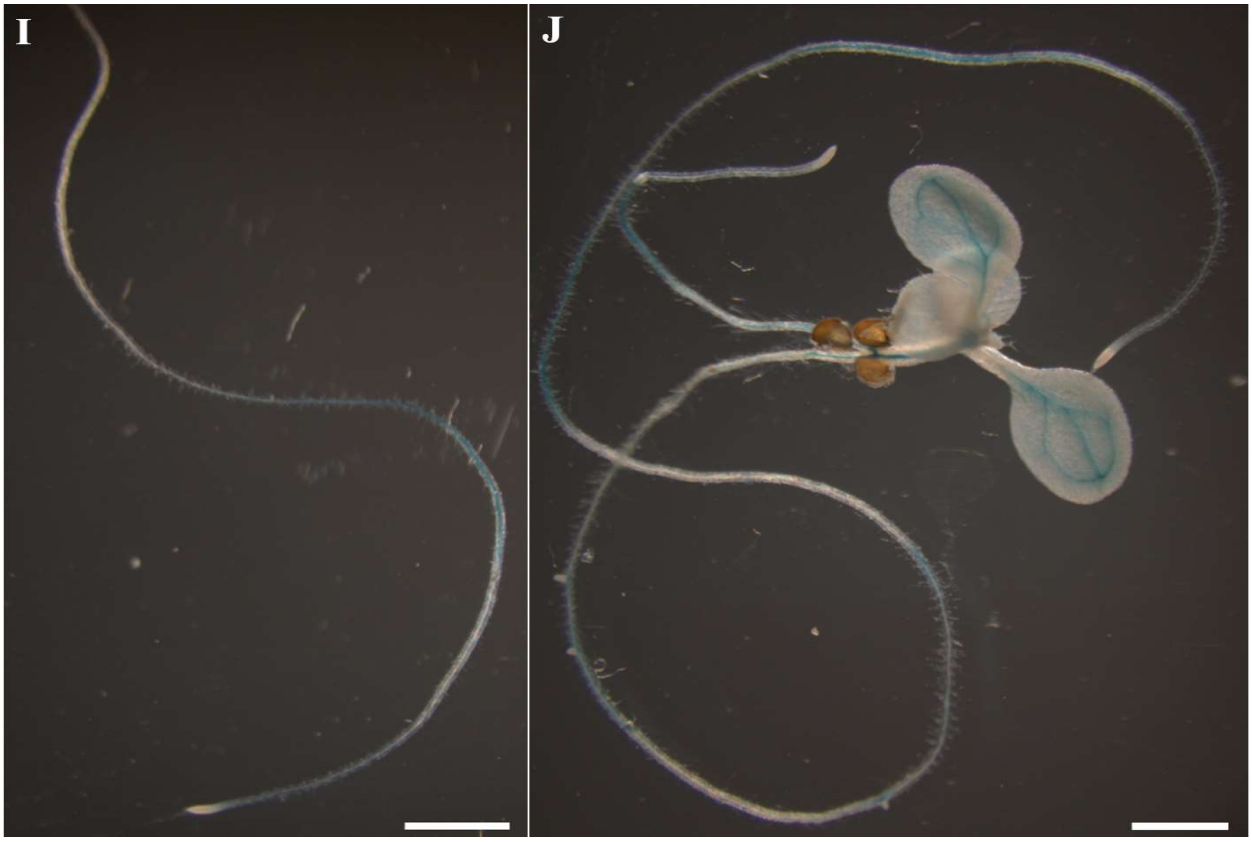


Figure 5. Promoter activity of *CLO4* gene under 2µM ABA stress and control condition. All plants were transferred to either control MS-Sucrose plates or MS-sucrose plates complemented with 2µM ABA 36 hours post germination. **A)** Arabidopsis plant 4 days post transfer to control plates. **B)** Arabidopsis plant 4 days post transfer 2µM ABA. **C)** Arabidopsis plant 5 days post transfer control. **D)** Arabidopsis plant 5 days post transfer 2µM ABA. **E)** Arabidopsis plant 6 days post transfer control. **F)** Arabidopsis plant 6 days post transfer 2µM ABA. **G & H)** Arabidopsis plant 7 days post transfer control. **I & J)** Arabidopsis plant 7 days post transfer to 2µM ABA. The scale bar represents 1mm.

2.4.3 Lateral Root Morphology

Arabidopsis lateral root morphology was assayed by evaluating lateral root length, number of lateral roots and lateral root elongation of each mutant line. Arabidopsis seedlings germinated on control MS media were transferred 36 hours post germination to either control plates or MS plates complemented with 2µM ABA. Morphology of the lateral roots was assayed for both ABA treated and control plants 10 days post transferring the seedlings.

The wild type Arabidopsis plant showed inhibition of the lateral root elongation in response to ABA treatment, showing reduction in total number of lateral roots, the number of lateral roots that grew more than 1 cm and consequently reduction of total lateral root length. The *clo4* mutant line did not show any significant difference compared to the wild type plants under both control and ABA treatments however over expression of *CLO4* gene led to a significant reduction in number of lateral roots that grew longer than 1 cm and total lateral root length under control conditions. Although single mutant line of *gpa1* did not show any significant difference compared to wild type plant, the *clo4 gpa1* double mutant Arabidopsis showed significant reduction in the number of lateral roots longer than 1cm and consequently reduced total lateral root length under control condition. The *clo4 gpa1* double mutant line also showed less reduction of length of the lateral roots in response to ABA treatments. The *CLO4* overexpressing line had a phenotype similar to the *clo4 gpa1* double mutant, i.e. it showed reduced lateral root development under control conditions and less of a reduction in lateral root development in response to ABA treatment (Figure 6).

It is unclear if *CLO4* and *GPA1* act in the same pathway or parallel signalling pathways. The observed phenotype for the single and the double mutant *clo4* and *gpa1* can suggest that the genes act in parallel pathways, or with the genes acting in the same pathway but with each single mutation resulted in only small and statistically insignificant effects.

Total number of lateral roots were not different in the different genotypes assayed in this study. The mutant lines and the *CLO4* over-expressing line did not show diffeent number of lateral roots from the WT under both control and ABA treatment conditions. The contrast between the counts for total roots and roots longer than 1 cm indicates that CLO4 and GPA1 are involved in lateral root elongation rather than formation of lateral roots or primordia. The results obtained for the number of lateral roots for the *gpa1* mutant line in this study agrees with the previous report by Pandey et.al (2006) and not Chen et al. (2006).

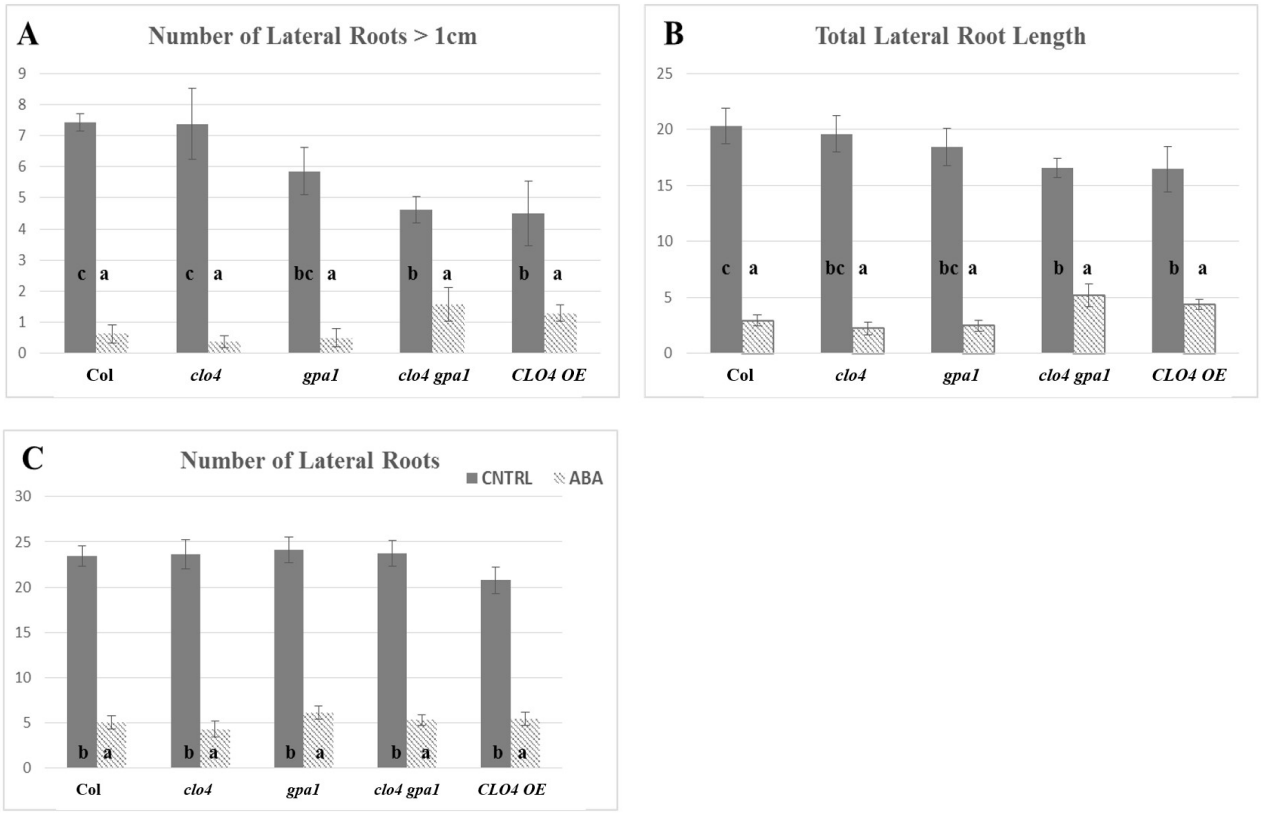


Figure 6. Effect of *clo4* and *gpa1* on lateral root architecture after 10 days of ABA treatment. A) Number of lateral roots longer than 1cm under control conditions or after 2 µM ABA treatment for 10 days. **B)** Total lateral root length for each genotype under control conditions and 2µM ABA treatment. **C)** Total number of lateral roots 10 days post transfer. The error bars indicate standard error of the mean. The a,b,c letters indicate the Duncans multiple range test. Bars that do not share a same letter are significantly different.

Table 6. Pairwise comparison of WT, mutant and *CLO4* overexpression lines for lateral roots in response to ABA treatment. *P-values* from two-way ANOVA. The *p values* ≤ 0.05 was considered statistically significant and highlighted on the table.

Genotypes	Total Number of Lateral Roots			Total Lateral Root Length			Number of Lateral Roots >1cm		
	Genotype	Treatment	Genotype *Treatment	Genotype	Treatment	Genotype *Treatment	Genotype	Treatment	Genotype *Treatment
WT- *clo4*	0.810	0.000	0.681	0.578	0.000	0.998	0.831	0.000	0.890
WT- *gpa1*	0.370	0.000	0.840	0.312	0.000	0.538	0.134	0.000	0.199
WT - *clo4 gpa1*	0.772	0.000	0.986	0.470	0.000	**0.006**	0.088	0.000	**0.001**
WT - *CLO4 OE*	0.306	0.000	0.161	0.392	0.000	0.060	0.113	0.000	**0.015**
clo4 - gpa1	0.328	0.000	0.577	0.704	0.000	0.559	0.330	0.000	0.252
clo4 gpa1 - CLO4 OE	0.233	0.000	0.191	0.740	0.000	0.793	0.761	0.000	0.905
CLO4 OE - clo4	0.505	0.000	0.118	0.737	0.000	0.068	0.236	0.000	**0.027**
clo4 gpa1 - gpa1	0.583	0.000	0.842	0.695	0.000	**0.039**	0.878	0.000	**0.036**
clo4 gpa1 - clo4	0.639	0.000	0.713	0.972	0.000	**0.011**	0.266	0.000	**0.008**

2.4.4 Primary root morphology

The effect of mutations and ABA responses among the Arabidopsis mutant lines used in this study was also evaluated for the primary root growth. The Columbia wild type plant, as well as all mutant and transgenic lines, showed reduction in primary root length under ABA treatment. The *clo4* mutant line did not show any difference from the wild type plant under both control and ABA treatments however the *CLO4 OE* showed statistically significant reduction of primary root length under ABA treatment. The *gpa1* mutant line also showed significant reduction of primary root length after ABA treatment and the double mutant *clo4 gpa1* showed a phenotype similar to the *gpa1* single mutant. The response observed for the double mutant *clo4 gpa1* was not different than the single mutants.

The mutant lines showed different percent reduction of primary root in response to ABA. The Columbia wild type showed 37.5 percent reduction in primary root length under ABA treatment. The *clo4* mutant line showed 43.66 percent reduction in primary root which is only about 6 percent more reduction than the WT and the observed difference is statisticaly insignificant and less of a reduction than the other mutants and the CLO4 over-expressing line. The *gpa1* mutant line showed significant reduction of 52.41 percent and the result agrees with Pandey et al.'s (2006) previous report for the percent reduction of primary root length in response to ABA for the Gα mutant. The *CLO4* over expressing and the *clo4 gpa1* double mutant lines showed the same trend as the *gpa1* single mutant line by showing 63.73 and 56.83 percent reduction in primary root length in response to ABA. The results also showed that the *clo4* mutant line and *CLO4* over expressor line have different phenotypes (Figure 7).

A B

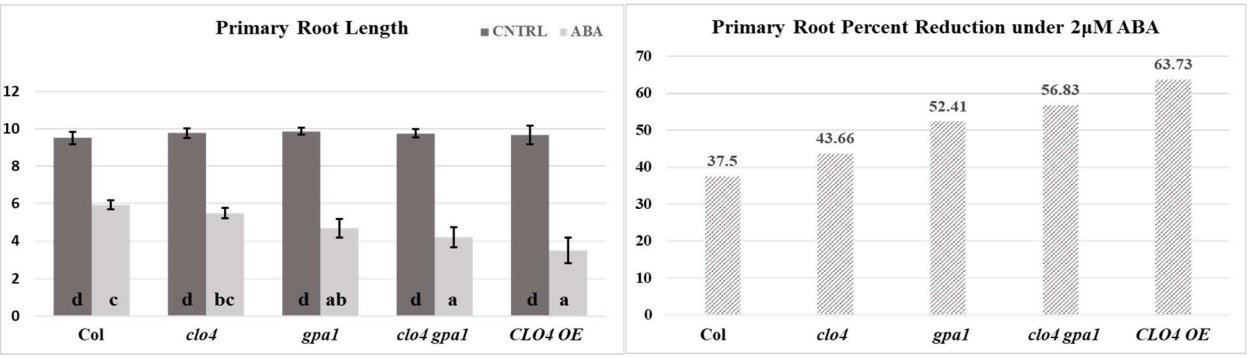


Figure 7. Effect of *clo4* and *gpa1* on Primary root length in response to ABA treatment. A. Effect of primary root length in response to ABA treatment for *gpa1* and *clo4* single and double mutants and *CLO4* over-expressing Arabidopsis lines in response to 10 days of ABA treatment. **B.** Percent reduction of primary root length in response to 2µM ABA. The error bars indicate standard error of the mean. The a,b,c letters indicate the Duncans multiple range test. Bars that do not share a same letter are significantly different.

Table 7. Pairwise comparison of WT, mutant and *CLO4* overexpression lines for primary root length in response to ABA treatment. P-values from two-way ANOVA. The *p values* ≤ 0.05 was considered statistically significant and highlighted in the table.

Genotypes	Genotype	Treatment	Genotype* Treatment
WT - *clo4*	0.763	0.000	*0.232*
WT - *gpa1*	0.203	0.000	***0.028***
WT - *clo4 gpa1*	0.049	0.000	***0.010***
WT - CLO4 OE	0.019	0.000	***0.009***
clo4 - gpa1	0.294	0.000	*0.194*
clo4 gpa1 - CLO4 OE	0.432	0.000	*0.547*
CLO4 OE - clo4	0.031	0.000	***0.050***
clo4 gpa1 - gpa1	0.480	0.000	*0.641*
clo4 gpa1- clo4	0.077	0.000	*0.082*

2.4.5 Stomatal aperture

Stomatal aperture was measured for the single mutant lines of *clo4* and *gpa1*, the *clo4 gpa1* double mutant and *CLO4* Over-Expressing line, as well as the WT. Leaves of 3-4 weeks old Arabidopsis plants were treated with stomatal opening solution followed by treatment with 5μM ABA for 2 hours. The wild type plants respond to ABA treatment with a significant reduction in stomatal opening. Although *clo4* mutant line had higher reduction in stomatal opening under ABA however the difference was not significantly different from the wild type in stomatal opening under control conditions, nor in the degree of stomatal closure in response to ABA treatment. The overexpression of *CLO4* led to significantly increased stomatal aperture in control conditions and the over expression of *CLO4* obliterated the ABA induced reduction of stomatal aperture. Both the *gpa1* mutant and the *clo4 gpa1* double mutant also has increased stomatal aperture and had a loss of ABA response; there were no differences observed between the double mutant and the *gpa1* single mutant. The comparison of the double mutants to the single mutants did not give insight to as to whether *GPA1* and *CLO4* act in the same or different signalling pathways (Figure 8).

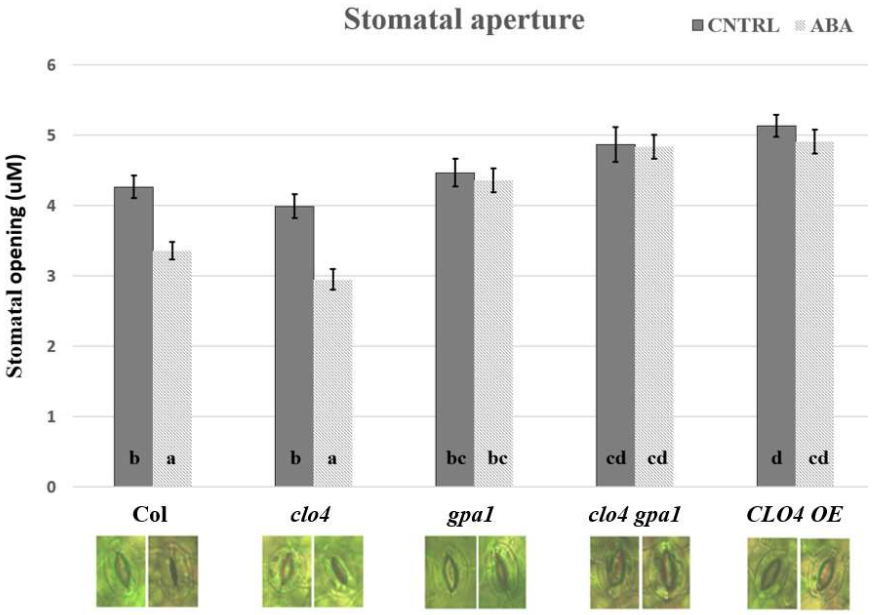


Figure 8. Effect of *clo4 and* and *gpa1* on stomatal aperture in response to ABA. Stomatal aperture was measured in detached leaves of different genotypes from 3-4 weeks old soil grown Arabidopsis after treatment with stomatal opening solution, and after subsequent 2-hour treatment with 5μM ABA. The error bars indicate standard error of the mean. The a,b,c letters indicate the Duncans multiple range test. Bars that do not share a common letter are significantly different while those that share the same letter are not significantly different.

Table 8. Pairwise comparison of WT, mutant and *CLO4* overexpression lines for stomatal aperture in response to ABA treatment. P-values are from two-way ANOVA. The *p values* ≤ 0.05 was considered statistically significant and highlighted in the table.

Genotypes	Genotype	Treatment	Genotype* Treatment
WT - *clo4*	0.024	0.000	*0.655*
WT - *gpa1*	0.001	0.005	***0.025***
WT - *clo4 gpa1*	0.000	0.010	***0.015***
WT - CLO4 OE	0.000	0.000	***0.027***
clo4 - gpa1	0.000	0.004	***0.007***
clo4 gpa1 - CLO4 OE	0.372	0.509	*0.613*
CLO4 OE - clo4	0.000	0.000	***0.011***
clo4 gpa1 - gpa1	0.041	0,746	*0.853*
clo4 gpa1- clo4	0.000	0.005	***0.007***

2.4.6 Stomatal Density

The wild type Columbia Arabidopsis showed reduction in stomatal density in response to ABA treatments. Mutation in *CLO4* and *GPA1* genes resulted in reduced stomatal density under control condition with less severe reduction of stomatal density under ABA than the WT. The double mutant *clo4 gpa1* showed the same phenotype as was observed for the single mutants of *gpa1* and *clo4*, that was a reduction of stomatal density under control condition and less severe reduction in stomatal density under ABA treatment than the WT. The double mutant analysis supports a model in which the genes act in the same pathway controlling stomatal development and ABA responses. The *CLO4* over-expressing Arabidopsis plants did not show a different phenotype than the wild type plant under control conditions while over expression of *CLO4* led to complete insensitivity to ABA; it showed no reduction of stomatal density. The opposite phenotypes observed for the *clo4* mutant and the *CLO4* over-expressing lines under control conditions and their responses to ABA treatment suggests that the gene increases stomatal density and plays a role in the reduction of stomatal density in response to ABA treatment (Figure 9A).

2.4.7 Stomatal Index

The wild type Arabidopsis plants have reduced stomatal index in response to ABA treatments. The *clo4* mutant line showed significant insensitivity to ABA reduction of stomatal index and the *gpa1* mutant line showed less severe reduction of stomatal index under ABA. The double mutant *clo4 gpa1* showed similar phenotype as that observed for the *gpa1* mutant line and the response to ABA did not differ from the *clo4* single mutant line suggesting that the genes act in the same pathway controlling stomatal index (Figure 9B).

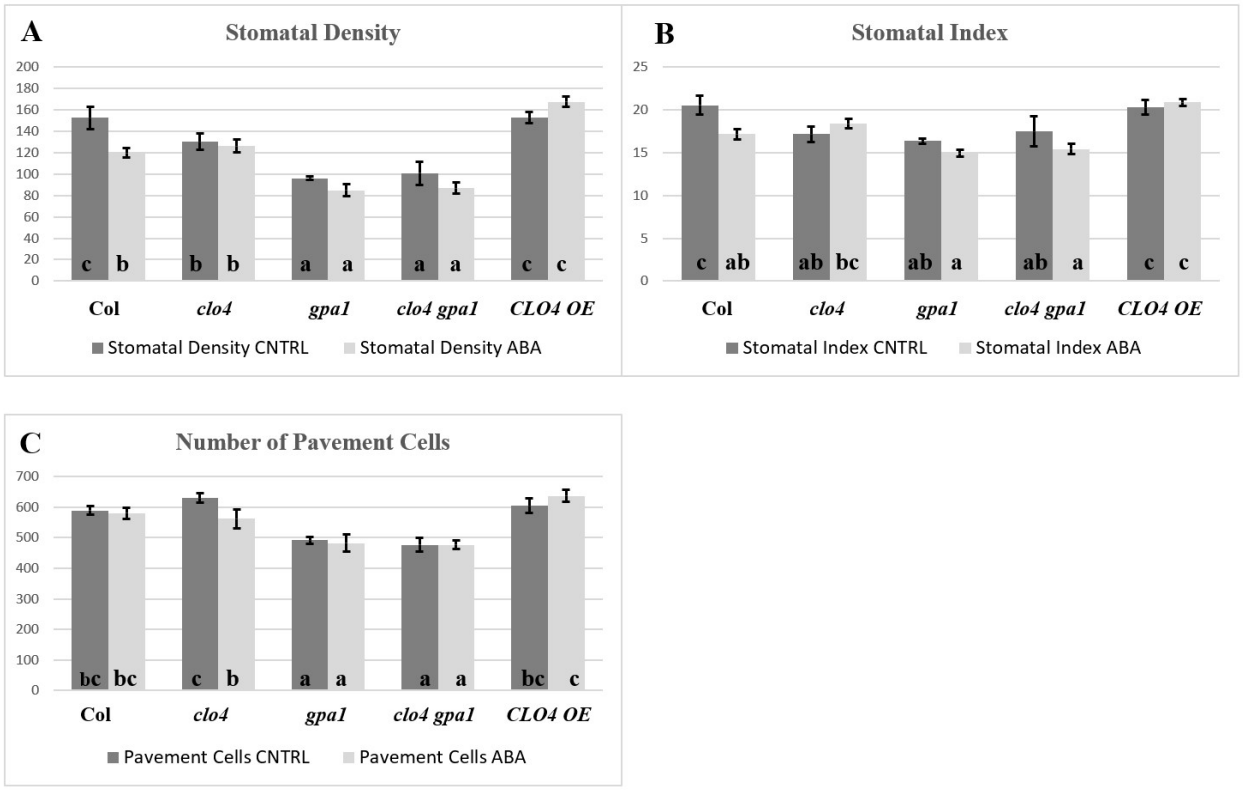


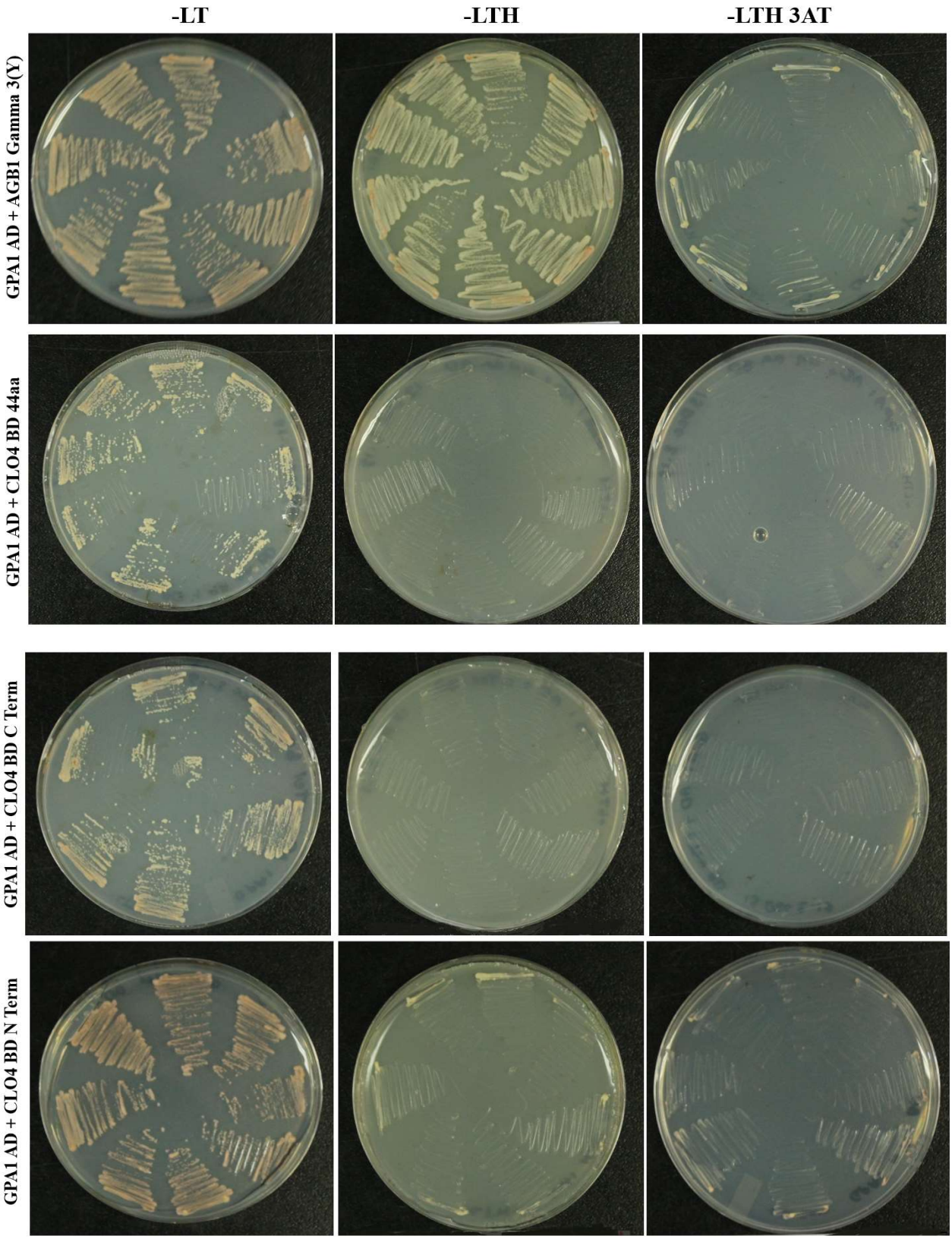
Figure 9. Effect of *clo4 and* and *gpa1* on stomatal density and index. First and second leaves from 15-day old soil grown plants treated 3 times per week with either 25µM ABA or control solution were TBO stained and stomata and pavement cells were counted. **A.** Stomatal Density. **B.** Stomatal Index. **C.** Total number of pavement cells. The error bars indicate standard error of the mean. The a,b,c letters indicate the Duncans multiple range test. Bars that do not share a common letter are significantly different while those that share the same letter are not significantly different.

Table 9. Pairwise comparison of WT, mutant and *CLO4* overexpression lines for stomatal density and index in response to ABA treatment. *P-values* are from two-way ANOVA. The *p values* ≤ 0.05 was considered statistically significant and highlighted in the table.

Genotypes	Stomatal Density			Stomatal Index		
	Genotype	Treatment	Genotype *Treatment	Genotype	Treatment	Genotype *Treatment
WT- *clo4*	0.290	0.022	0.068	0.202	0.201	**0.010**
WT- *gpa1*	0.000	0.003	0.107	0.000	0.002	0.152
WT- *clo4 gpa1*	0.000	0.010	0.263	0.042	0.023	0.558
WT- *CLO4 OE*	0.002	0.183	**0.002**	0.038	0.090	**0.018**
clo4 - gpa1	0.000	0.191	0.532	0.002	0.880	**0.035**
clo4 gpa1 - Clo4 OE	0.000	0.947	**0.045**	0.001	0.493	0.214
CLO4 OE - clo4	0.000	0.363	0.119	0.001	0.212	0.666
clo4 gpa1 - gpa1	0.392	0.071	0.840	0.417	0.084	0.739
clo4 gpa1 - clo4	0.000	0.253	0.525	0.226	0.703	0.138

2.4.8 Protein-protein interaction between either truncated versions of the CLO4 or GPA1

The results for the yeast two hybrid assay indicates that, neither the full length nor truncated versions of the CLO4 protein interact with the GPA1. Growth of the colonies on the double drop out plate indicates that the transformation was successful however the growth observed on the triple dropout plates did not differ from interaction of GPA1 fused to the AD domain of GAL4 tested with empty vector pGBKT7 which indicates that neither the full length nor truncated versions of the CLO4 gene interact with GPA1 compared to the interaction of the GPA1 with AGB1 Gamma3 (γ). On the other hand, Truncated versions of GPA1 did not show interaction with CLO4 using BiFC assay.


-LT
-LTH
-LTH 3AT
GPA1 AD + AGB1 Gamma 3(Y)
GPA1 AD + CLO4 BD 44aa
GPA1 AD + CLO4 BD C Term
GPA1 AD + CLO4 BD N Term

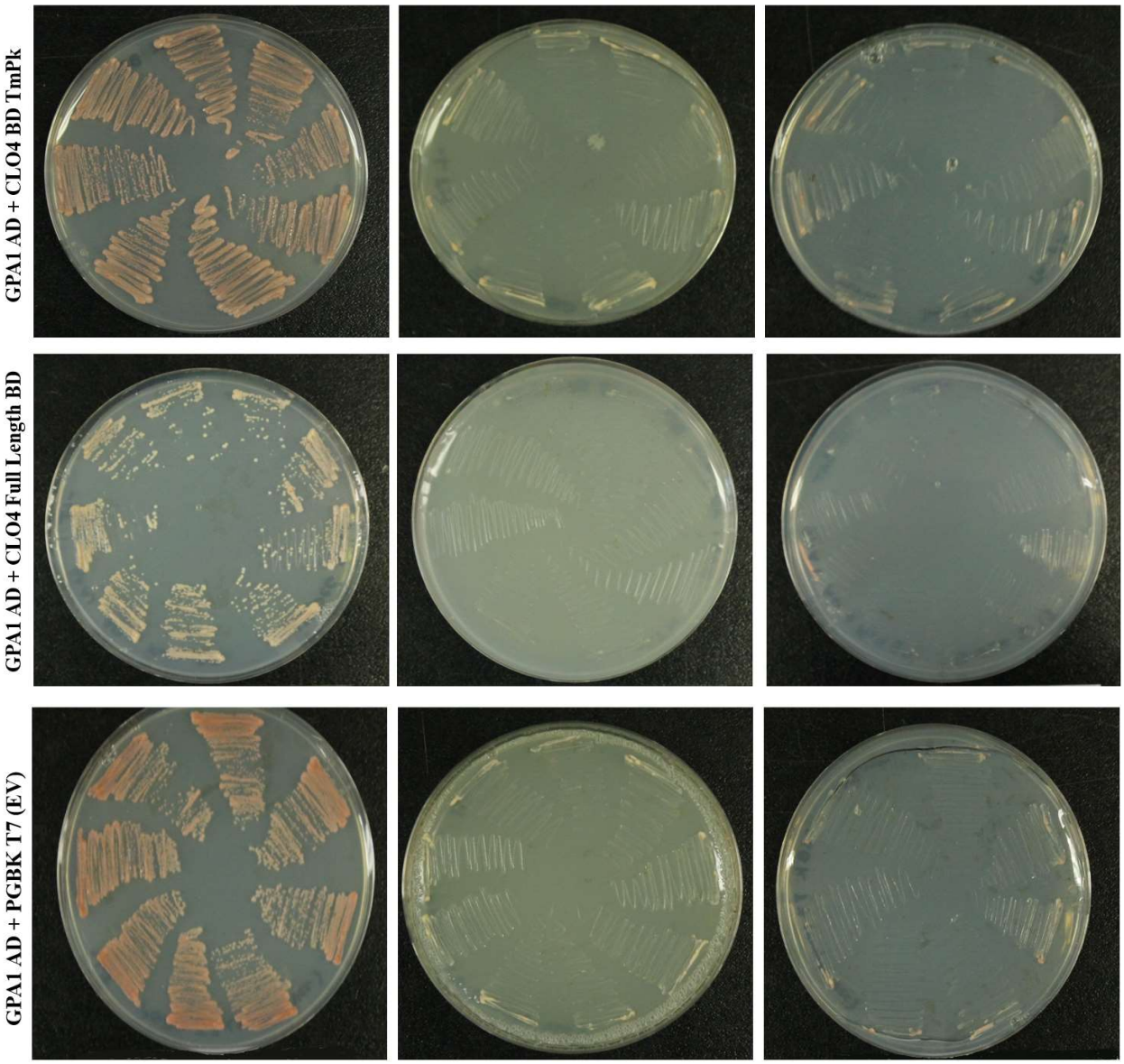
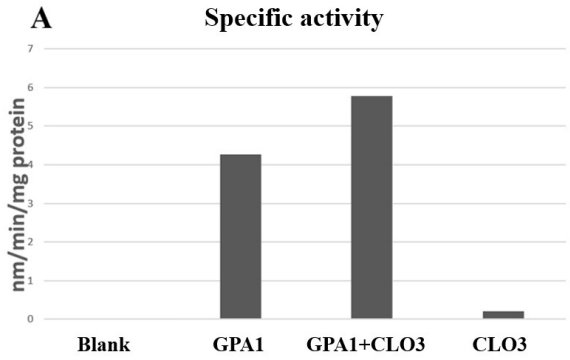


Figure 10. Yeast two hybrid experiment on truncated versions and full-length Arabidopsis CLO4 interaction assay with full length Arabidopsis GPA1. Different truncated versions of the CLO4 protein cloned in pGBKT7 vector are shown for interaction with the full length GPA1 cloned in pGADT7 using yeast strain AH109. Two versions of the N-terminal domain of the protein were used containing the calcium binding motif with either amino acids 1-44 (CLO4 BD 44aa) or N-terminal domain amino acids 1-79 (CLO4 BD N-terminal) which contained calcium binding motif, amphipathic alpha helix and the proline knot. The C-terminal domain construct included amino acids 80 to 195. The full length CLO4 construct, amino acid 1-195, was also assayed in parallel with the truncated versions.

2.4.9 Effect of Clo3/RD20 on intrinsic GTPase activity of GPA1

Both GPA1 and CLO3/RD20 proteins were expressed in *E. coli,* purified and used for an *in vitro* enzymatic assay to investigate possible GTPase activity of CLO3 toward GPA1. The intrinsic GTPase activity of GPA1 was measured and addition of RD20 to GPA1 did not affect GTPase activity of GPA1 that would be expected from a GAP. RD20 protein alone showed very low GTPase activity (Figure 11). In addition, the small increases in the Kcat value was very low and statistically insignificant showing that RD20 does not affect GTPase activity of GPA1.



B

Kcat for GTP hydrolysis (s-1)	
GPA1	0.005
GPA1 + RD20	0.006
RD20	0.000

Figure 11. CLO3/RD20 GTPase assay. A) Specific activity of GPA1 in presence and absence of CLO3. Specific activity of GPA1 in nm/min/mg of protein was shown to be increased for about 1.2-fold by addition of RD20/CLO3. The RD20/CLO3 protein was shown to have very low intrinsic GTPase activity when it was assayed independently. **B)** Kcat for GTP hydrolysis rate in presence of CLO3 and intrinsic GPA1 GTP hydrolysis. The Kcat for GTP hydrolysis showed that addition of RD20/CLO3 increases the value however the increased Kcat value was very low and statistically insignificant with the *p value* of 0.577

2.5 Discussion

2.5.1 Protein-protein interaction between CLO4 and GPA1

CLO4 protein was shown to physically interact with GPA1 using the BiFC *in vivo* assay and the interaction was localized to the plasma membrane. The interaction and localization of the two proteins in plant cells, about 43 hours post infiltration, in this study confirmed the previously reported interaction of the two proteins under longer incubation period (Rafeh 2016) under an improved protocol and with additional cellular markers. The previous work employed long incubation times of near 72 hours, to assure expression and visibility of the fluorescent fusion

protein, which might lead to mis localized signals. Tracking and early detection and the interaction of the two proteins between, CLO4 and GPA1, in this study (1) confirmed the reported interaction between the proteins, indicating that the observed signal was not due to over-expression of the proteins and possible non-specific assembly of the two halves of the YFP (2) precisely localized the interaction between GPA1 and CLO4 to the plasma membrane and not in tonoplast or ER. The plasma membrane and the vacuole membrane are adjacent to each other and the current work used higher levels of magnification and markers that could distinguish between the two membranes. Previous subcellular localization of full length CLO4 fused to GFP, showed that CLO4 is localized in the oil bodies in onion epidermal cells (Kim et al. 2011) and plasma membrane and tonoplast in *Nicotiana benthamiana* (Rafeh, 2016). On the other hand, the GPA1 protein has been reported to be localized in the plasma membrane (Huang et al. 2006). BiFC Assay did not show interaction of the truncated versions of GPA1 with full length CLO4 protein Figure 2. This finding can suggest that truncation of the GPA1 protein affects either proper folding pattern of the protein or affecting the important domain(s) for the interactions with CLO4 or just simply the truncated version gets degraded in the plant cells.

Truncation of GPA1 protein might not be the optimal approach for finding interacting domain with CLO4 particularly considering possible degradation of the truncated version in plant cells or improper folding pattern of the truncated versions. Neither the full length nor the truncated versions of the CLO4 protein were found to interact with full length GPA1 protein using yeast two hybrid assay; this might be due to presence of the transmembrane domain in CLO4 protein or degradation or misfolding of the truncated versions in yeast cells (Figure 1 & 10). It has been reported that GPA1 interaction with β and γ3 only grew on 3AT plates when the transmembrane domain of γ3 was removed by truncation (Chakravorty et al 2015). This can suggest that presence of the transmembrane domain in CLO4 protein might also interfere with the protein-protein interaction using yeast two hybrid assay by either mis-localizing the protein, interfering with protein folding pattern or solubility of the protein.

The interaction of the CLO4 protein with GPA1 is an important finding that led to further investigation of signaling pathways associated with ABA and its relationship with subunits of the heterotrimeric G protein complex since both CLO4 and GPA1 have been shown to play a role in the ABA signalling pathway. Previous reports on the role of GPA1 and CLO4 in developmental and physiological responses in plants indicated candidate characteristics to investigate the

interaction of GPA1 and CLO4. The *gpa1* mutant Arabidopsis has been reported to have higher percent reduction of primary root in presence of ABA (Pandey et al. 2006) and to be insensitive to ABA inhibition of stomatal opening (Wang et al. 2001; Perfus-Barbeoch et al. 2004). The *clo4* mutant line was reported to have greater stomatal closing in response to ABA (Kim et al. 2011). In addition to the morphological analysis of the *clo4* and *gpa1* mutant lines, the genes also have been reported to be negative regulators of certain ABA responsive genes; *ABI3* and *ABI5* transcripts have been reported to be up-regulated in *gpa1* mutant line (Pandey et al. 2006) and to have higher expression of *ABF3, ABF4* and *ABI1* in the *clo4* mutant line under ABA treatment conditions (Kim et al. 2011).

2.5.2 Histochemical GUS analysis of promoter activity of the *CLO4* gene

Promoter activity of the *CLO4* gene was monitored under multiple stress conditions by focusing on expression of the gene in primary and lateral roots. Compared to the control plants, the promoter activity was reduced in both lateral roots and primary root tips after six hours on media with 10 μM ABA as well as 7 days post transfer to 2μM ABA. This study showed that ABA downregulates and represses expression of *CLO4* gene in Arabidopsis root tissues by monitoring promoter activity of the gene under ABA stress condition. For plants grown under 2μM ABA for seven days post transfer, low activity of the promoter was observed in the root system compared to the plants under control condition of the same age which did show expression of the gene in vascular bundles. The result concludes transcriptional regulation of *CLO4* in root system under ABA treatment. The GUS reporter assay represented in this study complements the previously reported transcriptional downregulation of *CLO4* gene expression in wild type plant leaves under ABA (Kim et al. 2011), however the reported downregulation of the gene characterized expression in leaf tissues while the GUS assay in this study was focused on expression of *CLO4* in primary and lateral root tissues. Finding of this study and the previous report on ABA regulation of *CLO4* gene suggests that under elevated ABA, *CLO4* gene is downregulated in multiple plant organs. Under control condition, tissue specific expression of *CLO4* promoter fused to GUS reporter gene in this study agrees with Kim et al. (2011) and contradict with the previous report from our lab (Rafeh, 2016) with no promoter activity of the gene under control condition. The heterozygosity of the transgenic line with the *pCLO4:GUS*

construct used in the previous study and segregating plants may have lead to erroneous conclusions.

Previous report on immunolocalization of GPA1 showed that, GPA1 is present in most of Arabidopsis developmental stages and organs including primary and lateral roots (Weiss et al. 1993) and the gene had higher expression in Arabidopsis root system (Chen et al. 2006) which can suggest that the interaction of the two protein by the ectopic expression of GPA1 and CLO4 in *N. benthamiana* leaf epidermis can be relevant to tissues where the two genes are expressed in Arabidopsis.

2.5.3 Morphological analysis

Stomatal density analysis suggests that both *CLO4* and *GPA1* are in the same regulatory pathway with partial decrease of stomatal development in response to ABA. Although the double mutant of *clo4 gpa1* showed similar phenotype as single mutants *gpa1* and *clo4,* the phonotype was closer to *gpa1* single mutant Arabidopsis. The observed phenotypes indicate that both *GPA1* and *CLO4* play a role in establishing the stomatal density found in the WT and in decreasing the stomatal density in response to ABA treatment. The finding in this study indicates that CLO4 and GPA1 are both positive regulators of stomatal development and reduction of stomatal density in response to ABA in Arabidopsis. Results obtained in this study can suggest that, ABA negatively regulates *CLO4* which positively regulates GPA1 that in turn induces stomatal formation and development (Figure 12). The non-ABA treated *clo4* mutant Arabidopsis showed a phenotype that was similar to the Columbia wild type Arabidopsis under ABA treatment. The observed phenotype suggests that ABA reduces stomatal development through reduction of *CLO4* transcript in Arabidopsis leaves. The observed changes in stomatal density and index in the *gpa1* mutant line in this study agrees with that reported by Nilson and Assmann (2010). The observed changes in stomatal density in this study contradict previous report from our lab with no changes for *clo4* under both control and ABA treatment compared to WT which was performed on a different *clo4* mutant line while lower stomatal index observed in this study agrees with the previous report. (Rafeh 2016).

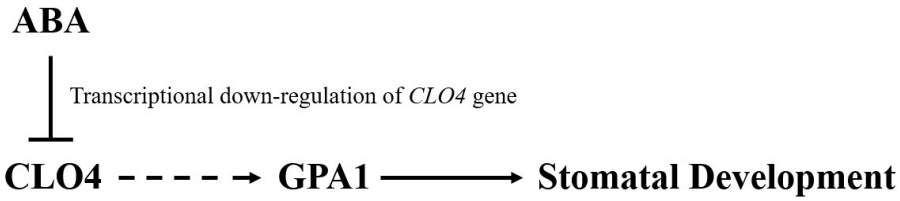


Figure 12. Proposed pathway for Stomatal development through heterotrimeric G protein complex.

The single mutations in neither *gpa1* nor *clo4* caused complete inhibition of lateral root development or elongation however the double mutant, *clo4 gpa1* had significantly fewer lateral roots that grew more than one centimetre than that of the wild type. This can be interpreted as additive effects of the two mutations and an indication that the genes act in different pathways. However, the physical interaction of the proteins *in vivo* can suggest that the genes act in the same pathway, and since neither mutation gives a null phenotype, their mutations may have additive effects while still acting in the same pathway. The results in this study indicate that the genes are not the only determinants of the lateral root elongation and the inhibition of lateral root elongation in response to ABA treatment. Since *CLO4* is a member of the caleosin gene family, a calcium binding gene family with six more members in Arabidopsis, there is a possibility that the phenotype of a single mutant caleosin is being partially rescued by other members of the gene family that also interact with GPA1. The root morphology for the *clo4* mutant line observed in this study agrees with previous report from our lab for both primary root and lateral roots (Rafeh 2016).

Considering GUS analysis that showed that ABA downregulates expression of *CLO4* gene in Arabidopsis root system, and that the two proteins, GPA1 and CLO4, physically interact and only the double mutant affected the lateral root elongation, it suggests that interaction of the two proteins could be the determinant of activation of downstream effectors of either the Gα subunit or by the Gβγ which is dissociated from the trimeric complex. It has been previously shown that the Gβγ dimer is negative regulator of Arabidopsis lateral root formation and the GTP bound GPA1 is positive regulator of cell division in primary root meristem (Chen et al. 2006). This study showed that *CLO4* and *GPA1* gens are not involved in lateral root initiation but play a role in regulation of lateral root elongation. GPA1 and CLO4 were shown to inhibit the elongation step more likely inhibitory mechanism toward meristem activation and inhibition

of lateral root elongation than in lateral root initiation. Formation of lateral roots in Arabidopsis is divided to five main steps; initiation of lateral root, formation of primordia, emergence, meristem activation and elongation and it is known that ABA inhibits meristem activation through mechanisms that include activation of *ABI5* and *ABI4* genes (Harris 2015).

It is also possible that each mutant line had effect on elevated expression of the ABA responsive genes that were not sufficient to cause statistically significant changes in the phenotype and response to ABA and the double mutation caused increased levels of expression of multiple ABA responsive genes causing the observed phenotype. Both GPA1 and CLO4 are negative regulators of ABA responsive genes; *gpa1* mutant Arabidopsis showed elevated levels of *ABI3* and *ABI5* transcripts (Pandey et al. 2006) and *clo4* mutant showed higher expression of *ABF3*, *ABF4* and *ABI1* under ABA treatment conditions (Kim et al. 2011).

Since *clo4* mutant line was not different than wild type for the percent reduction of primary root under ABA and stomatal aperture, the double mutant analysis cannot give insight to whether *GPA1* and *CLO4* act in the same or different pathways in regulation of the primary root elongation and stomatal aperture. Although the mutation in *clo4* did not affect the phenotype, over expression of *CLO4* showed higher percent reduction of primary root than wild type and increased stomatal aperture which also caused insensitivity to ABA induced reduction of stomatal aperture. The phenotypes observed for the *CLO4* over expressing Arabidopsis in this study compared to the *clo4* mutant line suggest that, caleosins other than *CLO4* may be affecting the traits. Over-expression and the mutation of *clo4* do not always show the opposite effects which suggests *CLO4* mimics the effects of other members of the gene family. The increased insensitivity of the *CLO4* overexpressing line under ABA treatment agreed with the previous report by Kim et al. (2011) however, the results obtained for the *clo4* mutant which showed the same response under ABA as was observed for the WT plant is not consistent with the previously reported increased ABA sensitivity of *clo4* mutant line (Kim et al. 2011). It has been reported that GPA1 is inhibitor of K^+ influx (Wang et al. 2001) and the insensitivity of ABA inhibition of stomatal opening for *gpa1* mutant plants observed in this study is consistent with that reported by Wang et al. (2001).

The results obtained from the potential GTPase accelerating activity of CLO3/RD20 toward GPA1, showed that this protein does not accelerate the GTPase activity of GPA1. The result would suggest different role of caleosins in regulation of the heterotrimeric G protein

complex other than GAP activity toward the α subunit. The Kcat value for GPA1 in presence of RD20 was 0.006 compared to Kcat value of 0.005 of GPA1 alone which is about 1.2 fold change compared to the RGS1; the previously reported GTPase accelerating protein which increases the GTPase activity of GPA1 by more than ten-fold (Willard & Siderovski 2004). In addition, the change in Kcat was not statistically significant, concluding that RD20 is not a GTPase accelerating protein toward GPA1. Moreover, the morphological analysis of *clo4* mutant line, double mutant *gpa1 clo4* and the *CLO4 OE*, in this study does not support negative regulation of GPA1 through interaction with CLO4 protein nor suggest that CLO4 acts as a GAP for GPA1.

Chapter 3

Characterization of Members of the Extra-Large G Protein Family in *Triticeae*

3.1 Abstract

Arabidopsis thaliana's genome contains three genes encoding Extra-Large G proteins named *XLG1*, *XLG2* and *XLG3*. Members of the Extra-Large G protein in Arabidopsis are reported to be involved in plant's biotic and abiotic stresses and to interact with the GβGγ subunits. The wheat, *Triticum aestivum,* hexaploid genome contains three genes encoding *Xlg* proteins per haploid genome with three homeologous copies of each gene in the hexaploid genome. This study annotated and confirmed coding sequences for all nine copies of the *Xlg* gene family members in *Triticum aestivum*. All coding sequences showed about 97% nucleotide sequence identity among homeologous copies of the gene. Tissue specific expression analysis using a bioinformatics approach showed expression of all members of the *Xlg* gene family in *Triticum aestivum* seed, root, stem, inflorescence and leaf. Different members and the homeologous copies of the members of the *Xlg* gene family in *Triticum aestivum* showed differential responses to biotic and abiotic stresses such as cold, drought, heat and *Fusarium graminearum* infection.

3.2 Introduction

The genome of *Arabidopsis thaliana* encodes only five core subunits of the heterotrimeric G protein complex; one Gα, *GPA1*, one Gβ, *AGB1*, and three Gγ's; *AGG1, AGG2* and *AGG3* (Urano et al. 2013). Presence of only one Gα subunit in the G protein complex in Arabidopsis and the proposed non-classical signaling model through the G protein complex and low GTP hydrolysis rate in addition to the GPCR-independent activation of Gα (Urano et al. 2012), have raised many questions regarding the signaling through the plant heterotrimeric G protein complex and the differences between heterotrimeric G protein signaling in plants and animals. One of these differences is the presence of only a single gene encoding a Gα subunit in many plant species whereas animal genomes contain many more, for example the human genome contains 23 genes encoding Gα subunits (Chakravorty et al 2015). Arabidopsis genome encodes three Extra-Large GTP binding proteins; *XLG1*, *XLG2* and *XLG3* which share some sequence similarities with the Gα subunit, GPA1 (Chakravorty et al. 2015) even though they are more than double the size of the Gα subunit. The genomic sequence of Arabidopsis heterotrimeric G protein α subunit, GPA1 has a 1152 bp CDS which translates 383 amino acids proteins with the molecular weight of 44,545.5 Daltons (Tair website; https://www.arabidopsis.org/). In contrast, the Extra-Large G protein 1 XLG1 gene (AT2G23460.1) has a 2667 CDS that codes for 888 amino acids protein with molecular weight of 98,795.2 Daltons (Tair website; https://www.arabidopsis.org/). Arabidopsis Extra Large G proteins 2 and 3 encode proteins of 861 and 848 amino acids respectively. Structurally, the three Arabidopsis Extra-Large G proteins share sequence similarities with GPA1 in their C-terminal domain also referred to as Gα like region; responsible for GTP binding and hydrolysis. Their N-terminal domain have putative Nuclear Localization signals (NLS) (Ding at al. 2008; Chakravorty et al. 2015; Heo et al. 2012). *In vitro* GTP binding and hydrolysis assays showed that XLG proteins bind and hydrolyze GTP though they lack specific conserved amino acids found in the GTP hydrolysis sites of mammalian G proteins. These binding and catalytic activities require Ca^{2+} while GPA1 and other Gα's have the Mg^{2+} cation as their preferred divalent ion required for GTP binding and hydrolysis (Heo et al. 2012).

The first Arabidopsis Extra Large G protein, *XLG1*, was cloned and reported in 1999 and showed high sequence similarities to other eukaryotic Gα's and expression in most plant tissues including roots and aboveground tissues (Lee & Assmann 1999). The Extra-Large G proteins are

shown to be involved in the G protein signaling by interacting with the GβGγ subunits; the Extra-Large G protein 3 which is structurally the closest XLG protein to GPA1, was shown to compete with GPA1 in binding to GβGγ dimer in yeast three hybrid assay (Chakravorty et al. 2015). The protein encoded by another member of the gene family, XLG2, has also been shown to interact with the GβGγ dimer in Arabidopsis and the interaction was localized in the plasma membrane (Maruta et al. 2015).

Despite presence of Gβ and Gγ subunits of the heterotrimeric G protein complex in the non-vascular plant *Physcomitrella patens,* the genome of the moss does not code for a Gα subunit and instead contains an Extra Large Gα protein (Hackenberg et al. 2016) which can also highlight the role of Extra Large G proteins in heterotrimeric G protein complex signaling. The null mutants of Arabidopsis plants in the core subunits of the G protein complex and XLG proteins added other evidence for the role of XLG proteins in heterotrimeric G protein signaling. The Arabidopsis *gpa1* mutants showed decreased in stomatal density (Nilson and Assmann 2010) while *agb1* mutant showing increased number of stomatal cells on leaf epidermis (Urano et. al. 2013). The *xlg1/2/3* triple mutant has higher stomatal density compared to the wild type Arabidopsis (Chakravorty et al. 2015) and the mutant showed similar phenotype to that of *agb1* mutants by having higher stomatal densities compared to the wild type plant. The Arabidopsis *xlg1/2/3/gpa1/agb1* also had higher number of stomatal cells compared to wild type plants which suggests the role of XLG proteins as negative regulators of stomatal density (Roy Choudhury et al. 2020). The mutant analysis of *xlg* mutant lines also showed that, mutations in genes encoding Extra Large G proteins and the GβGγ dimer caused similar changes to the responses to fungal and bacterial pathogens; the GβGγ mutants and *xlg* mutant lines showed more susceptibility to the pathogens *Fusarium oxysporum, Alternaria brassicicola* and *P. syringae* (Maruta et al. 2015).

The mutants of the Gα subunit, have shown altered phenotypes however it has been recently suggested that there is a possibility that the observed phenotypes for the Gα mutants could be the consequence of non-Gα bound GβGγ and interaction of GβGγ with XLG proteins which adds to the complexity of the G protein signaling in plant (Hackenberg et al. 2016). XLG proteins are involved in Arabidopsis salt stress response by interacting with the GβGγ dimer and enhancement of plant growth under NaCl stress (Liang et al. 2017). In addition to interaction of the XLG proteins with subunits of the heterotrimeric G protein complex, the interactome of

Extra-Large G proteins showed that the proteins encoded by the three members of the Arabidopsis XLG protein gene family interact with about 70 other proteins using yeast two hybrid or the Bimolecular Fluorescent Complementation assay (Liang et al. 2017). In addition to the interaction with the β subunit of the heterotrimeric G protein complex, all three members of the XLG proteins in Arabidopsis were shown to interact with PUB2 and PUB4 which are Plant U-Box protein (PUB) E3 ligases involved in cytokinin signaling pathway (Wang et al 2017). Interaction of the members of the Extra-Large G proteins with members of the G protein complex has been also reported in other plants. In rice the Extra-Large G protein 2, PXLG2, is the only XLG protein that interacts with the RGB1; the beta subunit of the heterotrimeric G protein complex in a yeast two hybrid assay (Cui et al. 2020).

3.3 Purpose of the study

The objectives of this study are to characterize the members of the gene family encoding the Extra-Large G proteins in *Triticum aestivum* by the aid of available sequence databases and to confirm the coding sequences, exon/intron junctions, and gene annotations for each *Xlg*, by comparison to TSA and EST databases in NCBI. The other objective of the study is to assay expression levels of the genes in *Triticum aestivum* in different tissues and in response to different biotic and abiotic stresses using RNA-Seq databases. This study will also analyze evolutionary relationships of XLGs among other monocots such as *Oryza sativa, Sorghum bicolor* and *Brachypodium distachyon*. Findings of this study will expand understanding of plant stress response at transcript level under biotic and abiotic stresses by evaluating expression level of *Xlgs* under cold, heat, drought stresses and *F. graminearum* infection using bioinformatic analysis.

3.4 Methods

3.4.1 Validation of Extra-Large G Protein sequences

The protein and the coding sequences for the three Arabidopsis Extra Large G proteins; *XLG1* (AT2G23460) , *XLG2* (AT4G34390) and *XLG3* (AT1G31930) were retrieved from Tair website (https://www.arabidopsis.org/) and used as initial sequences to search against *Aegilops tauschii* genome at the National Center for Biological Information (NCBI) by using both tblastn and nucleotide search with blastn restricted to transcriptome Shotgun Assembly (TSA) and *Ae.*

tauschii. This species is the diploid progenitor of the D genome of *T. aestivum.* The *Ae. tauschii* sequences for each Extra-Large G protein were then used to search in Ensembl Plant database for *T. aestivum* (https://plants.ensembl.org/Triticum_aestivum/Tools/Blast) to retrieve sequences for the D, A and the B copies of the genes from this hexaploid species. The *T. aestivum* sequences were verified using NCBI BLAST restricted to *T. aestivum* TSA database and then verified by EST sequences for coding sequences and their 3'UTR.

3.4.2 Expression analysis of XLG protein

The expression analysis was performed as previusly described by Brunetti et al. (2018). The RNA-seq libraries were retrieved from NCBI (https://www.ncbi.nlm.nih.gov/) by a search of the SRA database (Sequence Read Archive). RNA-SEQ datasets from libraries with replications were chosen and the IDs from SRA database were searched in EMBL-EBI (https://www.ebi.ac.uk/) to retrieve the FASTQ sequence files. The FASTQ sequences were converted to FASTA using FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/links.html). Due to high similarities in the coding sequences of the homeologs of *Xlg* protein genes, the 3' UTR sequences from each homoeolog, either with or without portions of the 3' end of the CDS, were used for CD-HIT-EST-2D for clustering sequence data (Fu et al. 2012). The 3′ UTR sequences were retrieved from the gene sequences on Ensembl Plant and corresponding EST sequences in the NCBI *T. aestivum* EST database were used to confirm the sequence to be used for expression analysis. The CD-HIT-EST-2D for clustering sequences with cut off of 99% identity was used to identify hits in the RNAseq libraries. The numbers of hits for each gene was normalized for the length of the query sequence and the size of each RNAseq library to the RPKPM format (Reads Per Kilobase Per Million) for comparison. The selected libraries had either two or three replicates. More details on the RNA-seq libraries used in this study is available at Brunetti, et al. (2018).

3.4.3 Statistical analysis

The statistical analyses for experiments showing differences between stress responses and tissue specific gene expression was done using IBM SPSS statistics. One-way ANOVA with post-hoc Duncan multiple range tests was used for each experiment to identify statistically significant differences observed among Extra-Large G protein genes in A, B and D genomes in

both control and under stress treatments and the results were shown on each graph. For simplicity of interpretation, the results from Duncan multiple range tests were shown in a separate table for the tissue specific expression of *Xlgs*.

3.4.4 Construction of the Phylogenetic tree

The Extra Large GTP binding protein sequences for *Oryza sativa, Sorghum bicolor* and *Brachypodium distachyon* were retrieved from NCBI using the protein sequence of the D-copy of each *Xlg* gene from *T. aestivum* using blastp restricted to each species. The phylogenetic tree and evolutionary analyses were conducted using in MEGA X (Kumar et al. 2018). Evolutionary analysis was performed using Maximum Likelihood method and the Whelan And Goldman model (Whelan and Goldman 2001). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the JTT model, and then selecting the topology with superior log likelihood value. This analysis involved 21 amino acid sequences. There was a total of 1055 positions in the final dataset.

3.5 Results

3.5.1 Annotation of *Xlg* gene family in *Triticum aestivum*

Total of nine coding sequences of Extra-Large G protein gene family sequences were found in the hexaploid genome of *Triticum aestivum*; three *Xlg* genes with three homoeologous copies on either A, B or D genomes. The size of the proteins ranged from 862 to 906 amino acids. The nucleotide sequence identity among homologous sequences ranged from 98.5% to 97.5%; there was approximately 70% sequence identity between Xlg2 and Xlg3 proteins, and approximately 45% sequence identity between Xlg1 proteins and Xlg2 and Xlg3's. The identity matrix for the proteins is shown in Tables 1A and 1B; the multiple sequence alignment is shown in Figure 1.

The *Xlg1* had homoeologous copies on chromosome five named *Xlg1-A*, *Xlg1-B* and *Xlg1-D*. The *Xlg2* gene had two copies located on the A genome chromosomes 4A and 7A in addition to the D copy on chromosome 7D. The *Xlg*2 does not have a gene copy located on a B genome chromosome. The gene copy on chromosome 4A was likely the original B copy that has been translocated from 7B. In addition, a pseudo gene with high similarity to the other *Xlg2*'s

was found on chromosome 7A between nt positions 5,184,384 to 5,180,089 which is quite distant from the intact *Xlg2* copy on 7A which starts at nt position 14,73,0462. The *Xlg3* genes were located on chromosome six with one copy on each corresponding chromosome in the A, B and D genomes. The Ensembl Plant identifiers, protein and coding sequence length and chromosomal locations are shown in Table 2. and exon/intron junctions are shown in Table 3.

Table 1. Identity matrix among members of Xlg gene family. A) Amino acid sequence identity among members of the Xlgs. **B)** Identity matrix among nucleic acid sequences of CDS of the members of the Xlg gene family

A	**Xlg-5A**	**Xlg-5B**	**Xlg-5D**	**Xlg-4A**	**Xlg-7D**	**Xlg-7A**	**Xlg-6B**	**Xlg-6A**	**Xlg-6D**
Xlg-5A	100	98.06	97.83	44.71	44.94	44.7	45.6	45.43	45.07
Xlg-5B	98.06	100	98.4	44.74	44.97	44.73	45.64	45.48	45.11
Xlg-5D	97.83	98.4	100	44.65	45.11	44.63	45.48	45.43	45.07
Xlg-4A	44.71	44.74	44.65	100	97.69	98.02	70.91	71.04	70.57
Xlg-7D	44.94	44.97	45.11	97.69	100	98.46	70.96	70.86	70.39
Xlg-7A	44.7	44.73	44.63	98.02	98.46	100	70.84	70.86	70.39
Xlg-6B	45.6	45.64	45.48	70.91	70.96	70.84	100	97.56	97.68
Xlg-6A	45.43	45.48	45.43	71.04	70.86	70.86	97.56	100	98.38
Xlg-6D	45.07	45.11	45.07	70.57	70.39	70.39	97.68	98.38	100

B	***Xlg-5A***	***Xlg-5B***	***Xlg-5D***	***Xlg-4A***	***Xlg-7D***	***Xlg-7A***	***Xlg-6B***	***Xlg-6A***	***Xlg-6D***
Xlg-5A	100	97.75	97.91	55.4	54.9	55.14	56.01	55.34	55.38
Xlg-5B	97.75	100	97.99	55.49	55.15	55.27	55.74	55.19	55.15
Xlg-5D	97.91	97.99	100	55.56	55.26	55.34	56.05	55.42	55.46
Xlg-4A	55.4	55.49	55.56	100	96.55	96.85	76.36	76.51	76.78
Xlg-7D	54.9	55.15	55.26	96.55	100	97.87	75.86	76.2	76.16
Xlg-7A	55.14	55.27	55.34	96.85	97.87	100	76.21	76.2	76.51
Xlg-6B	56.01	55.74	56.05	76.36	75.86	76.21	100	96.33	96.56
Xlg-6A	55.34	55.19	55.42	76.51	76.2	76.2	96.33	100	97.04
Xlg-6D	55.38	55.15	55.46	76.78	76.16	76.51	96.56	97.04	100

```
Xlg1-A      ------------------------MASAVAGDAEYSFAAEYDGPPLPYSLPRAIPLDLS   35
Xlg1-B      ------------------------MATAVAGDAEYSFAAEYDGPPLPYSLPRAIPLDLS   35
Xlg1-D      ----------------------MASAVTVAGDAEYSFAAEYDGPPLPYSLPRAIPLDLS   37
Xlg2-4A     --MAGATETATWEDMLRRMLPPGAAIPEGAAGNLDYSIALEYDGPPVAYEVPRIAPVDMA   58
Xlg2-7A     --MAGATETATWEEMLRRMLPPGTAIPEGAAGNLDYSIALEYDGPPVAYEVPRIAPVDMA   58
Xlg2-7D     --MSGATETATWEEMLRRMLPPGTAIPEGAAGNLDYSIALEYDGPPVAYEVPRIAPVDMA   58
Xlg3-B      MAEPEAADGGGWQEMMRRILPPGAPVPEEA-PNLDYSIALVYDGPPVPYDLPRVDPVEIP   59
Xlg3-A      MAEAEAADGGSWQEMMRRILPPGAPVPEEA-PNLDYSIALVYDGPPVPYDLPRVDPVEIP   59
Xlg3-D      MAEAESADGGSWQEMMRRILPPGAPVPEEA-PNLDYSIALVYDGPPVPYDLPRVDPVEIP   59
                                          .  : :**:*  *****: *.:**  *::::
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```
Xlg1-A      H-IPLAALSSSS-SPPPTGSSPLPVVRPLTPSSLCSAIHAQQHAAGPPRS--SGDP-AGG   90
Xlg1-B      H-IPLAALSSSS-SPPPTGSSPLPVVRPLTPSSLCSAIHAQQHAAGPPRSS-SGGP-ARG   91
Xlg1-D      H-IPLAALSSSS-SPPPTGSSPLPVVRPLTPSSLCSAIHAQQHAAGPPRS--SGGP-AGG   92
Xlg2-4A     -DVPTAEPVSASYGLLGNGGAAVPVAPVFRPP---ASR--ARARAEPPQA--RGGAGPAD   110
Xlg2-7A     -DVPTAEPVSASYGLLGNGGAAVPVAPVFRPP---ASR--A--RAEPPRA--RRGAGPAD   108
Xlg2-7D     -DVPTAEPVSASYGLLGNGGATVPVAPVFRPP---ASR--A--RAEPPQA--RRGAGPAD   108
Xlg3-B      AEIPTAEPASGP-QTLGSGGRGLPVAPVVEPIRLPVSR--IARCAEPAAAAAR----QGG   112
Xlg3-A      AEIPTAEAASAP-QGLGVGGRGLPVAPVVEPIRLPVSR--IARCAEPAAAARQGGDGDGG   116
Xlg3-D      AEIPTAEPASGP-QTLGGGGRGVPVAPVVEPIRLPVSR--IARCAEPAGAARQGGDGDGG   116
              :* *   *.       *.  :**.  . *     :       * *  :         .

Xlg1-A      GAAQAVADSP---------TSVIENHHAAAHHSAELPSSPSDADDDDKEEEE--------   133
Xlg1-B      VGAQAVADSP---------TSVIENHHAAAHHSAELPSSPSD--DDDKE-----------   129
Xlg1-D      GGAQAVADSP---------TSVIENHHAAAHHSAELPSSPSDADDDDKE-----------   132
Xlg2-4A     SAPRDEASRPAARARQEPPSPVQVRRSSEFAHSGPRNEGYSDSDDDSRRSVSRESAPSYR   170
Xlg2-7A     SAPRDEASRPATRARQEPPSPVQVRRSSEFAHSGPQNEGYSDSDDDSRRSVSRESAPSYQ   168
Xlg2-7D     SAPRDEASRPATRARQEPPSPVQVRRSSEFAHSGPQNEGYSDSDDDSRRSVSRESAPSYR   168
Xlg3-B      DGS------------------------SESVNSVLQNGEFDDEDDDSRSR-SHGSAQ---   144
Xlg3-A      GGS------------------------SGSVNSVLQNEEFDDEDDDSRSR-SHGSAQ---   148
Xlg3-D      GGS------------------------SESVNSVLQNGEFDDEDDDSRSR-SHGSAQ---   148
             .                          :   :*       .*  **.:

Xlg1-A      -----EEEGAGMPSVPHQPTVSFAETSCSILDSSEEEED--DDDEDETADEALPAAARSS   186
Xlg1-B      -----EEEGAGMPSVPHQPTVSFAETSCSLLESSEEEEDDDDDDEDETADEALPAAARSS   184
Xlg1-D      -----EEEGAGMPSVPHQPTVSFAETSCSLLESSEEEEE-EDDDEDETADEALPAAARSS   186
Xlg2-4A     GQSDGSRRAMAAPEGRRSHVVTFGLADDSKYDQSSELDD--------TRSEQFVAV----   218
Xlg2-7A     GQSDGGRHAMAAPEGRRSHVVTFGLADDSKYDQSSELDD--------TRSEQFVAV----   216
Xlg2-7D     GQNDGGRHAMAAPQGRRSHVVTFGLADDSKYDQSSELDD--------TRSEQFVAV----   216
Xlg3-B      -----SSPGPGSRDGRRAPVVTFGFTPDSKYGESG---D--------DMSEQYVAV----   184
Xlg3-A      -----SSPGPGSRDGRRAPVVTFGFTPDSKYGESG---D--------DMSEQYVAV----   188
Xlg3-D      -----SSPGPGSRDGRRAPVVTFGFTPDSKYGESG---D--------DMSEQYVAV----   188
                    . .  .  :  .*:*. :  *   .*    :          .*   *.

Xlg1-A      GSLSPAHFEAGNHQTGAKSRGCYRCGKGGGFWGRGDKEACLACGARYCSACVLRAMGSMP   246
Xlg1-B      GSLSPAHFEAGNHPTGAKSRGCYRCGKGGGFWGRGDKEACLACGARYCSACVLRAMGSMP   244
Xlg1-D      GSLSPAHFEAGNHPTGAKSRGCYRCGKGGGFWGRGDKEACLACGARYCSACVLRAMGSMP   246
Xlg2-4A     --------TRTEK----RGKTCDRCGKRK--W--ESKESCIVCDKRYCGYCLLRAMGSMP   262
Xlg2-7A     --------TRTEK----RGKTCDRCGKRK--W--ESKESCIVCDKRYCGYCLLRAMGSMP   260
Xlg2-7D     --------TRTEK----RGKTCDRCGKRK--W--ESKESCIVCDKRYCGYCLLRAMGSMP   260
Xlg3-B      --------TKQEKRRRRRRMACNRCGKRK--W--ESKEACIVCDARYCGYCVLRMMGSMP   232
Xlg3-A      --------TKQEKRRRRRRMACNRCGKRK--W--ESKEACIVCDARYCGYCVLRMMGSMP   236
Xlg3-D      --------TKQEKRRRRRRMACNRCGKRK--W--ESKEACIVCDARYCGYCVLRMMGSMP   236
                         ::    :   * ****    *   .**:*:.*. ***. *:** *****

Xlg1-A      EGRKCLSCIGRPVAESRRNALGRGSRVLRRLISAAEVDLVMRSERECAANQLRPEDIYVN   306
Xlg1-B      EGRKCLSCIGRPVAESRRNALGRGSRVLRRLISAAEVELVMRSERECAANQLRPEDIYVN   304
Xlg1-D      EGRKCLSCIGRPVAESRRNALGRGSRVLRRLISAAEVELVMRSERECAANQLRPEDIYVN   306
Xlg2-4A     EGRKCITCIGRPIYEGKRSKLGKSSRILSRLLSSLEVRQILKAEKECQANQLRPEQLIVN   322
Xlg2-7A     EGRKCITCIGRPIYEGKRSKLGKSSRILSRLLSSLEVRQILKAEKECQANQLRPEQLIVN   320
Xlg2-7D     EGRKCITCIGRPIYEGRRSKLGKSSRILSRLLSSLEVRQILKAEKECQANQLRPEQLIVN   320
Xlg3-B      EGRKCVSCIGGPIDESKRSKLGKSSRTLARLLSPLEVRQILKAEKECQANQLRPEQLIVN   292
Xlg3-A      EGRKCVSCIGGPIDESKRSKLGKSSRTLARLLSPLEVRQILKAEKECQANQLRPEQLIVN   296
Xlg3-D      EGRKCVSCIGGPIDESKRSKLGKSSRTLARLLSPLEVRQILKAEKECQANQLRPEQLIVN   296
            *****::*** *: *.:*. **:.** * **:*  **  ::::*:** *******:: **
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Xlg1-A      GAKLSPEELALLQGCSCPPSRLRPGFYWYDKVSGFWGKEGHKPHCIISANLNVGGALVQK   366
Xlg1-B      GAKLSPEELAFLQGCSCPPSRLRPGFYWYDKVSGFWGKEGHKPHCIISANLNVGGALVQK   364
Xlg1-D      GAKLSPEELALLQGCACPPSRLRPGFYWYDKVSGFWGKEGHKPHCIISANLNVGGALVQK   366
Xlg2-4A     GFPLCQEEMSDLLSCPRPPQNLKPGRYWYDKESGLWGKEGEKPNRVVSTNLNFNGKIQPN   382
Xlg2-7A     GFPLCQEEMSDLLSCPRPPQNLKPGRYWYDKESGLWGKEGEKPNRVVSTNLNFNGKIQPN   380
Xlg2-7D     GFPLCQEEMSDLLSCPRPPQNLKPGRYWYDKESGLWGKEGEKPNRVVSTNLNFNGKIQPN   380
Xlg3-B      GCPLRPEELTDLLSCSRPPQKLKPGRYWYDKESGLWGKDGQKPDRIVSSNLNFSGKLHAK   352
Xlg3-A      GCPLRPEELTDLLSCSRPPQKLKPGRYWYDKESGLWGKDGQKPDRIVSSNLNFSGKLHAK   356
Xlg3-D      GCPLRPEELTDLLSCSRPPQKLKPGRYWYDKESGLWGKDGQKPDRIVSSNLNFSGKLHAK   356
            *  *  **:: * .*  **..*:** ***** **:***:*.**. ::*:***..* :  :

Xlg1-A      ASNGSTGILVNGREITKSELQILKLAGVQCAGKPHFWLNADGSYQEEGQKTVKGKIWDKP   426
Xlg1-B      ASNGSTGILVNGREITKSELQILKLAGVQCAGKPHFWLNADGSYQEEGQKTVKGKIWDKP   424
Xlg1-D      ASNGSTGILVNGREITKSELQILKLAGVQCAGKPHFWLNADGSYQEEGQKTVKGKIWDKP   426
Xlg2-4A     ASNGNTQVYMNGREITSIERKILKFAQVQCPRDTHFWVYHDGGYEEEGQNNIKGKIWESP   442
Xlg2-7A     ASNGNTQVYMNGREITSIERKILRFAQVQCPRDTHFWVYHDGGYEEEGQNNIKGKIWESP   440
Xlg2-7D     ASNGNTQVYMNGREITSIERKILRFAQVQCPRDTHFWVYHDGGYEEEGQNNIKGKIWESP   440
Xlg3-B      ASNGNTQVYINGREITKSELKILKVAHVQCPRDTHFWVYDDGRYEEEGQNNIKGKIWESA   412
Xlg3-A      ASNGNTQVYINGREITKSELKILKVAHVQCPRDTHFWVYDDGRYEEEGQNNIKGKIWESA   416
Xlg3-D      ASNGNTQVYINGREITKSELKILKVAHVQCPRDTHFWVYDDGRYEEEGQNNIKGKIWESA   416
            ****.* : :******. * :**:.* ***  . ***:  ** *:****:.:*****:.

Xlg1-A      IVKLLSPVLSLPTPNKMTNQCGEE-AINVVNRPDYLEQRTTQKLLLVG---SGTSTILKQ   482
Xlg1-B      IVKLLSPVLSLPTPNKMTNQCGEE-AINMVNRPDYLEQRTTQKLLLVG---SGTSTILKQ   480
Xlg1-D      IVKLLSPVLSLPTPNKMTNQCGEE-AINVVNRPEYLEQRTTQKLLLVG---SGTSTILKQ   482
Xlg2-4A     LTRFVCTLVSLPVPPANSVEPRDDAPYSARTVPEYLDQKRIQKLLILGSPGAGTSTIFKQ   502
Xlg2-7A     LTRFVCTLVSLPVPPANSVEPRDDAPYSARTVPEYLDQKRIQKLLILGSPGAGTSTIFKQ   500
Xlg2-7D     LTRFVCTLVSLPVPPANSVEPRDDAPYSARTVPEYLDQKRIQKLLILGSPGAGTSTIFKQ   500
Xlg3-B      LTRFACALFSLPVPPGDSNGTKDEIPFVPRTVPDYLDQKRIQKLLLLGPPSAGTSTIFKQ   472
Xlg3-A      LTRFACALFSLPVPPGDSNGTKDEIPFVPRTVPDYLDQKRIQKLLLLGPPSAGTSTIFKQ   476
Xlg3-D      LTRFACALFSLPVPPGDSNGTKDEIPFVPRAVPDYLDQKRIQKLLLLGPPSAGTSTIFKQ   476
            :.:: . :.***.*   :    ::        *:**:*:  ****::*   :*****:**

Xlg1-A      AKFSYKSKPFSVDECEDLKLIIQSNIYRYIGILLEGRERFEEEVLADRRKLCQHDPSSSG   542
Xlg1-B      AKFSYKSKPFSVDECEDLKLIIQSNIYRYIGILLEGRERFEEEVLADRRKLCQHDPSSSG   540
Xlg1-D      AKFSYKSKPFSMDECEDLKLIIQSNIYRYIGILLEGRERFEEEVLADRRKLCQHDPSSSG   542
Xlg2-4A     AKLLYGS-RFTPEELDNIKLMIQSNMFKYLGILLEGRERFEEEALAISNNPNSEDEDTQ-   560
Xlg2-7A     AKLLYGS-RFTPEELDNIKLMIQSNMFKYLGTLLEGRERFEEEALAIPNNPNSEDGDTQ-   558
Xlg2-7D     AKLLYGS-RFTPEELDNIKLMIQSNMFKYLGILLEGRERFEEEALAISNNPNSEDEDTQ-   558
Xlg3-B      AKYLYGT-RFTQDELDAIKLMIQSNMFKYLGILLEGRERFEEEALSRLDQTTSEDEAAQ-   530
Xlg3-A      AKYLYGS-RFTQDELDAIKLMIQSNMFKYLGILLEGRERFEEEALSRLDQTIPEDEAAQ-   534
Xlg3-D      AKYLYGT-RFTQDELDAIKLMIQSNMFKYLGILLEGRERFEEEALSRLDQTIPEDEAAQ-   534
            **  * :  *: :* : :**:****:::*:* ***********.*:   :   .*  :.

Xlg1-A      RPESGFCDEEVTEYSIVPRLKAFSDWILKAMALGNLEDIFPAASREYAPLVEELWKDPAI   602
Xlg1-B      RPESGFCDEEVTEYSIVPRLKAFSDWILKAMALGNLEDIFPAASREYAPLVEELWKDPAI   600
Xlg1-D      RPESGFCDEEVTEYSIVPRLKAFSDWILKAMALGNLEDIFPAASREYAPLVEELWKDPAI   602
Xlg2-4A     QDGNESNGLNSCIYSINAKLKKFSDWLLDIIAMGDLDAFFPAATREYAPVVDELWKHPAI   620
Xlg2-7A     QDGNESSGLNSCIYSINAKLKKFSDWLLDIIAMGDLDAFFPAATREYAPVVDELWKHPAI   618
Xlg2-7D     QEENESSGLNSCIYSINAKLKKFSDWLLDIIAMGDLDAFFPAATREYAPVVDELWKHPAI   618
Xlg3-B      KDENKANGPDSCIYSINARLKKFSDWLLDIIAMGDLDAFFPAATREYAPFVEEMWKDPAI   590
Xlg3-A      KDENKANGADSCIYSINARLKKFSDWLLDIIAMGDLDAFFPAATREYAPFVDEMWKDPAI   594
Xlg3-D      KDENKANGRDSCIYSINARLKKFSDWLLDIIAMGDLDAFFPAATREYAPFVDEMWKDPAI   594
            :  .   . :   ***  :** ****:*. :*:*:*: :****:*****.*:*:**.***
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Xlg1-A       QATYRRRNELPFLPFAASYFLDRVVDISRTEYELCDTDILYADGITSSDGLASTDISFPQ   662
Xlg1-B       QATYRRRNELPFLPSAASYFLDRVVDISRTEYELCDIDILYADGITSSDGLASTDISFPQ   660
Xlg1-D       QATYRRRNELPFLPSAASYFLDRVVDISRTEYELCDIDILYADGITSSDGLASTDISFPQ   662
Xlg2-4A      QATYKRKDELYFLPDVAEYFLSRAIEVSSNEYEPSEKDVLYAEGVSQGNGLAFIDFTLDE   680
Xlg2-7A      QATYKRKDELYFLPDVAEYFLSRAIEVSSNEYEPSEKDVLYAEGVSQGNGLAFIDFTLDE   678
Xlg2-7D      QATYKRKDELYFLPDVAEYFLSRAIEVSSNEYEPSEKDVLYAEGVSQGNGLAFIDFTLDE   678
Xlg3-B       QATYKRKDELHFLPDVAEYFLSRAIEVSSNEYEPSEKDVIFAEGVTQGNGLSFIEFTLDD   650
Xlg3-A       QATYKRKDELHFLPDVAEYFLSRAIEVSSNEYEPSEKDVIFAEGVTQGNGLSFIEFTLDD   654
Xlg3-D       QATYKRKDELHFLPDVAEYFLSRAIEVSSNEYEPSEKDVIFAEGVTQGNGLSFIEFTLDD   654
             ****:*::** *** .*.***.*.:::* .*** .: *:::*:*::..:**:  :::: :

Xlg1-A       LAL-DVRVADE-LDPHDTLLRYQLIRINNKGLQENSKWLQMFDDVSLVIFCVAVSDYDEY   720
Xlg1-B       LAL-DVRVADE-LDPQDTLLRYQLIRINNKGLRENSKWLQMFDDVSLVIFCVAVSDYDEY   718
Xlg1-D       LAL-DVRVADE-LDPQDTLLRYQLIRINNKGLRENSKWLQMFDDVSLVIFCVAVSDYDEY   720
Xlg2-4A      RNPMSELYGDSHDPSSQAQNKYQLIRVNAKGLNEGCKWVEMFEDVRAVIFSIALSDYDQL   740
Xlg2-7A      RNPMSELYGDSHDPSSQAQNKYQLIRVNAKGLNEGCKWVEMFEDVRAVIFSVALSDYDQL   738
Xlg2-7D      RNPMSELYGDSHDPSSQAQNKYQLIRVNAKGLNEGCKWVEMFEDVRAVIFSVALSDYDQL   738
Xlg3-B       RSPMSEPYIDNPEAHSQPLTKFQLIRVSAKGMNDGCKWVEMFEDVRMVIFCVALSDYDLV   710
Xlg3-A       RSPMSEPYIDNPEAHSQPLTKFQLIRVSAKGMNDGCKWVEMFEDVRMVIFCVALSDYDLV   714
Xlg3-D       RSPMSEPYIDNPEAHSQPLTKFQLIRVSAKGMNDGCKWVEMFEDVRMVIFCVALSDYDLV   714
                 .    *.     :   ::****:. **:.:..**::**:**  ***.:*:****

Xlg1-A       YEDANGT---VVNKMIESRQLFESIALHPTFEQMNFLLLLTKFDLLEQKIGKSPLTACDW   777
Xlg1-B       YEDANGT---VVNKMIESRQLFESIALHPTFEQMNFLLLLTKFDLLEQKIGKSPLTACDW   775
Xlg1-D       YEDANGT---VVNKMIESKQLFESIALHPTFEQMNFLLLLTKFDLLEQKIGKSPLTACDW   777
Xlg2-4A      GAPASGSSRRLENKMIQSRDLFEATIRHPSFRDTPFVLVLNKFDIFEEKIGRSPLTACEW   800
Xlg2-7A      GAPASGNSRRLENKMIQSRDLFEATIRHPSFRDTPFVLVLNKFDIFEEKIGRSPLTACEW   798
Xlg2-7D      GAPASGTSRRLENKMIQSKDLFEATIRHPSFRDTPFVLVLNKFDIFEEKIGRSPLTACEW   798
Xlg3-B       GTPVNGN---LQNKMMQSKELFEATIRQPCFCDTPFVLVLNKYDLFEEKISRAPLTACEW   767
Xlg3-A       GTPVNGN---LQNKMMQSKELFEATIRQPCFCDTPFVLVLNKYDLFEEKISRAPLTACEW   771
Xlg3-D       GTPVNGN---LQNKMMQSKELFEATIRQPCFCDTPFVLVLNKYDLFEEKISRAPLTACEW   771
                ..*.   : ***::*::***:   :* * :  *:*:*.*:*::*:**.::*****:*

Xlg1-A       FAEFTPLVSRNLIDGTSRSKRGSQNGASLAQMAAHYIGVKFKRLFHSLTE-RKLYVSYVN   836
Xlg1-B       FAEFTPLVSRNLIDGTSRSKRGSHNGASLAQMAAHYIGTKFKRLFHSLTE-RKLYVSYVN   834
Xlg1-D       FAEFTPLVSRNLIDGTSRSKRGSHNGASLAQMAAHYIGVKFKRLFHSLTE-RKLYVSYVN   836
Xlg2-4A      FSDFDPLRTH--------------NNQSMAQQAFFYVAMKFKNLYAAHTGDRKLFVWQAR   846
Xlg2-7A      FSDFDPLRTH--------------NNQSMAQQAFFYVAMKFKNLYAAHTGDRKLFVWQAR   844
Xlg2-7D      FSDFDPLRTH--------------NNQSMAQQAFFYVAMKFKNLYAAHTGDRKLFVWQAR   844
Xlg3-B       FADFSPVRTQ-------------SNNQSLAQQAYYYVAMKFKEAYNEHTG-RKLFVWQAR   813
Xlg3-A       FSDFSPVRTQ-------------SNNQTLAQQAYYYVAMKFKEAYNDHTG-RKLFVWQAR   817
Xlg3-D       FGDFSPVRTQ-------------SNNQTLAQQAYYYVAMKFKEAYNDHTG-RKLFVWQAR   817
             *.:* *: ::              *. ::** * .*:. ***. :   *  ***:*  ..

Xlg1-A       ALDQQSVCSAIRYGREIVKWEEEKPV--FGSSETVYSGDEPSSYSH--------------   880
Xlg1-B       ALDQQSVCSAIRYGREIVKWEEEKPV--FGSSETVYSGDEPSSYSH--------------   878
Xlg1-D       ALDQQSVCSAIRYGREIVKWEEEKPV--FGSSETVYSGDEPSSYSH--------------   880
Xlg2-4A      ARDGPTVDEAFRYIREVLRWEDEKEYGGFCPDESFYSTTELSSSRLVEQDWQQHPAADQR   906
Xlg2-7A      ARDGPTVDEAFRYIREVLRWEDEKEYGGFCPDESFYSTTELSSSRLVEQDWQQHPAADQR   904
Xlg2-7D      ARDGPTVDEAFRYIREVLRWEDEKEYGGFCPDESFYSTTELSSSRLVEQDWQQHPAADQR   904
Xlg3-B       GRDRQTVDEAFKYIREVLKWEEEKDD-NYYQEESFYSTTEMSSSPFIRAE----------   862
Xlg3-A       GRDRQTVDEAFKYIREVLKWEEEKDE-NYYQEESFYSTTEMSSSPFIRAE----------   866
Xlg3-D       GRDRQTVDEAFKYIREVLKWEEDKDE-NYYQEESFYSTTEMSSSPFIRAE----------   866
             . *  :* .*::* **:::**::*    :  .*:.**  * **
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Xlg1-A        -----        880
Xlg1-B        -----        878
Xlg1-D        -----        880
Xlg2-4A       PGSGA        911
Xlg2-7A       QGST-        908
Xlg2-7D       QGST-        908
Xlg3-B        -----        862
Xlg3-A        -----        866
Xlg3-D        -----        866
```

Figure 1. Sequence alignment of Xlg proteins in *T. aestivum.* The sequence alignment shows that amino acid sequences are highly conserved among the three homeologous copies of each Xlg protein. The sequences were aligned using Clustal Omega.

Table 2. Annotation of the *Xlg* sequences in *Triticum aestivum*.

Gene Name	*Ensembl Plant identifier*	*Chromo-some*	*EST accession**	*EST 3' UTR*	*T. aestivum TSA**	**CDS Length**	**Amino acid Length**
Xlg1-A	TraesCS5A02G064400.1	5A	HX144562.1	CD873702.1	GAJL01283801.1	2643	880aa
Xlg1-B	TraesCS5B02G068300.2	5B	HX147190.1	HX147190.1	GEWU01239503.1	2637	878aa
Xlg1-D	TraesCS5D02G075200.1	5D	LU052528.1	HX144541.1	GFFI01006804.1	2643	880aa
Xlg2-7A	TraesCS7A02G033500	7A	CJ918770.1	CJ824990.1	GEWU01254644.1	2727	908aa
Xlg2-4A	TraesCS4A02G455800	4A	HX105721.1	LU010776	GEWU01257620.1	2736	911aa
Xlg2-D	TraesCS7D02G030100	7D	CJ789210.1	HX105721.1	GEWU01254643.1	2727	908aa
Xlg3-A	TraesCS6A02G007400	6A	CJ558977.1	LU043751.1	IAAL01003462.1	2601	866aa
Xlg3-B	TraesCS6B02G012700	6B	LU103643.1	LU050080.1	IAAL01004225.1	2589	862aa
Xlg3-D	TraesCS6D02G011700	6D	LU106084.1	LU018910.1	GFFI01005452.1	2601	866aa

* **EST and TSA accession numbers are from GenBank**

Table 3. Exon and Introns of the Extra-Large G proteins in *Triticum aestivum*.

3.1 Annotations of the homoeologous copies of the Extra-Large G protein 1.

Gene Name-Chromosome	***Xlg1-A***	***Xlg1-B***	***Xlg1-D***
Ensemble Plant identifier	TraesCS5A02G064400.1	TraesCS5B02G068300.2	TraesCS5D02G075200.1
Start Codon (bp)	69842212	77720180	74459176
Orientation	+/+	+/-	+/+
Exon 1	1032	1026	1032
Intron	1879	1873	1936
Exon 2	138	138	138
Intron	77	77	77
Exon 3	105	105	105
Intron	89	89	89
Exon 4	171	171	171
Intron	219	220	220
Exon 5	178	178	178
Intron	558	153	153
Exon 6	251	251	251
Intron	147	147	147
Exon 7	167	167	167
Intron	332	329	334
Exon 8	601	601	601

The table presents the position of the start codons of each gene on the *T. aestivum* whole genome annotation V1 at Ensembl Plants and the length of each exon and intron.

Table 3.2 Annotations of the homoeologous copies of the Extra-Large G proteins 2.

Gene Name-Chromosome	*Xlg2-7A*	*Xlg2-4A*	*Xlg2-D*
Ensemble Plant identifier	TraesCS7A02G033500	TraesCS4A02G455800	TraesCS7D02G030100
Start Codon (bp)	14730462	719168794	15829448
Orientation	+/+	+/-	+/+
Exon 1	1074	1080	1074
Intron	82	82	82
Exon 2	138	138	138
Intron	111	123	102
Exon 3	105	105	105
Intron	117	120	116
Exon 4	183	183	183
Intron	463	467	464
Exon 5	178	178	178
Intron	80	81	82
Exon 6	245	245	245
Intron	263	263	319
Exon 7	173	173	173
Intron	297	299	298
Exon 8	198	198	198
Intron	185	185	185
Exon 9	433	436	433

The table presents the position of the start codons of each gene on the *T. aestivum* whole genome annotation V1 at Ensembl Plants and the length of each exon and intron.

Table 3.3, Annotations of the homoeologous copies of the Extra-Large G proteins 3.

Gene Name-Chromosome	*Xlg3-A*	*Xlg3-B*	*Xlg3-D*
Ensemble Plant identifier	TraesCS6A02G007400	TraesCS6B02G012700	TraesCS6D02G011700
Start Codon (bp)	2952141	7943891	4629423
Orientation	+/+	+/-	+/+
Exon 1	1002	990	1002
Intron	94	97	108
Exon 2	138	138	138
Intron	1784	1796	2033
Exon 3	105	105	105
Intron	109	108	108
Exon 4	183	183	183
Intron	328	640	314
Exon 5	178	178	178
Intron	77	107	77
Exon 6	245	245	245
Intron	353	416	371
Exon 7	173	173	173
Intron	71	90	71
Exon 8	189	189	189
Intron	97	116	108
Exon 9	388	388	388

The table presents the position of the start codons of each gene on the *T. aestivum* whole genome annotation V1 at Ensembl Plants and the length of each exon and intron.

3.5.2 Tissue Specific gene expression of the Extra-Large G proteins in *Triticum aestivum*

Tissue specific gene expression of *Xlg*s were assayed using RNA seq database of the transcriptomics to study gene expression in different tissues including seed, leaf, root and inflorescence (Pingault, et al. 2015). The hits were normalized to Read Per Kilobase Per Million (RPKPM) for comparison. The *Xlg1-A* showed higher levels of expression in leaf and stem compared to seed, root and inflorescence. Although *Xlg1-B* copy showed higher levels of transcript in leaf and stem but only gene expression in stem was significantly higher than the seed, root and inflorescence. The *Xlg1-D* showed higher levels of expression in leaf and stem while only leaf had significantly higher expression compared to seed, root and inflorescence.

The *Xlg2-7A* had high levels of gene expression in leaf and seed which were significantly higher compared to root, stem and inflorescence. The other copy of the gene on the A chromosome, *Xlg2-4A*, and the D copy of the gene, *Xlg2-D*, showed expression in all assayed tissues while no significant differences in transcript levels in different tissues were observed.

The *Xlg3-A*, showed higher levels of expression in stem, inflorescence and seed compared to root and leaf tissue. For the *Xlg3-B* copy, inflorescence showed higher levels of the transcript compared to root and leaf tissues. The *Xlg3-D,* showed higher expression in the stem and inflorescence than the root and leaf, but was not statistically different than expression in the seed (Figure 2).

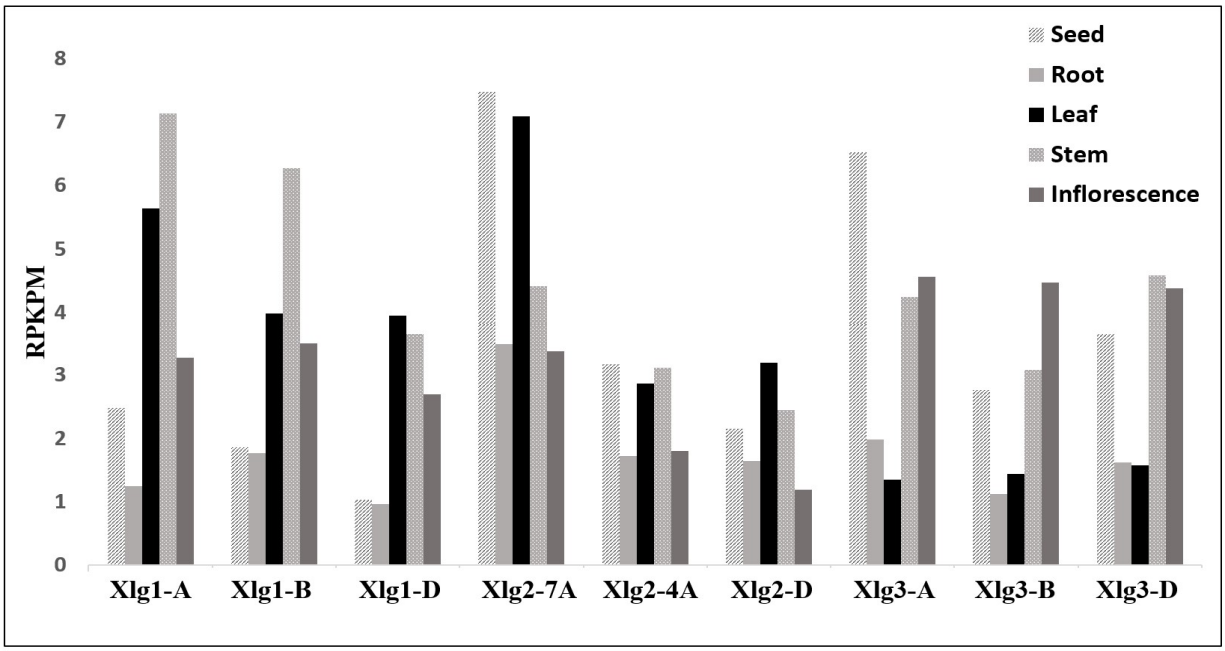


Figure 2. Tissue specific gene expression of *Xlg* proteins in *Triticum aestivum.* RNA-seq database obtained from seed, root, leaf stem and inflorescence tissues were used to search for hits for each *Xlg* gene. Tissue specific gene expression was normalized to RPKPM; Read Per Kilobase Per Million. Statistical analysis is shown in (Table 4) for simplicity.

Table 4. Statistical analysis showing Duncans Multiple range test values for Tissue specific gene expression of *Xlg* transcripts.

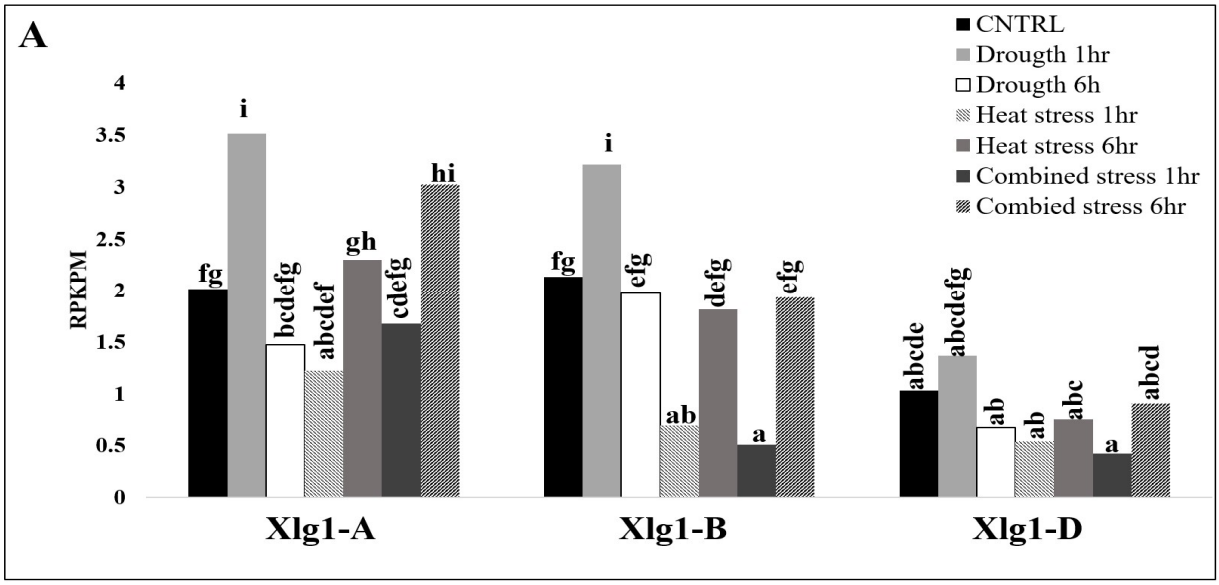
Gene Name	Tissue	Duncan's abc	Gene Name	Tissue	Duncan's abc	Gene Name	Tissue	Duncan's abc
Xlg1-A	Seed	abcdefg	Xlg2-7A	Seed	l	Xlg3-A	Seed	jkl
	Root	ab		Root	abcdefgh		Root	abcdefg
	Leaf	hijkl		Leaf	kl		Leaf	ab
	Stem	kl		Stem	defghij		Stem	cdefghij
	Inflorescence	abcdefgh		Inflorescence	abcdefgh		Inflorescence	fghijk
Xlg1-B	Seed	abcdefg	Xlg2-4A	Seed	abcdefgh	Xlg3-B	Seed	abcdefg
	Root	abcdef		Root	abcde		Root	a
	Leaf	bcdefghij		Leaf	abcdefgh		Leaf	ab
	Stem	ijkl		Stem	abcdefgh		Stem	abcdefgh
	Inflorescence	abcdefgh		Inflorescence	abcdefg		Inflorescence	efghij
Xlg1-D	Seed	a	Xlg2-D	Seed	abcdefg	Xlg3-D	Seed	abcdefghi
	Root	a		Root	abcd		Root	abcd
	Leaf	bcdefghij		Leaf	abcdefgh		Leaf	abc
	Stem	abcdefghi		Stem	abcdefg		Stem	ghijk
	Inflorescence	abcdefg		Inflorescence	ab		Inflorescence	cdefghij

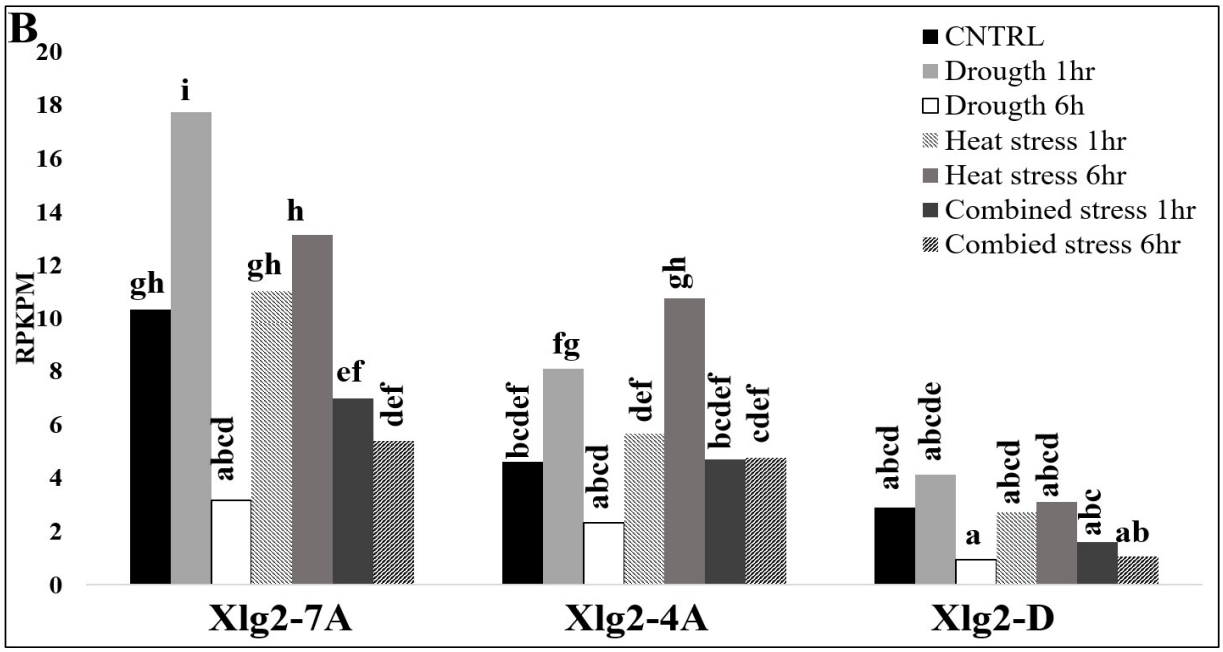
N.B Values for tissue samples within the analysis for each gene that share a common letter in the Duncan's multiple range test are not significantly different.

3.5.3 Expression of members of Extra-Large G protein family under drought, heat and combined stresses

RNA-seq libraries from seedlings grown under drought and heat treatment (Liu et al. 2015) were used for evaluation of *Xlg* gene expression under the stress conditions. The A copies of the *Xlg1*, *Xlg2* and *Xlg3* showed altered expression in response to either heat, drought or the combined stress. The *Xlg1-A* and *Xlg3-A,* was up-regulated in response to six hours of combined heat and drought stress while *Xlg2-7A* showed downregulation under similar condition. On the other hand, *Xlg1-A* and *Xlg2-7A,* showed up-regulation under one hour of heat treatment, while *Xlg3-A* did not alter gene expression. Among the B-copies of the *Xlg* transcripts, the *Xlg3-B* showed to be up-regulated under six hours of combined heat and drought stress while no alteration was observed for *Xlg2-4A* and *Xlg1-B*. The *Xlg4-A* transcript level was up-regulated under six hours of heat stress. The *Xlg1-B* showed up-regulation under one-hour drought while the gene was down-regulated under one-hour heat and one hour combined stress. The D copies of *Xlg1*, *Xlg2* and *Xlg3* did not alter expression in response to heat, drought and combined

stresses. The results suggest differential regulation of members of the *Xlg* gene family in *T.aestivum* (Figure 3).





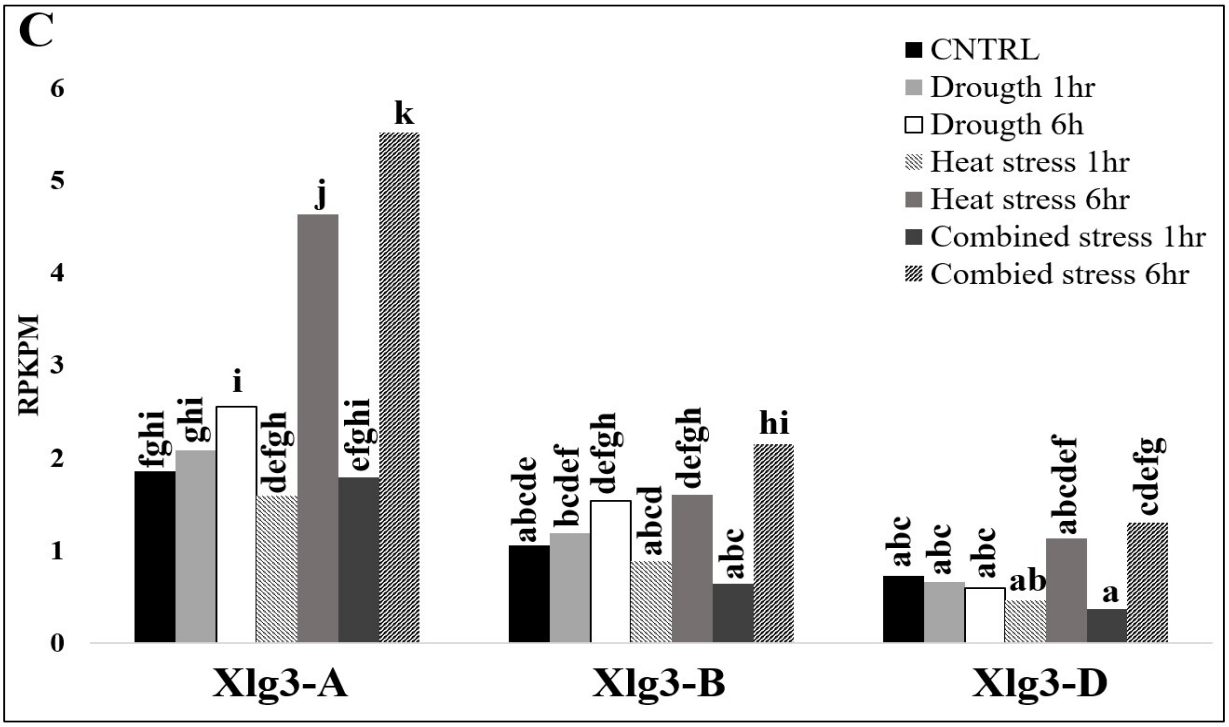


Figure 3. Extra-Large G protein expression in response to Heat, Drought and combined stress. A) Effect of drought, heat and heat and drought combined stress on expression level of *Xlg1* copies in A, B and D genome. **B)** Effect of drought, heat and drought and heat combined stress on expression level of the three homeologous copies of *Xlg2* in A and D genome. **C)** Effect of drought, heat and heat and drought combined stress on expression level of *Xlg3-A, Xlg3-B* and *Xlg3-D* copies. The data was normalized to Reads per Kilobases per Millions (RPKPM) for comparison. Duncans multiple range tests results are shown on the graph. Stress responses that share a common letter in the Duncan's multiple range test are not significantly different.

3.5.4 Expression of members of the *Xlg* gene family in response to cold treatment

Transcriptomic study of gene expression in response to two weeks cold treatment of seedlings assayed in leaf tissue libraries (Li et al. 2015) were used in CD-hit algorithm to find differential expression of *Xlgs* in *T. aestivum*. Under 4°C cold treatment for two weeks, *Xlg1* did not alter level of gene expression while *Xlg2-4A* showed more than 1.5-fold up-regulation. The *Xlg3* homoeologous copies showed no change in expression level under cold treatment compared to the control condition which was grown under 23°C. Under control condition, the two copies of the *Xlg2* on chromosome A, showed higher level of expression compared to the other homoeologous copy of the gene and *Xlg1* and the A and B copies of the *Xlg3* (Figure 4).

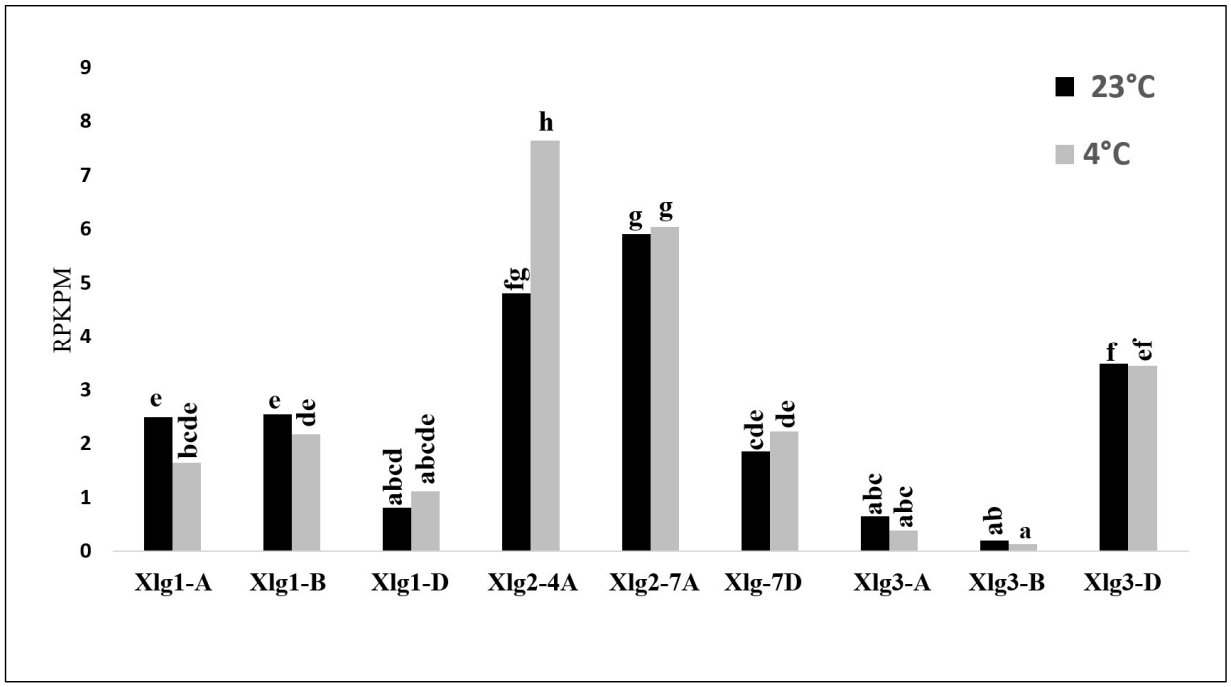


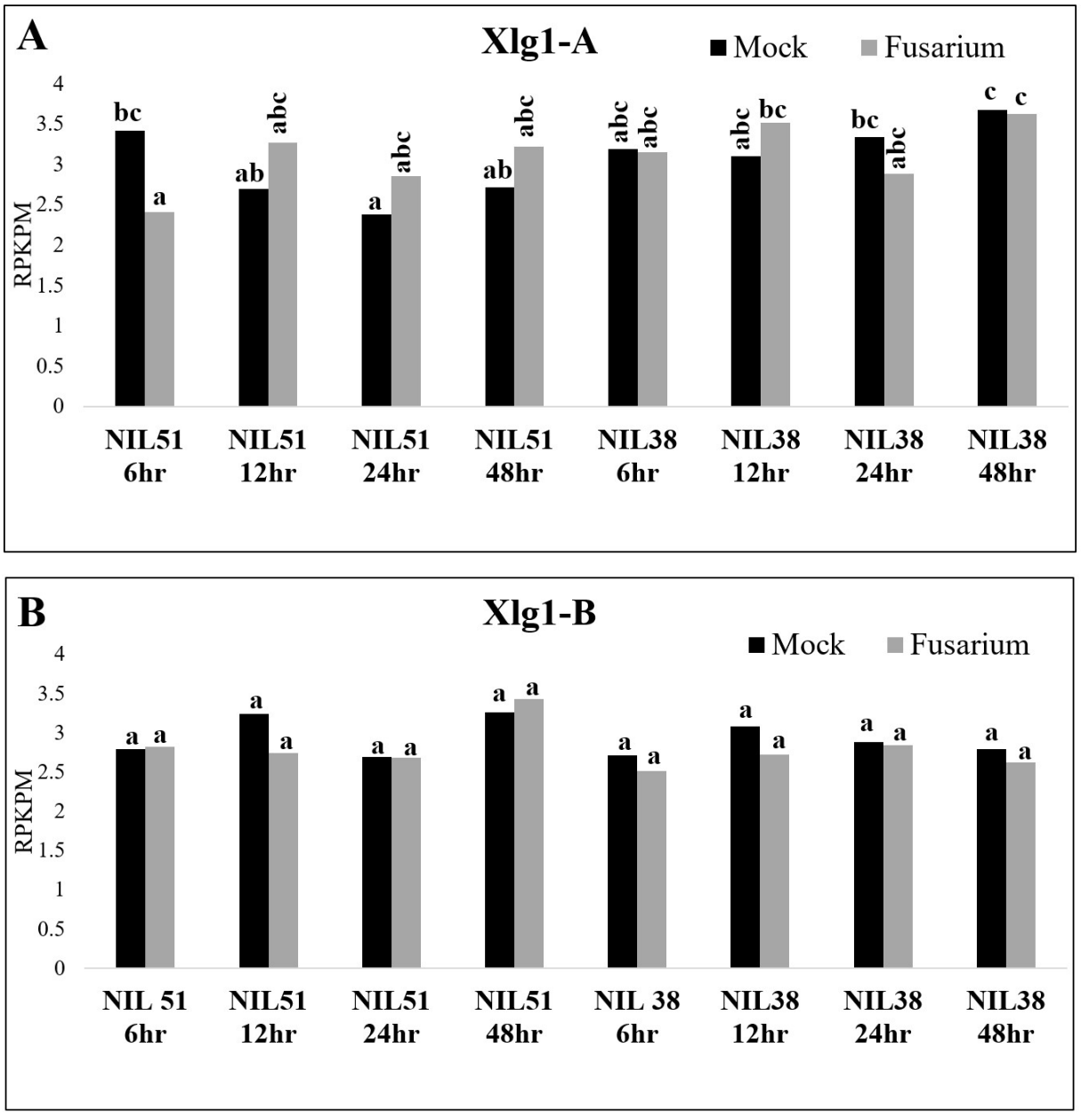
Figure 4. Extra-Large G protein expression in response to cold treatment. Different levels of expression for each of the Extra-Large G protein members are shown under control condition (23°C) and cold treatment (4°C). All hits were normalized to RPKPM. Duncans multiple range tests results are shown on the graph. Responses that do not share a common letter in the Duncan's multiple range test are significantly different.

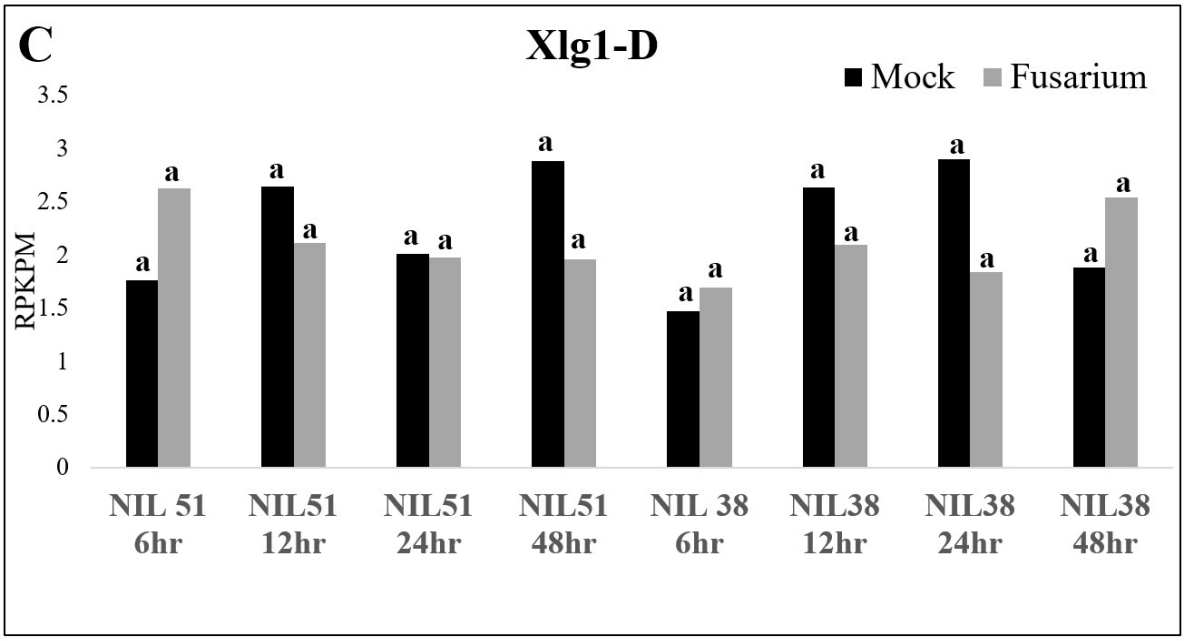
3.5.5 Expression of Xlgs in response to F. graminearum infection

The transcriptome libraries of the *F. graminearum* resistant line, NIL 38, and, the *F. graminearum* susceptible line, NIL 51, in developing spikes in response to *F. graminearum* infection was used in this study (Steiner et al. 2017). In the disease susceptible line NIL51, only the *Xlg1-A* copy showed immediate and significant response to Fusarium infection by decreasing gene expression six hours post infection while under longer incubation times gene expression was not significantly different from the control treatment. The disease resistant line, NIL38 did not show significantly altered expression levels of *Xlg1-A* copy. The *Xlg1-B* and *D* copies did not show statistically significant changes of the transcript abundant.

The NIL51, the *F. graminearum* susceptible line and the *F. graminearum* resistant line, NIL 38, showed up-regulation of the *Xlg2* transcript 48 hours post infection. Among copies of the *Xlg2,* the *Xlg2-7A* was transcriptionally up-regulated at earlier times post infection; elevated transcript levels were observed six hours post infection in the disease susceptible line and 24

hours post infection in both disease susceptible and disease resistant line. None of the *Xlg3* homoeologous genes had altered expression in response to Fusarium infection (Figure 5).

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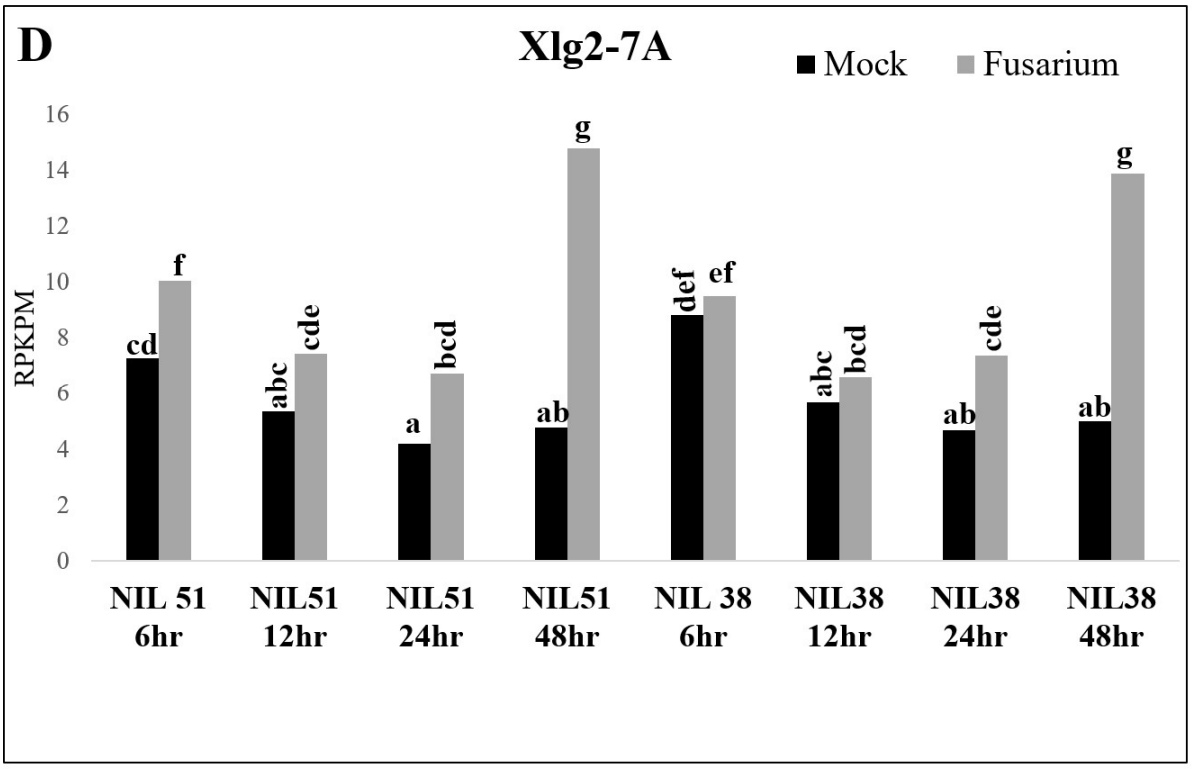
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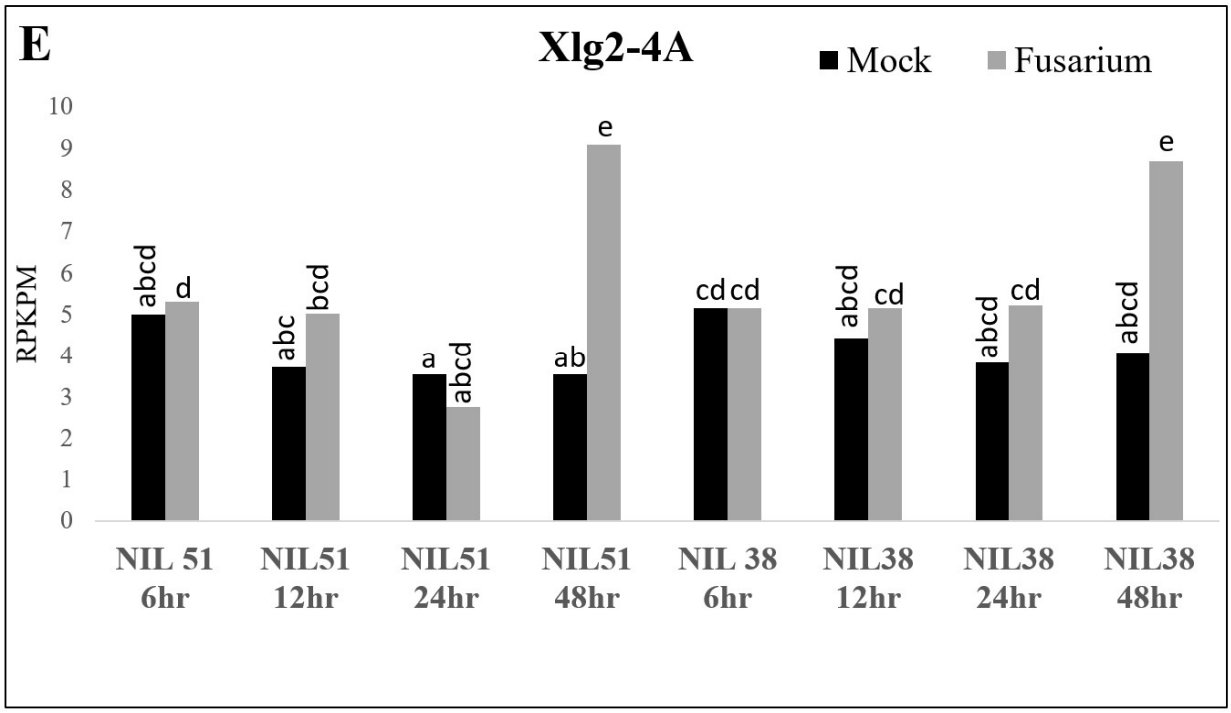
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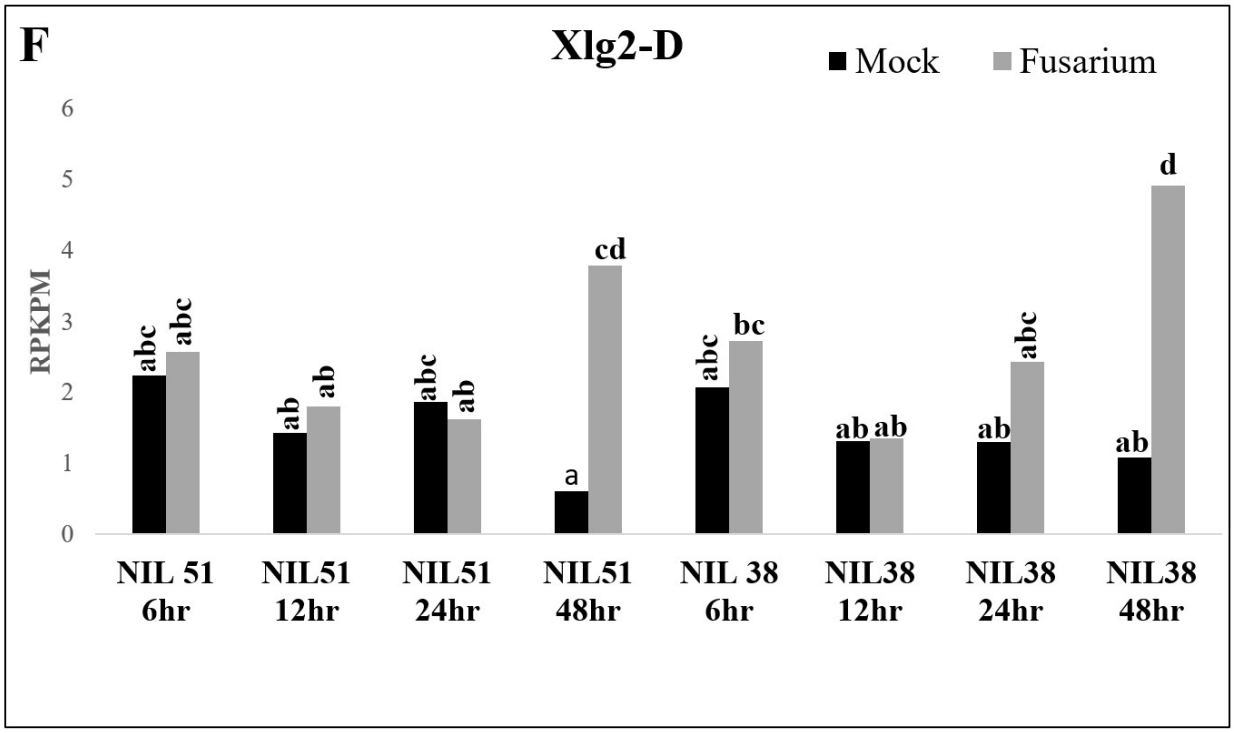
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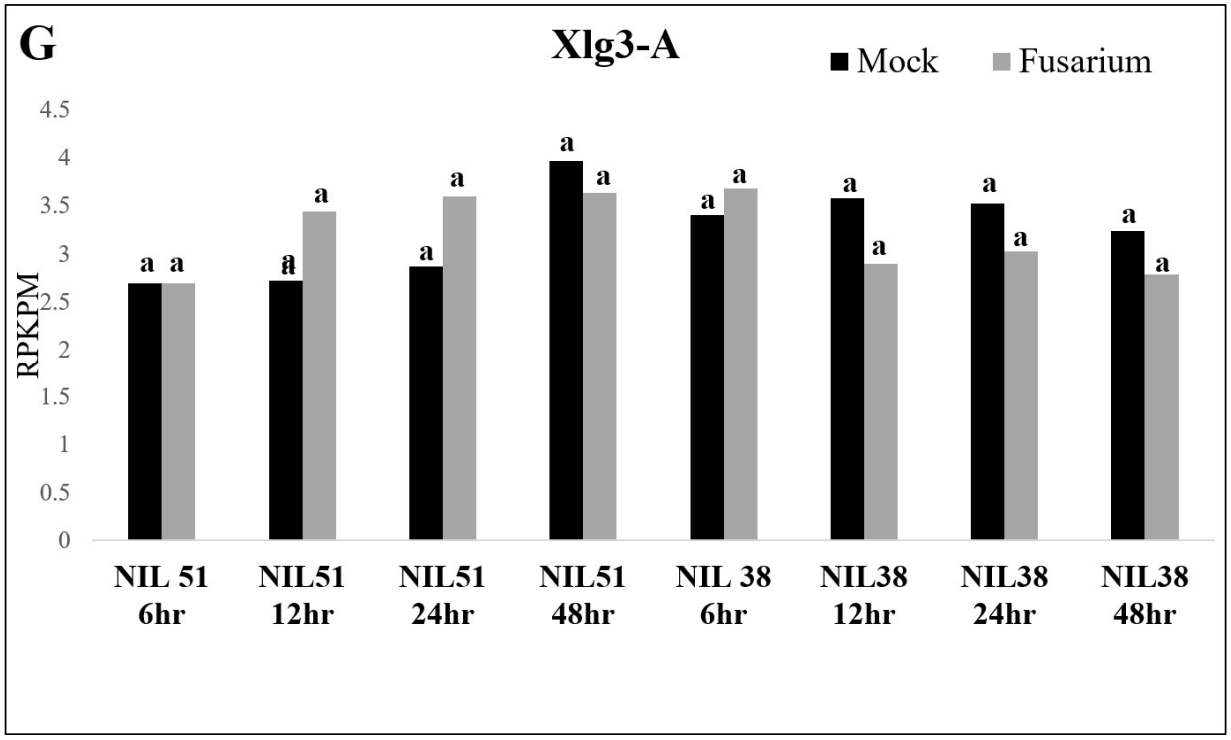
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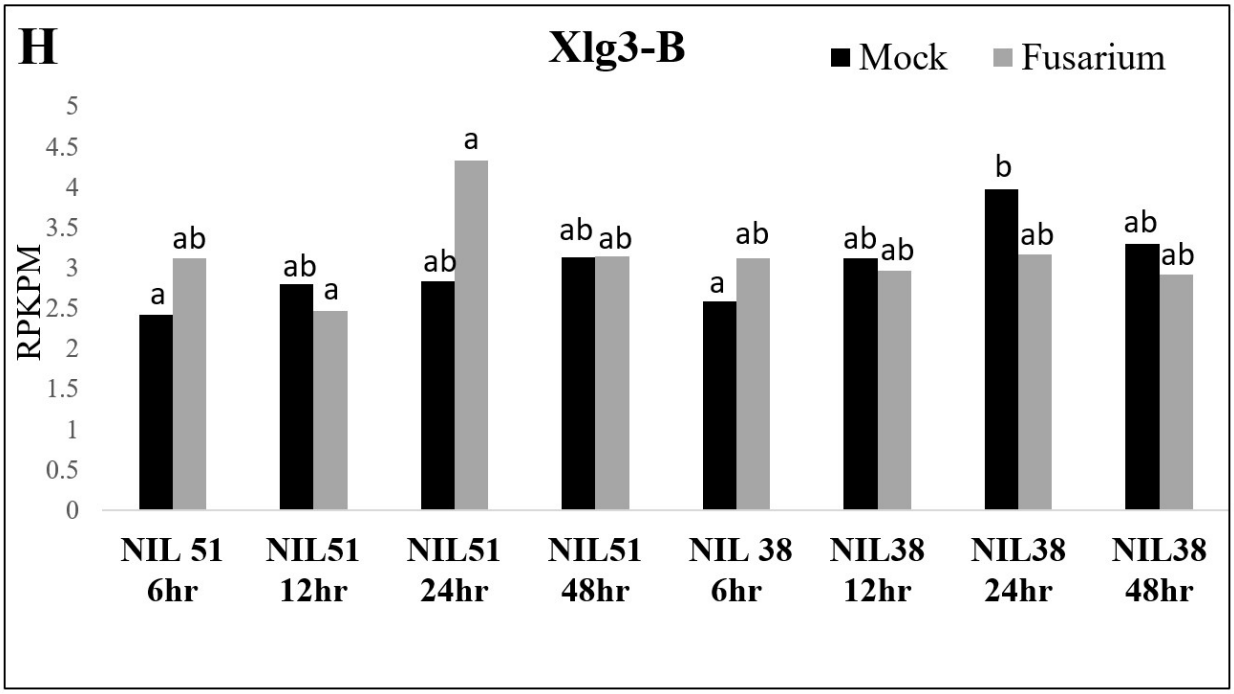
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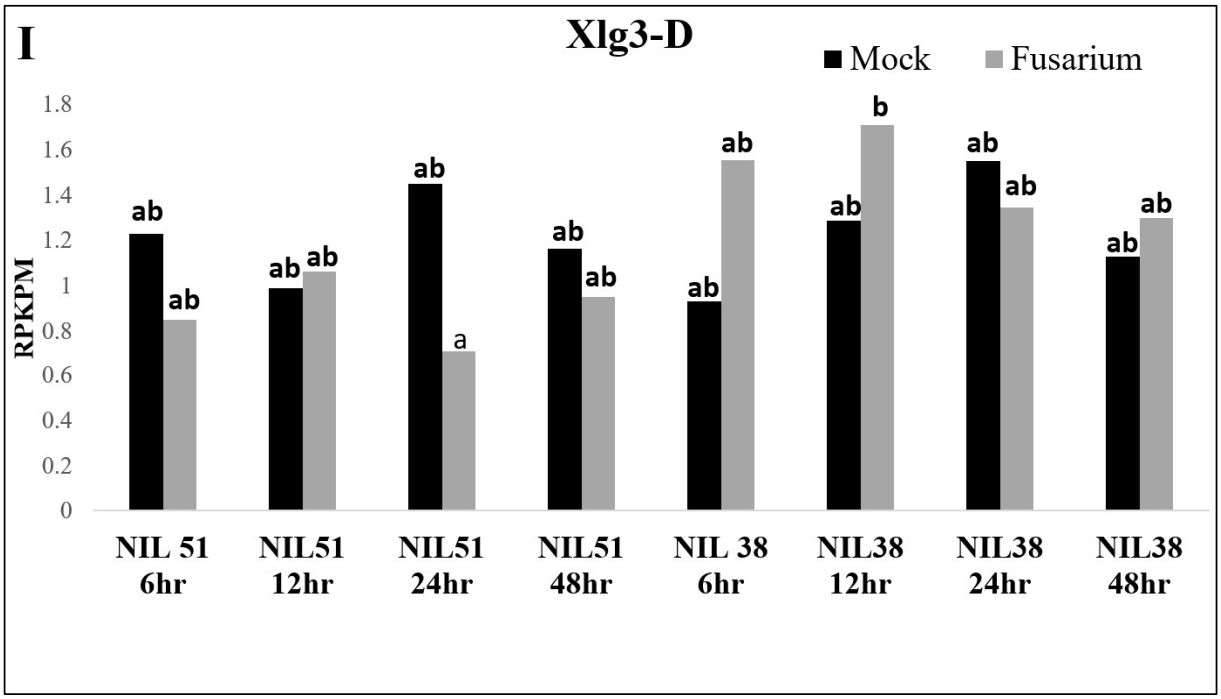

Figure 5. Transcriptional regulation of *Xlg* protein in response to *F. graminearum* infection. Developing spikes were sampled in response to Fusarium infection in the resistant line, NIL 38, and the Fusarium susceptible line NIL 51. The levels of transcripts were normalized to Reads Per Kilobases Per Millions (RPKPM) for comparison. Duncans multiple range tests results are shown on the graph.

3.5.6 Phylogenetic analysis of the Extra-Large G protein amino acid sequences.

Molecular phylogenetic analysis of the monocots; *Triticum aestivum*, *Brachypodium distachyon*, *Sorghum bicolor* and *Oryza sativa* showed that, the amino acid sequences for each member of the Xlg gene family is conserved among the monocots assayed in this study (Figure 6).

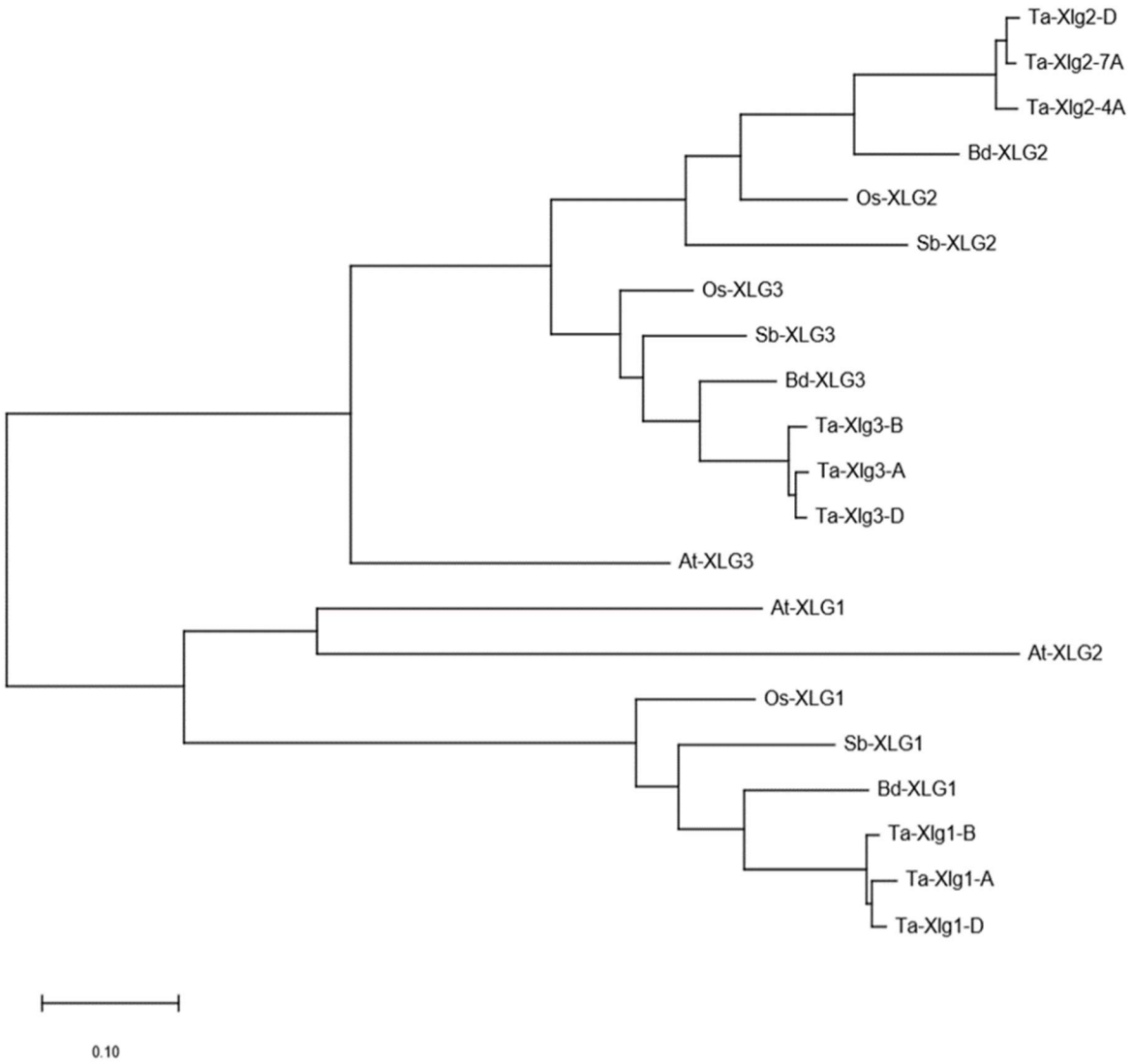

Figure 6. Molecular Phylogenetic analysis of the Extra-Large G protein amino acid sequences. The tree shows that the Extra-Large G protein family is conserved among monocots. The evolutionary history was inferred by using the Maximum Likelihood method and Whelan And Goldman model (Whelan and Goldman 2001). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The phylogenetic tree and evolutionary analyses were conducted using in MEGA X (Kumar et al. 2018). The tree shows evolutionary relationships between Ta (*Triticum aestivum*), Bd (*Brachypodium distachyon*), Sb (*Sorghum bicolor*), Os (*Oryza sativa*) and At (*Arabidopsis thaliana*) amino acid sequences.

3.6 Discussion

Automated annotation of sequences and determination of the exon and intron junctions might lead to incorrect exon designations and errors in the coding sequence and consequently wrong annotation of the predicted translated protein. Although at least one correct annotation was found for each of the *Xlg* genes for *T aestivum* in Ensembl Plant, some of the genes have alternate annotated versions that appear to be erroneous. The D-copy of the *Xlg1* coding sequence had two alternative predicted protein sequences based on different exon/intron junctions. The genes with the Ensembl Plant identifiers TraesCS5D02G075200.2 codes for a protein with 865 amino acids and TraesCS5D02G075200.1, codes for 880 amino acids. The coding sequence and the translated protein of (TraesCS5D02G075200.1), agreed with corresponding transcript sequences in the TSA gene transcript database and matched with about 97% nucleotide sequence identity with the other two homeologous copies of the gene; *Xlg1-A* and *Xlg1-B*. The differences between two annotated transcripts is an error in variant TraesCS5D02G075200.2, that leads to a deletion of 15 amino acids at position 377 of the protein in the variant. For *Xlg1-B* copy there were also two alternative transcript IDs; TraesCS5B02G068300.2 codes for 878 amino acids and TraesCS5B02G068300.1 that codes for 728 amino acids protein. The longer version (.2) was supported by transcript contigs in the *T. aestivum* TSA database and high sequence similarity to the other two homeologs. There was approximately 97% nucleotide sequence identity and 97-100% amino acid sequences identity within the CDS among the homoeologous copies of each *Xlg* coding sequence from the A, B and D genomes. The previous studies showed about 97% nucleotide sequence identity in coding sequences and about 99-100% amino acid sequence identity between homeologs among members of other gene families in *T. aestivum* including the Early salt induced 3 (*Esi3*) gene family (Brunetti, et al. 2018), the caleosin gene family (*Clo*) (Khalil et al. 2014) and alpha tubulins (Ridha Farajalla & Gulick, 2007).

The Extra-Large G protein family is a small gene family with only three paralogous members in *T. aestivum* named *Xlg1*, *Xlg2* and *Xlg3*. In the *T. aestivum* hexaploid genome, the *Xlg1* and *Xlg3* have one homeologous copy in each of the A, B and the D genome for total of three copies for each gene. The *Xlg2* was found to have two copies on A genome chromosomes with one copy on chromosome 4A and the other one on chromosome 7A and the third copy was found on the D genome of the 7th chromosome. The copy of *Xlg2* on chromosome 4A is

consistent with translocated B genome copy of the gene. The *T. aestivum* chromosome 4A is known to be the result of two reciprocal translocations between chromosomes 4A, 5A and 7B (Nelson et al. 1995). The other *Xlg2* copy on an A genome chromosome, 7A, has 99.6% nt sequence identity with a *Xlg2* like sequence in the genome of *T. urartu*, the A genome progenitor for *T. aestivum*, whereas the gene copy on chromosome 4A has only 97% sequence identity with the *T. urartu* gene. The D copy of *Xlg2* is located on chromosome 7 and the two flanking genes are homeologous to the genes flanking the *Xlg2* genes on the other two chromosomes, indicative of a block of genes from chromosome 7B being translocated to chromosome 4A. *T. turgidum*, the tetraploid progenitor of *T. aestivum*, also has copies of *Xlg2* on chromosomes 4A and 7A, and no gene copy on a B genome chromosome which indicates the translocation predates the second polyploidization event that gave rise to *T. aestivum*. In addition, a pseudo gene with high sequence identity with the other *Xlg2* genes was identified on chromosome 7A; comparison of the transcript from the gene identified in the TSA database had two regions of deletions, of 17 and 22 nt and one of an insertion 118 nt, all of which produced frameshifts in the CDS.

For all the expression analysis the 4A copy of the gene was treated as the B genome copy to perform CD-HIT algorithm to facilitate analysis, since homeologous sequences have to be run separately in the program, however consistency of the chromosomal location was kept in the gene designation with two copies in the A genome and one copy in the D genome for all the interpretations of the gene.

Phylogenetic analysis showed that, the Extra Large GTP binding proteins are conserved with no gene loss or duplications among the monocots analyzed in this study including: *Triticum aestivum, Sorghum bicolor, Oryza sativa and Brachypodium distachyon* compared to the dicot *Arabidopsis thaliana* by using amino acid sequences alignments (Figure 5).

The Extra-Large G proteins have been reported to be involved in Arabidopsis salt and drought stress responses through protein-protein interaction with other members of the G protein complex (Liang et al. 2017). This study reports that in addition to the protein-protein interaction reported in Arabidopsis, the A copies of the *Xlg* genes in *T. aestivum* are also regulated at transcriptional level under either drought, heat treatment or combined stress.

The expression analysis showed that other than *Xlg2-4A*, no other *Xlg* copy in *T. aestivum* is changed at the transcript level in response to cold stress. Previous studies showed that, Arabidopsis Gα, GPA1, is involved in plant cold stress response through interacting with

COLD1 protein, elevation of Ca^{2+}, and up-regulation of genes involved in cold stress responses (Wu & Urano 2018). On the other hand, another study reported that GTPase activity of the XLGs in Arabidopsis is dependent on presence of Ca^{2+} unlike other G proteins that are Mg^{2+} dependent (Ding at al. 2008; Chakravorty et al. 2015; Heo et al. 2012) which can suggest involvement of *Xlgs* in cold stress response through calcium ion dependent GTPase activity and not necessarily by regulation at the transcript level.

This study showed that, all three homoeologous copies of the *Xlg2* genes are transcriptionally regulated and that they are involved in *F. graminearum* infection response. Previously it has been reported that mutant lines of α subunit of the heterotrimeric G protein complex, *gpa1*, had enhanced resistance to *Fusarium oxysporum* (Trusov et al. 2006) while *xlg* mutants showed to be more susceptible to *Fusarium oxysporum* infection in Arabidopsis (Maruta et al. 2015). In addition, Arabidopsis *XLG2* and *XLG3* transcripts have been previously reported to be up-regulated under *Pseudomonas syringae* infection (Zhu et al. 2009) and *xlg* mutants in Arabidopsis showed to be more susceptible to pathogens (Chakravorty et al. 2015).

Chapter 4

Conclusions and Future work

4.1 Conclusions

The characterization of interaction of Arabidopsis CLO4 and GPA1 proteins expands the repertoire of interacting partners of the members of the heterotrimeric G protein complex in *Arabidopsis thaliana*. This study showed that *CLO4* is transcriptionally down-regulated in ABA treated Arabidopsis roots. Although the CLO4 gene is among ABA responsive genes, the mutant line of the *clo4* gene does not always alter the phenotype under control condition and in response to ABA; probably due to being a member of a multigene family. This study suggests that, Arabidopsis CLO4 is positive regulator of stomatal development and acts in the same pathway with GPA1; the mutant lines showed reduction of stomatal density with the double mutant showing a phenotype closer to *gpa1* single mutant and over expressing CLO4 in Arabidopsis was not sufficient to increase stomatal density.

RD20/CLO3, another member of the caleosin gene family was shown to have very low and insignificant GAP activity toward GPA1. Since members of the caleosin gene family showed interaction with GPA1, we hypothesized that RD20 may act as a GAP protein. This was not shown to be the case. The GTPase assay shown in this study suggests that caleosins regulate stress response through the G protein complex without affecting GTP hydrolysis rate of the Gα subunit.

T. aestivum contains nine gene encoding XLG proteins. The three paralogous genes; *Xlg1*, *Xlg2* and *Xlg3* showed different responses under cold treatment, higher temperature, drought and in response to plant pathogen. Interestingly, homeologous copies of each gene did not show the same response to the assayed stress conditions. This highlights complexity of wheat genome and the evolutional adaptation of the plant in response to varied stress. This study also highlighted how the Extra-Large G proteins are conserved among monocotyledons such as rice, wheat, brachypodium and sorghum.

The Extra-Large G proteins in Arabidopsis have been shown to be a stress response gene and to interact with members of the G protein complex (Chakravorty et al 2015). This study showed that some of the wheat *Xlg*s are transcriptionally regulated while some paralogous and homeologous copies did not have altered gene expression. These findings suggest these genes

may regulate signalling in the stress response at post-transcriptional and post translational levels such as protein-protein interactions.

4.2 Future Work

The interaction between CLO4 and GPA1 suggests that CLO4 could play a role in competitive binding between GPA1 and the Gβγ complex. Currently there is no well-established technique that can measure and evaluate the relative interaction strength of competitive interactions between protein complexes and other members of the caleosin gene family *in planta*. Experiments *in vitro* or *in vivo* by the aid of heterologous protein expression in *E. coli* or yeast, has limitations for caleosin gene family and the members of the G protein complex considering the proper folding pattern, localization of the proteins and presence of the amphipathic alpha helix domain in caleosins. One approach could be co-expression of the proteins to evaluate the competitive binding through transient expression of the genes using BiFC in *Nicotiana benthamiana* leaf.

This study showed that stomatal density of Arabidopsis was affected in the *clo4* mutant line in 15 day old plants. Further investigations could be performed on older and fully expanded Arabidopsis leaves to further evaluate the role of the gene in regulation of stomatal density with detailed analysis on stomatal initiation and development.

Expression level of the *GPA1* gene transcript in the *CLO4* over-expressing plants could be assayed to determine if over expression of *CLO4* can affect transcript level of the *GPA1* gene. In this study the observed phenotypes for the *CLO4* over-expressing plants was similar to the *clo4 gpa1* double mutant line in terms of lateral root elongation, primary root and stomatal aperture. One explanation of these results could be that overexpression of *CLO4* gene can affect expression level of *GPA1*. However transcriptional regulation of GPA1 by CLO4 is less likely to be the case since the two proteins interact; suggesting post translational regulation of GPA1 rather than transcriptional regulation with CLO4.

Identifying domains of the CLO4 and GPA1 proteins which are critical for the interaction would help with better understanding of how the heterotrimeric G protein complex is regulated by different proteins that are not members of the trimer themselves in Arabidopsis. Since the interacting domains between the α and the βγ subunits are known, protein interacting with the same domains might have been interfering with trimer formation. Such a study could be

expanded to the other members of the caleosin gene family. The GPA1 truncation did not show any interaction with the CLO4 protein using BiFC. Amino acid substitutions on GPA1 by site-directed mutagenesis could also be used to identify the interacting domains of GPA1 with CLO4. Another important finding would be characterization of the real structure and folding pattern of CLO4 or other members of the caleosin gene family. The CLO4 protein contains amphipathic alpha helix domain referred to as a transmembrane domain in most of the studies however this domain might not pass any membrane or may divide the protein in two sides of the organelle or just separate the two domains of the protein by anchoring to one side of the plasma membrane. Knowledge of the structure of the protein might pave the way for future studies of caleosins and probably other calcium binding proteins in plants and other organisms. Organelle purification and testing degradation with proteases could also help with identification of cytoplasmic domains.

Possible interaction between the two members of the caleosins gene family CLO4, CLO3 and members of the Arabidopsis XLG1, XLG2 and XLG3 could be assayed using Bimolecular Fluorescent Complementation assay (BiFC). In addition, interaction of the Clo3 protein from *T. aestivum* could be assayed with the three members of the *T. aestivum* Extra-Large G proteins using BiFC in *Nicotia benthamiana* leaf. Interaction of the proteins will expand the understanding of the heterotrimeric G protein complex signaling in plants and answer questions regarding G protein signaling. In addition, double and triple mutant analysis of *clo3*, *clo4* and *xlg* in Arabidopsis may answer many questions regarding regulation mechanism of caleosins gene family and the heterotrimeric G protein complex in *Arabidopsis thaliana*.

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10.4161/psb.6.4.14836. PubMed PMID: 21673513; PubMed Central PMCID: PMC3142386.

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