

8-19-2015

# Secondary Messengers Involvement in Plant Signal Transduction

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# Secondary Messengers Involvement in Plant Signal Transduction

Hsuan Chou, PhD

University of Connecticut, 2015

Secondary messengers play important roles in plant signal transduction. This dissertation focus on secondary messengers cyclic nucleotides (cAMP and cGMP) and  $\text{Ca}^{2+}$  and their involvement in different signaling pathways to affect Arabidopsis plant physiology. Leucine rich repeat receptor like kinase (LRR-RLK) CLAVATA1 (CLV1) and BRASSINOSTEROID INSENSITIVE 1 (BRI1) receptors are involved with shoot apical meristem (SAM) development and hormone signaling. The ligands for these receptors are peptide CLV3 and steroid hormone BR, respectively. CLV3 and BR binding to their receptors can cause cytosolic  $\text{Ca}^{2+}$  elevation and affect ligand dependent gene expression and phenotypes. Both CLV1 and BRI1 have a guanylyl cyclase (GC) domain. cGMP activated  $\text{Ca}^{2+}$  conducting ion channel cyclic nucleotide gated channel (CNGC) 2 is involved in both signaling pathways. A calmodulin (CaM) binding transcription activator (CAMTA) 3 is involved in the  $\text{Ca}^{2+}$ -dependent BR signaling pathway to decode and transmit  $\text{Ca}^{2+}$  signals into the nucleus to modulate gene expression. The level of cyclic nucleotides in plants are much lower than in animals and published data only show plant cAMP level *in vitro*. We developed a new biosensor that can help determine cAMP level change *in planta* based on luminescence light output change. Although published data suggest plants also have a functional cAMP-dependent signaling pathway system, the only annotated and experimentally confirmed cAMP-generating enzyme in plants is a maize pollen adenylyl cyclase (AC). Only a partial sequence was reported and the linkage of the gene with the cAMP-dependent phenotypes was never demonstrated in the plant. We used a bacteria screening method to search for potential Arabidopsis AC. The screening result suggests it is not a good method

because the potential gene that encodes an AC we identified does not belong to Arabidopsis but is a gene from *Salmonella enterica* (*S. enterica*) subspecies. The results in this thesis imply that there are cyclic nucleotide activities in plants, and the enzyme that generates cyclic nucleotides may be from nucleotide cyclases (NC)s or other unidentified signaling proteins.

**Secondary Messengers Involvement in Plant Signal Transduction**

**Hsuan Chou**

**B.S., National Chung Hsing University, 2010**

A Dissertation

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

at the

University of Connecticut

2015

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2015

APPROVAL PAGE

Doctor of Philosophy Dissertation

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## Acknowledgement

I would like to express my sincere appreciation to my advisor, Dr. Gerald Berkowitz. I have learned a lot from you in the past five years, not only how to be a good scientist, but also how to be a good mentor. Coming to the lab without a master's degree, I had little experience in research. You taught me with baby steps on how to do research when I first begin by giving me a lot of information. As I advanced, you taught me how to think indepdently by only giving me tips when I don't know the answers. You are always very encouraging and inspiring. You always remind me to think about what we have proven and what we have not proven on my research throughout the years of my studies. I would also like to thank you for helping me with my writing, from the simplest abstracts for conferences to my PhD dissertation. I learned how to be more specific and precise in writing (and in scientific presentations), and also how to write with better English. This dissertation would not have been completed without your insightful insturctions and opinions.

I would also like to dediacte my greatful thanks to my committee members, Dr. Yi Li, Dr. Charles Giardina and Dr. Huanzhong Wang. I really appreciate all of you providing materials for my research, spending time reading my dissertation and giving me helpful suggestions on how to become a better scientist. I would also like to thank Dr. Thomas Chen for attending my qualifying exam and dissertation proposal defense.

I thank all the previous and present members in the Berkowitz lab. Special thanks to Dr. Yi Ma and former lab member Dr. Yingfang Zhu for teaching me so much in doing bench work. I also thank Alice Zelman and former lab member Dr. Yichen Zhao for helping me so much throughout the years. I had a great time working with all of you.

I also thank the Taiwanese Student Association for bringing the small group of Taiwanese students at UConn altogether. Thank you Huang Chung, Steven, Ken for your help when I first came to UConn. Thank you Anna, Kristen, Yungching, Hsinbai, Chihong, Jieiu, Oliver and Matthew for being such a good friend to me. I feel really lucky to meet all of you here at UConn and I cherish this friendship very much.

The most important people I would like to thank are my parents Dr. Shihyu Chou and Linfang Yu and my boyfriend Dr. Chingchang Chung. I thank my parents for motivating me to study abroad and encouraging me to pursue a PhD. I am also truly thankful for your financial support and mental support. I thank my boyfriend for taking care of me and accompanying me these years at UConn. I also thank you for telling me not to give up and reminding me the passion I have in science when I feel burned out. Without the encouragement from you, I would never have reached this point.



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## Chapter 1 Overview

The research work presented in this dissertation is focused on cell cytosolic secondary messengers involved in Arabidopsis plant signal transduction. Environmental and hormonal signals regulate various physiological processes in plants via signal transduction pathways. Secondary messengers are molecules inside cells that act to transmit signals from a receptor (on the cell surface) to a target (in the cytoplasm or nucleus). Secondary messengers can have multiple downstream targets, thereby expanding the scope of signal transduction. The importance of studying secondary messengers is to understand the mechanism of how they signal and what is downstream from them. By understanding the complicated network of the signal transduction pathways, we will be able to alter plants' response to external stimuli (i.e. by using genetic or pharmacological approaches) and eventually apply the changes on crops, improving crop yield. Examples of secondary messengers in the plant kingdom include nitric oxide (NO), carbon monoxide (CO), 1,2-diacylglycerol (DAG), nicotinic acid adenine dinucleotide phosphate (NAADP) and cyclic ADP ribose (cADPR), cyclic nucleotides (adenosine 3',5'-cyclic monophosphate (cAMP) and guanosine 3',5'-cyclic monophosphate (cGMP)), inositol 1,4,5,-triphosphate ( $IP_3$ ), as well as possibly  $IP_6$  and  $Ca^{2+}$  (Volotovskii et al., 1998, Lee 2001; Xia and Yang, 2005; Ma et al., 2009; Dodd et al., 2010; Dong et al., 2012; Stael et al., 2012; Gayatri et al., 2013; Sarwat et al., 2013; He and He, 2014). The secondary messengers we study and present in this dissertation are cyclic nucleotides (cAMP and cGMP) and  $Ca^{2+}$ , and the role they play in physiology and development in the Arabidopsis plant.

Cyclic nucleotides are known as secondary messengers involved in signal transduction in animals, fungi and prokaryotes (Gehring, 2010). In animals, cAMP and cGMP are generated by enzymes adenylyl cyclase (AC) and guanylyl cyclase (GC). These enzymes utilize ATP and GTP

as substrates to generate cAMP and cGMP, respectively. In plants, the presence and the role of cyclic nucleotides is still open to debate. The reason why it is highly controversial is because the low levels of cyclic nucleotides in plants and the uncertainty of enzymes that generate cyclic nucleotide. The only annotated and experimentally confirmed cAMP-generating enzyme in plants is a maize pollen AC (only a partial sequence was reported and the linkage of the gene with the cAMP-dependent phenotypes never demonstrated in the plant) alleged to be associated with polarized pollen tube growth, which in turn depends on cAMP (Moutinho et al., 2001). In fact, the publication of the first AC sequence from a plant in *Nature* by the internationally-known plant molecular biologist Jeff Schell and colleagues (Ichikawa et al., 1997) and the subsequent retraction of this work may be among the most high-visibility cases of scientific fraud in plant biology over the last half-century and perhaps (some contend) cost Shell (a co-inventor of *Agrobacterium* for plant transformation) a Nobel Prize!

The first GC (AtGC1) in higher plants was discovered by Ludidi and Gehring (2003). Based on an alignment of conserved and functionally assigned amino acids in the catalytic center of annotated type III GCs from lower and higher eukaryotes (Liu et al., 1997; McCue et al., 2000), 14 amino acids were suggested to be the GC core motif (Ludidi and Gehring, 2003). More candidate GCs in *Arabidopsis* were annotated and several of them are leucine rich repeat receptor like kinases (LRR-RLKs); the putative GC domain identified lies within the cytosolic kinase domain. These LRR-RLKs include BRASSINOSTEROID INSENSITIVE 1 (BRI1), PLANT ELICITOR PEPTIDE RECEPTOR 1 (PEPR1) (which have already been shown to be catalytically active *in vitro*) (Kwezi et al., 2007; Qi et al., 2010), CLAVATA1 (CLV1) receptor and ERECTA receptor. However, a recent publication suggested, based on crystal structure analysis, that the amino acid residues proposed as comprising the GC catalytic center are buried



deep inside the hydrophobic core of the BRI1 kinase domain, making the GC domain unlikely catalytically competent (Bojar et al., 2014). The analyses from Bojar et al. (2014) are inconsistent with the proposed GC activity. None of the purported GCs have been shown to function as such *in vivo* by demonstration that a null mutation of the corresponding gene affects cGMP generation in the plant under any circumstances (either basal levels or a rise due to initiation of a signaling cascade).

Aside from the issues about nucleotide cyclases (NCs), cyclic nucleotide existence and functions in plants were also highly in debate several decades ago (Keates, 1973; Amrhein, 1974). The main reason for this was the low sensitivity of the methods employed to detect cAMP at the picomole level. The experimental procedures for studying cAMP in animals were not adapted for plants until the late 70s. After the mid 1980s, more studies of cyclic nucleotides in plants have been shown. It is clear that cyclic nucleotides are present in various plant tissues as well where they are involved in the regulation of several important plant processes, e.g. cation flux regulation (Maathuis and Sanders, 2001; Essah et al., 2003; Maathuis, 2006; Rubio et al., 2007), pathogen response (Durner et al., 1998; Ma et al., 2009), stomatal opening (Newton and Smith, 2004), cell cycle in tobacco BY-2 cells (Ehsan et al., 1998) and chloroplast development (Bowler et al., 1994). Although it is still unclear how cAMP or cGMP (cNMP) signals are relayed to downstream components in plants, studies have shown that both cell permeable 8-Bromo-cAMP (8-Br-cAMP) and stimulation of albeit unknown adenylyl cyclases (ACs) with forskolin are able to elicit concentration and time-dependent biological responses such as increases in  $\text{Ca}^{2+}$  influx across the plasma membrane (Kurosaki and Nishi, 1993; Kurosaki et al., 1993; Volotovskii et al., 1998). Both membrane permeable cAMP and cGMP are able to increase

cytosolic  $\text{Ca}^{2+}$  likely from activation of cyclic nucleotide gated channels (CNGCs) (Lemtiri-Chlieh and Berkowitz, 2004; Qi et al., 2010).

There are 20 CNGCs in Arabidopsis, all CNGCs except CNGC 20 are specifically localized to the plasma membrane (Mäser et al., 2001; Yuen and Christopher, 2013). Plant CNGCs consist a cyclic nucleotide binding domain (CNBD) and a calmodulin binding domain (CaMBD). Unlike animal CNGCs, which the CNBD and CaMBD are found in the opposite C and N termini, plant CNGCs have the CNBD and CaMBD both at the carboxyl terminus (Köhler et al., 1999; Arazi et al., 2000; Bridges et al., 2005; Kaplan et al., 2007). cAMP and cGMP have been shown to activate plant CNGCs, allowing cations to move into the cytosol (Leng et al., 1999, 2002; Lemtiri-Chlieh and Berkowitz, 2004; Ali et al., 2007; Qi et al., 2010). Plant CNGCs function as non-selective cation channels (Mäser et al., 2001; Talke et al., 2003; Kaplan et al., 2007). Some CNGCs have been linked to  $\text{Ca}^{2+}$  signaling (Leng et al., 1999; Ali et al., 2007; Frietsch et al., 2007; Urquhart et al., 2007; Chan et al., 2008; Guo et al., 2010; Gao et al., 2012; Tunc-Ozdemir et al., 2012; Gao et al., 2014). CNGCs are a molecular mechanism that links cyclic nucleotide and calcium signaling in plants. This is one basis for the focus of much of the work in this dissertation on CNGCs, and the role they play in some signaling pathways.

Calcium is a ubiquitous secondary messenger that plays an important role in numerous signal transduction pathways. All cells, regardless whether animal, plant or prokaryote, use elevation of cytosolic  $\text{Ca}^{2+}$  as a means of translating many external signals into altered cell function and gene expression. The changes of cytosolic  $\text{Ca}^{2+}$  in green algae is the first published examples of  $\text{Ca}^{2+}$  functioning as a secondary messenger in plants (Williamson and Ashley, 1982). Transient cytosolic free  $\text{Ca}^{2+}$  elevations in plants occur from the movement of  $\text{Ca}^{2+}$  through plasma membrane  $\text{Ca}^{2+}$  channels in response to signals such as pathogen elicitors, hormones,

abiotic stress, and herbivore attack/wounding stress (Qudeimat et al., 2008; Ma et al., 2009; Bose et al., 2011; Reddy et al., 2011). It has also been reported that the changes of the cytosolic  $\text{Ca}^{2+}$  concentration were triggered by cellular secondary messengers, for example, cAMP, cGMP, nicotinic acid adenine dinucleotide phosphate (NAADP) and cyclic ADP ribose (cADPR) (Volotovski et al., 1998, Lee 2001; Ma et al., 2009; Dodd et al., 2010). As a secondary messenger itself, cytosolic  $\text{Ca}^{2+}$  increase in plants due to external stimuli can then interact with CaM (a  $\text{Ca}^{2+}$  binding protein) and different families of kinases (i.e.  $\text{Ca}^{2+}$ -dependent protein kinases (CDPKs), CDPK-related kinases (CRKs),  $\text{Ca}^{2+}$  and calmodulin-activated kinases (CCaMKs), and Snf1-related kinases (SnRK3s)). The SnRK3 is also called calcineurin B-like protein interacting protein kinase (CIPK) (Harper et al., 2004). These proteins all utilize  $\text{Ca}^{2+}$  sensors that bind  $\text{Ca}^{2+}$  through multiple EF-hands (a helix-loop-helix structural domain that is found in a large family of  $\text{Ca}^{2+}$  binding proteins) and decode this signal by transmitting it to the nucleus to regulate gene expression (Harper et al., 2004; Hashimoto and Kudla, 2011; Reddy et al., 2011; Cheval et al., 2013).

The research described in chapter 2 focuses on elucidating a new signal transduction pathway. The new signal transduction pathway suggests  $\text{Ca}^{2+}$  is involved in mediating peptide/receptor regulated gene expression to control shoot apical meristem (SAM) development. CLAVATA 3 (CLV3) is a signaling peptide which binds to its leucine-rich-repeat receptor like kinase (LRR-RLK) CLV1 and regulates expression of *WUSHEL* (*WUS*) to control the SAM size. In our model, we hypothesize the guanylyl cyclase (GC) activity in the CLV1 receptor cytoplasmic domain is able to convert GTP into cGMP. cGMP activates CNGC channels and induces cytoplasmic  $\text{Ca}^{2+}$  elevation. The change of cytoplasmic  $\text{Ca}^{2+}$  level will mediate downstream gene regulation and affect the SAM size. Although the GC activity of this receptor

is still open to debate, our results indicate that cGMP and  $\text{Ca}^{2+}$  are involved in the CLV3/CLV1 signaling pathway regulating *WUS* gene expression.

Chapter 3 describes the development of a new tool to measure the change of cAMP level in response to an external stimulus e.g. chemicals and signaling peptides *in vivo* in plant protoplasts and also *in planta*. The pGloSensor™-22F cAMP Plasmid is a commercial plasmid. It is used to detect cAMP level change in Human Embryonic Kidney (HEK) 293 cells. The coding region of the plasmid consists of a firefly luciferase. The firefly luciferase is disrupted with an animal cAMP binding site. Upon cAMP binding to the binding site, it allows a conformational change of the firefly luciferase leading to emission of light. Measurement of the luminescence change can indicate the level of cAMP change. It will be useful to subclone the coding region of pGloSensor™-22F cAMP plasmid and express it in plants. We have successfully subcloned the gene into a plant expression plasmid and generated transgenic plants “GloSensor”. We have developed an assay to measure and analyze luminescence caused by cAMP level change in Arabidopsis transgenic plants. In our findings, the GloSensor transgenic leaves respond to exogenous cAMP and forskolin (an AC activator) by giving more light output. An adenylyl cyclase inhibitor prevents the effect of forskolin on GloSensor transgenic leaves, resulting in lower light output. Although more experiments need to be done to evaluate this new biosensor, our current results suggest that the pGloSensor™-22F cAMP coding region works in a plant system and can be used as a tool to measure plant cAMP level change.

Chapter 4 describes using a bacterial mutant screening method to search for potential Arabidopsis adenylyl cyclases. A library of synthesized Arabidopsis cDNA generated from total plant mRNA is ligated to the plasmid portion of  $\lambda$ YES vector and transferred into a *cyaA* mutant *Escherichia coli* (*E.coli*) bacteria SP850. *E.coli* SP850 is a *cyaA* deletion mutant and thus lacks

both adenylyl cyclase and cAMP. Due to the *cyaA* mutation, cAMP cannot be synthesized, strain SP850 cannot utilize lactose and will produce white colonies on MacConkey plates. On the contrary, a colony on MacConkey plates that is a potential AC will present a red color. This method was used to demonstrate that the *Prevotella ruminicola D31d* gene is an adenylyl cyclase (Cotta et al., 1998). We have found three colonies that showed in a red color throughout the screening process. With proper restriction enzyme digestion, all three cDNA fragments show the same size of 4.3 Kb. The three candidates were sequenced and were all identical. Unfortunately sequencing results did not indicate the cDNA is an Arabidopsis AC. In fact the cDNA fragment is not a gene from Arabidopsis but rather a gene from Salmonella sub species. This result could be explained by cDNA library contamination when it was made. Since the gene identified is not a Salmonella sub species AC, it suggests the complementation assay isn't a good assay for screening potential ACs. Therefore we decided not to invest future time on this project.

Chapter 5 describes linking cytosolic  $\text{Ca}^{2+}$  elevation to the molecular steps that modulate brassinosteroid (BR) responsive gene regulation in the BR signaling cascade. Previous work done in the lab indicates that a CNGC-dependent  $\text{Ca}^{2+}$  signal is involved in at least a component of the BR signal transduction pathway. Here we provide gene expression and phenotype results that show Calmodulin transcription activator (CAMTA) 3 may decode BR-induced  $\text{Ca}^{2+}$  elevations and transmit the signal to the nucleus to modulate gene expression.

## **Chapter 2: The CLAVATA signaling pathway mediating stem cell fate in shoot meristems requires calcium**

### **Abstract**

CLAVATA (CLV) 1 is a receptor protein expressed in the shoot apical meristem (SAM) that translates perception of a non-cell-autonomous CLV3 peptide signal into altered stem cell fate. CLV3 reduces expression of WUSHEL (*WUS*) and FANTASTIC FOUR 2 (*FAF2*) in the SAM. *WUS* and *FAF2* expression lead to the maintenance of undifferentiated stem cells in the SAM. CLV3 binding to CLV1 regulates expression of these genes and stem cell fate in the SAM through an unidentified signaling pathway. CLV1 has a putative cytosolic guanylyl cyclase (GC) domain and generation of cytosolic cGMP can open  $\text{Ca}^{2+}$ -conducting ion channels, elevating cytosolic  $\text{Ca}^{2+}$ . Hence, we propose that cytosolic  $\text{Ca}^{2+}$  elevation mediates CLV3 ligand/CLV1 receptor signaling that controls meristem stem cell fate. CLV3 application to Arabidopsis seedlings results in elevation of cytosolic  $\text{Ca}^{2+}$  and cGMP. CLV3 control of *WUS* and *FAF2* expression was prevented in genotypes lacking functional cGMP activated  $\text{Ca}^{2+}$  conducting channels, and in wild type plants treated with either a  $\text{Ca}^{2+}$  channel blocker or a GC inhibitor. When the CLV3-dependent repression of *WUS* is blocked, altered control of stem cell fate leads to an increase in SAM size; we observed larger SAM size in seedlings treated with the  $\text{Ca}^{2+}$  channel blocker. These results suggest that the CLV3 ligand/CLV1 receptor system initiates a signaling cascade that elevates cytosolic  $\text{Ca}^{2+}$ , and that this cytosolic secondary messenger is involved in the signal transduction cascade linking CLV3/CLV1 to control of gene expression and stem cell fate in the SAM.

## Introduction

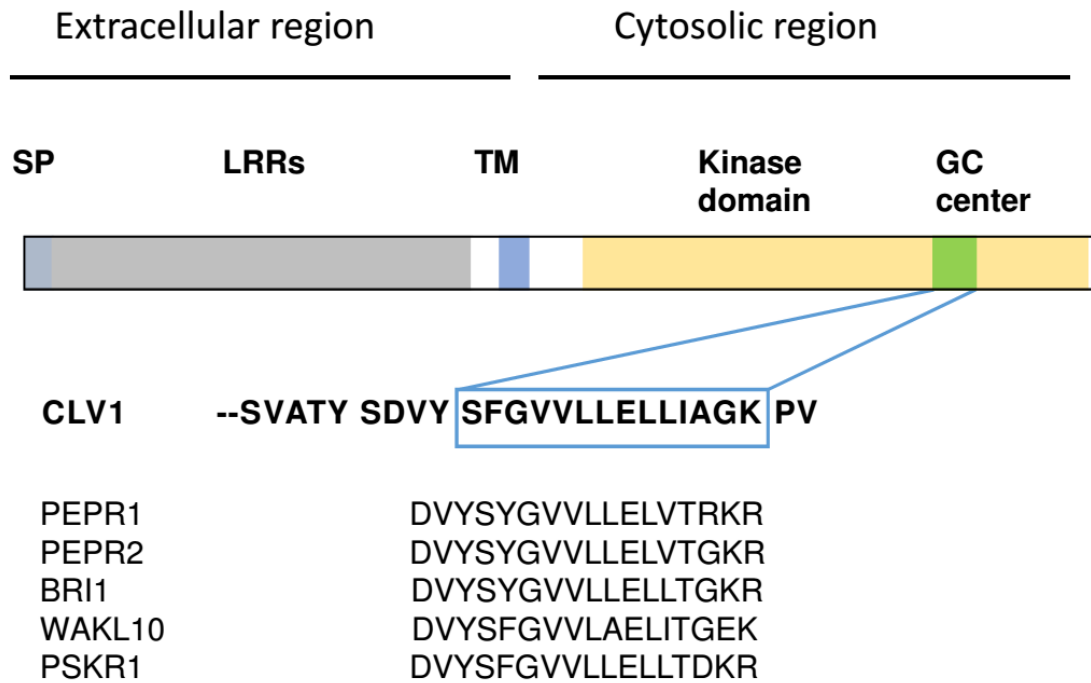
Calcium is a ubiquitous secondary messenger that plays an important role in numerous signal transduction pathways. All cells, regardless whether animal, plant or prokaryote, use elevation of cytosolic  $\text{Ca}^{2+}$  as a means of translating many external signals into altered cell function and gene expression. Transient cytosolic free  $\text{Ca}^{2+}$  elevations in plants occur in response to signals such as pathogen elicitors, hormones, abiotic stress, and herbivore attack/wounding stress (Qudeimat et al., 2008; Bose et al., 2011; Ma et al., 2011; Reddy et al., 2011). Leucine-rich repeat receptor like kinases (LRR-RLKs) are a large (approximately 235 members) protein family in plants. Many of the LRR-RLKs are plasma membrane-localized proteins that are involved in plant signaling pathways, including perception of these same aforementioned extracellular cues that evoke  $\text{Ca}^{2+}$  elevations as cytosolic secondary messages (Chaea et al., 2009).

A plethora of evidence suggests that the cytosolic kinase domain of LRR-RLKs (as well as autophosphorylation and transphosphorylation between LRR-RLK co-receptors) is involved in downstream signaling (Stone et al., 1998; Nam et al., 2002; Clouse 2011; Schwessinger et al., 2011). However, there is much less evidence at this time documenting that LRR-RLK phosphorylation of downstream proteins is the sole, or primary mechanism by which these receptors initiate cytosolic signaling. Alternatively, in the case of some specific LRR-RLKs, a cytosolic guanylyl cyclase (GC) domain of the receptor protein may be involved in a signaling pathway that involves generation of cytosolic free  $\text{Ca}^{2+}$  elevation downstream from receptor activation by the ligand. The (Arabidopsis) LRR-RLKs PLANT ELICITOR PEPTIDE RECEPTOR 1 (PEPR1), PEPR2, WALL ASSOCIATED KINASE-LIKE 10 (WAKL10), PHYTOSULFOKINE RECEPTOR 1 (PSKR1), and BRASSINOSTEROID INSENSITIVE 1

(BRI1), involved in pathogen defense (PEPR1 and PEPR2), stress response (WAKL10) and growth/development programs (PSKR1 and BRI1), respectively, share a conserved putative GC domain (Figure 1) (Ludidi et al., 2003; Kwezi et al., 2007; Meier et al., 2010; Qi et al., 2010; Kwezi et al., 2011). The cytosolic portion of these receptors has been shown to generate cGMP *in vitro* (affinity-purified recombinant protein) and *in vivo* (Ludidi et al., 2003; Kwezi et al. 2007; Qi et al., 2010; Kwezi et al., 2011; Ma et al., 2012). Ligand binding to the receptor may lead to cyclic nucleotide elevation (either directly by the receptor or some other mechanism); this may facilitate downstream signaling by activation of  $\text{Ca}^{2+}$ -conducting plasma membrane-localized cyclic nucleotide gated cation channels (CNGCs) (Qi et al., 2010; Tunc-Ozdemir et al., 2013).

CLAVATA1 (CLV1) is a LRR-RLK that shares a putative GC domain with these other LRR-RLKs (Figure 1). CLV1, along with the signaling peptide CLAVATA3 (CLV3) controls stem cell fate in the shoot apical meristem (SAM). CLV3 binding to the CLV1 receptor initiates a signaling cascade that acts to repress expression of WUSCHEL (WUS), a homeodomain transcription factor that regulates stem cell differentiation in the SAM (Laux et al., 1996). WUS expression in the SAM maintains the stem cell population and increases the size of the SAM. CLV3/CLV1 signaling represses WUS expression and results in the generation of differentiated daughter cells and thus limits the size of the SAM. At present, not much is known about the signal transduction cascade that links CLV3 peptide ligand perception by the CLV1 receptor to the downstream targets: regulation of WUS expression, influence of stem cell fate and control of SAM size. Here, we provide results that demonstrate CLV3 peptide binding to CLV1 causes cGMP and cytosolic free  $\text{Ca}^{2+}$  elevation, and that this CLV3-dependent  $\text{Ca}^{2+}$  signal is a component of the CLV3/CLV1 signaling cascade that influences WUS expression and stem cell fate.





**Figure 1. CLV1 has a putative GC domain similar to that found in some other LRR-RLKs.** The structural domains typically found in LRR-RLKs are portrayed in the cartoon at the top; these include a signal peptide (SP) and (a variable number of) leucine-rich repeats (LRRs) in the extracellular region of the receptors, and a cytosolic kinase domain. Also shown is the catalytic GC center that is within the cytosolic kinase domain of some LRR-RLKs (Wong and Gehring, 2013). The 14 amino acid residues making up the putative GC catalytic center of CLV1 are shown in a box below the cartoon. The GC catalytic center and some flanking residues thought to be involved in GC activity (see Ludidi et al., 2003 for analysis) of PEPR1, PEPR2, BRI1, WAKL10 and PSKR1 are shown at the bottom of the Figure.

## Results

### CLV1 expression

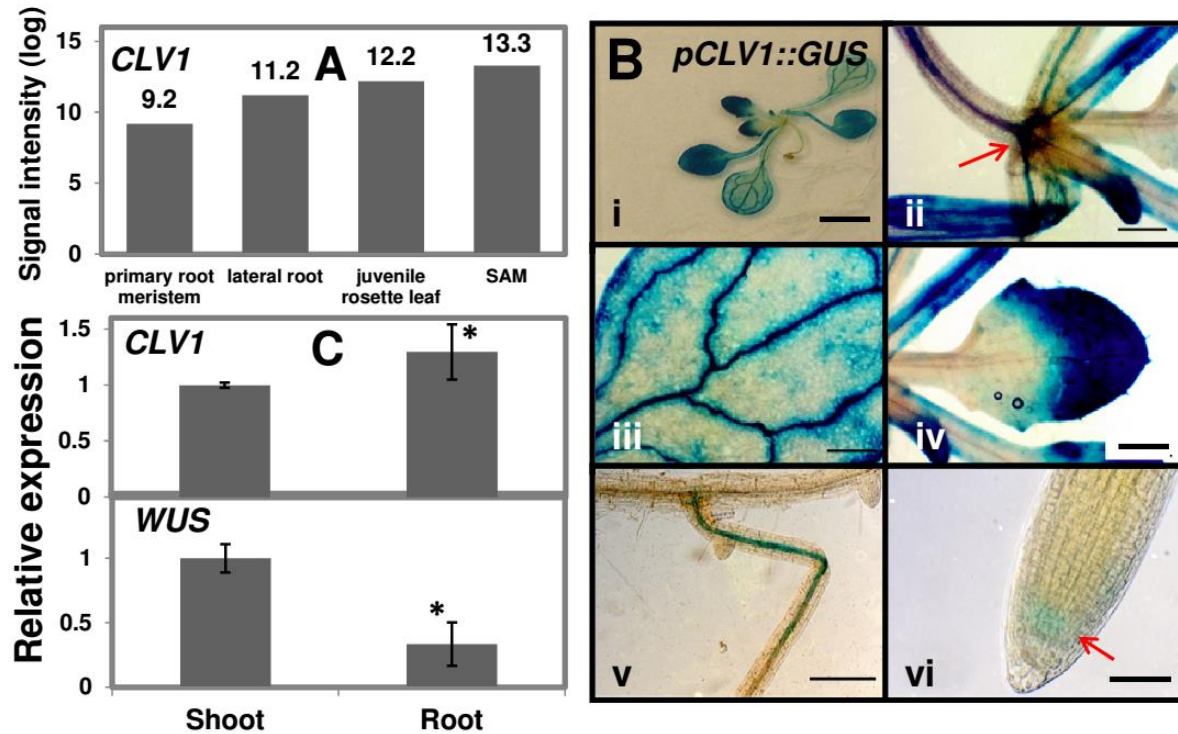
The focus of the work presented in this report is to test the hypothesis that cytosolic free  $\text{Ca}^{2+}$  elevation is involved in the signal transduction cascade that links CLV3 perception by CLV1 to downstream regulation of gene expression and stem cell fate in the SAM. One aspect of our experimental approach to test this hypothesis was to examine the CLV1-dependent signaling cascade evoked in seedlings by exogenous application of CLV3 to various genotypes of Arabidopsis plants. We reasoned that changes in cytosolic secondary messengers occurring in response to CLV3 application could be diagnostic for this signaling pathway in any tissues in which CLV1 is expressed; i.e. not necessarily limited to the SAM. Studies of CLV1 function in signaling cascades have typically focused on genes controlling stem cell fate in the SAM. Studies and reviews (Boscá et al., 2011; Durbak and Tax, 2011) portray CLV1 receptor function as occurring in cells of the SAM, and later, during vegetative-to-reproductive developmental transition, in the inflorescence and floral meristems. However, recent work points to a role for CLV1 also in the primary root meristem (Stahl et al., 2013; Williams and De Smet, 2013; Araya et al., 2014). Analysis of publically-available expression profiles such as Genvestigator indicate that although CLV1 expression is maximal in the SAM, this receptor is expressed in many tissues of Arabidopsis plants and, relative to expression in the root meristem, expression is greater in lateral roots and in leaf tissue (Figure 2A). We also evaluated GUS expression patterns in various tissues of Arabidopsis plants transformed with a *pCLV1::GUS* construct (Figure 2B). Results of these GUS expression studies also suggest a broad pattern of CLV1 expression in multiple tissues of Arabidopsis plants (Figure 2B, panel (i)). Strong GUS staining was evident in the region of the seedlings corresponding to the SAM (ii). However, the CLV1 promoter was

active in cotyledons (iii), rosette leaves (iv), and lateral roots (v). Faint GUS staining was also found in the root apical meristem (RAM) (vi). A third approach to evaluate relative CLV1 expression in the SAM as compared to other tissues in Arabidopsis plants was taken in the work shown in Figure 2C. Relative level of CLV1 message was evaluated in a portion of the shoot including the SAM, and roots of Arabidopsis seedlings (Figure 2C). Results of this qPCR analysis of CLV1 expression indicate that the relative expression level of CLV1 in roots was as great as the expression level in regions of the shoots including leaf petioles and the SAM. In contrast, expression level of WUS, a gene specifically expressed in the SAM (Laux et al., 1996) was significantly less in roots than in shoots. Thus, it can be concluded from the analyses presented in Figure 2 that CLV1 receptor protein is present in numerous tissues of Arabidopsis plants, even though its function has primarily been studied up to now in the SAM.

### **CLV3 signaling involves generation of the cytosolic secondary messenger molecules cGMP and Ca<sup>2+</sup>**

As mentioned above in the ‘Introduction’ section, some LRR-RLKs with a GC domain similar to that (presumably) present in CLV1 have been shown to generate cGMP, and activate CNGC channels that facilitate Ca<sup>2+</sup> entry into the cell cytosol as a component of signaling cascades. Here, we examine whether these cytosolic secondary signaling molecules are also involved in signaling downstream from CLV3 perception by the CLV1 receptor.

We used plants constitutively (35S CaMV promoter) expressing a construct generated by fusion of a circularly permuted (enhanced expression) GFP sequence to a portion of an animal cGMP-activated protein kinase resulting in a **fluorescence indicator** of **cGMP** (FlinG) reporter protein (Isner and Maathuis, 2011) to monitor *in vivo* cGMP levels in intact root tips of

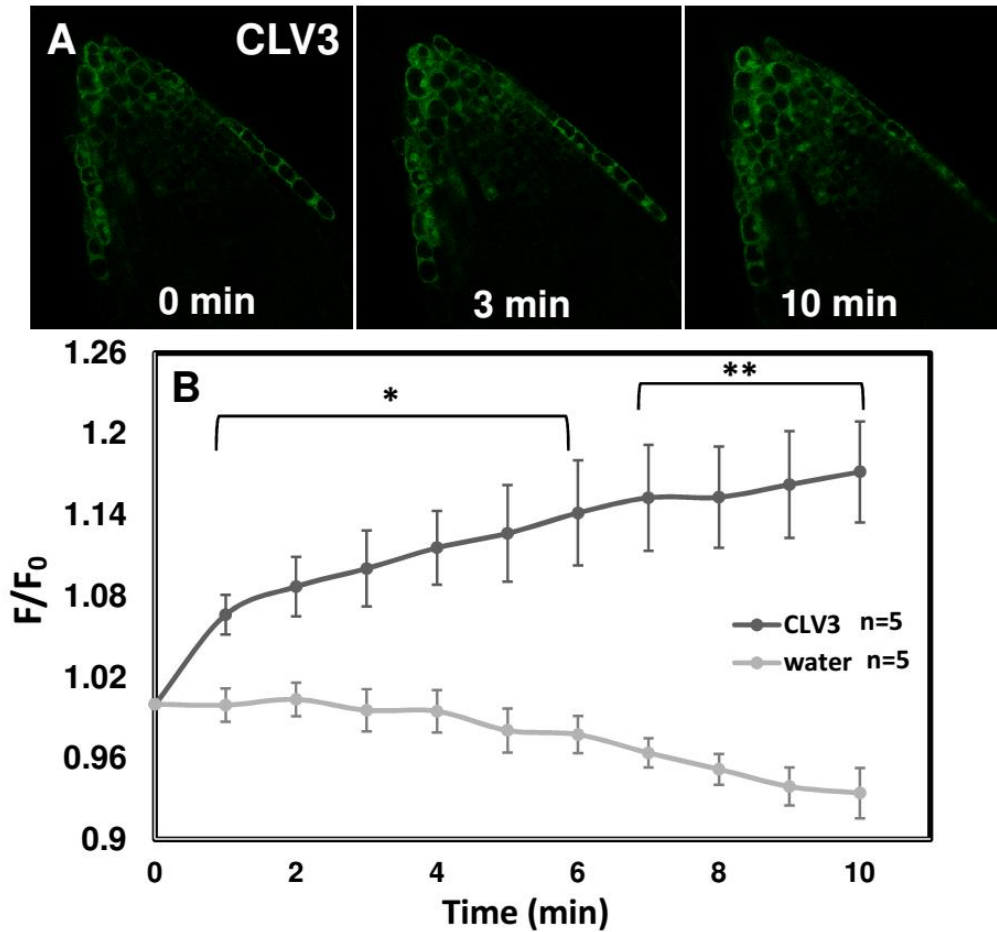


**Figure 2. CLV1 expresses in multiple tissues throughout the Arabidopsis plant.** (A) Analysis of publicly available microarrays accessed through the Genevestigator database (Zimmermann et al., 2004) shows relative *CLV1* expression in the primary root meristem, lateral root, juvenile rosette leaf and SAM. Numbers above bars denote relative expression level (log units). (B) GUS reporter protein analysis of *CLV1* promoter activity in different tissues of 9-d-old Arabidopsis (Col) seedlings expressing *pCLV1::GUS*: (i) whole seedling, (ii) SAM, (iii) cotyledon, (iv) juvenile rosette leaf, (v) lateral root, and (vi) primary root meristem. Bars represent 2 mm (i), 200  $\mu$ m (ii to iv), 10  $\mu$ m (v), and 5  $\mu$ m (vi). Arrow highlights the general region of the SAM in (ii), and the RAM in (vi). This experiment was repeated three times; representative images of plant organs and tissue are shown in each panel. (C) qPCR analysis of relative expression level of *CLV1* and *WUS* in Arabidopsis (Col) root tissue as compared to a region of the shoot containing the SAM. For the 'shoot', leaves and roots were removed, leaving the SAM with as little surrounding tissue as possible. For the 'roots', whole roots were used. Results shown are means  $\pm$  SE (n = 3) normalized to the transcript level in the shoot. ANOVA analysis was used to evaluate means separation between the values recorded from shoot and root tissue. Asterisks above the bars representing root tissue indicate means were significantly different ( $P < 0.05$ ) than values obtained for shoot tissue.

Arabidopsis seedlings. Root tips are typically considered the best tissue for live-cell fluorescence imaging (Groover and Jackson, 2007) and prior studies from our lab (Ma et al., 2012; Zhao et al., 2013) have used this system successfully. Here, we observed a time-dependent increase in cytosolic cGMP in root tips of Arabidopsis seedlings upon addition of exogenous synthetic, biologically-active (see Materials and Methods for information about the peptide) CLV3 peptide that did not occur when water alone was added (Figure 3). The extent of fluorescence increase (reflecting elevation in cytosolic cGMP) occurring in response to CLV3 application in the experiment shown in Figure 3 is at least as great as that observed in response to treatments (such as hormones and mobile signaling molecules (nitric oxide)) that are known to induce cGMP increases in plants as a component of signal transduction cascades (Isner and Maathuis, 2011; Isner et al., 2012; Ma et al., 2012).

We used aequorin-expressing Arabidopsis plants to monitor *in vivo* cytosolic free  $\text{Ca}^{2+}$  in intact seedlings. A previous review suggests that cytosolic free  $\text{Ca}^{2+}$  elevations occurring in tissues such as whole leaves, roots, or intact seedlings can be evaluated using aequorin-expressing plant material (McAinsh and Pittman, 2009); however, the sensitivity of this  $\text{Ca}^{2+}$ -monitoring assay could not be expected to detect  $\text{Ca}^{2+}$  changes specifically in the few cells of the SAM (Kudla et al., 2010). As CLV1 is expressed in many tissues of the Arabidopsis seedling (Figure 2), we presumed that application of the CLV3 peptide to whole seedlings could evaluate the evocation of signaling cascades dependent on CLV1 perception of the CLV3 ligand.

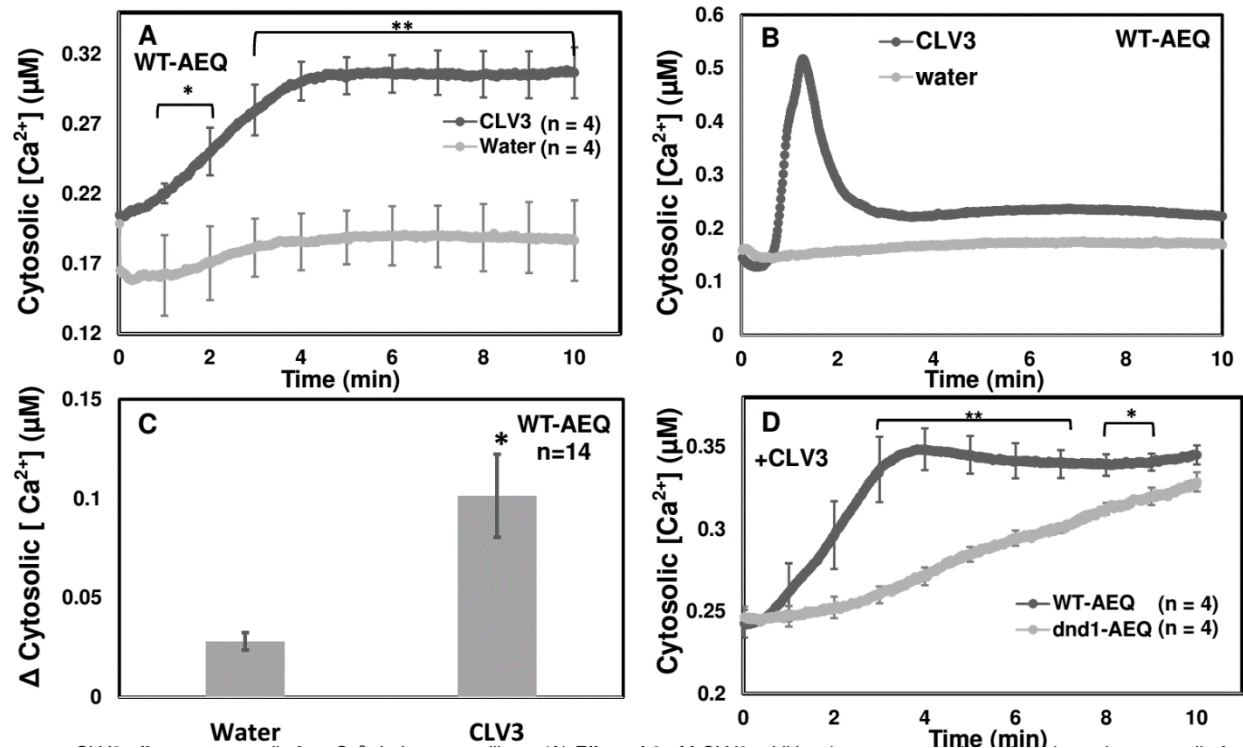
Application of exogenous CLV3 to wild type (WT) (Col) seedlings resulted in a modest elevation of cytosolic free  $\text{Ca}^{2+}$  above that occurring when water is added (Figure 4). The CLV3-dependent elevation we observe (e.g. Figure 4A) is less than that evoked upon addition of other peptide ligands (e.g. Ma et al., 2012), perhaps because the CLV3 receptor, CLV1, is not strongly



**Figure 3. Effect of exogenous CLV3 on in vivo cytosolic cGMP generation in FlincG-expressing Arabidopsis seedlings.** Results were recorded from root tips, as they are typically considered the best tissue for live-cell fluorescence imaging (Groover and Jackson, 2007). Individual WT Arabidopsis seedlings were placed in 60  $\mu$ L of water in a recording chamber set up on an inverted stage of a confocal microscope. The root tip was located under bright field illumination, and observation of background GFP fluorescence was made. After adjustment of the perfect focus, 6  $\mu$ M CLV3 (or water as a control) was applied at time “0”. After application of 6  $\mu$ M CLV3 peptide (or water as control) to root tips of seedlings, fluorescence images were taken over time. (A) Representative images of the root tip 0, 3, and 10 min after CLV3 addition are shown. (B) Quantitative analysis of mean ( $n = 5$ )  $\pm$  SE change in fluorescence ( $F/F_0$ ) over time is shown for 6  $\mu$ M CLV3-, and water-treated seedlings. ANOVA analysis was used to evaluate means separation between control (water) and CLV3 treatment at each time point. An asterisk, or two asterisks above the brackets indicates fluorescence change after CLV3 application was significantly different (at  $P < 0.05$  or  $P < 0.01$ , respectively) than the water control for that time point.

expressed in all tissues types as noted in Figure 2B). Further, it is known that water addition alone to aequorin-expressing leaves can cause elevations in cytosolic free  $\text{Ca}^{2+}$  due to slight changes in temperature, osmolality, or other factors (e.g. Carpanto et al., 2007; Ranf et al., 2008). We therefore undertook further analysis of the difference in the change in cytosolic free  $\text{Ca}^{2+}$  evoked by addition of CLV3 and water. Figure 3A shows the mean (of four replicates) of CLV3-dependent elevation of  $\text{Ca}^{2+}$  as a broad peak over time. Individual treatment replications typically (but do not always) show a sharper ‘spike’ in cytosolic free  $\text{Ca}^{2+}$  upon addition of CLV3 (Figure 4B). In consideration of the variability in the time at which the spike representing an increase in cytosolic free  $\text{Ca}^{2+}$  occurred in response to CLV3, we compared the maximal and minimal level of cytosolic free  $\text{Ca}^{2+}$  measured over the first five minutes after addition of CLV3 or water in a large number of biological replications (n=14) (Figure 4C). Consistent with results shown in Figure 4A and 4B, this more refined analysis of CLV3-dependent change in cytosolic free  $\text{Ca}^{2+}$  again shows a statistically significant ( $P<0.01$ ) difference between effect of CLV3 (mean maximal increase over the first five min of recording was 100 nM) or water (30 nM increase) addition on cytosolic free  $\text{Ca}^{2+}$ .

Further experiments indicated that the cytosolic free  $\text{Ca}^{2+}$  elevation occurring in response to CLV3 was impaired in the DEFENSE NO DEATH 1 (*dnd1*) mutant, which lacks a functional CNGC2 polypeptide (Figure 4D). Ion channel proteins that are formed at least in part by CNGC2 polypeptide subunits in both native Arabidopsis cell membranes as well as upon expression in heterologous systems (cultured human embryonic kidney cells) have demonstrated cyclic nucleotide activated  $\text{Ca}^{2+}$  conductance (Leng et al., 1999; Ali et al., 2007). Thus, results presented in Figure 4D raise the possibility that CLV3 binding to CLV1 may activate CNGC channels in a similar manner.



**Figure 4. Exogenous CLV3 effects on cytosolic free  $\text{Ca}^{2+}$  in intact seedlings.** (A) Effect of 2  $\mu\text{M}$  CLV3 addition (or water control) on time-dependent cytosolic free  $\text{Ca}^{2+}$  levels in WT-aeq plants. (B) An example showing individual recordings of single (WT-aeq) seedling responses to addition of CLV3 or water. The difference between the value recorded prior to CLV3 (or water) addition and the maximal  $\text{Ca}^{2+}$  level recorded from a plant over the first five minutes was used as an individual treatment replicate to generate the mean values shown in (C). (C) Bars represent the average maximal change in cytosolic free  $\text{Ca}^{2+}$  that occurred over the first five min after CLV3 (or water) addition to seedlings; results from recordings made from 14 individual seedlings were averaged for each treatment mean (shown  $\pm$  SE). ANOVA analysis indicated the treatment means in (C) were significantly different ( $P \leq 0.05$ ), as indicated by the asterisk. (D) Effect of 2  $\mu\text{M}$  CLV3 addition to WT-aeq and dnd1-aeq plants. Results in (A) and (D) are presented as means ( $n = 4$ )  $\pm$  SE calculated every min. ANOVA analysis was used to evaluate treatment means separation. An asterisk, or two asterisks above the brackets indicates the treatment means of cytosolic free  $\text{Ca}^{2+}$  were significantly different (at  $P < 0.05$  or  $P < 0.01$ , respectively) at that time point.



In prior studies from our lab, it was noted that elevations of free  $\text{Ca}^{2+}$  in response to ligands other than CLV3 were impaired but not completely blocked in the *dnd1* mutant (Qi et al., 2010) as is the case with the results shown in Figure 4D. These responses might be due to the possibility (as is the case with animal CNGCs; Biel, 2009) that plant CNGC channel complexes are formed from multiple CNGC gene products (Abdel-Hamid et al., 2013). In the *dnd1* mutant, the absence of CNGC2 may result in the assembly of nonnative, partially functional channel complexes formed from other CNGC polypeptides.

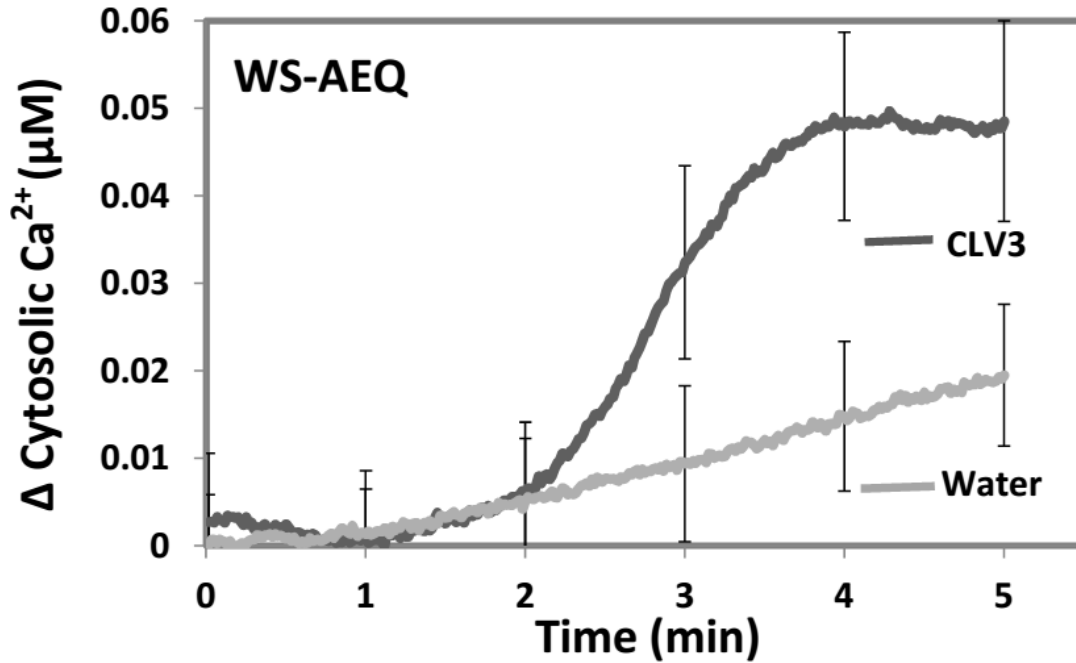
Some evidence suggests that, in addition to the flagellin peptide (present in many microbial pathogens infecting plant tissue), the LRR-RLK FLAGELLIN-SENSITIVE 2 (FLS2) (flagellin receptor) might also bind CLV3 (Lee et al., 2011). Other researchers, however, have questioned the physiological significance of this assertion (Lee et al., 2012; Mueller et al., 2012; Segonzac et al., 2012). Mueller et al. (2012) raised the possibility that some effects of CLV3 demonstrated by Lee et al. (2011) could be due to contamination of synthetically-generated CLV3 peptide preparations by flg22 (a peptide derived from flagellin that binds to FLS2). We evaluated this possibility in our work (see discussion and mention of additional experimental results in the ‘CLV3 peptide’ section of the ‘Materials and Methods’ below). In any case, this point is germane to our evaluation of CLV3 signaling because flagellin binding to FLS2 activates a signaling cascade leading to cytosolic free  $\text{Ca}^{2+}$  elevation (Jeworutzki et al., 2010). It is relevant, therefore, to the work presented here to determine if application of CLV3 to Arabidopsis plants lacking FLS2 could still lead to  $\text{Ca}^{2+}$  signaling (i.e. through CLV1). We evaluated this possibility by using wild type Arabidopsis plants of the ecotype Wassilewskija (WS) that express aequorin (WS-aeq) (Zhao et al., 2013). WS Arabidopsis has a natural mutation in the FLS2 gene, does not express functional FLS2 receptor protein, and lacks responses to

flagellin peptide (Gómez-Gómez et al., 1999). Experiments shown in Figure 4 monitor exogenous CLV3 effects on cytosolic free  $\text{Ca}^{2+}$  in Col-aeq plants. In further experiments, we evaluated if CLV3 application to WS-aeq plants resulted in cytosolic free  $\text{Ca}^{2+}$  elevations above that generated with controls (water application). Results presented in Figure 5 confirm that in the absence of FLS2, CLV3 is still capable of generating a cytosolic free  $\text{Ca}^{2+}$  elevation in WT (i.e. in these WS ecotype) plants.

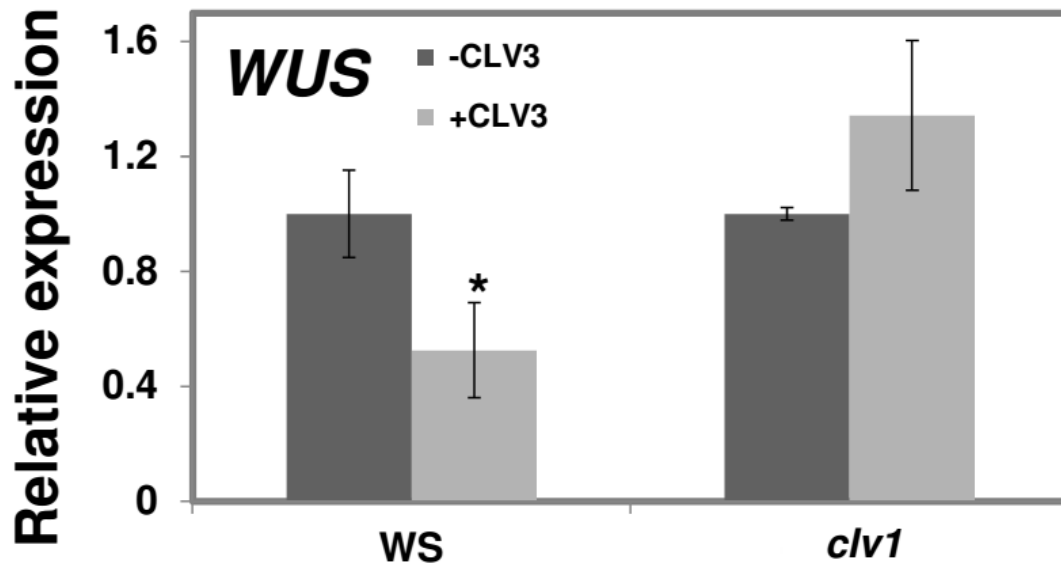
### **Involvement of CLV1-, and CLV3-dependent $\text{Ca}^{2+}$ generation in the transcriptional regulation of genes associated with stem cell fate in the SAM.**

As mentioned in the ‘Introduction’, CLV3 binding to CLV1 leads to a restriction of WUS expression and a limitation of stem cell proliferation in the SAM. Reduction of SAM size and WUS expression levels have been used to monitor CLV3 and CLV1 function in the SAM (e.g. Brand 2000; Schoof et al., 2000; Lenhard and Laux, 2003; Fiers et al., 2006; Müller et al., 2006; Ohyama et al., 2009; Lee et al., 2011). Here, we also use changes in WUS expression level to monitor CLV3 signaling in the SAM, specifically to identify components of the signaling cascade linking the signaling peptide to its effects on the SAM. The T-DNA insertional mutant allele *clv1-13* (in the WS background) has been shown to lack CLV1 message and is a null mutant genotype (Diévert et al., 2003). A comparison was made between exogenous CLV3 effects on WUS expression in WT (WS) and *clv1-13* null mutants (Figure 6). WUS expression was reduced in WT (WS) plants due to exogenous CLV3 application; this effect of exogenous CLV3 was absent from *clv1-13* plants (Figure 6).

In addition to CLV1, current reviews point out the possibility that a number of other receptors might contribute to CLV3 signaling in the SAM (either in association with CLV1 or as components of independent receptor protein complexes) as well as in other tissues and meristems



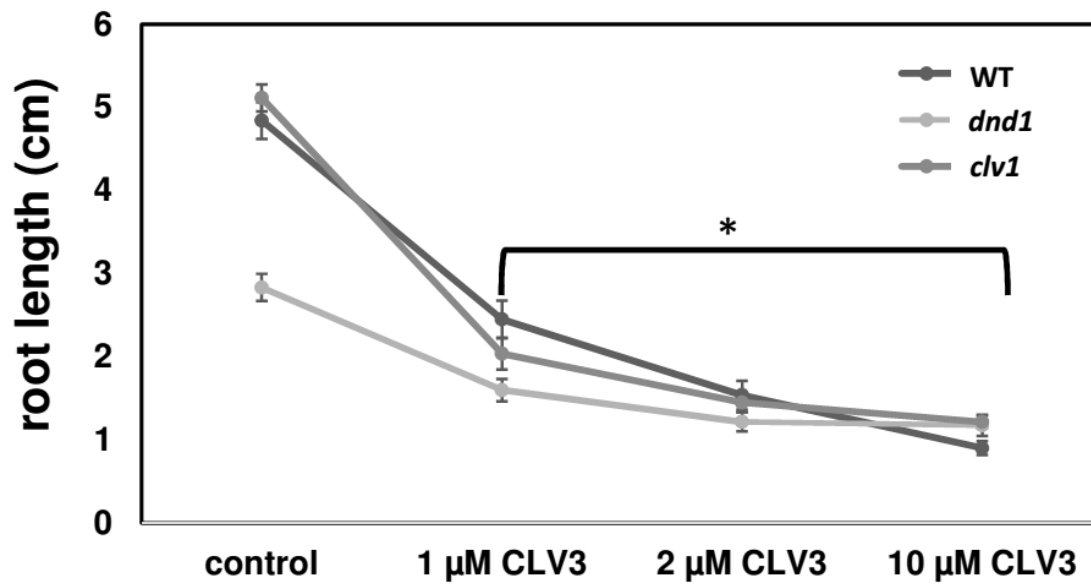
**Figure 5. Exogenous CLV3 causes cytosolic free  $\text{Ca}^{2+}$  elevation in Wassilewskija (WS) ecotype *Arabidopsis* plants.** CLV3 (2 $\mu\text{M}$ ) or water (control) was applied to WS-aeq seedlings; this experiment and the analysis of the results (shown here as means ( $n = 4$ )  $\pm$  SE) are similar to that shown in Figure 4A with Columbia ecotype seedlings, except the results are shown as changes in free cytosolic  $\text{Ca}^{2+}$  from initial levels (prior to addition of CLV3 or water).



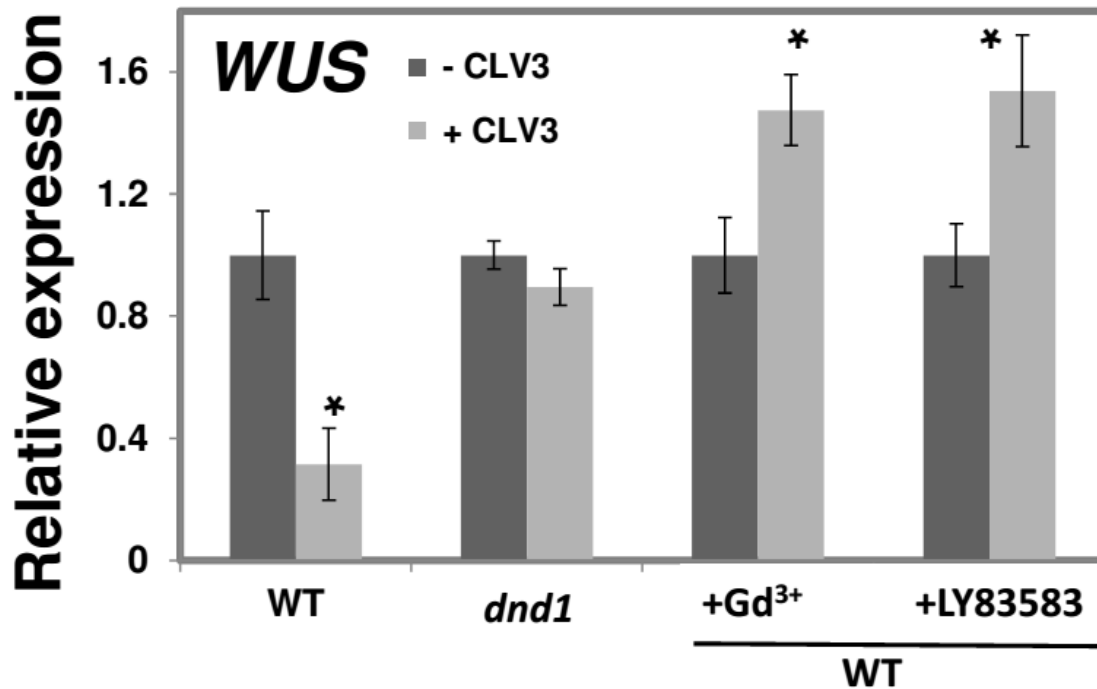
**Figure 6. Relative expression of WUS in response to application of 1  $\mu$ M CLV3 and water ('-CLV3' or control) to WT (WS) and *clv1* mutant plants.** q-PCR analysis of WUS transcript levels were undertaken; results are shown as means ( $n = 3$ )  $\pm$  SE normalized to the transcript level of the control treatment ('-CLV3') for each genotype. ANOVA analysis was used to evaluate means separation between the treatments for each genotype. An asterisk above the bar representing the '+ CLV3' treatment indicates the value was significantly different (at  $P < 0.05$ ) than the control value for that genotype. Gene expression experiments employing similar technical approaches (q-PCR) and analyzed in a similar fashion as the work shown here are presented in Figures 8, 9, and 10.

(DeYoung and Clark 2008; Müller et al., 2008; Bleckmann et al., 2010; Kinoshita et al., 2010; Lee et al., 2011; Mandel et al., 2014). These other receptor proteins include: CORYNE (CRN), CLV2, BARELY ANY MERSITEM 1 (BAM1), BAM2, BAM3, RECEPTOR-LIKE PROTEIN KINASE 2 (RPK2), ERECTA and (perhaps) FLS2. Thus, it is significant for our study that in the *clv1* null mutant, effects of exogenous CLV3 on the SAM, as monitored by decreased WUS expression, are prevented (Figure 6). Whether or not CLV3 signals through other receptors, results presented in Figure 6 indicate that CLV3 signaling cascades in the SAM that control WUS expression have, as a step in the pathway, CLV1 receptor function. The results in Figure 6 also have pertinence to the issue of the use of assays of root length inhibition as a means of evaluating whether a peptide is biologically active (e.g., our results shown in Figure 7). We find that addition of CLV3 peptide inhibited root length of *clv1-13* and *dnd1* seedlings, in addition to WT (Figure 7). These results suggest that the physiological mechanism by which peptides such as CLV3 inhibit root length may not be mediated by the same signal transduction pathway steps that facilitate the peptide's function in 'native' signaling (e.g., in the case of CLV3, regulating stem cell fate in the SAM). The results shown in Figure 6 also provide a basis for further experiments included in this study that involve using genetic and pharmacological approaches to test the involvement of cytosolic secondary signaling molecules ( $\text{Ca}^{2+}$ , and cyclic nucleotides) in the signaling cascade linking CLV3 perception by CLV1, and control of gene expression related to stem cell fate in the SAM.

As shown in Figure 4, CLV3-dependent elevation of cytosolic free  $\text{Ca}^{2+}$  elevation was impaired in the *dnd1* (CNGC2 loss of function) mutant. Results presented in Figure 8 indicate that CNGC2 loss of function also prevents CLV3 mediated reduction in WUS expression. With (Col) WT plants, exogenous CLV3 significantly reduced WUS expression but CLV3 had no



**Figure 7. Growth of Arabidopsis seedlings on medium containing increasing synthetic CLV3 indicates the peptide is biologically active.** Results shown are the (primary) root length of WT, *dnd1* and *clv1* seedlings grown vertically for 12 d on media containing 0, 1, 2, or 10  $\mu$ M CLV3. Data represent means ( $n > 4$ )  $\pm$  SE. ANOVA analysis was used to evaluate means separation between the control (no CLV3 added) and different CLV3 concentrations for each genotype. For all genotypes tested, root length was significantly reduced ( $P \leq 0.01$ ) from control values at all CLV3 concentrations tested (indicated by the '\*' above the bracket).

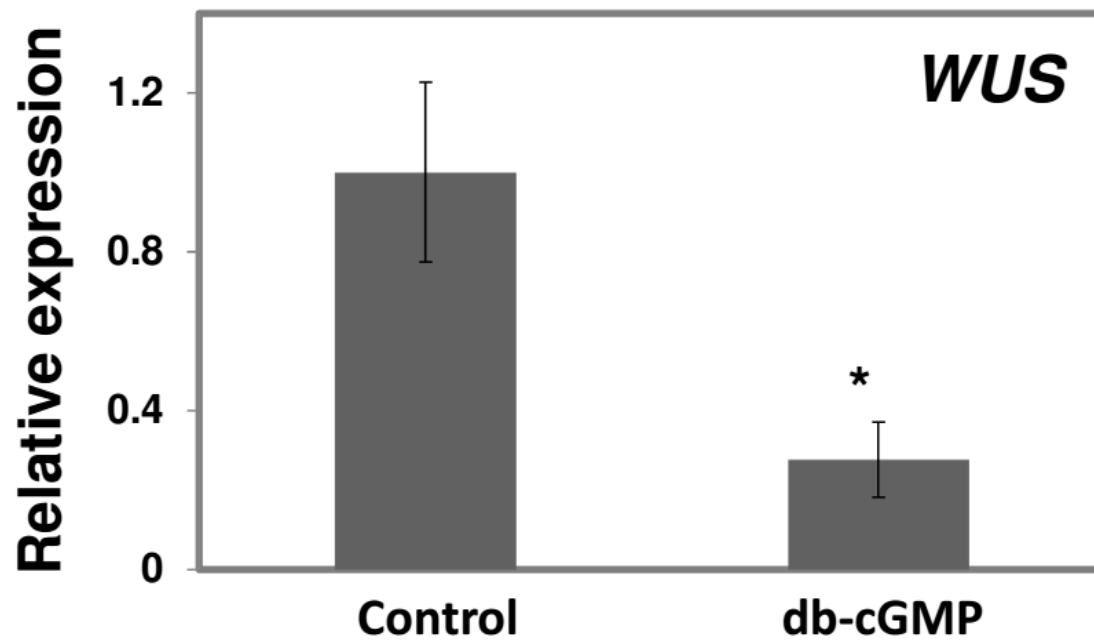


**Figure 8. Exogenous CLV3 effects on *WUS* expression involve the secondary messengers cytosolic free  $\text{Ca}^{2+}$  and cGMP.** *WUS* expression was monitored in WT plants treated with water or 1  $\mu\text{M}$  CLV3 with or without the  $\text{Ca}^{2+}$  channel blocker  $\text{Gd}^{3+}$  or the GC inhibitor LY83583. Results are shown as means ( $n = 3 \pm \text{SE}$ ) for a genotype or treatment. Effect of exogenous CLV3 on *WUS* expression was also evaluated in *dnd1* mutants, which lack a cGMP activated  $\text{Ca}^{2+}$ -conducting ion channel. ANOVA analysis was used to evaluate means separation between control (water or ‘-CLV3’) and CLV3 application for each genotype and treatment. An asterisk above the bar representing the CLV3 treatment indicates gene expression was significantly different (at  $P < 0.05$ ) than the water control for that genotype or treatment. In the experiment shown here, qPCR analysis indicated that application of CLV3 to seedlings treated with 150  $\mu\text{M}$   $\text{Gd}^{3+}$  or 20  $\mu\text{M}$  LY83583 resulted in a modest increase in *WUS* expression. We note that our (qualitative) GUS staining assay of *WUS* expression showed a similar trend (see Figure 7). We speculate that this might be due to the effect of the channel blocker or GC inhibitor on signaling facilitated by endogenous CLV3 (in addition to effects of the treatments on exogenous CLV3; the main point of our study).

significant effect on *WUS* expression in the *dnd1* genotype. In addition to the genetic intervention in CLV3 signaling that occurred with the *dnd1* genotype, pharmacological treatments known to alter CNGC-mediated  $\text{Ca}^{2+}$  signaling had corresponding effects on *WUS* expression in the experiment shown in Figure 8. The  $\text{Ca}^{2+}$  channel blocker  $\text{Gd}^{3+}$  and the guanylyl cyclase inhibitor 6-anilino-5, 8-quinolinequinone (LY83538) have been shown to abolish CNGC-dependent cytosolic free  $\text{Ca}^{2+}$  elevations occurring in response to addition of peptide ligands that activate the LRR-RLK PEPR1 (Qi et al., 2010; Ma et al., 2012). These same treatments, i.e., application of CLV3 along with either the  $\text{Ca}^{2+}$  channel blocker  $\text{Gd}^{3+}$ , or the GC inhibitor LY83538, both prevent the reduction in *WUS* that occurs in WT plants upon addition of CLV3 alone (Figure 8). We also note from the work shown in Figures 4, 5, 6, and 8 that application of CLV3 peptide to either Col or WS ecotype Arabidopsis plants results in both cytosolic free  $\text{Ca}^{2+}$  elevation and reduction in *WUS* expression; these results support the physiological significance of the  $\text{Ca}^{2+}$  signal in the pathway linking CLV3 to gene expression in the SAM.

Further evidence consistent with the involvement of cyclic nucleotides and  $\text{Ca}^{2+}$  conducting cyclic nucleotide gated channels in CLV3/CLV1 signaling is presented in Figure 9. In this experiment, CLV3 was not supplied to plants. Rather, a comparison was made of *WUS* expression in the presence and absence of an exogenous lipophilic analog of cGMP (dibromo-cGMP). Addition of this lipophilic analog of the cyclic nucleotide to Arabidopsis leaves results in an elevation of cytosolic free  $\text{Ca}^{2+}$  that is impaired in leaves of the *dnd1* mutant (Qi et al., 2010), these results were interpreted as indicating that elevation of cytosolic cGMP can activate CNGCs leading to the generation of a  $\text{Ca}^{2+}$  signal. As is the case with exogenous CLV3 application (Figure 8), *WUS* expression was significantly reduced by addition of dibromo-cGMP.





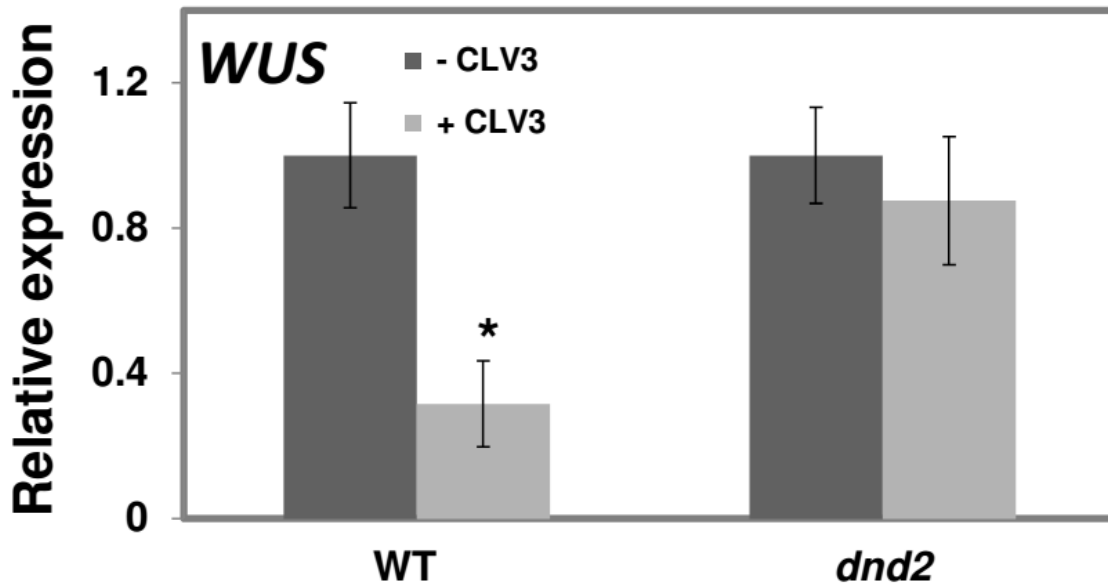
**Figure 9. Application of a lipophilic analog of cGMP reduces *WUS* expression.** Relative expression of *WUS* in WT plants treated with water (control) or 150  $\mu$ M db-cGMP is shown as means ( $n = 3$ )  $\pm$  SE normalized to the transcript level measured under the control treatment. ANOVA analysis was used to evaluate means separation; an asterisk above the bar representing the db-cGMP treatment indicates it was significantly different (at  $P < 0.05$ ) from the control treatment.

It appears that adding an exogenous source of (lipophilic) cGMP (in the absence of added CLV3 peptide) mimics the effect of the signaling peptide.

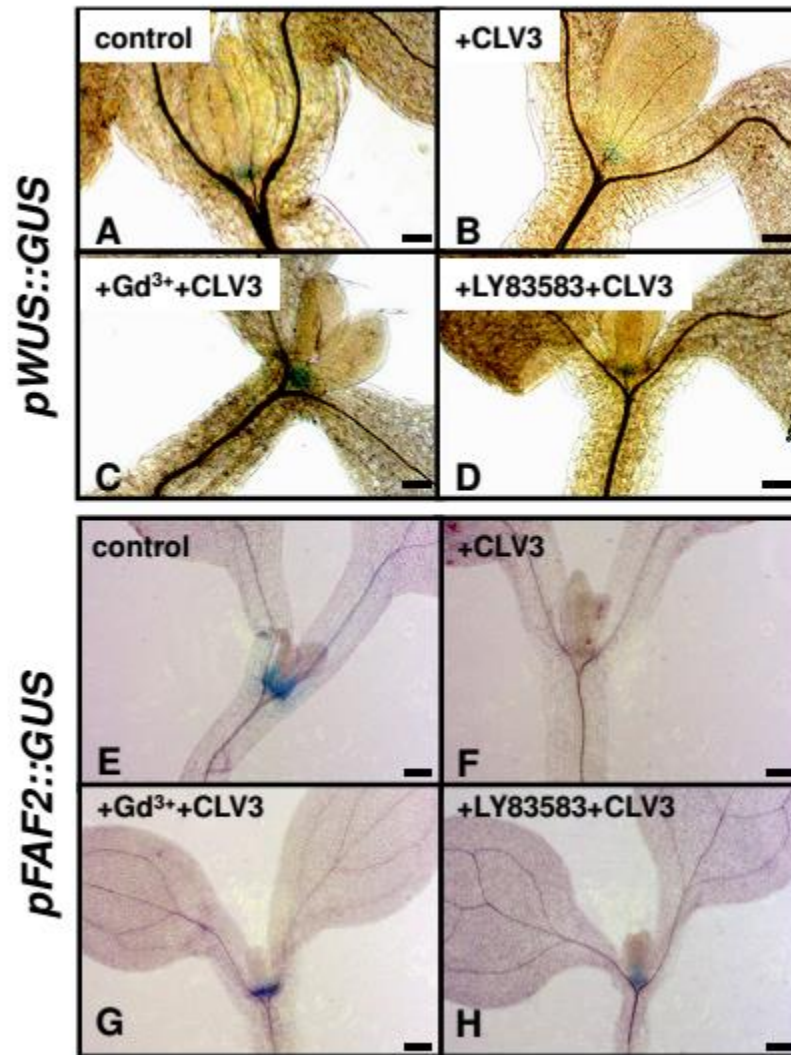
As mentioned above with regards to CLV3-dependent cytosolic free  $\text{Ca}^{2+}$  elevations in leaves of WT and *dnd1* plants, native CNGC channel complexes in plants may be formed from translation products of more than one *CNGC* gene. Some experimental evidence suggests that Arabidopsis *CNGC2* and *CNGC4* might be subunits of the same channel complex (Abdel-Hamid et al., 2013; also see discussion of this issue in Ma et al., 2012). Mutations in either of these channel subunit-encoding genes, therefore, have similar effects on  $\text{Ca}^{2+}$  signaling-related phenotypes such as pathogen defense or heat tolerance (Clough et al., 2000; Balagué et al., 2003; Jurkowski et al., 2004; Finka et al., 2012). Therefore, we investigated if CLV3 regulation of *WUS* expression was also affected in a *CNGC4* null mutant (DEFENSE NO DEATH 2, *dnd2*). Results of the experiment, shown in Figure 10, indicate that the down-regulation of *WUS* expression that occurs in WT (Col) plants is prevented in a genotype (*dnd2*) that has a null mutation in the *CNGC4* gene. This result is similar to that shown in Figure 8 for the effects of *CNGC2* on CLV3 regulation of *WUS* expression in the *dnd1* mutant.

### **Histological analysis of CLV3 effects on gene expression in the SAM using plants expressing *GUS* reporter constructs.**

In the experiments shown in Figure 11, we took a complimentary approach to evaluating CLV3 signaling in the SAM from the studies (Figs. 6, 8 and 9) using qPCR analysis of *WUS* expression. Arabidopsis plants expressing the reporter *GUS* gene under control of the *WUS* promoter were used to analyze CLV3 control of gene expression related to stem cell fate in the SAM. We also used another research tool, plants expressing *GUS* under control of the FANTASTIC FOUR 2 (*FAF2*) promoter to evaluate CLV3 signaling in the SAM. Like *WUS*,



**Figure 10. *CNGC4* is required for CLV3 signaling in the SAM.** Expression of *WUS* in WT and *dnd2* (null mutation in *CNGC4*) seedlings treated with water (‘-CLV3’ or control) or exogenous CLV3 was evaluated (qPCR analysis). Results shown are means ( $n = 3$ )  $\pm$  SE normalized to the transcript level of the control treatment for each genotype. ANOVA analysis was used to evaluate means separation between the control and 1  $\mu$ M CLV3 treatments for each genotype. An asterisk above the bar representing the ‘+ CLV3’ treatment indicates a significant difference ( $P < 0.05$ ) from the control for that genotype.

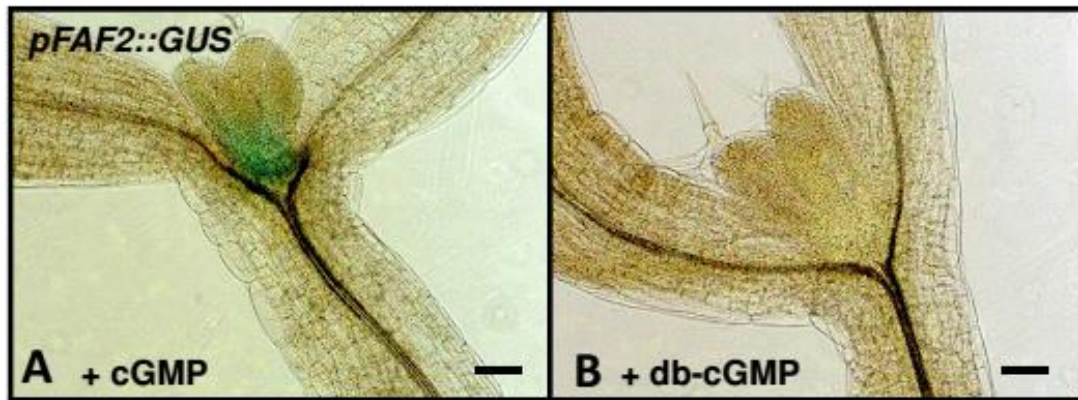


**Figure 11. Effects of exogenous CLV3, alone or in combination with  $Gd^{3+}$  or LY83583 on gene expression in the SAM.** *WUS* promoter (A-D) and *FAF2* promoter (E-H) activity was evaluated in seedlings treated with water (control), 1  $\mu$ M CLV3 alone, or 1  $\mu$ M CLV3 with either 150  $\mu$ M  $Gd^{3+}$  or 20  $\mu$ M LY83583. At least 6 *WUS*::GUS seedlings and 10 *FAF2*::GUS seedlings were tested for each treatment; images shown are representative. Bars represent 100  $\mu$ m (A and B) or 200  $\mu$ m (C-H).

*FAF2* expression in the SAM is under negative control by *CLV3*; *FAF2* has been identified as another modulator (besides *CLV3* and *WUS*) of the genetic circuit regulating stem cell fate in the SAM (Wahl et al., 2010). Expression of *GUS* under the control of both the *WUS* and *FAF2* promoter has been used to evaluate *CLV3* control of gene expression in the SAM (Wahl et al., 2010; Lee et al., 2011).

We note that in the qPCR experiments shown in Figures 6 and 8, exogenous *CLV3* application to *Arabidopsis* seedlings reduced *WUS* expression 48% and 67% respectively. Although not analyzed quantitatively, we note a modest reduction in *GUS* staining in the SAM of *pWUS::GUS* seedlings upon addition of *CLV3* (Fig. 11; compare panels A and B). A comparison of *GUS* staining in the SAM of *pWUS::GUS* seedlings exposed to exogenous *CLV3* indicated that application of either  $Gd^{3+}$  or the GC inhibitor along with the signaling peptide prevented *CLV3*-dependent down-regulation of *WUS* expression in the SAM (Fig. 11; compare panels C and D with B). Similar results of *CLV3*,  $Gd^{3+}$  and the GC inhibitor LY83538 on *FAF2* expression were observed in *pFAF2::GUS* seedlings (Fig. 11; panels E-H). The results shown in Figure 11, then, are consistent with results of qPCR analyses of *CLV3* control of gene expression in the SAM; i.e. the effect of the signaling peptide is impaired when seedlings are exposed to either a  $Ca^{2+}$  channel blocker or GC inhibitor.

As shown in Figure 9, qPCR analysis indicated that application of lipophilic cGMP reduced *WUS* expression. In the experiment shown in Figure 12, we compared *GUS* staining in the SAM of *pFAF2::GUS* seedlings in the presence of either (nonlipophilic) cGMP or (lipophilic) db-cGMP. Results indicated that application of an exogenous cyclic nucleotide that is not lipophilic results in a greater expression of *FAF2*, a gene under tight regulation by *CLV3* in the SAM (Wahl et al., 2010) than if the exogenously applied cyclic nucleotide is lipophilic. These



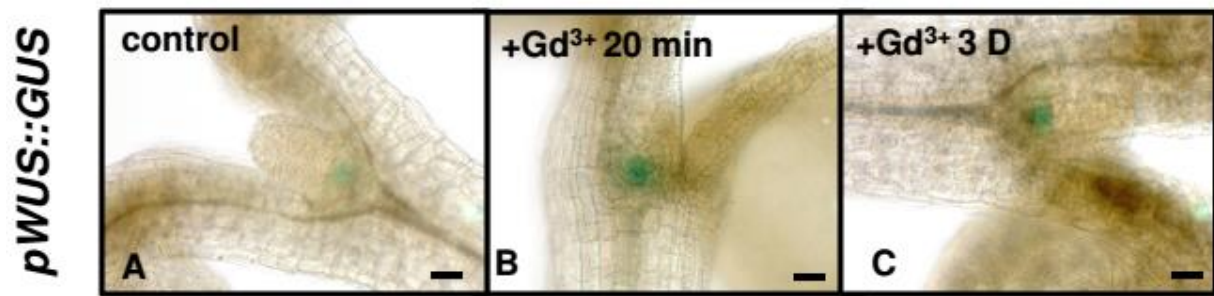
**Figure 12. Effects of 150  $\mu$ M (nonlipophilic) cGMP (A) or a lipophilic analog of the cyclic nucleotide (db-cGMP) (B) on *FAF2* promoter activity in 6-d-old seedlings. Bars represent 200  $\mu$ m. At least 10 seedlings were tested for each treatment; images shown are representative.**

results, along with results shown in Figures 8 and 9, suggest that the application of this secondary messenger might mimic the effect of CLV3 on gene expression in the SAM only if it is able to penetrate plant cells and reach its site of action; i.e. the cytosol of stem cells in the SAM.

### **Ca<sup>2+</sup> involvement in the regulation of meristem by endogenous CLV3**

Experimental results presented in this report up to this point are consistent with a link between cytosolic free Ca<sup>2+</sup> as a secondary messenger in plant cells, and signaling downstream from exogenous CLV3 peptide application to seedlings. Results presented in Figure 13 extend this work. In this case, the effect of exogenous Gd<sup>3+</sup> addition on *WUS* was evaluated in the absence of added CLV3 peptide. Exposure of 7-day-old seedlings to the channel blocker on either a short-term (20 min) or long term (3 d) basis increased *WUS* expression (compare panels B and C with A in Figure 13). We speculate that application of Gd<sup>3+</sup> to seedlings blocked endogenous CLV3 action; as *WUS* expression is negatively regulated by CLV3 in the meristem. The results shown in Figure 13 do not necessarily prove that the effect of Gd<sup>3+</sup> on *WUS* expression is mediated by impairment of CLV3 signaling. However, the results shown in Figure 13 are consistent with the model developed in this report, that cytosolic Ca<sup>2+</sup> elevation is involved in the signaling pathway linking CLV3 perception in the SAM by CLV1, and the downstream regulation of *WUS* expression.

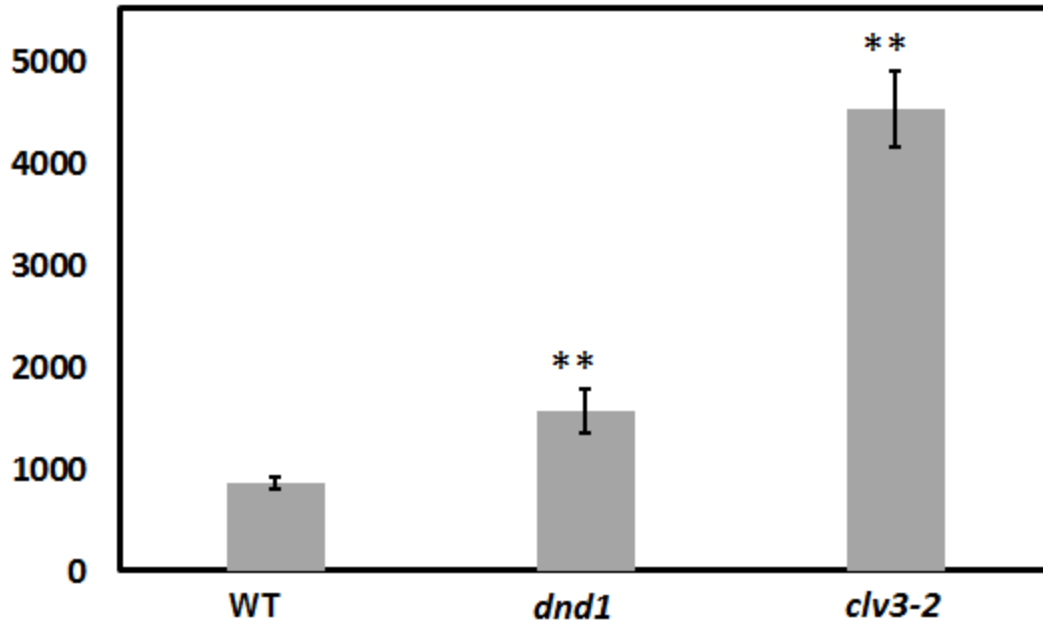
Current models of the interactive cross-control of *CLV3* and *WUS* (and possibly *FAF2*) expression in the SAM posit that the downstream ‘read-out’ of this meristem-controlling signal transduction cascade is the regulation of stem cell fate. The interplay of non-cell-autonomous CLV3 perception in the central zone (CZ) (L1-L3 layer) of the SAM with *WUS* expression in the organizing center (OC) leads to control of stem cell fate which ultimately manifests as the



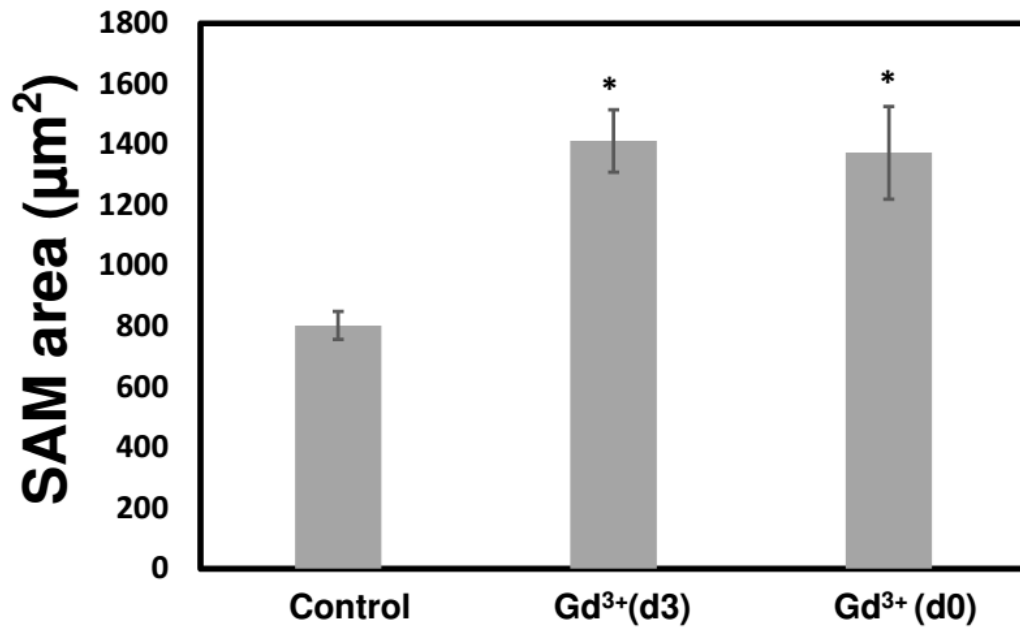
**Figure 13. Effects of 150  $\mu\text{M}$   $\text{Gd}^{3+}$  on *WUS* promoter activity in 6-d-old WT seedlings.** (A), Control (water addition). (B), Seedlings were incubated with  $\text{Gd}^{3+}$  20 min (B) or 3 d (C) prior to harvesting and analysis for GUS expression. Bars represent 100  $\mu\text{m}$  (A-C). At least 4 seedlings were tested for each treatment.



size of the meristem (Schoof et al., 2000; Fiers et al., 2006; Ohyama et al., 2009). The experimental basis presented in these papers for this model is work showing that in the null *clv3-2* mutant lacking endogenous CLV3 peptide (or in *clv1* null mutants), the size of the SAM is much enlarged compared to WT. Further, when exogenous CLV3 is applied to *clv3-2* seedlings, the size of the SAM is reduced from the enlarged dome of cells in this mutant. We reasoned that the genetic and pharmacological arrest of CLV3 signaling through control of CLV3-dependent cytosolic  $\text{Ca}^{2+}$  elevation as presented in our work might result in similar effects on SAM size as demonstrated in these aforementioned reports that used the *clv3* and *clv1* mutants. The experimental approach we used to test this hypothesis was to employ Nomarski optics to visualize the width and height of the SAM in individual seedlings. Results of the experiment are shown in Figure 14. As compared to (Col) WT seedlings, the SAM size (i.e., measured as the two-dimensional maximal face of the SAM ‘dome’) was increased three to five-fold in *clv3-2* seedlings. These results are similar to aforementioned previous reports (Fiers et al., 2006; Ohyama et al., 2009). We also examined SAM size in the  $\text{Ca}^{2+}$  channel mutant *dnd1* seedlings. SAM size in *dnd1* was larger than that measured in WT seedlings (Figure 14). The differences between WT and *dnd1* were less dramatic although they were significant (at  $P \leq 0.01$ ). A second technological approach to blocking CLV3-induced  $\text{Ca}^{2+}$  signals was used in the experiment shown in Figure 15. In this case, the  $\text{Ca}^{2+}$  channel blocker  $\text{Gd}^{3+}$  was added to growth medium at either the time of germination, or after 3 d. As compared to WT seedlings grown under control (no  $\text{Gd}^{3+}$ ) conditions, application of the  $\text{Ca}^{2+}$  channel blocker at either germination (‘D0’) or three days after germination (‘D3’) resulted in ~ 75% increase in SAM size. These differences were about similar to the differences between WT and the *dnd1* genotype shown in Figure 14.



**Figure 14. SAM area of 7-d-old WT, *dnd1* and *clv3-2* Arabidopsis seedlings.** The area of the SAM was measured by calculating the area above the straight line between the basal edges of two opposite leaf primordia. Data represent means (  $n > 16$ )  $\pm$  SE. ANOVA analysis was used to evaluate means separation between control (WT), *dnd1* and *clv3* genotypes. An asterisk or two asterisks above the bar representing a genotype indicates SAM size was significantly different (at  $P < 0.01$ ) than WT.

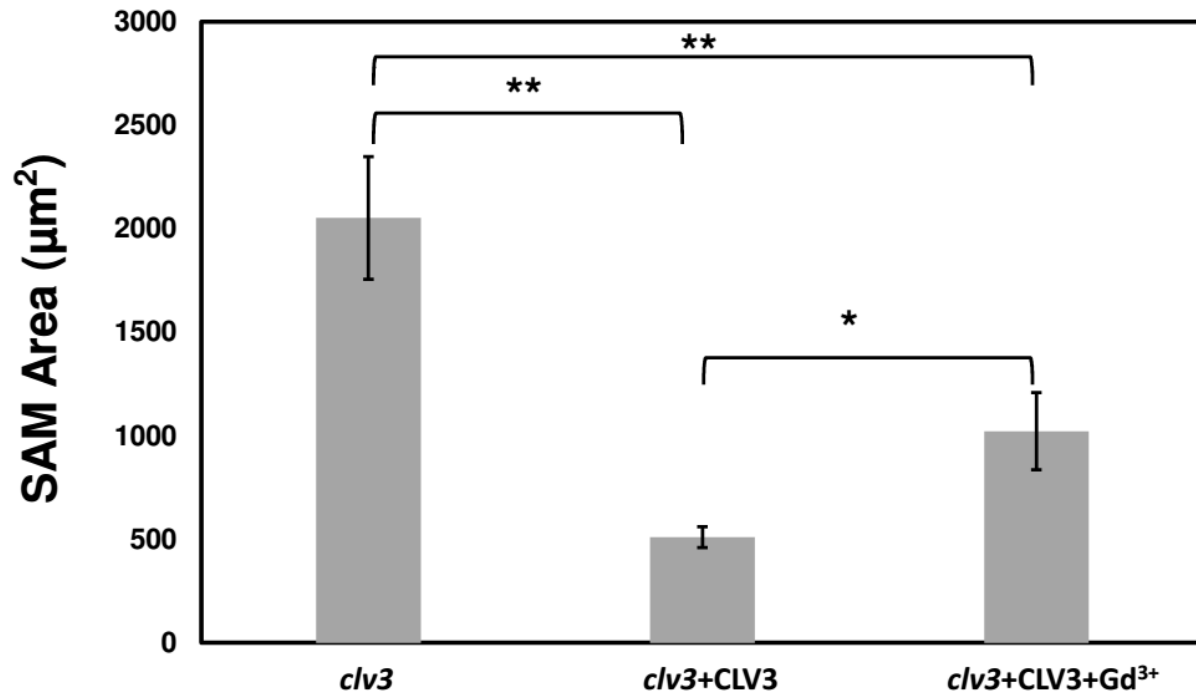


**Figure 15.** SAM area of 7-d-old WT *Arabidopsis* seedlings grown on standard medium (Control), or medium supplemented with Gd<sup>3+</sup> (150 μM) on day 3 or on day 0. Results shown are mean values ( $n > 11$ )  $\pm$  SE for each treatment. An asterisk above the bar representing the Gd<sup>3+</sup> treatment indicates SAM size was significantly different (at  $P < 0.01$ ) than the control.

A third, and complementary (to the work shown in Figs. 14 and 15) experimental approach was employed to investigate the relationship between CLV3,  $\text{Ca}^{2+}$  signaling, and SAM size in the experiment shown in Figure 16. In the experiments shown in Figures 14 and 15, impairment of  $\text{Ca}^{2+}$  signaling was (presumably) impacting SAM size due to interaction with endogenous CLV3-mediated stem cell fate programming. In the experiment shown in Figure 12, we used the *clv3-2* mutant, which lacks endogenous CLV3. In this experiment, a CLV3 phenotype was demonstrated by application of exogenous CLV3 to the *clv3-2* mutant. As was the case in previous studies, exogenous CLV3 reduced SAM size in the mutant (Figure 16). The addition of the  $\text{Ca}^{2+}$  channel blocker partially prevented the signaling in the SAM caused by exogenous CLV3. SAM size of *clv3-2* seedlings provided with CLV3 and  $\text{Gd}^{3+}$  was significantly larger than in *clv3-2* seedlings exposed to CLV3 alone (Fig. 16). It should be noted, though, that SAM size of seedlings treated with  $\text{Gd}^{3+}$  were still smaller than in *clv3-2* seedlings grown in the absence of either CLV3 or  $\text{Gd}^{3+}$  (Figure 16). Nonetheless, the results of the experiment shown in Figure 16 are consistent with the central model of our work; impairment of  $\text{Ca}^{2+}$  signaling affects the signal transduction pathway downstream from CLV3 in the SAM. The results of the work shown in Figures 14, 15, and 16, then, provide experimental evidence consistent with the involvement of  $\text{Ca}^{2+}$  signaling in the cell processing of CLV3 perception that controls gene expression, stem cell fate, and, ultimately, size of the meristem in the shoot apex.

## Discussion

Dissection of the signal transduction cascades initiated by receptors and non-cell autonomous peptide ligands that control stem cell fate in meristematic regions of plant organs is an active area of research. Much new information has been generated recently to expand our relatively extensive understanding of the signaling systems controlling stem cell fate in the SAM



**Figure 16. SAM area of 7-d-old *clv3* Arabidopsis seedlings treated with water (control), CLV3, or CLV3 and Gd<sup>3+</sup>. CLV3 and Gd<sup>3+</sup> were applied on day 0. Results are shown as means ( $n > 7$ )  $\pm$  SE. ANOVA analysis was used to evaluate means separation between the CLV3 treatment, or the CLV3 and Gd<sup>3+</sup> treatment, and the control. An ANOVA analysis was also performed to evaluate means separation between the ‘CLV3’, and the ‘CLV3 and Gd<sup>3+</sup>’ treatments/ An asterisk or two asterisks above the bar connecting two treatments indicates SAM size was significantly different (at  $P < 0.05$ ; or  $P < 0.01$ , respectively) between the two treatments.**

to other tissues such as the RAM (e.g. Stahl et al., 2013; Williams and De Smet, 2013). Current work has also focused on delineating the interplay between receptors that may act in concert or convergently to impact stem cell homeostasis (Betsuyaku et al., 2010). Nonetheless, little is known about the molecular steps that link receptor perception of a signaling peptide to downstream control of the gene expression in stem cells that controls meristem homeostasis. We believe work presented in this report provides some new insights into this signaling paradigm.

Much information about *CLV3* interaction with *WUS* and resultant control of stem cell fate in meristems has been generated from monitoring meristem size and *WUS* expression in meristems of plant genotypes with altered expression of *CLV3*, or that have null mutations in the various *CLV3* receptors such as *CLV1* (Brand et al., 2000; Schoof et al., 2000; Lee et al., 2011). Many of the experiments included in this report used an alternative experimental approach; application of exogenous *CLV3* peptide to *Arabidopsis* seedlings. This allowed for the analysis of ligand-dependent changes in secondary cytosolic signaling molecules ( $\text{Ca}^{2+}$  and cGMP). This experimental system also allowed us to evaluate components of the *CLV3/CLV1* signaling pathway through use of pharmacological agents (channel blockers, enzyme inhibitors and the secondary messenger cGMP) as well as genetic approaches (*clv1*, *clv3*, *cngc2*, and *cngc4* null mutants). Our analysis of *CLV3/CLV1* signaling used, in some cases, a ‘read-out’ of meristem-specific genes that have been previously shown to be responsive to *CLV3*; *WUS* and *FAF2*. We evaluated treatment effects on *CLV3* signaling two h after ligand application. The efficacy of this assay, in terms of waiting a sufficient time to allow for exogenous *CLV3* to reach the SAM is supported by previous studies (Lee et al., 2011), which demonstrated effects of exogenous *CLV3* on expression of genes within the SAM one h after ligand addition. These studies of *WUS* and *FAF2* expression would likely be influenced only by *CLV3* effects on transcription, rather

than a change in the population of stem cells (which might take longer times to show responses to CLV3) in various zones of the meristem (Beemster and Baskin, 1998; Müller et al., 2006). Conclusions based on these studies are limited to an evaluation of CLV3/CLV1 control of gene expression in the SAM. However, previous reports (Kondo et al., 2006; Kinoshita et al., 2010, Lee et al., 2011) have used a similar experimental approach (exogenous CLV3 application to plants) and the information generated from these studies did have relevance for control of stem cell fate by the ligand/receptor signaling system. In our report, we extended our studies of CLV3-dependent generation of  $\text{Ca}^{2+}$  signals, and the involvement of  $\text{Ca}^{2+}$  signals in CLV3 control of gene expression in the SAM to also probe the involvement of  $\text{Ca}^{2+}$  signals in CLV3 control of SAM size.

We cannot definitively link all of the CLV3 signaling exclusively with CLV1 function in our studies. However, we do show that in our experimental system (i.e. exogenous CLV3 application to seedlings and measurement of *WUS* expression by qPCR), the loss-of-function mutation of CLV1 abolishes signaling. We also find that impairment of  $\text{Ca}^{2+}$  signaling only partially reverses CLV3-dependent changes in SAM size. We speculate that this could be associated with any of several possibilities as follows. (1) SAM size is a more complex CLV3-associated phenotype than regulated expression of individual genes. The impaired  $\text{Ca}^{2+}$  signaling in the *dnd1* (and *dnd2*) mutant has many pleiotropic effects on Arabidopsis seedlings, such as constitutively high salicylic acid levels (Clough et al., 2000). These pleiotropic manifestations of the CNGC mutation may influence SAM size in unknown ways. And,  $\text{Gd}^{3+}$  is a general  $\text{Ca}^{2+}$  channel blocker and may affect the cell in ways other than targeting CNGCs. (2) Stem cell fate and SAM size may be affected by signal transduction cascades initiated by CLV3 beyond that controlled by CLV3/CLV1 interaction. Within the context of the aforementioned limitations due

to the nature of our experiments, we present the following conclusions that provide some new insights into CLV3/CLV1 signaling. CLV3 perception by CLV1 leads to elevation of cytosolic cGMP and  $\text{Ca}^{2+}$ . This likely occurs in any cells/tissues where CLV1 is expressed; recent studies point to an expansion of tissues where CLV1 acts in signaling cascades to include the root (Williams and De Smet, 2013). CLV3-dependent  $\text{Ca}^{2+}$  elevation is impaired in *cngc* mutants, suggesting that the cGMP elevation caused by CLV1 perception of CLV3 leads to the generation of a cytosolic free  $\text{Ca}^{2+}$  signal by activation of a CNGC channel. These molecular events initiated by CLV3 interaction with CLV1 contribute to the signal transduction cascade initiated by the ligand/receptor complex that impacts stem cells in a meristem. Genetic (use of the *dnd1* and *dnd2* mutants) and biochemical (application of a  $\text{Ca}^{2+}$  channel blocker or GC inhibitor) interventions block downstream signaling (effects on WUS and FAF2 expression). Application of exogenous lipophilic cyclic nucleotide mimics (to some extent) effects of CLV3 signaling. Since we propose here that the CLV3/CLV1 signaling cascade involves cyclic nucleotide elevation in the cytosol that would activate  $\text{Ca}^{2+}$  conductance through CNGC channels, this result is consistent with the proposed model of CLV3/CLV1 signaling.

One report in the literature suggests that mitogen activated kinase (MAPK) signaling cascades are involved in CLV3 signaling through CLV1 and other receptors (Betsuyaku et al., 2010). (However, in this study, a reduction in MAPK phosphorylation was shown to be downstream from CLV3/CLV1 signaling). In any case, involvement of protein phosphorylation and MAPK signaling in CLV3/CLV1 signaling cascades would not be inconsistent with the conclusions presented here. Examples can be found (Boudsocq et al., 2010) of other peptide ligand/LRR-RLK receptor-dependent cytosolic free  $\text{Ca}^{2+}$  elevations leading to effects on MAPK signaling through activation by the  $\text{Ca}^{2+}$  signal of calcium-dependent protein kinases, which act



upstream from phosphorylation of MAPKs. It should also be noted that the specific molecular steps identified here as involved with CLV3/CLV1 signaling do not preclude CLV3 and CLV1, acting together or independently (e.g. with other signaling ligands or receptors) to affect gene expression in meristems and, by extension, stem cell fate, through entirely different signaling systems. Finally, we caution that our studies do not conclusively identify the source of CLV3-dependent cGMP generation; it may not necessarily involve the putative GC activity of the CLV3 receptor CLV1. Another hypothesis to be considered is that the CNGC channel is activated upon CLV3/CLV1 interaction by a mechanism other than cGMP elevation (and the mimicking of some CLV3 phenotypes in our work by dibromo-cGMP application is fortuitous). Nonetheless, the work presented in this report does identify cyclic nucleotide activated  $\text{Ca}^{2+}$ -conducting channels and  $\text{Ca}^{2+}$  signaling as an important component linking CLV3 signaling peptide perception by the CLV1 receptor, to downstream control of gene expression and stem cell fate in the SAM.

## Materials and Methods

### Plant materials

All Arabidopsis lines used in the reported work are in the Columbia (Col) background except the *clv1* null mutant *clv1-13* (At1g75820); the *clv1-13* mutation is in the Wassilewskija (WS) background. Col or WS plants were used as controls as appropriate. The aequorin-expressing lines Col-aeq, WS-aeq and *dnd1*-aeq (Qi et al., 2010) were used to monitor treatment effects on cytosolic free  $\text{Ca}^{2+}$  concentration. Col plants expressing the cGMP reporter protein  $\delta$ -FlnG (Isner et al., 2011) were used for *in vivo* cGMP determinations. *pCLV1::GUS*, *pWUS::GUS* and *pFAF2::GUS* were used to monitor expression patterns of the respective genes.

Arabidopsis seeds were surface-sterilized by first washing the seeds in 70% (v/v) ethanol, 20% (v/v) bleach and 0.02% (v/v) Triton X-100, shaking at 300 rpm for 10 min, and then rinsing with 95% (v/v) ethanol 3 to 4 times.

For all measurements, seeds were planted on ½ strength Murashige and Skoog (MS) medium (Caisson Labs, Logan, UT) supplemented with 1% (w/v) suc and solidified with 1% (w/v) agar. Seeds were stratified (4 °C) for 2 to 3 d after plating to break dormancy. Plants used for cytosolic free  $\text{Ca}^{2+}$  measurements were grown by germinating seeds grown on agar plates for 10 d at 16 h light/ ( $\sim 100 \text{ mol m}^{-2} \text{ s}^{-1}$ )/8 h dark and 25 °C. For *in vivo* cGMP measurement and GUS staining assays, the seeds were set vertically on square plates containing solid agar medium for 5 to 6 d. For qPCR measurement of gene expression, seeds were grown on solid medium for 7 d and transferred to liquid ½ MS medium (5 mL medium for 12 seedlings) for 5 d for additional treatments.

### **CLV3 peptide.**

Exogenously-added, synthetic CLV3 peptide was used for many of the experiments examining the CLV3 signaling pathway. For these studies, we used a synthetic (GenScript, Piscataway, NJ) 13-mer peptide (RTVPSGPDPLHH) exactly corresponding to one (referred to as ‘MCLV3’) shown to be maximally active by Kondo et al. (2006). Biological activity of peptides affecting plant growth, development, and response to environmental stresses has been characteristically evidenced by demonstrating a peptide-dependent inhibition of germinating seed radicle length (Krol et al., 2010; Kondo et al., 2006; Gómez-Gómez et al., 1999). In our hands, root length of 12 d-old WT (Col) seedlings was inhibited on medium containing 1-10  $\mu\text{M}$  of this synthetic CLV3 peptide (Figure 7), demonstrating biological activity of the peptide. Other evidence documenting the biological activity of the CLV3 peptide we used in our work is as

follows. A critical step in the CLV3 signaling pathway that was the focus of the experiments in this report is the repression of *WUS* expression by exogenous synthetic CLV3 peptide. As a negative control, we checked the effect of exogenous flg22 peptide on *WUS* expression and found no repression of its expression (data not shown). A positive control for the biological activity of our synthetic CLV3 was undertaken in the following experiment. We obtained the synthetic CLV3 peptide used by Lee et al. (2011) in their work showing repression of *WUS* expression. Their synthetic CLV3 was generated from a different source (Mass. General Hospital Peptide Core Facility) than that used for our CLV3 synthesis. In our hands, our CLV3 peptide had as strong or stronger effects than the peptide we obtained from Lee et al. when used in similar experiments (data not shown).

### **Cytosolic free $\text{Ca}^{2+}$ measurement**

The method from (Qi et al., 2010) was used with slight modification for cytosolic free  $\text{Ca}^{2+}$  measurements using aeq-expressing plants. See Qi et al. (2010) for details of methods. Here we use Col-aeq, *dnd1*-aeq and WS-aeq seedlings for experiments. CLV3 peptide (2  $\mu\text{M}$  final concentration) was added to whole 10-d-old Arabidopsis seedlings expressing cytosol-localized  $\text{Ca}^{2+}$ -dependent chemiluminescent apoaequorin protein reconstituted with coelenterazine-cp (CTZ-cp AAT Bioquest Inc, Sunnyvale, CA). Individual seedlings were placed in a capless 2 mL centrifuge tube containing 300  $\mu\text{L}$  water. For each tube, 1  $\mu\text{L}$  CTZ-cp was added (10  $\mu\text{M}$  final concentration in 0.2 % (v/v) ethanol). The seedling in the water was vacuum infiltrated for 15 s and incubated at room temperature in the dark for 1 to 2 h to allow coelenterazine incorporation into tissues. As the CTZ-cp is a light sensitive reagent, all preparatory steps after adding the CTZ-cp were carried out in dark; tubes were covered with foil paper.

For luminescence recordings, the centrifuge tube was placed into a luminometer (TD-20/20; Turner Designs) and kept in the dark for 2 to 3 min for recovery from handling and dissipation of touch induced  $\text{Ca}^{2+}$  spikes. Before application of ligand, the background luminescence of the leaf was recorded for 30 s (one data point per second frequency). The seedling was considered ready for monitoring ligand effects on cytosolic free  $\text{Ca}^{2+}$  if the background luminescence reading was stable over the recording time and was no less than 10-fold greater ( $>3,000$  luminescence events/s) than (and typically several orders of magnitude greater than) background luminescence recorded from seedlings of plants not transformed with the apoaequorin gene ( $\sim 300$  luminescence events/s). When background luminescence was stable, 300  $\mu\text{L}$  of water containing ligand (at 2 X final concentration) was added to the tubes containing seedlings by gently pipetting the solution against the interior wall of the centrifuge tube. After recording luminescence from a treatment replicate, the remaining aeq (i.e. not bound to  $\text{Ca}^{2+}$ ) in an assay tube was discharged by adding 500  $\mu\text{L}$   $\text{Ca}^{2+}$  release buffer (2 M  $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$  in 30% (v/v) ethanol) with continued recording for  $\sim 10$  min, until the instantaneous luminescence level dropped below 2000. Values obtained for aeq discharge were used to convert luminescence readings to cytosolic free  $\text{Ca}^{2+}$  concentration for each treatment replicate using an algorithm as described by Qi et al. (2010). Results shown in figures are mean values calculated from a minimum of at least 3 biological replicates

### ***In vivo* cGMP measurement**

Arabidopsis plants expressing the fluorescent cGMP reporter protein FlnG (Isner and Maathuis 2011) were grown on  $\frac{1}{2}$  MS solid medium vertically and used after five d to measure *in vivo* cytosolic cGMP. To avoid effects of chlorophyll autofluorescence, which would impede the monitoring of treatment effects on FlnG fluorescence intensity, studies were done on root

tips. Working with plants grown vertically on plates allowed for use of seedlings that were pulled off the surface of the solid agar medium without much damage to the root tissue. Seedlings were placed in 60  $\mu$ L water on a 24 X 40 mm cover glass and covered with a 22 X 22 mm cover slip. The small cover slip was taped (on two opposite sides) onto the larger one (forming a chamber) and then the chamber with a seedling inside was taped onto the stage of a confocal microscope (Nikon A1R, Nikon; Melville, NY). Baseline fluorescence was measured after sealing a seedling in the recording chamber on the microscope stage prior to addition of CLV3 peptide.

Seedlings with roots having high levels of GFP expression were chosen for use. The root tip was located under bright field illumination, observation of background GFP fluorescence was made, and after adjustment of the perfect focus, CLV3 was applied from one side of the glass slide and a filter paper cut in to a triangular shape was put on the other side of the chamber to wick excess water that was generated as CLV3 (or water as a control) was added to the other open side of the chamber. CLV3 peptide (in water) was added to the chamber near the seedling root tip; initial addition of CLV3 was counted as time “0” min. Fluorescence images were recorded every 30 s using 480/ 20-nm excitation wavelength using the microscope’s high-performance optical offset (Perfect Focus System) to facilitate real-time correction of focal drift. Fluorescence intensity at the root tip was quantified as relative brightness (within a defined range of 256 shades of gray per unit area) using NIH ImageJ processing/analysis software.

The efficacy of this experimental system for *in vivo* measurement of cGMP changes in plant cells is supported by the following points. First, a positive control experiment indicated that application of a lipophilic analog of cGMP (db-cGMP) resulted in similar fluorescence changes in root tips as we observed when CLV3 peptide was applied to the recording chamber near the root tip (data not shown). Second, this same db-cGMP treatment results in similar changes in the

SAM that occurred in response to exogenous CLV3 application using two other experimental systems. As shown in Figure 6, qPCR analysis showed db-cGMP regulation of *WUS* expression. Also, application of db-cGMP (but not the non-lipophilic cGMP) to *pFAF2::GUS* seedlings down-regulated expression of *FAF2* (see Figure 8). Third, Isner and Maathuis (2011) found that application of db-cGMP to Arabidopsis and rice protoplasts resulted in similar levels of fluorescence changes as reported here.

It should be noted that the CLV3 concentration used in the FlnG experiments reported here (6  $\mu$ M, see Figure 2) is higher than the CLV3 concentration used in our experiments monitoring effects on cytosolic  $\text{Ca}^{2+}$  or gene expression in the SAM (1-2  $\mu$ M). In other work (not shown) we found a greater extent of cGMP generation (i.e. change in fluorescence of FlnG seedling root tips) when 10  $\mu$ M CLV3 was added to the FlnG seedling recording chamber, but the differences between 2  $\mu$ M CLV3 addition and water (control) were not significant. As shown in the Figures included in this report, 1-2  $\mu$ M CLV3 was found to have an effect in these other assay systems used to monitor  $\text{Ca}^{2+}$  or gene expression. One possible explanation for this apparent discrepancy might be the technical limitations of the FlnG assay system we developed. We add CLV3 to the recording chamber near the root tip and wick solution away from the chamber at the other side of the chamber. However, we cannot know the precise concentration of ligand equilibrated in the chamber as the ligand-containing solution added to the chamber is diluted to some extent by the water already in the chamber. We can only report that a solution containing (for example) 6  $\mu$ M CLV3 is added to the recording chamber near the root tip; the actual ligand concentration in the chamber must be lower. We provide the following information to readers of this report as a basis for evaluating the concentration of exogenous CLV3 used in our studies (i.e. 1, 2, and 6  $\mu$ M). Prior studies of the effects of exogenous CLV3 peptide on

physiology of Arabidopsis seedlings have used a similar range of ligand concentrations; i.e. 10  $\mu\text{M}$  (Fiers et al., 2006, Ogawa et al., 2008; Betsuyaku et al., 2011), 5  $\mu\text{M}$  (Kinoshita et al., 2010; Ishida et al., 2014), and 1  $\mu\text{M}$  (Kondo et al., 2006; Lee et al., 2011),

### **Evaluation of gene expression using GUS-expressing plants.**

Seeds of Arabidopsis plants expressing GUS under control of the *WUS*, *FAF2*, or *CLV1* promoter were grown vertically on solid  $\frac{1}{2}$  MS medium square plates for up to 6 d, and seedlings were then transferred (one seedling/tube) to 1.5 mL centrifuge tubes for further treatments. These Arabidopsis genotypes have been characterized and used in prior studies as follows: *pCLV1::GUS* (Durbak and Tax, 2011), *pWUS::GUS* (Lee et al., 2011) and *pFAF2::GUS* (Wahl et al., 2010). The GC inhibitor LY-83583 (6-anilino-5, 8-quinolinequinone; EnzoLife Sciences; Farmingdale, NY) (at 20  $\mu\text{M}$  final concentration), the  $\text{Ca}^{2+}$  channel blocker  $\text{GdCl}_3$  (at 150  $\mu\text{M}$  final concentration) (Sigma-Aldrich) or cGMP (at 150  $\mu\text{M}$  final concentration) were added to tubes and the seedlings were exposed to these compounds for 20 min. Then, seedlings were transferred to another new 1.5 mL tubes containing 1  $\mu\text{M}$  CLV3 (in 1 mL water) (or just water as a control) and incubated an additional 2 h, followed by GUS staining using methods similar to the aforementioned studies with these *GUS*-expressing Arabidopsis lines.

### **qPCR analysis**

Treatment and genotype effects on the expression level of CLV3-responsive genes were evaluated using q-PCR analysis. Arabidopsis seedlings were grown on solid  $\frac{1}{2}$  strength MS medium first then transferred to  $\frac{1}{2}$  strength MS liquid medium on a shaker (180 rpm) with 24 h illumination ( $\sim 90 \text{ mol m}^{-2} \text{ s}^{-1}$ ) at 22 °C for further growth and were used after a total of 12 d. The CLV3 ligand (at 1  $\mu\text{M}$  final concentration) (or lipophilic cGMP) (at 150  $\mu\text{M}$  final

concentration) was added to the liquid medium and the seedlings were incubated for 2 h. For experiments involving addition of inhibitors, LY83583 (at 20  $\mu$ M final concentration) or  $GdCl_3$  (at 150  $\mu$ M final concentration) were added to the medium containing plants 20 min prior to addition of CLV3. Total RNA was isolated from the SAM region of the 12-d-old Arabidopsis as follows. Fine forceps were used to remove roots, cotyledons, and hypocotyls from seedlings, leaving the SAM with as little surrounding tissue as possible. After immediately killing seedlings in liquid nitrogen, tissue was stored at -80 °C until use. RNA was extracted following the manufacturer's protocol (Macherey-Nagel; Bethlehem, PA). SAM regions from 12 seedlings were pooled and used as a biological treatment replicate. During the RNA extraction process, tissue was treated with rDNase (Macherey-Nagel) to remove potential genomic DNA from samples.

After extraction, 400 ng of total RNA was used for reverse transcription by using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems (ABI); Carlsbad, CA). The synthesized cDNA was diluted 1:4 (v/v) in water, and 1  $\mu$ L of the diluted cDNA was used for each qPCR reaction. qPCR assays were performed using the ABI 7900 HT Real-time PCR system with the SYBR Green gene expression assay (ABI). Treatment effects on the expression level of *WUS* were examined. *Tubulin* was used as an endogenous control. The primers used for these analyses were as follows: *WUS* (Forward) 5'- TGCAAGCTCAGGTACTGAATG -3', *WUS* (Reverse) 5'- ATGATCCATGTTTGCCCATC -3'; and *Tubulin* (Forward) 5'- GAG CCT TAC AAC GCT ACT CTG TCT GTC -3', *Tubulin* (Reverse) 5'- ACA CCA GAC ATA GTA GCA GAA ATC AAG -3'.

For each analysis, 3 mechanical replications were tested on one plate, and each treatment mean was generated from analysis of at least 3 biological replications from separate RNA



isolated from different individual seedling. ANOVA of corresponding threshold cycle (CT) values was used for evaluation of means separation amongst treatments in an experiment (as noted in Figure legends), and to generate standard errors of the means for control treatments (Schmittgen et al., 2008). In many cases, experiments involving qPCR analysis of gene expression involved the evaluation of CLV3 effects on different genotypes. In these cases, gene expression in the absence of exogenous CLV3 peptide was normalized to '1' for each genotype in a given experiment (as noted in figures). In these cases, the relative amount of gene expression in the absence of CLV3 for each mutant line used in an experiment (i.e. in a given figure) is compared to the expression level in WT plants for that experiment. This information is provided in the corresponding Figure legend.

### **SAM size measurement**

The methods used for SAM size measurement followed those of Ohyama et al., 2009) with modifications as noted below. Arabidopsis seeds were grown in ½ strength MS liquid medium on a shaker (180 rpm) with 24 h illumination ( $\sim 90 \text{ mol m}^{-2} \text{ s}^{-1}$ ) at 22 °C and were used after growing for 7 d. All treatments including exposure of seedlings to CLV3 ligand (at 1  $\mu\text{M}$  final concentration) and  $\text{GdCl}_3$  (at 150  $\mu\text{M}$  final concentration) were added to the liquid medium at d 0 (in one of the experiments,  $\text{GdCl}_3$  was added at d 3 for comparison with effect of d 0 addition). After 7 days, individual seedlings were laid sideways under a dissecting microscope. The roots, cotyledons and the oldest leaf primordia were removed with a sharp razor blade. To prevent morphology change, the dissected tissue was quickly moved into a fixing solution (acetic acid-ethanol (1:9, v/v)) and incubated overnight at room temp. The next day the fixing solution was removed and replaced with 90% ethanol. Samples were put on a shaker for up to 1 h, after which the 90% ethanol was removed and replaced with 70% ethanol; seedlings were put back on

the shaker for an additional 1 h. Seedlings were then removed from the fixing solution and placed on a glass slide with a drop of clearing solution (chloral hydrate, glycerol and water (8:1:2, w/v/v)) and observed under a microscope equipped with Nomarski optics (MICROPHOT-FXA; Nikon, Tokyo, Japan). Photographic images of the individual seedlings were recorded at the focal plane corresponding to the median optical section of the SAM. The width and the height of the SAM were measured by drawing a straight line between the basal edges of two opposing leaf primordia on each side of the SAM dome. A line connecting the points was generated to represent the base of the SAM dome. A perpendicular line was generated connecting the midpoint of the SAM base to the top of the SAM dome. The area of the SAM was determined by calculating the area above the straight line that represented the width of the base of the SAM. All areas were measured using the infinity analyze program (Lumenera, Ottawa, Canada).

### **Growth inhibition assay**

Arabidopsis seedlings were grown vertically on ½ strength solid MS medium with or without additional CLV3 peptide (final concentration 1, 2 or 10 µM) for 12 d. Root length was measured from the border of the hypocotyl and root to the root tip.

# **Chapter 3: A new tool to measure the change of cAMP level *in vivo* in plant protoplasts and *in planta*- Glosensor-cAMP subcloning, protoplast transient expression and plant stable expression**

## **Abstract**

The molecule 3'-5'-cyclic adenosine monophosphate (cAMP) is a secondary messenger converted from ATP via an enzyme named adenylyl cyclase (AC). Despite that no canonical AC has been identified in plants and that cAMP levels in plants are shown to be very low when compared to animals, studies suggest that plants also have a functional cAMP-dependent signaling pathway system. Published data only show plant cAMP level *in vitro*, and there has been no demonstration of a tool that could measure cAMP levels for plants *in vivo* and *in planta*. The pGloSensor™-22F cAMP Plasmid is a biosensor that encodes a cAMP binding domain fused to a mutated form of *Photinus pyralis* luciferase, Upon binding to cAMP, conformational changes occur which promote large increases in light output when pGloSensor™-22F cAMP is expressed in animal HEK293 cells. We have subcloned the coding region of this commercial cAMP reporter plasmid into a protoplast expression vector for transient expression and a plant expression vector for stable expression. We did not get any results from transient expression in Arabidopsis protoplast; perhaps the reporter protein was not expressed at a high enough level to function as a biosensor when it is transiently expressed in protoplasts. However, in transgenic plants, we are able to detect a 3-fold change of light output when given either membrane permeable cAMP or forskolin (an AC activator). Both membrane permeable cAMP and forskolin result in a dose dependent response on the pGloSensor™-22F cAMP transgenic leaves. An adenylyl cyclase inhibitor prevents the effect of forskolin on pGloSensor™-22F cAMP

transgenic leaves, resulting in lower light output. As expected, this AC inhibitor did not affect light generated in response to dibutyl-cAMP addition. These results suggest that the pGloSensor<sup>TM</sup>-22F cAMP coding region works in plant system and can be used as a tool to measure plant cAMP level change.

## Introduction

The molecule 3'-5'-cyclic adenosine monophosphate (cAMP) is a secondary messenger and signaling molecule significant in animals and lower eukaryotes (Gehring, 2010). Adenylyl cyclase (AC) is an enzyme that catalyzes the conversion of ATP to cAMP. cAMP affects many different physiological and biochemical processes in plants and animals (Bindschedler et al., 2001; Pietrowska-Borek and Nuc, 2013). The studies with plants have primarily monitored phenotypes of plants, plant protoplasts, or plant cell cultures responding to exogenous addition of cAMP; not much work links changes in endogenous cAMP to plant signaling pathways at this point in the published literature. The importance of ACs and cAMP has drawn the attention of plant scientists to understand if the signaling system is universal (Assmann, 1995). However, cAMP levels in plants appeared to be low compared to animals. Reported cAMP levels in plants are typically < 20 pmol/g fresh weight whereas animal values are typically > 250 pmol/g wet weight (Gehring, 2010). Given these facts, conclusions have not been drawn from plant scientists. Despite the uncertainty of physiological function and low levels of cAMP in plants, published data suggests that plants also have a functional cAMP-dependent signaling pathway system (Kurosaki and Nishi 1993; Kurosaki et al., 1993).

Studies have shown that there are cNMP activities in plants by *in vitro* assays (Cooke et al., 1994; Moutinho et al., 2001; Kwezi et al., 2007; Ma et al., 2009; Meier et al., 2010; Qi et al., 2010; Kwezi et al., 2011; Irving et al., 2012). Recently an Arabidopsis transgenic plant which contains an animal cGMP binding site inserted in a green fluorescent protein was developed to measure the level of cGMP change in response to stimulus *in vivo* in protoplasts and *in planta* (Isner and Matthuis, 2011). However, there has been no demonstration of a tool that could measure cAMP levels for plants *in vivo* and *in planta*. Therefore, developing a biosensor that can

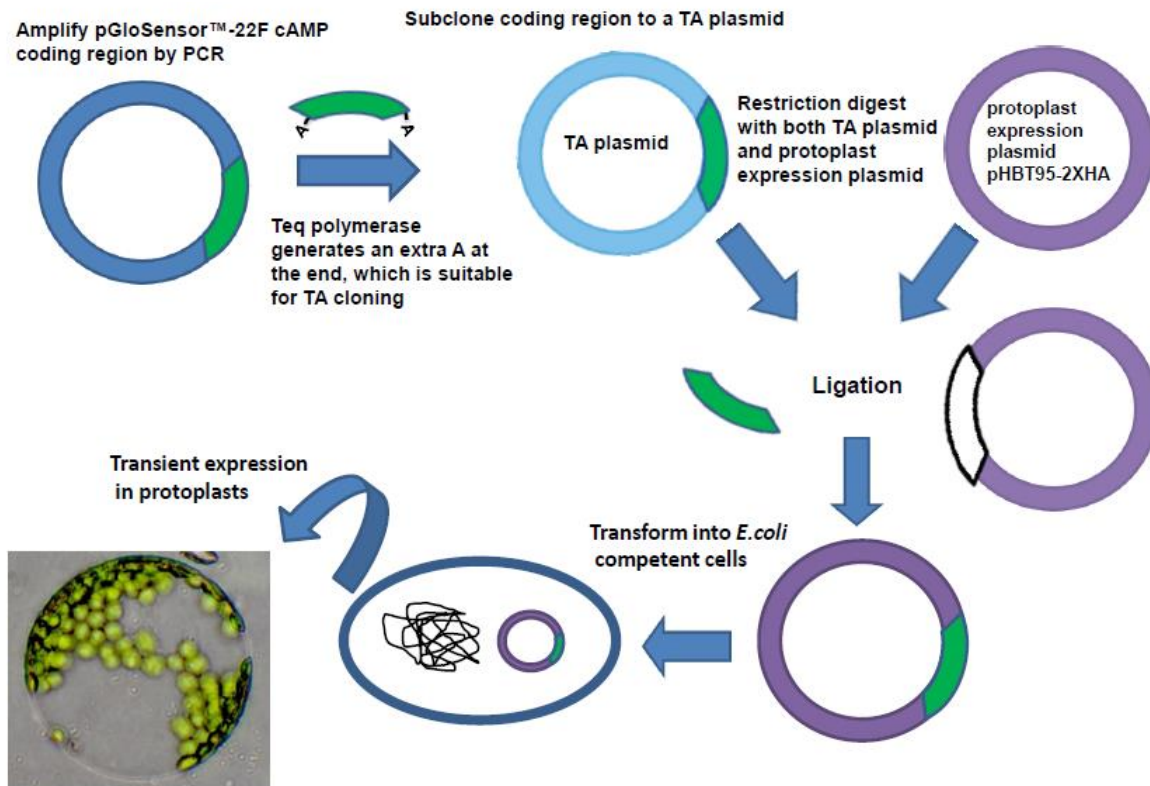
be used as a tool to measure cAMP *in vivo* or *in planta* can definitely bring contribution to the field.

The pGloSensor™-22F cAMP Plasmid (Promega, catalog number E2301) is a biosensor that encodes a cAMP binding domain fused to a mutated form of *Photinus pyralis* luciferase (Fan et al., 2008). Upon binding to cAMP, conformational changes occur which promote large increases in light output in animal HEK293 cells (Binkowski et al., 2011). This biosensor has been expressed in animal HEK293 cells with chemicals that can result in cAMP level changes, e.g. forskolin, which can activate adenylyl cyclase to increase cAMP, and isoproterenol, a chemical that activates  $\beta$ 2-adrenergic receptor (a class of G protein-coupled receptors, GPCRs)) (Binkowski et al., 2011). A more recent study has used this biosensor to study metabotropic glutamate receptors (GPCRs that inhibit adenylyl cyclase via activation of  $G\alpha_{i/o}$ ) in Chinese hamster ovary (CHO) cells (DiRaddo et al., 2014). We have subcloned this commercial cAMP reporter gene that is used to monitor cAMP levels in animal cells into a protoplast expression vector for transient expression and a plant expression vector for stable expression.

## Results

### Subcloning pGloSensor™-22F cAMP coding region in to a protoplast expression vector

In order to transiently express the pGloSensor™-22F cAMP plasmid in Arabidopsis protoplasts, the coding region of pGloSensor™-22F cAMP Plasmid (2.1 Kb) is subcloned into a protoplast expression vector pHBT95-2XHA under the control of the 35S CaMV constitutive promoter. Figure 17 shows a schematic representing the subcloning strategy. NcoI and StuI restriction sites are added to the forward and reverse primers for PCR subcloning. Forward primer: "CCA TGG CAA TGC CTG GCG CAG TAG GCA AG"; reverse primer "AGG CCT



**Figure 17.** A schematic representation of the procedure used to transiently express glosensor-cAMP- pHBT95-2XHA in leaf mesophyll protoplasts. The green color area represents the pGloSensor™-22F cAMP coding region fragment. The pHBT95-2XHA is represented in purple. The pCR® 2.1-TOPO® plasmid (TA plasmid) that is used as a shuttle is shown in light blue.

TTA AAC CCC TTC TGG AGT GAT CAG AAT GGC G". The NcoI restriction site is embedded with a start codon ATG and will affect the reading frame of the coding sequencing. Thus, two additional bases (CA) are added at the forward primer before the start codon. Taq DNA polymerase is used for PCR to generate adenine (A) overhang. The 2.1 Kb coding region (Figure 18) is first subcloned into pCR® 2.1-TOPO® plasmid with the TOPO® TA Cloning® Kit (Invitrogen, K4500-02). The pCR® 2.1-TOPO® plasmid is already linearized and has a single thymine (T) residue at the 3' end. Blue white screening is used to detect positive colonies which contain plasmid with the gene of interest.  $\beta$ -galactosidase is a protein produced by the lacZ gene from the lac operon. The lacZ gene in the pCR® 2.1-TOPO® plasmid only carries partial residues of the  $\beta$ -galactosidase ( $\alpha$ -peptide) and is inactive. The One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen, C4040-03) are cells that carry the lacZ deletion mutant which contains a  $\omega$ -peptide which is also inactive. When the plasmid is transformed into TOP10 competent cells, it will form a functional  $\beta$ -galactosidase. With the presence of Isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-gal), cells containing active  $\beta$ -galactosidase will produce blue color. If the pGloSensor™-22F cAMP coding region is inserted into pCR® 2.1-TOPO® plasmid, it will disrupt the  $\beta$ -galactosidase function. Cells that are transformed with the recombinant DNA will produce white colonies. The positive pCR® 2.1-TOPO® plasmid which contains the pGloSensor™-22F cAMP coding region and the pHBT95-2XHA are both cut out by NcoI and StuI restriction enzymes (Figure 19), the released fragments of interest are purified then ligated, with NcoI and StuI providing compatible cohesive ends. Plasmid is then transformed into One Shot® TOP10 chemically competent *E. coli* cells and cultured overnight on antibiotic selection plates. Colonies are picked out the next day with an overnight liquid culture followed by a plasmid mini prep. Positive plasmids are

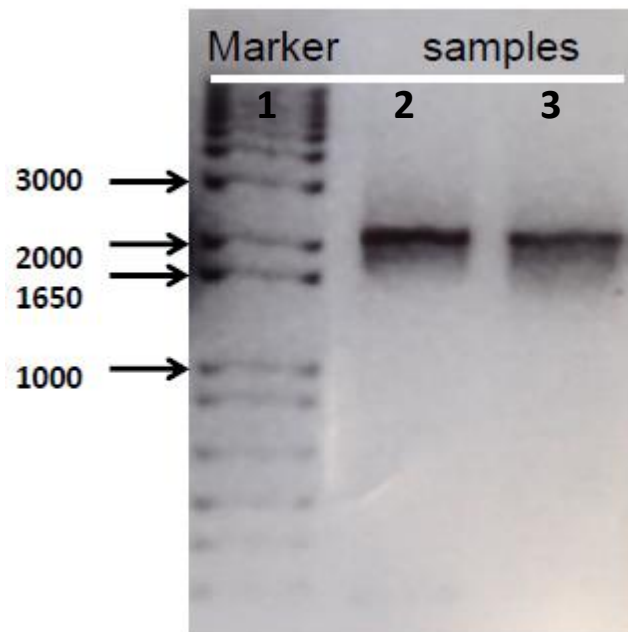


confirmed by both double digestion (Figure 20) and sequencing (Figure 21). A maxi prep is done in order to get high enough concentration for protoplast transient expression. Protoplasts are extracted from leaves of 3 to 4 week-old healthy Arabidopsis plants. pHBT95-2XHA encoding the pGloSensor™-22F cAMP coding region is transfected into protoplasts by following Dr. Jen Sheen's protocol (Yoo et al., 2007).

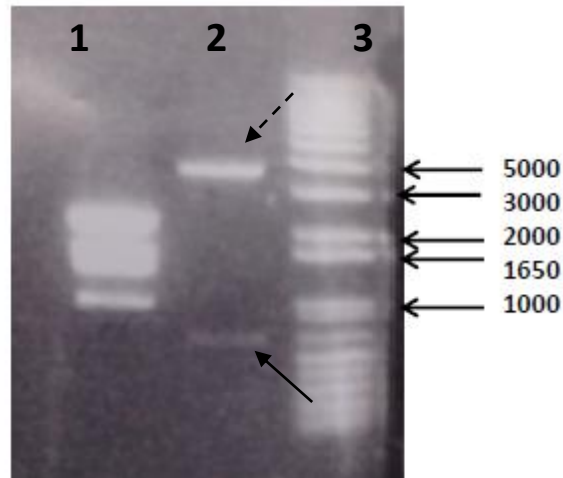
Several attempts were made to transiently express pHBT95-2XHA encoding the pGloSensor™-22F cAMP coding region in Arabidopsis protoplasts. ATP (final conc. 0.1 mM) and  $Mg^{2+}$  (final conc. 0.8 mM) were applied before measurement ligand-dependent light emission (i.e. cAMP generation in intact protoplasts) due to the lack of ATP synthesis caused by protoplast incubation in dark overnight. However, we were not able to detect cAMP level change by giving membrane permeable dibutyl (db)-cAMP to Arabidopsis protoplasts. We suspect that it is likely the system doesn't work in protoplasts or the expression level of cAMP is not strong enough for detection. In order to overcome this problem and have stable expression, we switched to subcloning the pGloSensor™-22F cAMP coding region into a plant expression plasmid.

### **Subcloning pGloSensor™-22F cAMP coding region in to a plant expression vector**

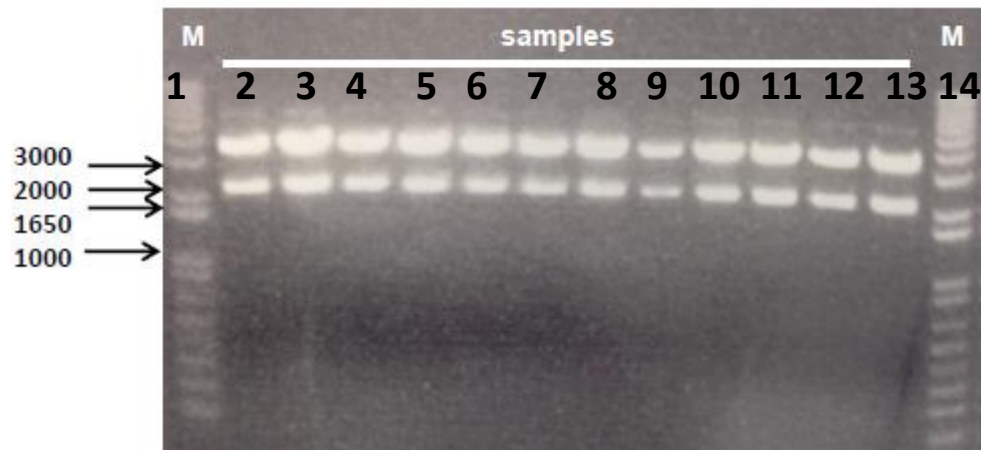
To express the pGloSensor™-22F cAMP coding region stably in Arabidopsis plants, it is necessary to generate transgenic plants. In order to efficiently subclone the gene, cloning work is done by using the Gateway system (Invitrogen). Figure 22 shows a schematic representing the subcloning strategy. The coding region of pGloSensor™-22F cAMP is amplified by PCR with primers- Forward: “CACC AT GCC TGG CGC AGT AGG C”, Reverse: “TTA AAC CCC TTC TGG AGT GAT CAG AAT GGC GCT G”. The iProof™ HF Master Mix (Bio-Rad, 172-5310) is used to amplify the coding region. The 2.1 Kb PCR product (Figure 23) is run on a 1% agarose



**Figure 18. PCR amplification of pGloSensor<sup>TM</sup>-22F cAMP coding region.** Lane 1 is a 1 kb+ ladder. Lanes 2 and 3 are PCR products presenting the 2.1 kb pGloSensor<sup>TM</sup>-22F cAMP coding region.



**Figure 19. Restriction enzyme digestion analysis of pCR<sup>®</sup> 2.1-TOPO<sup>®</sup> plasmid encoding the pGloSensor<sup>™</sup>-22F cAMP coding region and destination plasmid pHBT95-2XHA using NcoI and StuI (and also BsaI in pCR<sup>®</sup> 2.1-TOPO<sup>®</sup> plasmid encoding the pGloSensor<sup>™</sup>-22F cAMP coding region).** Lane 1 presents restriction digestion result of pCR<sup>®</sup> 2.1-TOPO<sup>®</sup> plasmid encoding the pGloSensor<sup>™</sup>-22F cAMP coding region. Lane 2 represents restriction digestion result of vector pHBT95-2XHA. Lane 3 represents a 1 kb plus DNA ladder. The pGloSensor<sup>™</sup>-22F cAMP coding region (2.1 kb) is located slightly above the 2 kb band in the DNA ladder. Additional BsaI digestion is used to separate out the fragment which should be the same size of the pGloSensor<sup>™</sup>-22F cAMP coding region due to NcoI site in pHBT95-2XHA. pHBT95-2XHA was digested into 2 fragments (0.7 kb (black arrow) and 3.6 kb (dashed arrow)).



**Figure 20. Restriction digestion to confirm that the pGloSensor<sup>TM</sup>-22F cAMP coding region has been successfully subcloned into the pHBT95-2XHA plasmid.** Restriction digestion was performed with NcoI and StuI restriction enzymes. Results show 2 strong bands, one representing the pGloSensor<sup>TM</sup>-22F cAMP coding region (2.1kb) and the other representing the pHBT95-2XHA plasmid (3.6kb). Lanes 1 and 14 are 1 kb+ ladder. Lanes 2 to 13 are samples.

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Sequence ID: lc|46129 Length: 2106 Number of Matches: 1

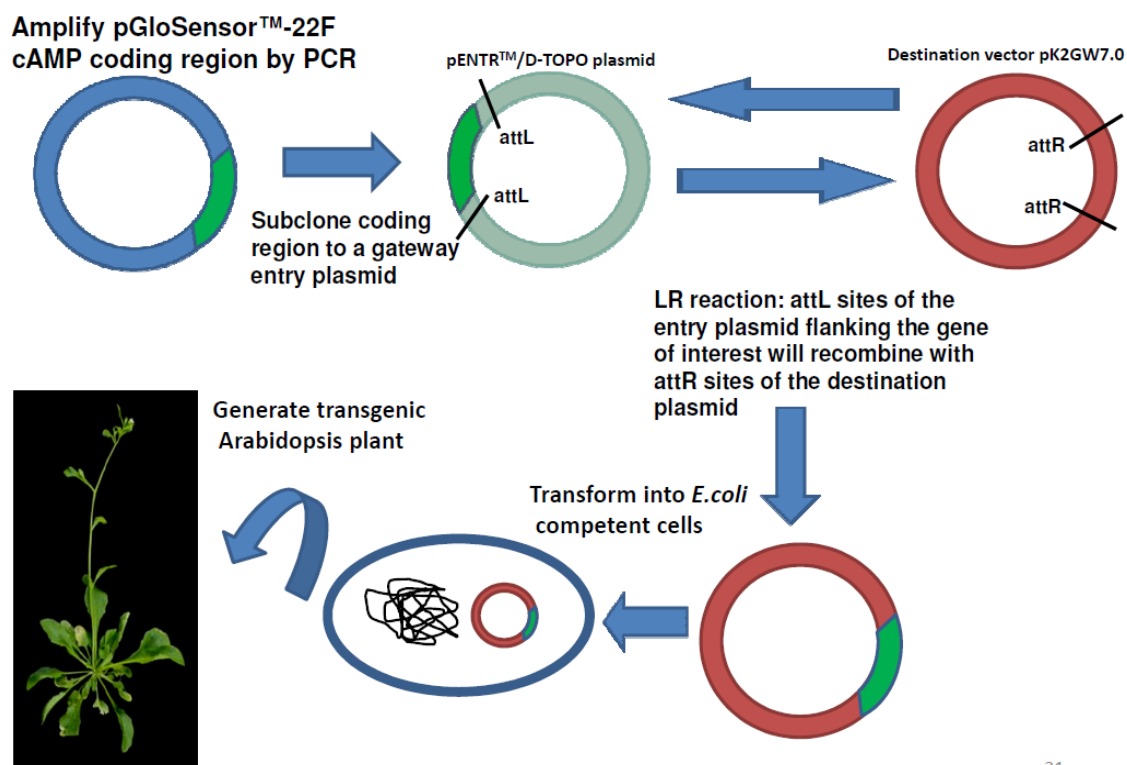
Range 1: 28 to 1202 [Graphics](#)

[Next Match](#) [Previous Match](#)

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Sbjct 28	CCCTTCATCGAGGCTAAGGTGGTGGACTTGGACACTGGTAAGACACTGGGTGTGAACCAG	87		
Query 66	CGCGGCGAGCTGTGCGTCCGTGGCCCCATGATCATGAGCGGCTACGTTAACAACCCCGAG	125		
Sbjct 88	CGCGGCGAGCTGTGCGTCCGTGGCCCCATGATCATGAGCGGCTACGTTAACAACCCCGAG	147		
Query 126	GCTACAAACGCTCTCATCGACAAGGACGGCTGGCTGCACAGCGCGACATCGCCTACTGG	185		
Sbjct 148	GCTACAAACGCTCTCATCGACAAGGACGGCTGGCTGCACAGCGCGACATCGCCTACTGG	207		
Query 186	GACGAGGACGAGCACTTCTTCATCGTGGACCGGCTGAAGAGCCTGATCAAATACAAGGGC	245		
Sbjct 208	GACGAGGACGAGCACTTCTTCATCGTGGACCGGCTGAAGAGCCTGATCAAATACAAGGGC	267		
Query 246	TACCAAGTAGCCCCAGCCGAACCTGGAGAGCATCCTGCTGCAACACCCCAACATCTTCGAC	305		
Sbjct 268	TACCAAGTAGCCCCAGCCGAACCTGGAGAGCATCCTGCTGCAACACCCCAACATCTTCGAC	327		
Query 306	GCCGGGTCGCCGGCCTGCCGACGACGATGCCGGCGAGCTGCCGCCCGCAGTCGTCGTG	365		
Sbjct 328	GCCGGGTCGCCGGCCTGCCGACGACGATGCCGGCGAGCTGCCGCCCGCAGTCGTCGTG	387		
Query 366	CTGGAACACGGTAAAACCATGACCGAGAAGGAGATCGTGGACTATGTGGCCAGCCAGGTT	425		
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Sbjct 928	ATGGATGTGCAAGCATTGAAAGGCTTCTGGGACCTTGCATGGGAAATTATGAAAGGGA	985		
Query 966	ACATCGCTACCTATGAAGAACAGTTAGTTTGCCTGTATGGGAACGAACATGGGATATTG	1025		
Sbjct 986	ACATCGCTACCTATGAAGAACAGTTAGTTTGCCTGTATGGGAACGAACATGGGATATTG	1042		
Query 1026	TAGCCAAAAACATTAAGA--GGCCCCAGCGCCATTCTACCCACTCGA-GAC-GGACCGC	1081		
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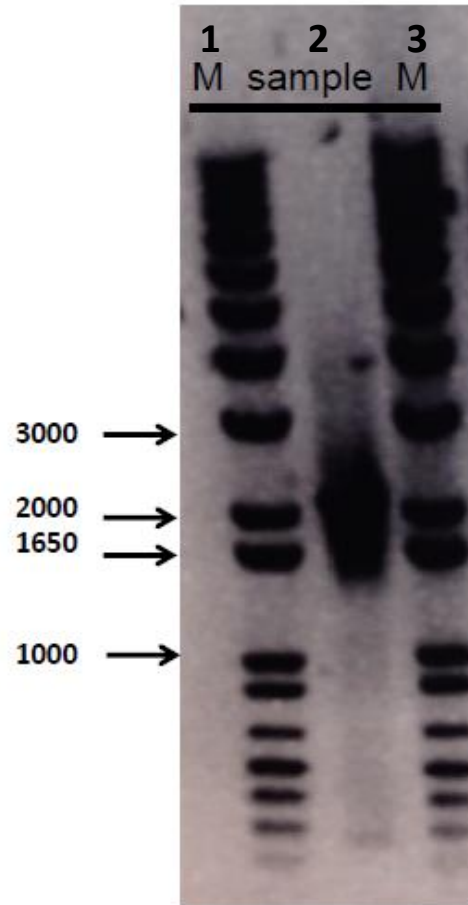
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Sbjct 2013	1954	GGCCTCACCTACCTCCTTGCTGAGCGGCGCCCGCCGCTGGCGATCTCGTGCAAGTTGCT		
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Sbjct 1893	1834	CAGGGCAGATTGAACTTTATAGTCTTGCAAGCTGCGCAAGAATAGCTCCTCCTCGAAGCG		
Query 243	302	GTACATGAGCAGACCCGAAAGCCGAGATCAAGTAGCCAGCGTGGTGAACATGCCGAA		
Sbjct 1833	1774	GTACATGAGCAGACCCGAAAGCCGAGATCAAGTAGCCAGCGTGGTGAACATGCCGAA		
Query 303	362	GCCGTGGTGAAATGGCACCACGCTGAGGATAGCGGTGTCGGGGATGATCTGTTGCCGAA		
Sbjct 1773	1714	GCCGTGGTGAAATGGCACCACGCTGAGGATAGCGGTGTCGGGGATGATCTGTTGCCGAA		
Query 363	422	GATGGGGTCGCGGGCATGACTGAATCGGACACAGCGGTGCGGTGCGGTAGGGCTACGCC		
Sbjct 1713	1654	GATGGGGTCGCGGGCATGACTGAATCGGACACAGCGGTGCGGTGCGGTAGGGCTACGCC		
Query 423	482	CTTGGGCAATCCGGTACTGCCACTACTGTTTCATGATCAGGGCGATGGTTTTGTCCCGTC		
Sbjct 1653	1594	CTTGGGCAATCCGGTACTGCCACTACTGTTTCATGATCAGGGCGATGGTTTTGTCCCGTC		
Query 483	542	GAAGCTCTCGGGCAGCAAGTCGTACTCGTTGAAGCCGGTGGCAATGGGAAGTCACGAA		
Sbjct 1593	1534	GAAGCTCTCGGGCAGCAAGTCGTACTCGTTGAAGCCGGTGGCAATGGGAAGTCACGAA		
Query 543	602	GGTGATACATGCTTTGGAAGCCCTGGTAGTCGGTCTTGCTATCCATGATGATGATCTTTTG		
Sbjct 1533	1474	GGTGATACATGCTTTGGAAGCCCTGGTAGTCGGTCTTGCTATCCATGATGATGATCTTTTG		
Query 603	662	TATGATCGGTAGCTTCTTTTGCACGTTGAGGATCTTTTGCAGCCCTTTCTTGCTCACGAA		
Sbjct 1473	1414	TATGATCGGTAGCTTCTTTTGCACGTTGAGGATCTTTTGCAGCCCTTTCTTGCTCACGAA		
Query 663	722	TACGACGGTGGGCTGGCTGATGCCCATGCTGTTTTCAGCAGCTCGCGCTCGTTGTAGATGTC		
Sbjct 1413	1354	TACGACGGTGGGCTGGCTGATGCCCATGCTGTTTTCAGCAGCTCGCGCTCGTTGTAGATGTC		
Query 723	782	GTTAGCTGGGGCCACAGCCACACCGATGAACAGGGCACCCAAACAGGGCATGAAGAACTG		
Sbjct 1353	1294	GTTAGCTGGGGCCACAGCCACACCGATGAACAGGGCACCCAAACAGGGCATGAAGAACTG		
Query 783	842	CAAGCTATTCTCGCTGCACACCAGATCCGATGGTTTGTATTACGCCATAGCGCTTCAT		
Sbjct 1293	1234	CAAGCTATTCTCGCTGCACACCAGATCCGATGGTTTGTATTACGCCATAGCGCTTCAT		
Query 843	902	AGCTTCTGCCAGCCGAACGCTCATCTCGAAGTACTCGGCGTAGGTAATGTCCACCTCGAT		
Sbjct 1233	1174	AGCTTCTGCCAGCCGAACGCTCATCTCGAAGTACTCGGCGTAGGTAATGTCCACCTCGAT		
Query 903	962	ATGTGCGTCGGTAAAGGCGATGGTGCCGGGACACAGGGCGTAGCGCTTCATGGCTTTGTG		
Sbjct 1173	1114	ATGTGCGTCGGTAAAGGCGATGGTGCCGGGACACAGGGCGTAGCGCTTCATGGCTTTGTG		
Query 963	1022	CAGCTGCTCGCCCGGCGGTCCCGTCTTCGAGTGGTAGAATGCGCTGGGCCCTTCTTAA		
Sbjct 1113	1055	CAGCTGCTCGCC-GGCGGTCCCGTCTTCGAGTGGTAGAATGCGCTGGGCCCTTCTTAA		
Query 1023	1077	TGTTTTTGGCTAC-ATATCCATGTTTCGTTCCATAACAGGGCA-CTA-CTGT-CT-CATA		
Sbjct 1054	997	TGTTTTT-GGCTACAATATCCATGTTTCGTTCCATA-CAGGGCAACTAACTGTTCTTCATA		
Query 1078	1126	G-TAGCGAIGTGC-TTT-CATA-TTTCATGCA-G-TCC-AGAAGC-TT-CAA-TGCT-G		
Sbjct 996	937	GGTAGCGAIGTTCCTTTTCATAATTTCATGCAAGGTCCCAGAAGCCTTTCAAATGCTTG		

**Figure 21. Sequence analysis of pGloSensor™-22F cAMP coding region in pHBT95-2XHA plasmid with both forward and reverse primers.** The obtained sequence (query) were aligned with the already known pGloSensor™-22F cAMP coding region sequence (subject). The alignment was done by using Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI) website. Sequence alignment indicated that the nucleotides are almost nearly identical (97%), suggesting that the pHBT95-2XHA plasmid sent for sequencing does contain the pGloSensor™-22F cAMP coding region sequence.



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**Figure 22.** A schematic representing the procedure used to generate the stable expression glosensor-cAMP plasmid glosensor-cAMP-pK2GW7.0. The green color area represents the pGloSensor™-22F cAMP coding region fragment. The pK2GW7.0 is represented in red. The pENTR™/D-TOPO vector that is used as a shuttle for GATEWAY cloning is shown in light green.



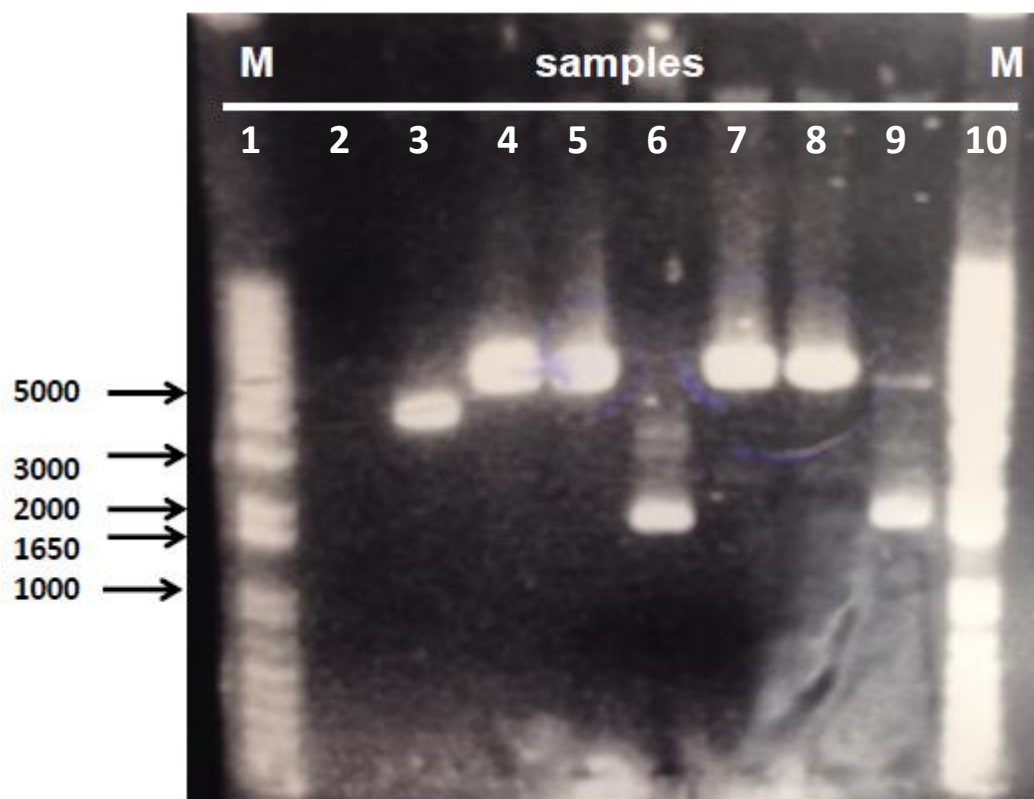
**Figure 23. PCR amplification of pGloSensor™-22F cAMP coding region.** Lane 1 and 3 is a 1 kb+ ladder. Lane 2 is the product presenting the 2.1 kb pGloSensor™-22F cAMP coding region



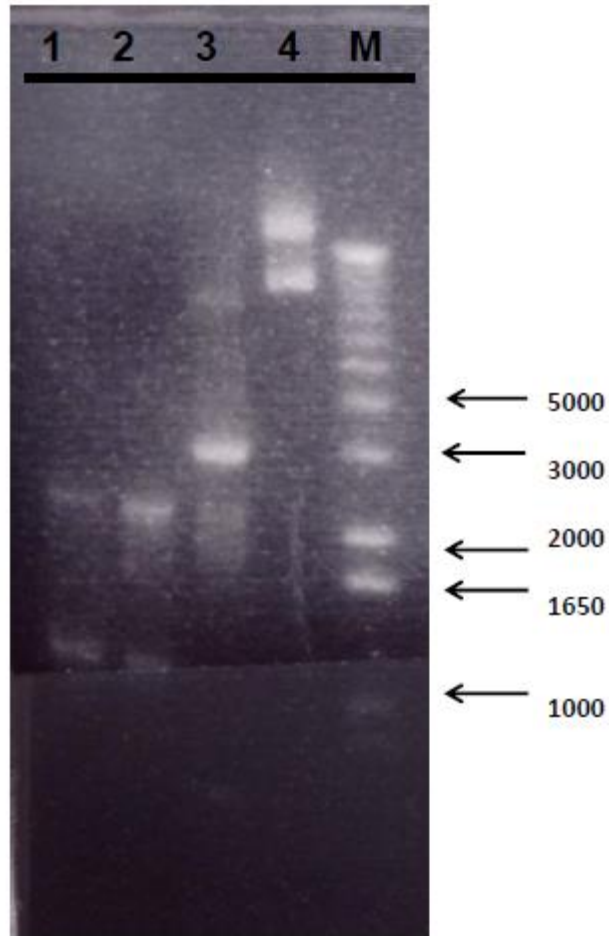
gel and purified using a gel extraction kit (Macherey-Nagel; Bethlehem, PA) then subcloned into pENTR<sup>TM</sup>/D-TOPO plasmid (entry plasmid) with pENTR<sup>TM</sup> Directional TOPO<sup>®</sup> Cloning Kit (Invitrogen, K2400-20). The plasmid is transformed into One Shot<sup>®</sup> TOP10 chemically competent *E.coli* and cultured overnight on Kanamycin antibiotic selection plates. A plasmid mini prep is done after identifying positive clones by PCR. Restriction enzyme HindIII is used to confirm that the pGloSensor<sup>TM</sup>-22F cAMP coding region has been successfully subcloned into the pENTR<sup>TM</sup>/D-TOPO plasmid (Figure 24). The isolated pENTR<sup>TM</sup>/D-TOPO plasmids that contain the pGloSensor<sup>TM</sup>-22F cAMP coding region are continued with LR reaction by using Gateway<sup>®</sup> LR Clonase<sup>®</sup> II enzyme mix (Invitrogen, 11791-020). The LR clonase catalyzes the *in vitro* recombination between an entry clone (containing the gene of interest flanked by *attL* sites) and a destination vector (Here we use pK2GW7.0, a plant expression plasmid with the size about 11 Kb) (containing *attR* sites) to generate an expression clone. The plasmid is transformed into One Shot<sup>®</sup> TOP10 chemically competent *E.coli* and cultured overnight on Spectromycin antibiotic selection plates. pK2GW7.0 containing the gene of interest- the pGloSensor<sup>TM</sup>-22F cAMP coding region- was confirmed by PCR (Figure 25), restriction digest (Figure 26) and sequencing (Figure 27).

### **Generation of pGloSensor<sup>TM</sup>-22F cAMP transgenic plants**

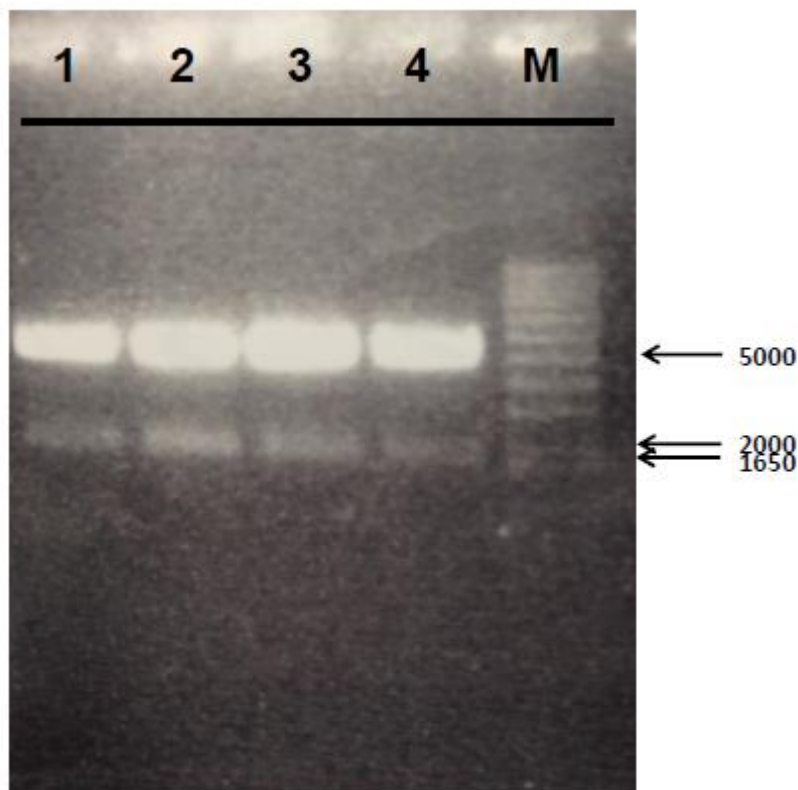
The method used to transform Arabidopsis plants with pGloSensor<sup>TM</sup>-22F cAMP-pK2GW7.0 plasmid was modified from the protocol described by Zhang et al. (2006). The pGloSensor<sup>TM</sup>-22F cAMP- pK2GW7.0 plasmid was extracted from an overnight grown cell culture. 200 ng of the plasmid was then electro-transformed into the competent AgL-1 *Agrobacterium tumefaciens* cells. An empty pK2GW7.0 plasmid was also transformed into another tube of AgL-1 cells as a control. 400 µL was added immediately after transformation and



**Figure 24. Restriction digest with enzyme HindIII from isolated pENTR plasmid after subcloning the pGloSensor™-22F cAMP coding region into gateway entry pENTR plasmid.** Lane 1 and 10 represents 1 kb plus ladder. Lane 2 to 9 represent result of HindIII restriction digest. Restriction digest results shown in lane 4,5 and 7,8 suggest the pGloSensor™-22F cAMP coding region (2.1 kb) is subcloned into the pENTR plasmid (2.6 kb), presenting one band at about 5 kb after linearizing the plasmid containing the pGloSensor™-22F coding sequence using a single restriction enzyme that cuts the plasmid at one site..



**Figure 25. PCR amplification analysis to examine if the pGloSensor™-22F cAMP coding region has been successfully subcloned into pK2GW7.0.** First lane represents the coding region from the original pGloSensor™-22F cAMP plasmid. Second and third lanes represent pK2GW7.0 plasmid isolated after LR reaction. Results suggest plasmid from the second lane contains the pGloSensor™-22F cAMP coding region (fragment slightly above 2 kb). Results in the third lane suggests contamination in the pK2GW7.0 as there is nonspecific priming and PCR amplification. Forth lane represents empty pK2GW7.0 plasmid as a negative control. Fifth lane represents a 1 kb plus ladder.



**Figure 26. Digestion with restriction enzymes BsaI and XhoI to check if the pGloSensor™-22F cAMP coding region has been successfully subcloned into the plant expression plasmid pK2GW7.0.** There are two BsaI sites located in the pK2GW7.0 plasmid, one at 210bp, the other at 6608bp. XhoI is located in the cAMP-glosensor coding region (at 881 bp). Therefore, if the LR reaction is successful, the attL1, attL2 sites from the pENTR plasmid that contains the cAMP-glosensor coding region (2.1 kb) will substitute the attR1, attR2 sites (about 1.7 kb) from the pK2GW7.0 plasmid. Although three cuts were performed, only 2 bands were expected because two of the expected three bands are predicted to have almost the same size. The band shown at 5 kb has much greater expression than the band at 2 kb, indicating that there are more DNA fragments at 5 kb, which is expected.

Score	Expect	Identities	Gaps	Strand
2089 bits(1131)	0.0	1144/1149(99%)	5/1149(0%)	Plus/Minus
Query 2	6TTGTTTCTGTC-GGCCGTAGCCCTGGCGGATGCCTGGTAGGTGGAAGCGTTTGGCC-CG	59		
Sbjct 2072	6TTGTTTCTGTCAGGCCGTAGCCCTGGCGGATGCCTGGTAGGTGGAAGCGTTTGGCCACG	2013		
Query 60	GCCTCACCTACCTCCTTGTCTGAGCGGCGCCCCGCCGCTGGCGATCTCGTGCAAGTTGCTT	119		
Sbjct 2012	GCCTCACCTACCTCCTTGTCTGAGCGGCGCCCCGCCGCTGGCGATCTCGTGCAAGTTGCTT	1953		
Query 120	AGGTCGTAAGTTGTCTGATGAGAGTGCTCTTAGCGAAGAAAGCTAAATAGTGTGGGCACCAAGC	179		
Sbjct 1952	AGGTCGTAAGTTGTCTGATGAGAGTGCTCTTAGCGAAGAAAGCTAAATAGTGTGGGCACCAAGC	1893		
Query 180	AGGGCAGATTGAATCTTATAGTCTTGCAAGCTGCGCAAGAAAGCTCCTCCTCGAAGCGG	239		
Sbjct 1892	AGGGCAGATTGAATCTTATAGTCTTGCAAGCTGCGCAAGAAAGCTCCTCCTCGAAGCGG	1833		
Query 240	TACATGAGCAGCAGCCGAAAGCCGAGATCAAGTAGCCGAGCGTGGTGAACATGCCGAAG	299		
Sbjct 1832	TACATGAGCAGCAGCCGAAAGCCGAGATCAAGTAGCCGAGCGTGGTGAACATGCCGAAG	1773		
Query 300	CCGTGGTGAATGGCACCACGCTGAGGATAGCGGTGTCGGGGATGATCTGGTTGCCGAAG	359		
Sbjct 1772	CCGTGGTGAATGGCACCACGCTGAGGATAGCGGTGTCGGGGATGATCTGGTTGCCGAAG	1713		
Query 360	ATGGGGTCGCGGGCATGACTGAATCGGACACAAGCGGTGCGGTGCGGTAGGGCTACGCCC	419		
Sbjct 1712	ATGGGGTCGCGGGCATGACTGAATCGGACACAAGCGGTGCGGTGCGGTAGGGCTACGCCC	1653		
Query 420	TTGGGCAATCCGGTACTGCCACTACTGTTTCATGATCAGGGCGATGGTTTTGTCCCAGTCG	479		
Sbjct 1652	TTGGGCAATCCGGTACTGCCACTACTGTTTCATGATCAGGGCGATGGTTTTGTCCCAGTCG	1593		
Query 480	AAGCTCTCGGGCAGCAAGTCGTACTCGTTGAAGCCGGGTGGCAATGGGAAGTCACGAAG	539		
Sbjct 1592	AAGCTCTCGGGCAGCAAGTCGTACTCGTTGAAGCCGGGTGGCAATGGGAAGTCACGAAG	1533		
Query 540	GTGTACATGCTTTGGAAGCCCTGGTAGTCGGTCTTGCTATCCATGATGATGATCTTTTGT	599		
Sbjct 1532	GTGTACATGCTTTGGAAGCCCTGGTAGTCGGTCTTGCTATCCATGATGATGATCTTTTGT	1473		
Query 600	ATGATCGGTAGCTTCTTTTGACGTTGAGGATCTTTTGACAGCCCTTTCTTGCTCAGCAAT	659		
Sbjct 1472	ATGATCGGTAGCTTCTTTTGACGTTGAGGATCTTTTGACAGCCCTTTCTTGCTCAGCAAT	1413		
Query 660	ACGACGGTGGGCTGGCTGATGCCCATGCTGTTTCAGCAGCTCGCGCTCGTTGTAGATGTCG	719		
Sbjct 1412	ACGACGGTGGGCTGGCTGATGCCCATGCTGTTTCAGCAGCTCGCGCTCGTTGTAGATGTCG	1353		
Query 720	TTAGCTGGGGCCACAGCCACACCGATGAACAGGGCACCCAAACAGGGCATGAAGAACTGC	779		
Sbjct 1352	TTAGCTGGGGCCACAGCCACACCGATGAACAGGGCACCCAAACAGGGCATGAAGAACTGC	1293		
Query 780	AAGCTATTCTCGCTGCACACCGATCCGATGGTTTGTATTAGGCCCATAGCGCTTCATA	839		
Sbjct 1292	AAGCTATTCTCGCTGCACACCGATCCGATGGTTTGTATTAGGCCCATAGCGCTTCATA	1233		
Query 840	GCTTCTGCCAGCCGAACGCTCATCTCGAAGTACTCGGCGTAGGTAATGTCCACCTCGATA	899		
Sbjct 1232	GCTTCTGCCAGCCGAACGCTCATCTCGAAGTACTCGGCGTAGGTAATGTCCACCTCGATA	1173		
Query 900	TGTGCGTCGGTAAAGGCGATGGTGCCGGGCAACAGGGCGTAGCGCTTCATGGCTTTGTGC	959		
Sbjct 1172	TGTGCGTCGGTAAAGGCGATGGTGCCGGGCAACAGGGCGTAGCGCTTCATGGCTTTGTGC	1113		
Query 960	AGCTGCTCGCCGGCGGTCCCCTCTTCGAGTGGGTAGAAATGGCGCTGGGCCCTTCTTAATG	1019		
Sbjct 1112	AGCTGCTCGCCGGCGGTCCCCTCTTCGAGTGGGTAGAAATGGCGCTGGGCCCTTCTTAATG	1053		

**Figure 27. Sequence analysis of pGloSensor™-22F cAMP coding region in pK2GW7.0 plasmid with reverse primer.** The obtained sequence (query) was aligned with the already known pGloSensor™-22F cAMP coding region sequence (subject). The alignment was done by using Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI) website. The result showed that the nucleotides are almost nearly identical (99%), suggesting that the pK2GW7.0 plasmid sent for sequencing does contain the pGloSensor™-22F cAMP coding region sequence.

recovered in the shaker for 3-4 hours before growing on LB solid plates. The transformed cells were grown on LB medium at 28 °C for 2 days with appropriate antibiotics (100 µg/mL rifampicin, 100 µg/mL Ampicilin and 100 µg/mL Spectromycin). PCR undertaken on selected colonies confirmed that the plasmid could be recovered from competent *E. coli* cells. A single colony is selected and cultured in 5 mL LB liquid media for 2 more days and transferred to a flask containing 50 mL LB liquid media and cultured until O.D. reaches between 0.5-1.0. The cell culture was harvested and resuspended in inoculation buffer (1/2 MS liquid medium, 40 nM 6-benzylamino purine (BAP), 5% (w/v) sucrose, 0.005% Silwet L-77, pH 5.7). The cell culture in the buffer was dipped onto Arabidopsis flower buds. After dipping, transfected plants were covered with plastic wrap in order to maintain the flowers in a moist environment. The plastic wrap was removed 2 d after the transfection. The seeds of the transfected plants were harvested after about 2 weeks. To identify the plants that contain the reconstructed plasmid, Arabidopsis seeds were grown on ½ strength MS medium plates containing 30 µg/mL kanamycin. To separate out homozygous transgenic plants from heterozygous transgenic plants by segregation rate, Arabidopsis seedlings were self-crossed and grown on kanamycin selection ½ strength MS medium plates for 3 generations before performing further experiments.

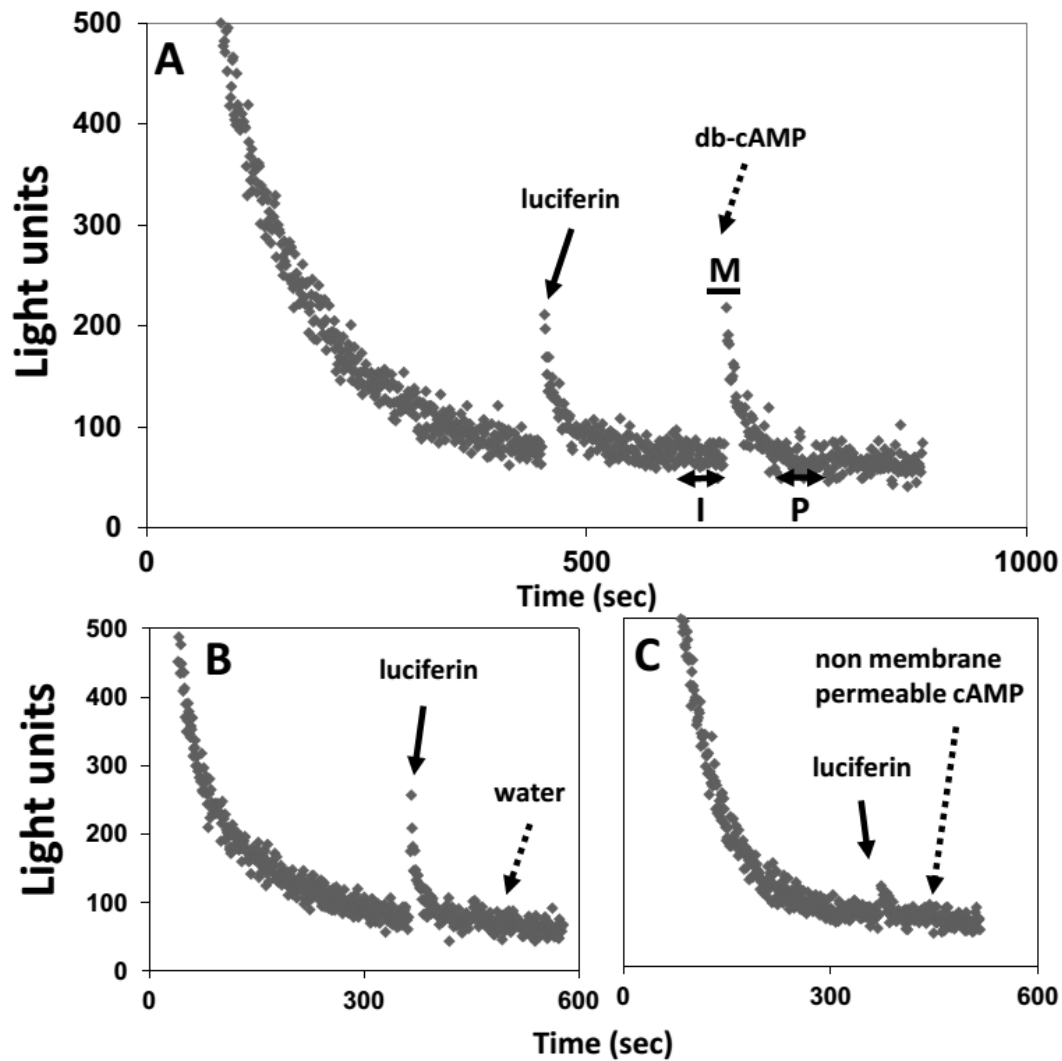
### **Membrane permeable cAMP causes light output increase in the pGloSensor™-22F cAMP transgenic plants**

In order to evaluate cAMP level change with our newly developed biosensor-pGloSensor™-22F cAMP (**GloSensor**) transgenic Arabidopsis plants, we applied membrane permeable dibutyl (db)-cAMP and record the changes of light output directly. The membrane permeable analog of cAMP has been used to evaluate cAMP as a secondary messenger in different signaling pathways in several studies (Ma et al., 2009; Gao et al., 2012; Pietrowska-

Borek and Nuc, 2013; Ordoñez et al., 2014). Figure 28A shows experimental results from such an experiment; transient light emission upon luciferin and db-cAMP addition to the GloSensor transgenic leaves. To analyze our data, we average the last 5 data points (1 data point/sec) before cAMP addition (“I”, initial, Figure 28A) and the 5 data points 40 sec after cAMP addition (i.e., a recording time during which the numbers remain at a steady level) (“P”, post, Figure 28A), and use the mean of these ‘before’ and ‘after’ ligand addition light emission values as a baseline. We subtract this baseline value from the peak value (“M”, maximum, Figure 28A) for each individual replicate for any specific treatment (typically a ligand addition). This subtraction gives us the net change of light output (N). This can be written into a simple equation:  $N = M - (I + P) / 2$ .

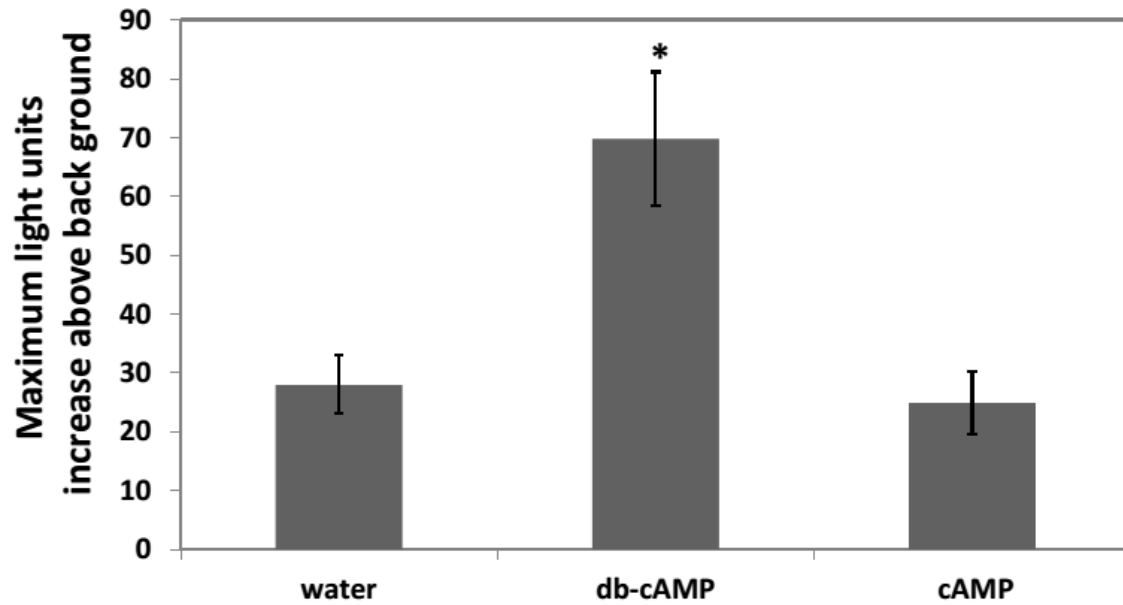
2. Non membrane permeable cAMP and water are used as negative controls in the experiment, and the analyses are done the same as db-cAMP treatment to leaves. The result is represented in Figure 29, and we compare the increase of light output of both non membrane permeable cAMP and (lipid permeable) db-cAMP to leaf discs in water. A student’s T test suggests that when compared to water, db-cAMP treatment shows a significant increase of light output whereas non membrane permeable cAMP does not. In addition, the results shown in Figure 30 suggest that the increased light output caused by db-cAMP is dose responsive. These results indicate that given exogenous db-cAMP, it binds to the cAMP binding site in the GloSensor transgenic Arabidopsis and causes light output increase. Therefore the more light output generated, the more cAMP. However, the results do not reveal whether this biosensor system is sensitive enough to respond to endogenous cAMP level changes in Arabidopsis. Therefore, the next experiment is to measure endogenous cAMP level change.

### **Using forskolin to measure endogenous cAMP level change**

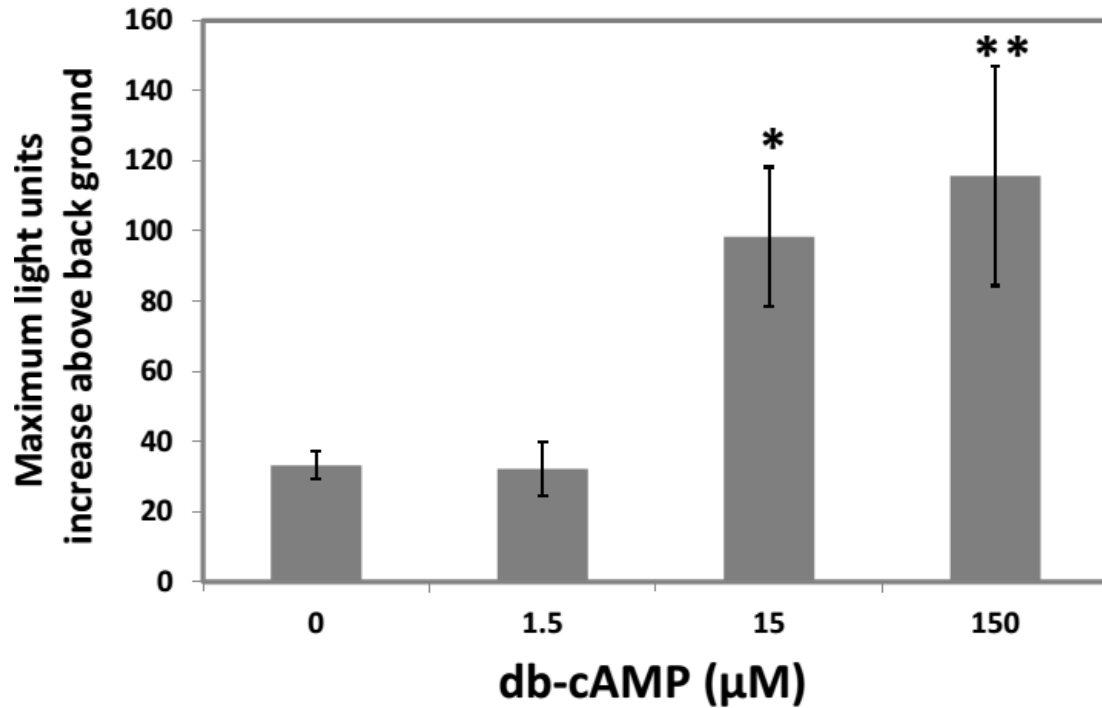


**Figure 28. A representation of raw data showing transient light emission upon luciferin addition and subsequent additions of either db-cAMP, water, or non-membrane permeable cAMP to leaf disks from GloSensor transgenic leaves. In the Figure, the black arrow shows the peak of luciferin addition to GloSensor transgenic leaves; the dashed arrow shows the peak of db-cAMP (A), water (B) and non-membrane permeable cAMP (C) addition to disks of GloSensor transgenic leaves.**



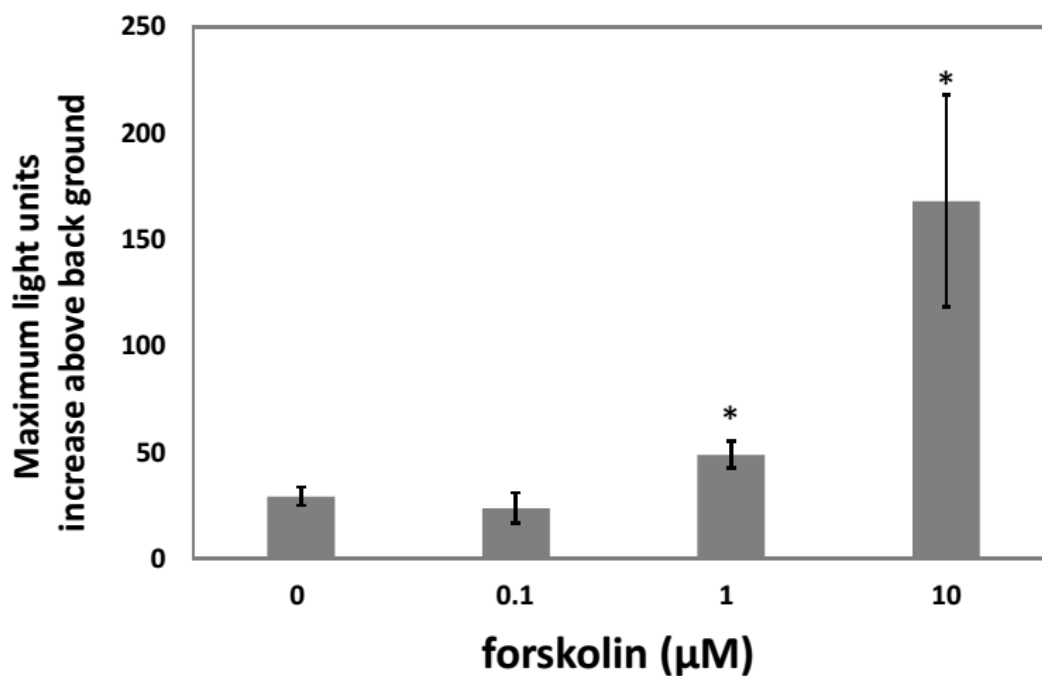


**Figure 29. Membrane permeable cAMP shows a significant increase of light output over baseline whereas non membrane permeable cAMP and water does not.** The relative increase of light output with both membrane permeable db-cAMP and non-membrane permeable cAMP treatments in GloSensor transgenic leaves are compared to water (control). Results are shown as means ( $n = 4$ )  $\pm$  SE. ANOVA analysis is used to evaluate means separation; bars with an asterisk above them indicate the treatment is significantly different (at  $P < 0.01$ ) than the control (water).



**Figure 30. Membrane permeable cAMP shows a dose-dependent response of light output.** The relative increase of light output in GloSensor transgenic leaves is compared to “0” (water control). Results are shown as means ( $n \geq 3$ )  $\pm$  SE. ANOVA analysis is used to evaluate means separation; bars with an asterisk or 2 asterisks above them indicate the treatment is significantly different (at  $P < 0.05$ ;  $P < 0.01$ ) than the control (water)

Forskolin is a natural plant extract from *Plectranthus barbatus* and is used in traditional Indian medicine for the treatment of heart failure, bronchial asthma, and alimentary diseases (Metzger and Lindner, 1981; Ammon and Müller, 1985; Nilani et al., 2009; Alasbahi and Melzig, 2010; Alasbahi and Melzig, 2012). Forskolin has been shown as a direct and rapid activator of adenylyl cyclase (AC) (ExPASy ENZYME entry: EC 4.6.1.1). AC uses ATP as a substrate to generate the enzymatic product cAMP (and diphosphate) in mammalian membranes, broken cells preparation and intact tissues (Seamon et al., 1981; Daly et al., 1982; Green and Clark, 1982; Birnbaumer et al., 1983; Purdy et al., 1991; Alasbahi and Melzig, 2012; Amaro-Ortiz et al., 2014). Forskolin activates adenylyl cyclase and allows conversion of ATP into cAMP. Although the only annotated and experimentally confirmed partial AC sequence in higher plants is a *Zea mays* pollen protein capable of generating cAMP, evidence suggests forskolin increases endogenous cAMP levels in Arabidopsis defense response to *Verticillium* toxins (Jiang et al., 2005). Forskolin has been used to test the effectiveness of the GloSensor as a biosensor for cAMP *in vivo* in animal HEK 293 and Chinese Hamster Ovary (CHO) cells (Binkowski et al., 2011; DeRaddo et al., 2014). To test whether endogenous cAMP level in Arabidopsis is sufficient enough for our newly developed tool to work, we apply 10  $\mu$ M forskolin to GloSensor transgenic Arabidopsis leaves. This level of forskolin is typically used as a test of the biosensor when the GloSensor cAMP reporter protein is expressed in animal (e.g. HEK 293 and CHO) cells. In some animal studies, increasing forskolin concentration up to 100  $\mu$ M resulted in increasing cAMP generation (DiRaddo et al., 2014). For experiments involving forskolin addition to leaves, we use the organic solvent DMSO as a negative control; the level of DMSO in the control and forskolin treatments is identical. Results represented in Figure 31 indicate that forskolin is able to generate a significant light output increase when compared to DMSO control.

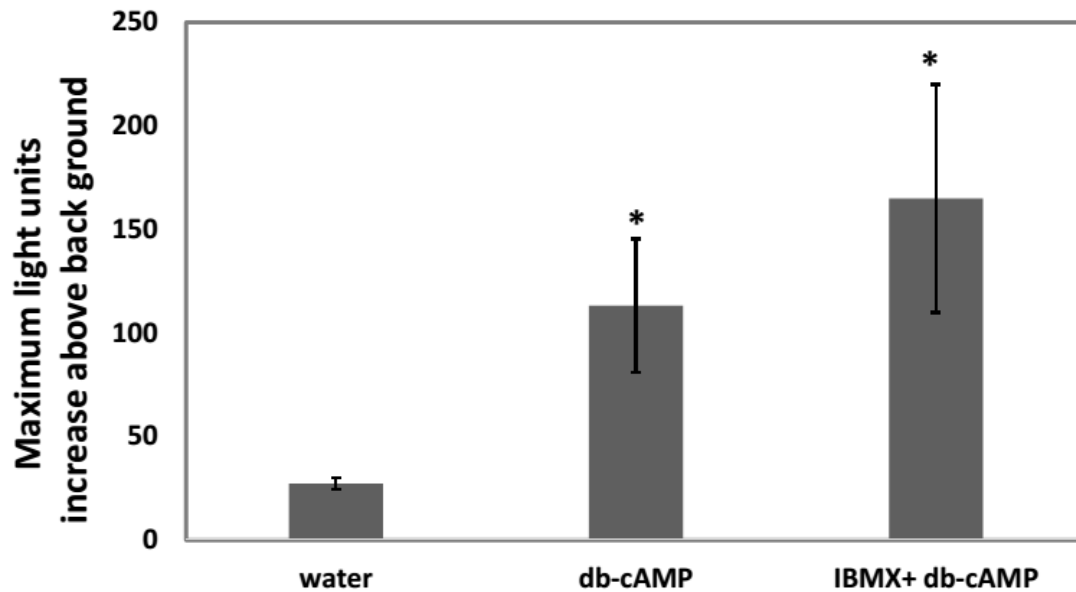


**Figure 31. Forskolin shows a dose-dependent response of light output.** The relative increase of light output in GloSensor transgenic leaves exposed to forskolin is compared to “0” (DMSO control). Results are shown as means ( $n \geq 4$ )  $\pm$  SE. ANOVA analysis is used to evaluate means separation; bars with an asterisk above them indicate the treatment is significantly different (at  $P < 0.05$ ) than the control (DMSO).

We also observed that the effect of forskolin on transgenic plants is dose-dependent. The results suggest that not only does our newly developed transgenic plant respond to exogenous cAMP, but is also sensitive enough to detect endogenous cAMP level changes in transgenic *Arabidopsis* leaves.

### **IBMX and papaverine do not have an effect on GloSensor transgenic plants**

Cyclic nucleotide phosphodiesterase (PDE) hydrolyzes cAMP to AMP, switching off the signal (Robison et al., 1971) that we presume leads to CNGC activation in numerous signaling cascades. PDE activities were first reported in pea seedlings (Lieberman and Kunishi, 1969). We suspect that in plants, there is higher ambient levels of cyclic nucleotide phosphodiesterase than in animals. This higher level of cyclic nucleotide phosphodiesterase present in plants could result in a more rapid cAMP break down during signaling cascades (and perhaps lower ambient levels of this cytosolic secondary messenger), resulting in lower levels of cAMP in plants. However, until now, there have not been many published studies of cyclic nucleotide phosphodiesterase in plants (Genschik et al., 1997; Abel et al., 2000). In order to test whether the transient nature of the db-cAMP-induced light output increase is due to the rapid breakdown of cAMP (Figure 28), a phosphodiesterase inhibitor is added to the lumenometer tube containing a sample leaf prior to any measurement and incubated for 30 min. 3-isobutyl-1-methylxanthine (IBMX) is a nonselective phosphodiesterase inhibitor well known from its use in animal systems (Tsai and Beavo, 2011; Muravyov and Tikhomirova, 2012; Buell et al., 2015); some studies suggest it may also prevent cAMP breakdown in plants (Li et al., 1994; Maurel et al., 1995; Lichter and Mills, 1998; Temkitthawon et al., 2008; Ma et al., 2009; Gao et al., 2012). Results presented in Figure 32 indicate that the GloSensor transgenic plants treated with db-cAMP generate a significantly greater increase of light output when compared with the application of water to the GloSensor



**Figure 32. IBMX does not have a significant effect on increasing cAMP-dependent light generation.** The relative increase of light emission in GloSensor transgenic leaves is compared to water (control). Results are shown as means ( $n \geq 5$ )  $\pm$  SE. ANOVA analysis is used to evaluate means separation; bars with an asterisk above them indicate the treatment is significantly different (at  $P < 0.05$ ) than the control (water).

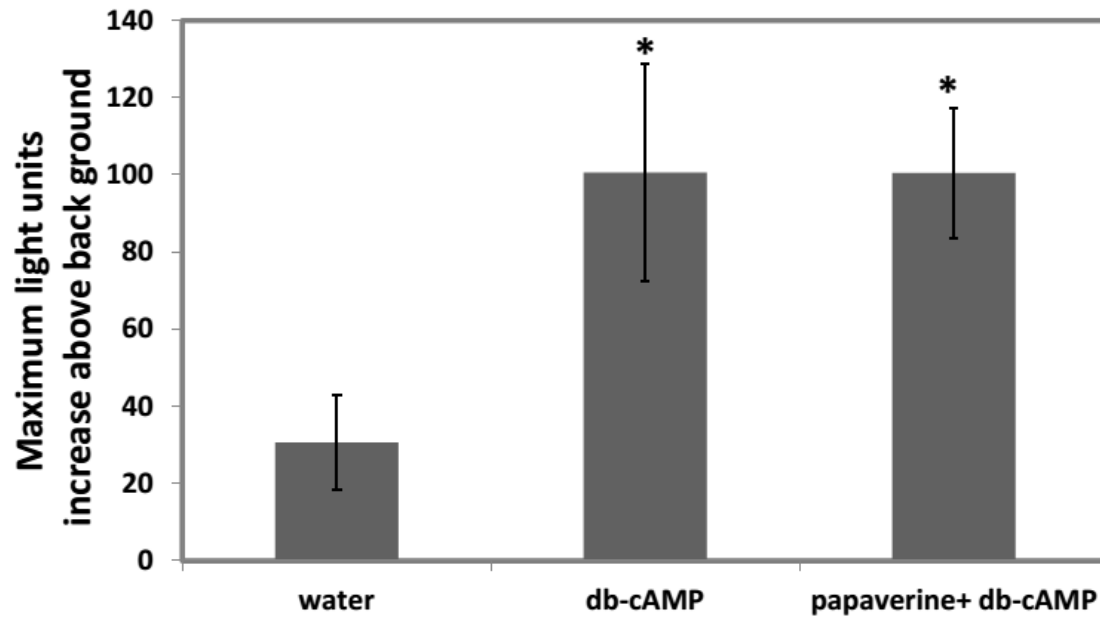
transgenic plants (control). Just like the db-cAMP treatment, transgenic leaves treated with IBMX prior to db-cAMP also show a significant light emission increase when compared with water control. However, when we compare db-cAMP treatment with IBMX addition prior to db-cAMP in the GloSensor transgenic leaves, a Tukey analysis indicates the difference of increased light emission between transgenic leaves pretreated with or without IBMX were not statistically significant. This result indicates that IBMX might not inhibit the phosphodiesterase that breaks down cAMP in the transgenic plant. (These results are also consistent with the possibility that cyclic nucleotide phosphodiesterases are not influencing the responses we are observing of GloSensor leaves to forskolin or db-cAMP). A third possible explanation for this result is that the inhibition of cAMP breakdown might only influence the system at lower levels of the messenger. Since IBMX is known to affect cGMP phosphodiesterases to a greater extent than cAMP phosphodiesterases in animal systems (Soderling and Beavo, 2000), we tried a phosphodiesterase inhibitor with more specificity for cAMP phosphodiesterases to test the hypothesis. Phosphodiesterases (PDE) can be classified into different categories: PDE1, PDE2, PDE3, PDE4, PDE5, PDE7 and PDE 10 (Ahmad et al., 2014). The different classes of PDEs can have more specificity for cAMP or cGMP. Some PDE inhibitors have differential effects on some classes of PDE's more than others. Papaverine is a selective phosphodiesterase 10 inhibitor (Ahmad et al., 2014). The mechanism of papaverine action on cyclic nucleotide phosphodiesterase is not clear, but it is shown that application of papaverine to animal tissue inhibits cyclic nucleotide phosphodiesterase and increases cell cAMP (Pösch and Kukovetz, 1971; Kedia et al., 2012). We incubate the GloSensor transgenic leaves with 100  $\mu$ M papaverine for 30 min before measuring light emission generated by exogenous cAMP. Unfortunately, we do not find a higher number of light output with papaverine treatment prior to cAMP addition. The increase of light output

generated by papaverine addition prior to cAMP treatment is almost the same extent as cAMP treatment to the GloSensor transgenic leaves alone (Figure 33). We acknowledge that these phosphodiesterase inhibitor do not work, in our case. However, we cannot conclude that phosphodiesterase inhibitor will not slow down the pace of cAMP break down in plants. Cyclic nucleotide phosphodiesterase activities are demonstrated in plants (Lieberman and Kunishi, 1969; Lin and Varner, 1972; Ashton and Polya, 1975; Brown et al., 1977; Endress, 1979; Kurosaki and Kaburaki, 1995). However, since there are only a few studies on plant cyclic nucleotide phosphodiesterase, a lot of information still remains unknown. Also, the db-cAMP concentration used in the experiment is 150  $\mu$ M, which is relatively high. Therefore, even the phosphodiesterase inhibitors were able to slow down the pace of cAMP break down in GloSensor transgenic leaves, we might still not have found an effect because of the high db-cAMP concentration (150  $\mu$ M) used in these experiments. We lowered the db-cAMP concentration added to transgenic leaves (1.5  $\mu$ M and 15  $\mu$ M, data not shown), but we did not see a significant effect of IBMX preventing cAMP break down, and as a result, generating more light output. It is unclear why IBMX did not work in the GloSensor transgenic plants, as we were unable to observe more light output when transgenic leaves were pretreated with IBMX before db-cAMP addition.

#### **AC inhibitor has an effect on preventing forskolin to work in GloSensor transgenic Arabidopsis**

2',3'-Dideoxyadenosine (ddA) is a specific adenylyl cyclase (AC) inhibitor and has been used to study cAMP activated signaling in Arabidopsis (Jiang et al., 2005; Kwaaitaal et al., 2011). In order to study whether blocking endogenous cAMP generation will affect the cAMP-dependent biosensor signal (i.e. generation of light output in leaves of plants expressing pGloSensor<sup>TM</sup>-22F), we pretreated leaves with 1 mM ddA for 1 h prior to giving forskolin to the



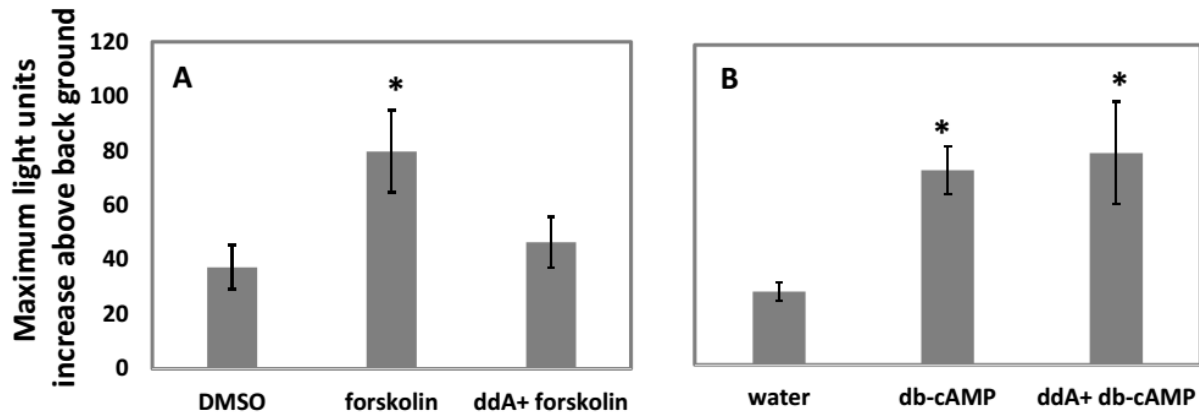


**Figure 33. Papaverine does not have a significant effect on increasing cAMP-dependent light generation.** The relative increase of light output in GloSensor transgenic leaves is compared to water (control). Results are shown as means ( $n \geq 5$ )  $\pm$  SE. ANOVA analysis is used to evaluate means separation; bars with an asterisk above them indicate the treatment is significantly different (at  $P < 0.05$ ) than the control (water).

leaf. We observed that the forskolin-dependent signal is affected by pretreatment of leaves with the AC inhibitor ddA. (Figure 34A). Our result indicates that ddA has an effect on inhibiting forskolin as we no longer see a significant light output increase when we compare transgenic leaves applied with forskolin and ddA pretreatment with DMSO control. A Tukey analysis also suggests that there is significant difference between Arabidopsis pretreated with or without ddA before addition of forskolin. To our expectation, ddA does not inhibit exogenous light generated by GloSensor leaves in response to db-cAMP addition (Figure 34B). This suggests that ddA only has an effect on inhibiting endogenous cAMP generation, but has no effect on cAMP-dependent GloSensor light emission when the biosensor is activated by supplying exogenous (lipophilic) cAMP. Since ddA is a specific adenylyl cyclase inhibitor, we suspect that there can either be more ACs or ACs that function differently than animal ACs in Arabidopsis plants.

#### **ATP, $Mg^{2+}$ and forskolin together do not generate more light output in GloSensor transgenic Arabidopsis**

Since it is known that ATP is a substrate for cAMP generation (Helmreich et al., 1976), we hypothesize that increasing the level of ATP in the cytosol of the leaf cells could increase the level of cAMP generation. In order to test the hypothesis, we give external ATP and  $Mg^{2+}$  (2 mM and 1 mM final concentration, respectively) to GloSensor transgenic Arabidopsis leaves. From the result presented in Figure 35, we do not see a significant increase when we compare ATP (with  $Mg^{2+}$ ) treatment to control on transgenic leaves. We are able to observe a higher light output number when we compare ATP,  $Mg^{2+}$  and forskolin added together with control in the transgenic leaf. However, a Tuckey analysis showing comparison between ATP,  $Mg^{2+}$  and forskolin added together and forskolin added alone in transgenic leaves show no significant light output difference. These results indicate that exogenous ATP and  $Mg^{2+}$  alone may not be



**Figure 34. AC inhibitor affects endogenous cAMP-increased light output but not exogenous cAMP-increased light output in GloSensor transgenic Arabidopsis.** The relative increase of light output in GloSensor transgenic leaves is compared to DMSO (A) or water (B) as they are the control for the treatments. (A) and (B) are experiments done at the same time. Results are shown as means ( $n \geq 5$ )  $\pm$  SE. ANOVA analysis is used to evaluate means separation; bars with an asterisk above them indicate the treatment is significantly different (at  $P < 0.05$ ) from the control treatment.

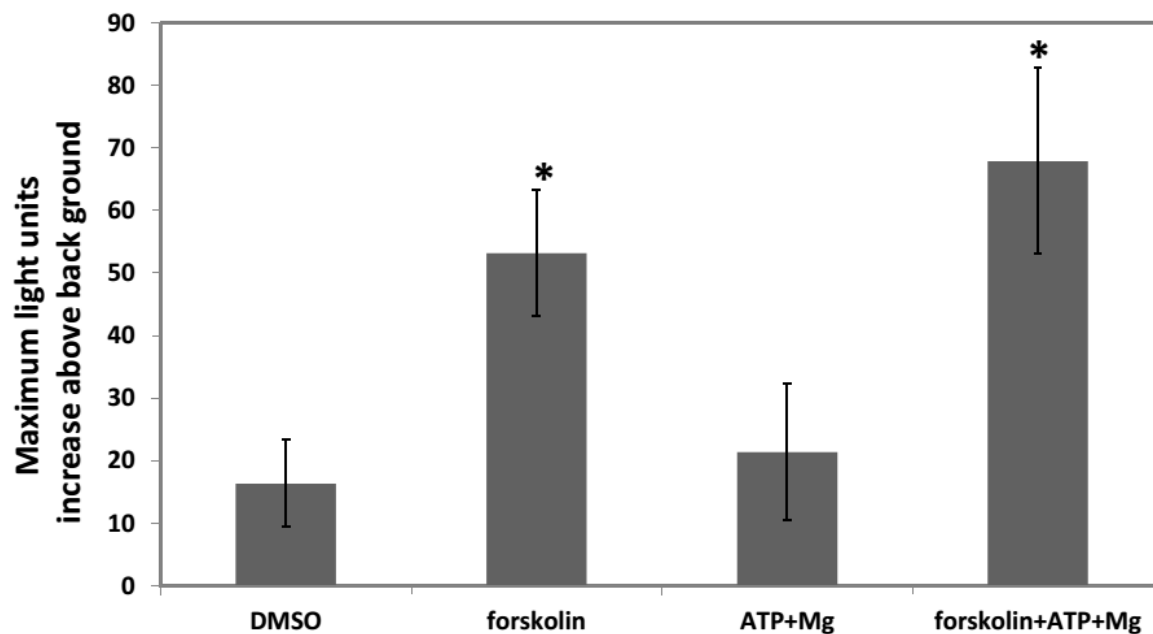
sufficient for cAMP generation in Arabidopsis transgenic leaves. The results also indicate that enzymatic activity (e.g. adenylyl cyclase) may not be limited by substrate (ATP) concentration.

### **Pst DC3000 has an effect on the GloSensor transgenic leaves**

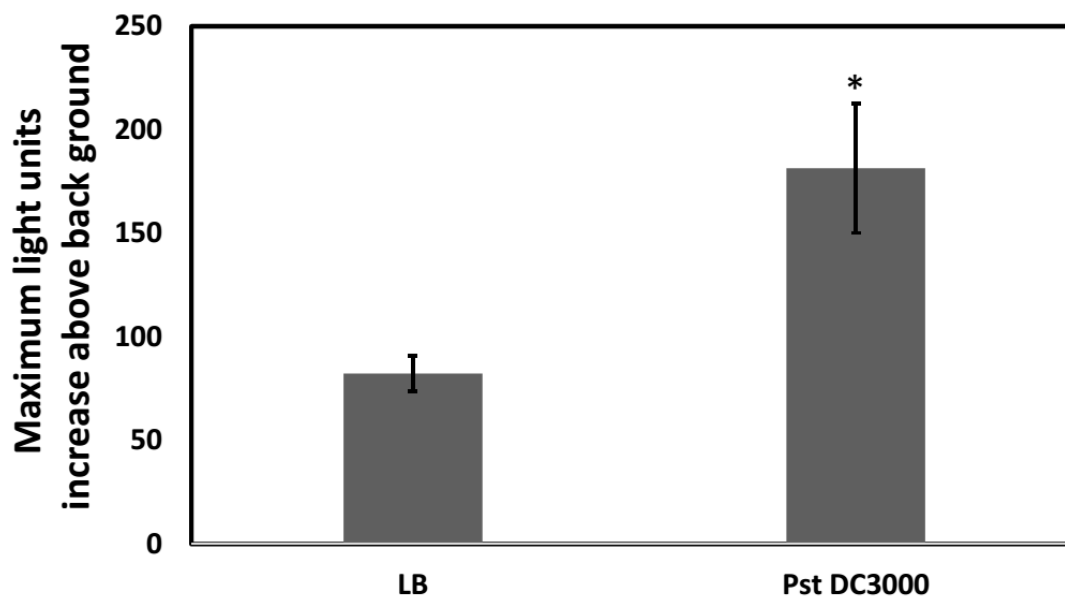
Studies have shown that cNMPs may act in plant immune signaling pathways (Ma et al., 2009; Ordoñez et al., 2014). Ma et al. (2009) showed ELISA (Enzyme-Linked Immunosorbent Assay) results that suggest pathogen inoculation to Arabidopsis leaves can induce cAMP level increase. Furthermore, Ma et al. (2009) linked cytosolic cAMP elevation to  $\text{Ca}^{2+}$ -dependent pathogen response signaling and hypersensitive response (HR) in Arabidopsis plant. Here, we find an effect of *Pseudomonas syringae* pv tomato (Pst) DC3000 on the GloSensor transgenic leaves. The result in Figure 36 suggests Pst DC3000 application induces endogenous cAMP level increase, therefore generating more light output than a control treatment. The result is consistent with Ma et al. (2009) and indicates the GloSensor transgenic plants work under conditions where the reporter protein is responding to cAMP changes that occur in the plant cell during signaling cascades that are thought to involve changes in cAMP. The reporter protein, therefore, is demonstrated to be capable of monitoring not just an increase in cytosolic cAMP (i.e., in response to db-cAMP addition) (Figs. 29 and 30), or an increase in cytosolic cAMP that occurs due to the activation of endogenous systems that generate cytosolic cAMP (i.e., in response to forskolin) (Fig. 31), but also is sensitive enough to respond to natural elevations in cAMP that occur during plant cell signal transduction cascades..

## **Discussion**

Adenylyl cyclase (AC) activity in animal cells has been well known to play important roles in signal transduction pathways, and this has led plant scientists to speculate that plants



**Figure 35. ATP,  $Mg^{2+}$  and forskolin together do not generate more light output.** The relative increase of light output in GloSensor transgenic leaves is compared to DMSO (control). Results are shown as means ( $n \geq 3$ )  $\pm$  SE. ANOVA analysis is used to evaluate means separation; bars with an asterisk above them indicate the treatment is significantly different (at  $P < 0.05$ ) from the control (DMSO) treatment.



**Figure 36. Pst DC3000 has an effect on the GloSensor transgenic leaves.** The relative increase of light output in GloSensor transgenic leaves upon addition of an aliquot of an overnight liquid culture of *Pst* DC3000 is compared to the light generation occurring when LB liquid medium alone is added to discs of GloSensor leaves (control). Results are shown as means ( $n = 6$ )  $\pm$  SE. ANOVA analysis is used to evaluate means separation; bars with an asterisk above them indicate the treatment is significantly different (at  $P < 0.01$ ) from the control (LB) treatment.

may possibly have the same, or similar signaling system. Unfortunately until now there has been no canonical ACs identified in plant genomes that have been fully sequenced. However, published data still suggest plants have a functional cAMP-dependent signaling pathway system. cAMP activity measurement in plants were mostly done by *in vitro* assays e.g. ELISA. The competitive ELISA assay is not an ideal assay because it shows inverse relationship between the signal obtained and the concentration of the target. The competitive displacement of an enzyme-linked cAMP molecule of the ELISA assay yields negative signals. Also, the ELISA assay requires cells or tissues to be disrupted for cAMP quantification. Rather than measuring localized cAMP changes, the ELISA measures cAMP changes throughout the whole cell. Therefore, localized cAMP changes which could have been high may be averaged out after measuring signaling changes throughout the whole cell. Moreover, the ELISA assay is highly sensitive and has several drawbacks, such as edge effects and cross contamination of samples (Corey, 2008). An alternative method for cAMP measurement will be mass spectrometry. However, in order to measure cAMP changes in plant tissue that occur during a signaling cascade using mass spectrometry analysis, samples also need to be extracted from tissues. It is not possible to measure localized cAMP elevation through mass spectrometry. Thus, it will be helpful if we develop a biological tool that can detect cAMP level change in response to stimulus *in vivo* in plants.

The GloSensor plasmid developed by Promega was designed to screen for new drugs that target G-protein coupled, seven-transmembrane (7-TM) receptors (GPCRs) in animal cells to identify ligand effects on GPCRs. Adenylyl cyclases (ACs) are activated by GPCRs, and therefore the cAMP level changes will indicate which drug affects the GPCR signal transduction pathway. Because it is a luciferase reporter protein (the RII $\beta$ B cAMP binding domain from

protein kinase A coding region fused to a mutated luciferase), it is highly sensitive and can work at a wide dynamic range (Binkowski et al., 2009; Allard and Kopish, 2008). Luciferase can be used effectively at much lower expression levels than fluorescent proteins, thereby minimizing their potential influence on cellular physiology. Moreover, the GloSensor method measures cAMP production in real time in a non-invasive manner. Thus it is to our advantage to utilize the GloSensor plasmid developed by Promega by subcloning the cAMP binding protein coding region along with the luciferase coding sequence into appropriate plant (and protoplast) expression plasmids and generate Arabidopsis transgenic plants.

The pGloSensor™-22F cAMP plasmid expressed in HEK cells can show up to 500-fold changes in light output in response to stimulus. However, the transgenic plants that continuously express the coding region of pGloSensor™-22F cAMP plasmid showed much less fold change in light emission dependent upon either db-cAMP (final conc. 150  $\mu$ M) or forskolin (final conc. 10  $\mu$ M) addition. This anomaly requires some speculative explanation. We could explain this based on the model that plants have more cyclic nucleotide phosphodiesterase activity than animal cells, causing the rapid breakdown of cAMP into AMP. However, we did not see a significant effect of PDE inhibitor (IBMX and papaverine) pretreatment on cAMP-dependent light emission in the GloSensor transgenic Arabidopsis leaves. Perhaps these two inhibitors were not the best choice to affect PDE activity since IBMX is a nonselective phosphodiesterase inhibitor (and has been used to specifically block cGMP breakdown more commonly than cAMP breakdown), and papaverine is not described to work in the plant system. Nevertheless, we do not exclude the possibility that plant phosphodiesterase is the reason why the light output is only temporary. The different levels and duration of light output change we observed from the GloSensor transgenic plant suggests that in plants, cAMP levels are much less than in animals and that this may be due



to plant cyclic nucleotide phosphodiesterase activity. From our results, we are able to observe a dose dependent response to exogenous cAMP in our assay system with leaf disks of transgenic plants. The concentration we used to observe a cAMP-dependent light output response may seem rather high compared to the physiological cAMP levels in plant cells from information in prior publications (Gilman 1970; Rosenberg et al., 1982; Kurosaki and Nishi, 1993; Cooke et al., 1994; Witters et al., 1996; Witters et al., 1997; Witters et al., 1999; Jiang et al., 2005; Ma et al, 2009; Lomovatskaya et al., 2011). However, we cannot over look that in the cytoplasm there are cAMP-activated CNGC channels, possibly physically associated with receptors that are involved in activating the CNGCs during signaling cascades. These receptors may generate a relatively high concentration of localized cAMP for activation of the channels to initiate a signaling cascade in the plant cell cytosol (Taskên and Stokka, 2006; Dai et al., 2009; Kunzelmann and Mehta, 2013; Nicol and Gaspar, 2014; Schmitz et al., 2014). Perhaps there is a chance that the cAMP concentration required for activating these receptors can go as high to the micromolar level. Volotovskii et al. (1998) were able to see a cytosolic  $\text{Ca}^{2+}$  spike when they gave 10  $\mu\text{M}$  db-cAMP to *Nicotiana plumbaginifolia* protoplasts. In our db-cAMP titration results of evaluating GloSensor transgenic leaf light emission, we are able to see a significant increase of light emission when we apply 15  $\mu\text{M}$  db-cAMP to transgenic leaves (Figure 30). This suggests that our data does have physiological significance meaning.

We show in our result that Pst DC3000 induces cAMP level changes by having an effect on the GloSensor transgenic leaves (Figure 36). This suggests the GloSensor transgenic *Arabidopsis* is biosensor sensitive enough to respond to cAMP changes that occur during plant cell signal transduction cascades. The light output increase over background by Pst DC3000 application to GloSensor transgenic leaves shows almost the same increase as application of 150

$\mu\text{M}$  db-cAMP (Figure 30). Ma et al. (2009) linked cytosolic cAMP elevation to  $\text{Ca}^{2+}$ -dependent pathogen response signaling and HR in Arabidopsis plant. The GloSensor transgenic plant not only can be used as an ideal tool to measure pathogen-induced cAMP level changes but also indicate the receptors involved may generate a relatively high concentration of localized cAMP for activation of the channels to initiate a signaling cascade in the plant cell cytosol (Taskên and Stokka, 2006; Dai et al., 2009; Kunzelmann and Mehta, 2013; Nicol and Gaspar, 2014; Schmitz et al., 2014).

We have tested the GloSensor transgenic plants under conditions where it is responding to cAMP changes that occur in plant cell signaling cascades involving cyclic nucleotides (Pst DC3000-induced cAMP level changes). However, more experiments need to be done to fully understand how sensitive our biosensor is and whether or not the biosensor will respond to cAMP changes that occur during other plant cell signal transduction cascades, i.e. cAMP level changes in pollen (Moutinho et al., 2001). Continuation of this project is beyond the scope of my dissertation research.

## **Materials and Methods**

### **Plants material**

All transgenic Arabidopsis used in the reported work are in the Columbia (Col) background. Arabidopsis seeds were surface-sterilized by first washing the seeds in 70% (v/v) ethanol, 20% (v/v) bleach and 0.02% (v/v) Triton X-100, shaking at 300 rpm for 10 min, and then rinsing with 95% (v/v) ethanol 3 to 4 times.

For all measurements, seeds were planted on  $\frac{1}{2}$  strength Murashige and Skoog (MS) medium (Caisson Labs, Logan, UT) supplemented with 1% (w/v) suc and solidified with 1%

(w/v) agar. Seeds were stratified (4 °C) for 2 to 3 d after plating to break dormancy. Seedlings were grown on ½ MS plates for 10 d at 16 h light/ ( $\sim 100 \text{ mol m}^{-2} \text{ s}^{-1}$ )/8 h dark and 25 °C and then transferred to artificial potting mix.

### **cAMP level measurement**

Leaves of four to six week old GloSensor transgenic Arabidopsis plants are used to evaluate cAMP level change. Leaves are cut with a hole punch (#4, 7 mm diameter) to ensure that all leaf samples used for assays had the same size. Afterwards, leaf disks are incubated in a tube (1 disk/tube) containing 400  $\mu\text{L}$  water (for between 0 and  $\sim 10$  min), and then put into the luminometer (TD-20/20; Turner Designs) to record initial readings. Lights are turned off before putting samples into the luminometer to prevent any interference. When the recorded light emission numbers remained at a steady level (in between 50-70), 2X luciferin (final concentration 10  $\mu\text{M}$ ) in water is added to the tube and the numbers are recorded immediately. A small luminescence peak showing an average of 2-fold change occurred for several seconds after addition of luciferin. We have found that unlike animal cells where samples need to be incubated with luciferin for 2 h before measuring light activity, the GloSensor transgenic plants can use up luciferin after 2 h, and we no longer see an effect of db-cAMP on the transgenic plants (results not shown). After the light output numbers returned to a low, steady-state level, we applied ligands (i.e. membrane permeable dibutyl (db)-cAMP (Sigma-Aldrich) (final concentration 150  $\mu\text{M}$ ), and record the changes of light output directly. Each time 2 leaf discs are prepared at the same time, one directly put into the luminometer, while the other is left on the bench. The order of samples recorded does not affect the light emission results.

Unless indicated, all treatments of db-cAMP (Sigma-Aldrich,) and cAMP (Sigma-Aldrich, A3262) are done at 150  $\mu\text{M}$ ; forskolin (Fisher scientific, BP2520) treatment is used at

10  $\mu$ M to transgenic leaves. Except for forskolin treatment, which its control leaf discs are treated with 0.05% (v/v) DMSO, all other treatments are compared with leaf discs in water. ATP (Sigma-Aldrich, A2383) and  $MgCl_2$  (Sigma-Aldrich, M8266) are given at 2 mM and 1 mM final concentration, respectively to GloSensor transgenic Arabidopsis leaves. For experiments involving addition of inhibitors, IBMX (Santa Cruz Biotechnology, SC-201188, 1 mM), papaverine (Sigma-Aldrich P3510, 100  $\mu$ M) or ddA (Sigma-Aldrich, D1285, 1 mM) were added to the tube containing a leaf disk at room temp. 30 min (ddA was added for 1 h) prior to addition of luciferin and forskolin or db-cAMP.

### **Growth of *Pseudomonas syringae***

*P. syringae* pv. tomato 'DC3000' (Pst DC3000) was grown at 28 °C on low-salt (1 g/L NaCl) Luria-Bertani (LB) (Fisher Scientific; Pittsburg, PA) 2% (w/v) agar medium containing 100 mg/L rifampicin for about 2 d. Colonies from these plates were used for liquid overnight cultures (medium composition was the same as the plates except no agar was used). Bacteria were grown to  $10^9$  colony-forming units/ mL. We apply 200  $\mu$ L of bacteria to the tube to measure cAMP-induced light emission change.

## Chapter 4: Identification of an adenylyl cyclase gene in *Arabidopsis* using the *cyaA* *E.coli* mutant as a tool for screening

### Introduction

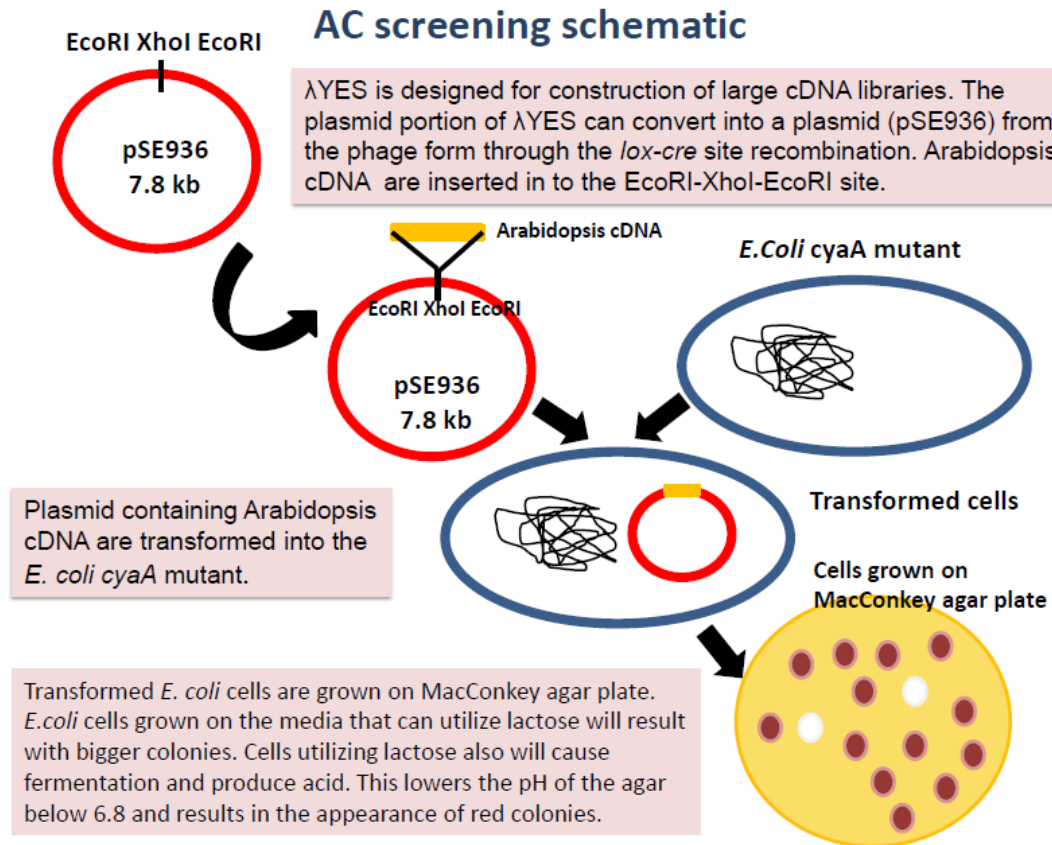
cAMP is known as a secondary messenger involved in signal transduction in both animal and lower eukaryotes. It has been established that adenylyl cyclase in animal cells catalyzes the conversion of ATP into cAMP and pyrophosphate. cAMP activates protein kinase A (PKA) and further affects many different physiological and biochemical processes, such as regulating glycogen, sugar and lipid metabolism. cAMP levels appeared to be much lower in plants compared to the level found in animals (Gehring, 2010). Biochemical evidence suggests plants show biological responses such as increasing  $\text{Ca}^{2+}$  influx across the plasma membrane by cell permeant 8-Br-cAMP and forskolin (Kurosaki and Nishi, 1993; Kurosaki et al., 1993). Forskolin activates adenylyl cyclase and increases intracellular levels of cAMP in animals ((Seamon et al., 1981; Daly et al., 1982; Green and Clark, 1982; Birnbaumer et al., 1983; Purdy et al., 1991)). This indicates that regardless of the low cAMP level, this cyclic nucleotide may still be involved in signal transduction in plants. However, up until now there has been no canonical AC identified in plants that functions correspondingly to animal adenylyl cyclase (Gehring, 2010). So far, there is only a partial gene sequence encoding a pollen tissue specific signaling protein PSiP in *Zea mays* that is considered to be a soluble AC (Moutinho et al., 2001). The partial gene sequence encoding PSiP is considered to be a soluble AC because of its competence for production of cAMP when expressed in *Escherichia coli* (*E. coli*)(Moutinho et al., 2001).

Bacteria *E.coli* SP850 was characterized to have a total deletion of the adenylyl cyclase gene (*cyaA*) (Shah and Peterkofsky, 1991). The difference between wild type *E.coli* and the

*cyaA* mutant is its utilization of lactose when grown on MacConkey agar plates. *E.coli* SP850 cannot undergo lactose fermentation due to its *cyaA* mutant (Cotta et al., 1998). When the *cyaA* mutant is expressed with a heterologous system which encodes a putative AC gene, the AC gene will complement the *cyaA* mutant and allow strain SP850 to undergo lactose fermentation. Therefore, the colonies grown on MacConkey agar plates will result like wild type *E.coli*. Here, we use a method based on screening Arabidopsis cDNA library in *E.coli* SP850 to look for putative adenylyl cyclase gene in Arabidopsis

The cAMP/ Catabolite activator protein (CAP) feed-back mechanism is influenced by glucose and lactose. At low levels of glucose and in the presence of lactose, cAMP levels are high. High levels of cAMP are more readily able to interact with CAP and together they bind to the Lac operon. CAP facilitates the binding of RNA polymerase to the Lac operon to transcribe the  $\beta$ -galactosidase gene. The MacConkey medium contains lactose and a neutral red dye. *E.coli* cells grown on this medium that can utilize lactose will result with bigger colonies. Cells utilizing lactose also will cause fermentation and produce acid. This lowers the pH of the agar below 6.8 and results in the appearance of red colonies. *E.coli* SP850 is a *cyaA* deletion mutant and thus lacks both adenylyl cyclase and cAMP. Due to the *cyaA* mutation, cAMP cannot be synthesized, strain SP850 cannot utilize lactose and will not cause fermentation. Without fermentation, the pH in the media will not change and will produce white color colonies on MacConkey lactose agar. Therefore, screening for Arabidopsis cDNA library that can complement *E.coli* SP850 and produce red colonies will be the goal. This tool has been utilized to demonstrate that the *Prevotella ruminicola D31d* gene encodes an adenylyl cyclase (Cotta et al., 1998).

$\lambda$ YES is designed for construction of large cDNA libraries (Elledge et al., 1991). The plasmid portion of  $\lambda$ YES can be converted into a plasmid (pSE936) from the phage form through the lox-cre recombination site. Fragments of Arabidopsis cDNA are inserted into the EcoRI-XhoI-EcoRI site (Elledge et al., 1991). Plasmid containing Arabidopsis cDNA is then transformed into the *E. coli* SP850 *cyaA* mutant to screen for a putative adenylyl cyclase gene on MacConkey lactose agar media. Figure 37 is a schematic representing the procedure used to screen for potential Arabidopsis adenylyl cyclase.

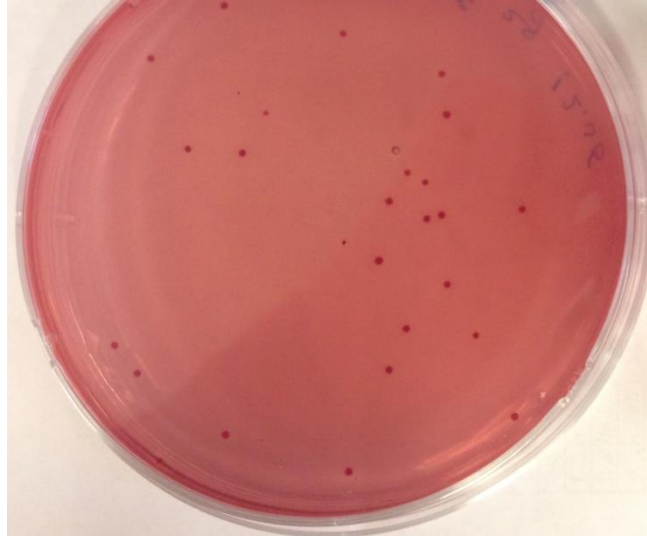


**Figure 37. A schematic representing the procedure used to screen for potential Arabidopsis adenyl cyclase.** The red circle represents pSE936 plasmid. The orange fragment represents Arabidopsis cDNA. The blue ovule represents *E. coli* SP850 which contains the *cyaA* mutant. Transformed cells grown on MacConkey agar plate (orange circle) that can utilize lactose will result in red colonies. Transformed cells that contain a potential AC fragment will complement the *cyaA* mutant, and will not undergo lactose fermentation. The cells will result in white colonies.

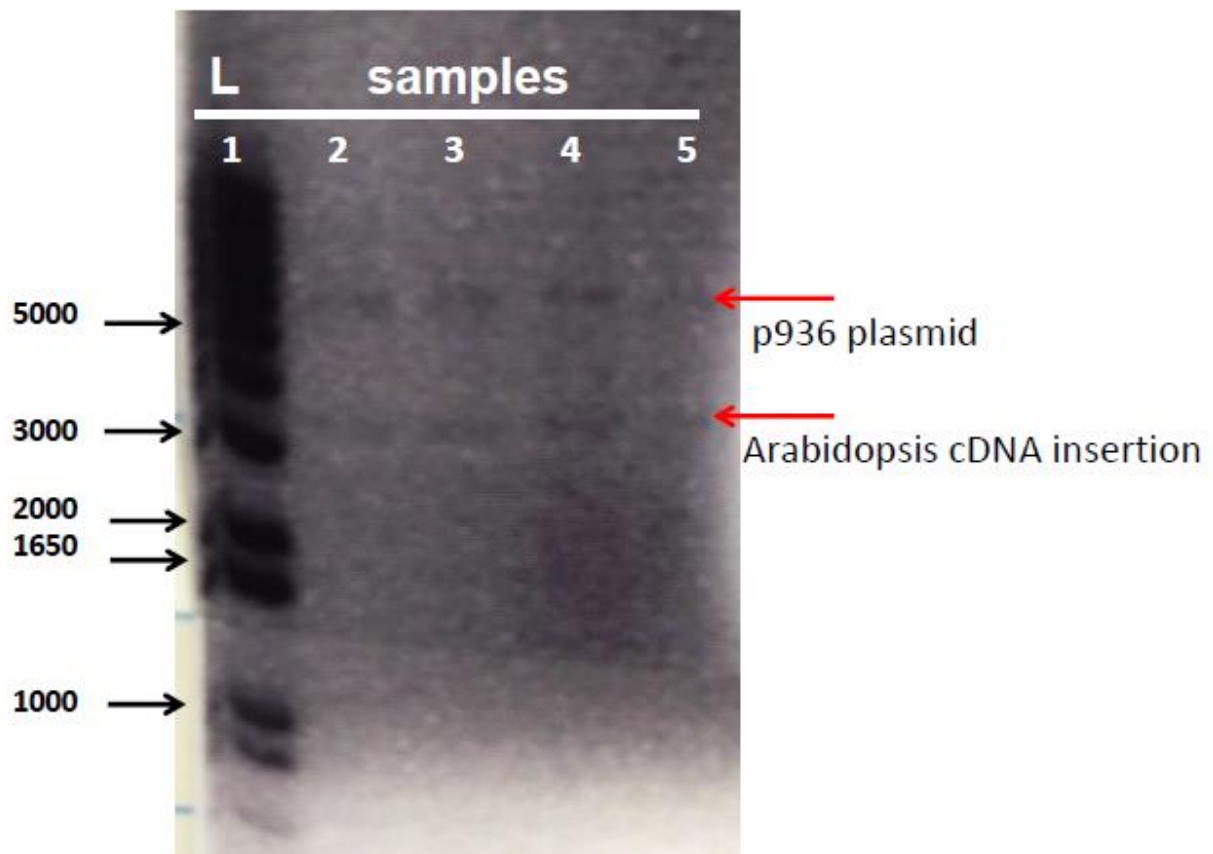


## Results

cAMP plays an important role in lactose utilization in *E.coli* cells. The *cyaA* mutant we use here as a host cell for experiment lacks a functional AC, therefore no cAMP will be synthesized. This will result in the inability of *E.coli* to undertake lactose fermentation. Cells that can't utilize lactose will appear in white colors instead of red on MacConkey plates. Throughout the screening, three colonies have been found on the MacConkey lactose agar media with a deep red color, suggesting lactose fermentation and complementation of the adenylyl cyclase deletion. In order to confirm the complementation was not false positive, all three colonies with the putative adenylyl cyclase gene from Arabidopsis cDNA insert were re-streaked on MacConkey lactose agar media as shown in Figure 38. The colonies were also cultured and pSE936 plasmid was isolated. A 4.3 kb cDNA insert was found in all three plasmids by using EcoRI endonuclease for restriction digest (Figure 39). All three pSE936 plasmids were sequenced to identify putative adenylyl cyclase gene from the cDNA insert. The p936 plasmid sequence approximately 1kb from the insertion site (EcoRI-XhoI-EcoRI) is modified from pSE937 plasmid. Since only the pSE937 full sequence is available, we started sequencing from position 6857 of the p936 plasmid (full length approximately 8 kb) and into the insertion site. Complete sequence of full length cDNA insertion result in a size of about 4.3 kb (Figure 40), all three candidates show the same sequence and it is not an Arabidopsis gene. By searching the sequence from the nucleotide database, result shows that the cDNA fragment is a gene from *Salmonella enterica* (*S. enterica*) subspecies. This result could be explained by contamination during the time the Arabidopsis cDNA library was generated.



**Figure 38. pSE936 containing a putative Arabidopsis adenylyl cyclase is transformed back into E.coli SP850 cyaA mutant to confirm the complementation assay.** After the red colony was selected from the screening plate, the cell was cultured in Lysogeny broth (LB) full name liquid medium (containing 50  $\mu\text{g/ml}$  kanamycin and 100  $\mu\text{g/ml}$  ampicillin) overnight at 37  $^{\circ}\text{C}$ . The cell culture was restreaked onto the fresh MacConky plate for confirmation. The colonies that can ferment lactose appear as red on the MacConkey plate.



**Figure 39. Restriction digest to confirm the size of the Arabidopsis cDNA insertion in p936 plasmid.** Restriction digest was performed with EcoRI restriction enzyme. Result shows 2 bands, one slightly above 3kb represents the Arabidopsis cDNA insertion; the other between 7 and 8 kb represents the p936 plasmid. Lane 1 is a 1 kb+ ladder (L). Lanes 2 to 5 are samples.

ECORI **GAATTC**GGCCGAATTCCGGACACGAGCTCCTGCATCGCCTGCAGATAAAAAATCCTCTCCCGGACCGCTACGGCGCGCGGGCA  
GTGTTATCACCAGCGCATCGACATCCAGCAGAGCGTCAAGATCGTCCGCTCAGACACCAACTCCGGCTCCAGACGACGCGGATAG  
CTTTCAATTCACCTCATACGGGCGCGTTCACACCCCGCTCAAGGGTGGTTTTGCTGCCGGTAACCTGCCAGCCCTCTCGCTGCCA  
GCGACATTTGCCAGCGGCATGCTTAACCAACCTAAACCGACAATTGCGACCTTTTTTCATCCATTTTCTCTGACTTTAACGCCTTAT  
ACATAAGGCTACGCCAGCCGCGTGACTGACCAATTACATAATAATCGAAATGAAAAAGCGCTTGTCTTTAAGCGTAAAAAG  
GTTTAGGTTAAAGGTATCAAAATGAATAGCAATTCATCGGAATTTTTATGACACGGGTTCAATTTAAACACCACCATCATACCA  
TCATCCTGACTAGTCTTTTACGGCGATGTGTGCTGGAAGACATTAGATCTTCCAGTGGTGCATGAACGCATGAGAAAGCCCCCGG  
AAGATCATCTTCCGGGGGCTTTTTTTTTTTTGGCGCGGATACAGACCCGGTTCAGACAGGATAAAGAGGAACGCAGAATGTTAGAC  
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ATTAGCCTTGAATACATTACTGGTAAGGTAAACGCCATTGTGACAAATTGATCCAAGAGAACCAACTTAAAGCTTCACGCTGCCG  
CAAGCACTCAGGGCGCAAGGCTGCTAAAGGGAAGCGGAACACGTAGAAAGCCAGTCCGCAGAAACGGTGCTGACCCCGGATGAA  
TGTCAGCTACTGGGCTATCTGGACAAGGGAACGCAAGCGCAAAGAGAAAAGCAGGTAGCTTGCAGTGGGCTTACATGGCGATAGC  
TAGACTGGGCGGTTTTATGGACAGCAAGCGAATCGGAATTCGCCAGTGGGGCGCCCTCTGGTAAGTTGGGAAGCCCTGCAAAATG  
AACTGGATGGCTTTCTTGGCGCCAAGGATCTGATGGCGCAGGATCAAGATCTGATCAAGAGACAGGATGAGGATCGTTTCGCATG  
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GCTGCTCTGATGCCGCCGTGTTCCGGCTGTGACGCGAGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGTCCGGTGCCCTGAAT  
GAACTGCAGGACGAGGACGCGCGGCTATCGTGGCTGGCCACGACGGGCGTTCTTTCGCGAGCTGTGCTCGACGTTGTCTACTGAAGC  
GGGAAGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTATCTACCTTGCTCCTGCCGAGAAAGTATCCATCA  
TGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCGGCTACCTGCCCATTCGACCACCAAGCGAAACATCGCATCGAGCGGACGA  
CGTACTCGGATGGGAAGCCGCTTTGTGCTGATCAGGATGATCTGGACGAAGAGCATCAGGGCTCGCGCAGCCGAACTGTTCGCCAG  
GCTCAAGGCGCGCATGCCGACGCGGAGATCTCGTGTGACCCATGGCGATGCCGTGCTTGGCGAAATATCATGGTGGAAATGGC  
CGCTTTTCTGGATTATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGA  
AGAGCTTGGCGCGCAATGGGCTGACCGCTTCTCTGTGCTTTACGGTATCGCCGCTCCCGATTGCGAGCGCATCGCCTTCTATCGCC  
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TCCACCGCGCCTTCTATGAAAGGTTGGGCTTCGGAATCGTTTTCCGGGACGCGCGCTGGATGATCTCTCCAGCGCGGGGATCTCAT  
GCTGGAGTTCTTCCGCCACCCCGGGCTCGATCCCCTCGCGAGTTGGTTTACGCTGCTGCTGAGGCTGGACGACCTCGCGAGTTCT  
ACCGGCAGTGCAAATCCGTCCGCATCCAGGAAACAGCAGCGCGCTATCCGCGCATCCATGCCCGGCAACTGCGAGTCTATACTGT  
TGCGGAAAGCTGGACGATCTTTTATGCTTGCTTTTCAAAGGCCTGCAGGCAAGTGACACAAACAATACTTAAATAAAATACTACTC  
AGTAATAACCTATTTCTTAGCATTTTTGACGAAATTTGCTATTTTGTAGAGTCTTTTACACCATTGTCTCCACACCTCCGCTTA  
CATCAACACCAATAACGCCATTTAATCTAAGCGCATACCAACATTTTCTGGCGTCAGTCCACCAGCTAACATAAAATGTAAGCTT  
TCGGGGCTCTCTTGCTCTCCAACCCAGTCAGAAATCGAGTTCCAATCCAAAAGTTACCTGTCCCACCTGCTTCTGAATCAAACAA  
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TGTTGATCATTGAACCAATTTTCAGTGATCATTTGCTGTTGACAAAGGGAATCATAGATCATATGACAGATGTGTATCTACCTTAA  
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GCCGGGCCGTCTGAAGTACTGGTGATCGCAGACAGCGGCGCAACACCGGATTTTCGTCGCTTCTGACCTGCTCTCCCAGGCTGAGCA  
 CGGCCCGGATTCCCAGGTGATCCTGCTGACGCCTGATGCTGACATTGCCCCGCAAGGTGCGAGCGTAGAACGTCAACTGGCGAACTG  
 CCGCGCCGCGGACACCGCCGAGCCCTGGAGCGCCAGTCGTCCTGATTGTGACCAAGATTAGCGCCAGTGCGTCGCATTTTCTTATC  
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 CTGGTCGCGCGAATCCGCCGGTGATTACGCTTCCGGAACCAACCATGTTTTACCGACCTATGGCTATACTGCTACCTGTTCCAGCC  
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 TTGGCGGCGGCAGAACGTCTGACCGCCCATAAAAATGCCGTGACCCTGCGCGTAAACGCCCTCAAGGAGCAAGCATGAGCACTGAA  
 AACACTCTCAGCGTCGCTGACTTAGCCCGTGAAAAATGTCCGCAACCTGGTACCGTATCAGTCTGCCCGCCGTCTGGGCGGTAACGG  
 CGATGTCTGGCTGAACGCGAATGAATTC EcoRI

**Figure 40. Full length sequence of Arabidopsis cDNA insertion.** Sequence was performed from the end of p936 plasmid then subsequently into the cDNA insertion due to some unknown sequence modification from the original p937 plasmid. EcoRI restriction site is used to identify the start and the end of the Arabidopsis cDNA insertion. By searching the sequence from the nucleotide database, result shows that the cDNA fragment is not from Arabidopsis but a gene from *S. enterica* subspecies.

## Discussion

cAMP has been established as an important secondary messenger in animals and lower eukaryotes. However, the presence and the biological role of cAMP in plants still remain open to debate and no Adenylyl cyclases have been identified in plants. Thus it is reasonable to perform an experiment to screen for a gene that is responsible to conduct AC activities in Arabidopsis. In the experiment, *E.coli cyaA* fail to utilize lactose due to deletion of its own AC genes. The plan for this experiment is to use an Arabidopsis cDNA library and find cDNA insertions that can complement the inability of lactose fermentation in *E.coli cyaA* mutant. We assume that the cDNA insertion that is capable of recovering lactose fermentation in *E.coli* mutant is due to AC activity that synthesizes cAMP. However, it should be noted that the experiment doesn't directly test cAMP production in the transformed mutant cells. We cannot rule out the possibility that the genes being transformed and expressed in *E.coli* cells may also be related to lactose fermentation and not involved in AC activity. An adenylyl cyclase ELISA assay should be used to evaluate cAMP formation by the *E. coli* mutant expressing the putative Arabidopsis adenylyl cyclase for further confirmation. Only by measuring cAMP synthesis and comparing cAMP synthesis level of the candidate cells with *E.coli cyaA* mutant can we prove the AC activity is really increased in the cell.

Unfortunately, this evaluation is not necessary since the three genes identified throughout the screening process are neither ACs nor Arabidopsis genes but all present the same gene from *S. enterica* subspecies. The gene from the *S. enterica* subspecies only covered 9% of the sequence when we aligned it with the *S. enterica* subspecies adenylyl cyclase. This indicates the *S. enterica* subspecies gene we identified is nowhere close to be an AC. This result of a gene from *S. enterica* subspecies appearing in Arabidopsis cDNA fragment could be explained by

contamination during the time the Arabidopsis cDNA library is generated. MacConkey agar is a culture medium designed to grow Gram-negative bacteria and differentiate them for lactose fermentation. Unlike *E.coli*, *S. enterica* doesn't utilize lactose for fermentation; hence the colony should have appeared to be white instead of red. This can be explained by the *cyaA* mutation of *E.coli* SP850 being abolished; reversion to wild type. The *E.coli* with abolished *cyaA* will allow cAMP synthesis again and will change the color of the colony on MacConkey agar media. We do not know what the probability is for the *cyaA* mutation to be reversed back into wild type since all the other colonies except the three we found remained white. We assume throughout the screening, the white colonies are due to *S. enterica* contamination in the Arabidopsis cDNA library and the red colonies are a result of the abolished *cyaA* in *E.coli*. The abolished *E.coli* *cyaA* mutant complements the *S. enterica*'s gene inability to undergo lactose fermentation, resulting in red colonies. This explains why the *E.coli* containing a *S. enterica* subspecies gene that is supposed to show a white colony resulted in red instead. Given the chances that the *E.coli* mutant may be abolished, the screening assay to look for putative ACs is not a good assay. Therefore we decide not to invest future time in this project.

## **Materials and Methods**

### **Preparation of *cyaA* mutant *E. coli* competent cells**

The *cyaA* mutant *E. coli* strain was obtained from the *E. Coli* Genetic Stock Center in Yale University (Coil Genetic Stock Center, New Haven, CT). The *E. coli* strain was spread on Luria-Bertani (LB) broth (10 g tryptone, 5 g yeast extract, 10 g NaCl, 1% Agar) petri dish containing 50 µg/mL kanamycin. The petri dish was incubated at 37 °C for 12-16 h. After incubation, a single colony was picked from the plate and grown in 5 mL LB liquid medium containing 50 µg/mL kanamycin. The single colony was cultured overnight shaking at 250 rpm

at 37 °C for 16 h. After 16 h, 1 mL of the liquid culture was inoculated into the 100 mL fresh LB medium containing 50 µg/mL kanamycin, and shaken continuously at 37 °C for 2-3 h or until absorbance at 600 nm reached 0.36. The culture was then transferred into a pre-chilled tube and placed on ice for 15 min then centrifuged at 4 °C, 4,000 rpm for 10 min. From this step everything was done in 4 °C cold room. The cell pellet from the culture was resuspended in 40 mL pre-chilled CaCl<sub>2</sub> (0.1 M). After adding the CaCl<sub>2</sub>, the cell culture was kept on ice again for 30 min, and then centrifuged at 4 °C, 4,000 rpm for 10 min. The cell pellet was resuspended again in 6 mL 0.1 M CaCl<sub>2</sub> plus 15% glycerol (v/v). The resuspended cell culture was pipetted into 1.5 mL micro-centrifuge tubes (500 µL /tube) freeze on dry ice and stored at -80 °C.

### **Plasmid Recovery**

The Arabidopsis cDNA expression library was generated by Dr. Elledge (Elledge et al., 1991). The cDNA library was expressed on the λ YES vector (Elledge et al., 1991). This vector is capable of replicating as a λ phage, a plasmid lysogen in *E. coli*, or as a centromere plasmid in yeast (Elledge et al., 1991). The plasmid can be looped out from the vector by site specific recombination of the cre protein and lox sites in the vector (Elledge et al., 1991). The method from the U.S patent of Richard Gaber (Gaber, 1993) was used to recover the plasmid from the vector.

*E. coli* strain BNN132 was grown overnight in LB liquid medium containing maltose (0.2% (w/v)) and kanamycin (50 µg/ml). The cells were harvested and resuspended in λ dilution buffer (10 mM Tris-HCl buffer, 5 mM MgSO<sub>4</sub>, 200 mM NaCl and 0.1% (w/v) gelatin, pH 7.4). The λ phage library was added to the cell culture, and the cell culture with the phage library was incubated at 37 °C for 20 min. After incubation, the cells were grown in LB medium for 30 min. The cell culture was then plated on the LB ampicillin plate (ampicillin 100 µg/ml). The plates



with the cell culture were incubated overnight at 37 °C. The plasmid recovered from the phage was resistant to ampicillin, which can be used to select for cells that are infected with the  $\lambda$  phage from the ones that are not. After overnight incubation, cells were harvested from the plate and plasmids were extracted.

### **Screening for the mutants that recover the AC activity**

A plasmid library transformed with an Arabidopsis cDNA library was transfected into the *cyaA* mutant competent cells. The transformed cells were incubated overnight at 37 °C on MacConkey agar (Becton, Dickinson and Company, Sparks, MD) plates that contained kanamycin (50 µg/ml) and ampicillin (100 µg/ml). After incubation, colonies that complemented the *cyaA* mutation can be identified from the MacConkey agar plate by color. A colony that can ferment lactose shows bright red and the one that cannot shows white.

## **Chapter 5: Linking $\text{Ca}^{2+}$ to the brassinosteroid signal transduction cascade: molecular steps that modulate BR responsive gene expression**

### **Abstract**

Brassinosteroid (BR) hormones bind to the receptor BRI1 (BRASSINOSTERIOD INSENSITIVE 1) and control growth and development through a phosphorylation/dephosphorylation signaling cascade which mediates downstream gene regulation. Previous work from our lab indicates that cytosolic  $\text{Ca}^{2+}$  elevation is involved in the BR signal transduction cascade. A recent report shows that in the presence of  $\text{Ca}^{2+}$ , Calmodulin (CaM) 7 binds to the BRI1 cytoplasmic domain (Oh et al., 2012), consistent with  $\text{Ca}^{2+}$  involvement in BR signaling. CaM-binding transcription activators (CAMTAs) are activated by CaM binding and facilitate  $\text{Ca}^{2+}$ -dependent gene expression. INDOLE-3-ACETIC ACID-INDUCIBLE1 (*IAA1*) and PHYTOCHROME B ACTIVATION-TAGGED SUPPRESSOR1 (*BAS1*) are two genes up-regulated by BR in a  $\text{Ca}^{2+}$ -dependent pathway (Zhao et al., 2013). Here qPCR shows BR no longer has an effect on *IAA1* and *BAS1* in Arabidopsis *camta3* mutant seedlings. BR-dependent expression of SMALL AUXIN UP RNA1 FROM ARABIDOPSIS THALIANA ECOTYPE COLUMBIA (*SAUR-AC1*), a gene known to respond to BR directly through the phosphorelay cascade, is not impaired in the *camta3* mutant. Treatment of wild type (WT) seedlings with a CaM antagonist W7 prior to BR addition impaired *BAS1* expression but did not affect expression of *SAUR-AC1*. Both the CaM antagonist and *camta3* results are consistent that CaM is acting in the BR induced,  $\text{Ca}^{2+}$ -dependent signaling pathway, and that  $\text{Ca}^{2+}$ /CaM is acting to regulate gene expression through the action of one or several CAMTAs.

qPCR results of *cpk5, 6, 11* triple mutant with BR treatment show no impaired expression of *BAS1* and *SAUR-AC1*, suggesting at least CPK5, 6, 11 are not the molecular steps mediating  $\text{Ca}^{2+}$ -dependent BR responsive gene expression. Exogenous BR causes a de-etiolated hypocotyl phenotype in the dark. Our results show that BR effect on the hypocotyl length is less pronounced in *camta3* seedlings. These results suggest that *CAMTA3* may decode BR-induced  $\text{Ca}^{2+}$  elevations and transmit this signal to the nucleus to modulate the expression of at least some BR responsive genes and control some BR-dependent phenotypes.

## Introduction

Brassinosteroids (BRs) are hormones ubiquitous in the plant kingdom. BRs bind to its receptor BRI1 (BRASSINOSTERIOD INSENSITIVE 1) and are involved in development, growth and immunity (Belkhadir et al., 2012; Clouse 2011; Yang et al., 2011; Wang, 2012). Physiological studies show that BRs have a strong effect on cell division and cell expansion (Gonzalez et al., 2010; González-García et al., 2011; Hacham et al., 2011 Oh et al., 2012). Studies also show that BRs can enhance tolerance to environmental stresses and resistance to pathogen infections, therefore increasing crop yield (Divi and Krishna, 2009). Mutants that are BR-deficient or insensitive display various growth defects, including dwarfism, dark-green and curled leaves, delayed flowering and male sterility (Szekeres et al., 1996). BRI1 is a receptor like kinase (RLK) that belongs to the largest class of plant cell membrane-localized RLK, the leucine-rich-repeat receptor like kinase (LRR-RLK). A great number of studies have described the steps of a protein phosphorylation/ dephosphorylation (phosphorelay) cascade which mediates the BR signal transduction pathway to affect downstream gene regulation (Clouse 2011; Yang et al., 2011). BRs binding to receptor BRI1 will initiate BRI1 and co-receptor Brassinosteroid associated receptor kinase 1 (BAK1) phosphorylation. BAK1 forms a ligand-independent heterodimer on the cell surface through interactions between extracellular and intracellular domains. This leads to dephosphorylation of BRASSINOSTERIOD-INSENSITIVE 2 (BIN2). BIN2 is a negative regulator of transcription factors (TFs) BRASSINAZOLE-RESISTANT (BZR) 1 and 2 (BZR2 is also known as BRI-EMS SUPPRESSOR1 (BES1). BR binding to BRI1 causes BIN2 degradation. BIN2 degradation leads to stabilization of BZR1 and 2, movement of BZR1/2 to the nucleus and BR-dependent gene expression. BR-dependent gene expression is regulated through the binding of BZR1 and/or 2 directly to promoter elements of

genes encoding enzymes, or the promoters of other transcription factors. BZR1 and/or 2 affecting some genes directly and some indirectly leads to BR regulation of the expression of ~1000 genes.

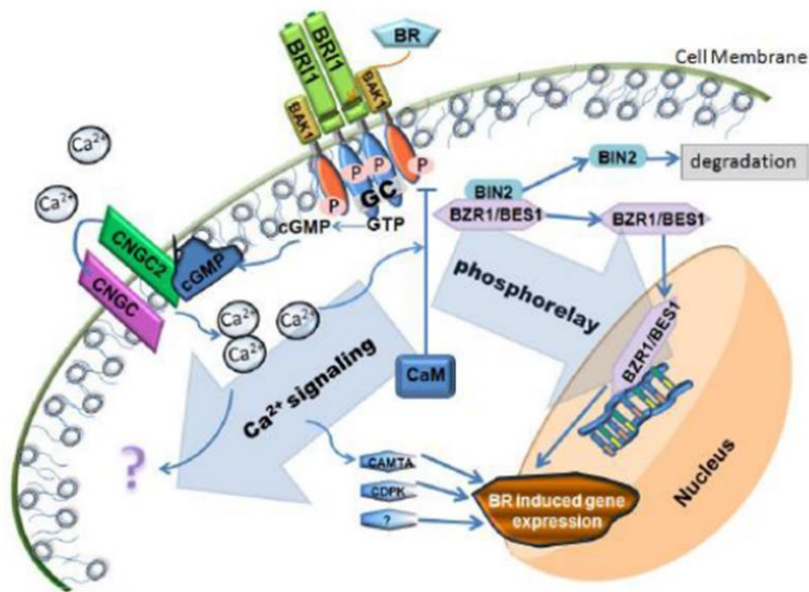
Cytosolic  $\text{Ca}^{2+}$  functions as a ubiquitous secondary messenger in plants. Elevation of cytosolic  $\text{Ca}^{2+}$  represents the core of the complex network in the plant signal transduction pathway.  $\text{Ca}^{2+}$  is also involved in LRR-RLK receptor dependent signal transduction. A recent work from our lab has provided evidence showing cytosolic  $\text{Ca}^{2+}$  is involved in the BR signaling pathway (Zhao et al., 2013). The work shows BR can induce cytoplasmic  $\text{Ca}^{2+}$  elevation immediately in *Arabidopsis aequorin* (aeq) seedlings but does not affect cytosolic  $\text{Ca}^{2+}$  concentration in *bri1-5* aeq (*brassinosteroid insensitive 1-5* mutant, which is the most fertile of the *bri1* weak allele mutants and has a reduced sensitivity to BR (Noguchi et al., 1999)). Moreover, BR induced cytosolic  $\text{Ca}^{2+}$  increase is impaired in *dnd1*-aeq (*defense no death 1* mutant, which lacks the function of the CYCLIC NUCLEOTIDE-GATED CHANNEL2 (CNGC2)). CNGC2 is a plasma membrane localized, inwardly conducting  $\text{Ca}^{2+}$ -permeable channel activated by cGMP. The work also provide genetic and pharmacological evidence that support  $\text{Ca}^{2+}$  involvement in the BR signal transduction pathway and show two genes, *INDOLE-3-ACETIC ACID-INDUCIBLE1* (*IAA1*) and *PHYTOCHROME B ACTIVATION-TAGGED SUPPRESSOR1* (*BAS1*) that are BR responsive in a  $\text{Ca}^{2+}$ -dependent manner. *Arabidopsis* grown in dark causes an etiolated hypocotyl phenotype and BR can impair this phenotype (Xu et al., 2008; Gou et al., 2012). Zhao et al. (2013) show that BR does not impair the phenotype in the *dnd1* mutant, nor does it have an effect on WT seedlings treated with  $\text{GdCl}_3$ , a  $\text{Ca}^{2+}$  channel blocker, supporting  $\text{Ca}^{2+}$  involvement in the BR signal transduction cascade. All these results provide new insights and show a revised model other than the phosphorelay cascade in the

delicate BR signaling pathway (Figure 41). However, it is still unclear what the molecular steps are involved in decoding  $\text{Ca}^{2+}$  signals that transmits to the nucleus to modulate BR-induced downstream gene expression.

There are three major types of  $\text{Ca}^{2+}$ -sensor proteins in plants: calmodulin (CaM)/CaM-like proteins, calcium-dependent protein kinases (CDPKs), and calcineurin B-like proteins. Calmodulin (CaM) is a conserved  $\text{Ca}^{2+}$  binding protein found in all eukaryotes. A recent report demonstrated physical and functional interactions between BRI1 and CaM (Oh et al., 2012). Oh et al. (2012) shows that in the presence of  $\text{Ca}^{2+}$ , CaM7 is bound to the BRI1 cytoplasmic domain protein. The work also demonstrated that co-expression of CaM and BRI1 in *Escherichia coli* (*E. coli*) inhibits both autophosphorylation of BRI1 and phosphorylation of several bacteria proteins (of unknown function). These results not only support the involvement of  $\text{Ca}^{2+}$  in the BR signaling cascade, but also raises a possibility that the elevation of cytosolic  $\text{Ca}^{2+}$  might act through the  $\text{Ca}^{2+}$ -dependent binding of CaM to BRI1.

CaM-binding transcription activators (CAMTAs) are a family of proteins identified by Bouché et al. (2002) more than 10 years ago and there are six genes in Arabidopsis (*AtCAMTA1*-*AtCAMTA6*). CAMTAs contain a variable number of IQ motifs that are known to be associated with binding of CaM and CaM-like proteins (Bouché et al., 2002; Rhoads and Friedberg, 1997; Song et al., 2006). Therefore, CAMTAs are possible candidates downstream from  $\text{Ca}^{2+}$  to decode and transmit signals and mediate BR-induced downstream gene regulation.

Here we provide results that suggest *CAMTA3* may decode BR-induced  $\text{Ca}^{2+}$  elevations and transmit this signal to the nucleus to modulate the expression of at least some BR responsive genes and control some BR-dependent phenotypes. Results with CaM antagonist W7 are



**Figure 41. A model showing a bifurcated BR dependent signal transduction pathway.** It has been known that the phosphorylation/dephosphorylation (denoted as ‘phosphorelay’) signaling cascade mediates BR responsive downstream gene regulation. Recent work from our lab (Zhao et al., 2013) provides evidence that cytosolic Ca<sup>2+</sup> elevation is involved in the BR signal transduction cascade. This cartoon is generate by Yichen Zhao.

consistent with this model of CaM and CAMTA involvement downstream from BR-dependent  $\text{Ca}^{2+}$  elevation in this component of BR signaling.

## Results

### **CAMTA3 involvement in BR induced $\text{Ca}^{2+}$ -dependent gene expression**

Galon et al. (2008) suggests it is possible that  $\text{Ca}^{2+}$  signaling through CAMTA3 is part of a signaling cascade mediating biotic defense response. Here, studies are undertaken to test the hypothesis that CAMTAs are the molecular steps that link BR-dependent  $\text{Ca}^{2+}$  signals to downstream modulation of BR-induced gene expression. We grow WT Arabidopsis and all the *camta* (1-6) mutants (data not shown) to two week old and apply epibrassinolide (eBL, one of the most biologically active compound of the many BRs; see Material and Methods) (1  $\mu\text{M}$  final concentration). qPCR is performed to evaluate BR induced gene expression. INDOLE-3-ACETIC ACID-INDUCIBLE1 (*IAA1*) and PHYTOCHROME B ACTIVATION-TAGGED SUPPRESSOR1 (*BAS1*) are two genes up-regulated by BR in a  $\text{Ca}^{2+}$ -dependent manner (Zhao et al., 2013). qPCR results in Figure 42 show that BR-dependent expression of these gene in WT Arabidopsis are impaired in *camta3* mutant. *IAA1* controls expression of some auxin-regulated genes (Kim et al., 1997). BR is known to interact with auxin signaling in an ill-defined manner. Since the effect of BR on *IAA1* expression in WT Arabidopsis is very modest, we do not evaluate *IAA1* gene expression in later experiments.

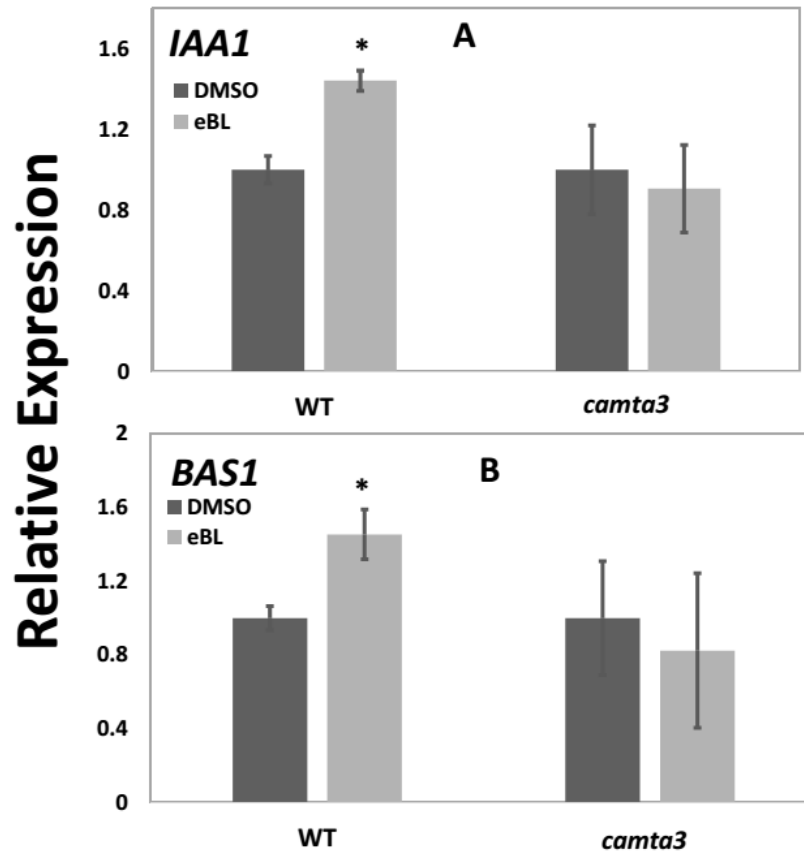
*SMALL AUXIN UP RNA1 FROM ARABIDOPSIS THALIANA ECOTYPE COLUMBIA* (*SAUR-AC1*) is a gene experimentally confirmed to respond directly to BR through the phosphorelay cascade (Yin et al., 2005). BZR2 is transcription factors that interacts with a basic helix-loop-helix (bHLH) transcription factor, BZR2-interacting Myc-like 1 (BIM1) in the BR induced phosphorelay signaling cascade. BZR2/BIM1 heterodimer can activate the SAUR-AC1



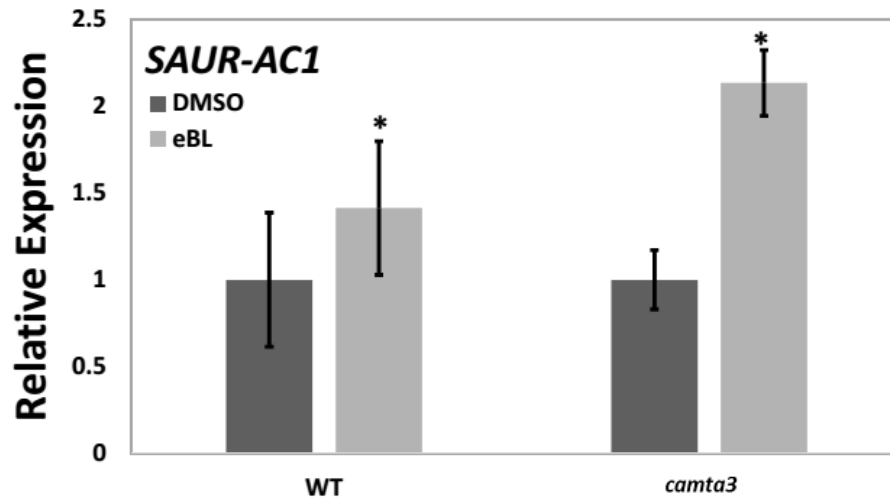
promoter both in a transient expression system and in plants (Yin et al., 2005). Zhao et al. (2013) show *SAUR-AC1* gene expression is increased in WT and *dnd1* treated with BR, consistent with Yin's work that BR effect on *SAUR-AC1* expression is  $\text{Ca}^{2+}$ -independent. Our qPCR result shown in Figure 43 demonstrates that application of BR to WT and *camta3* Arabidopsis still show significant effect on *SAUR-AC1* expression. This supports our assertion that  $\text{Ca}^{2+}$  involvement in the BR signaling pathway is independent to the phosphorelay pathway. It also supports that the phosphorelay pathway functions separately due to *SAUR-AC1* expression upregulated in both WT and *camta3*.

### **Blocking endogenous BR does not affect exogenous BR induced gene expression in both the $\text{Ca}^{2+}$ signaling pathway and phosphorylation pathway**

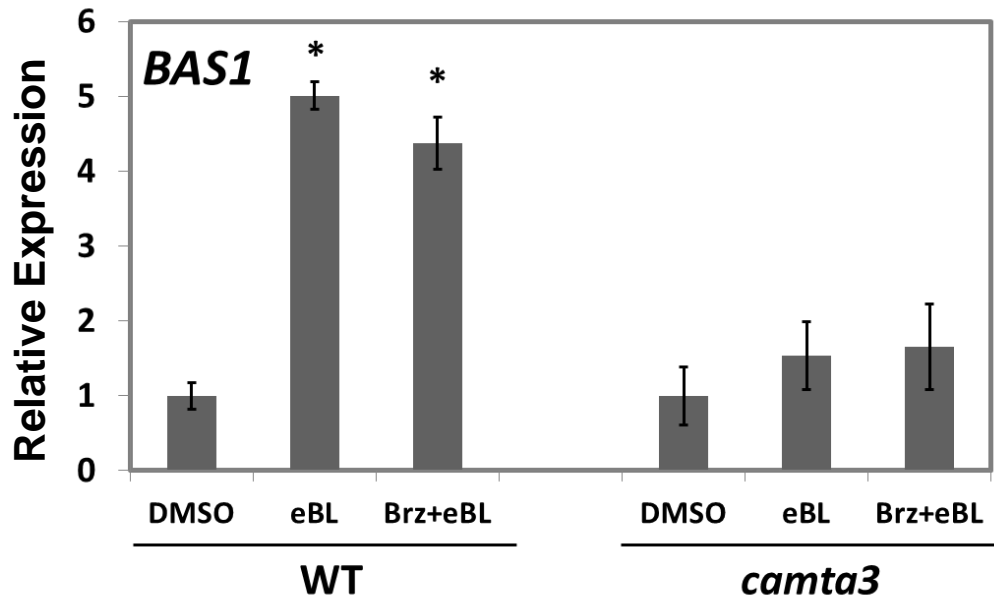
Brassinazole (Brz) is a commonly used brassinosteroid biosynthesis inhibitor. Arabidopsis seedlings grown in light appear to result in a dwarf phenotype with Brz application and this phenotype can be rescued by brassinolide (BL) (Asami et al., 2000). Brz also has an effect on Arabidopsis seedlings grown in dark, inducing short hypocotyl and open cotyledon. This BR-deficient mutant-like phenotype can be rescued by application of BL (Asami et al., 2000). Exogenous BR has a very significant effect on gene expression and phenotype. We hypothesize that blocking endogenous BR should not affect exogenous BR induced gene expression in both the  $\text{Ca}^{2+}$ -dependent pathway and phosphorylation pathway. In order to test this hypothesis, we monitor the effects of DMSO (control), eBL alone and Brz addition with eBL on *BAS1* and *SAUR-AC1* gene expression with both WT and *camta3* Arabidopsis seedlings. qPCR results show that eBL can rescue the effect of Brz on BR induced *BAS1* and *SAUR-AC1* gene expression in both WT and *camta3* seedlings (Figures 44 and 45). These results suggest eBL induced *BAS1* and *SAUR-AC1* gene expression mainly relies on exogenous eBL and blocking endogenous BR



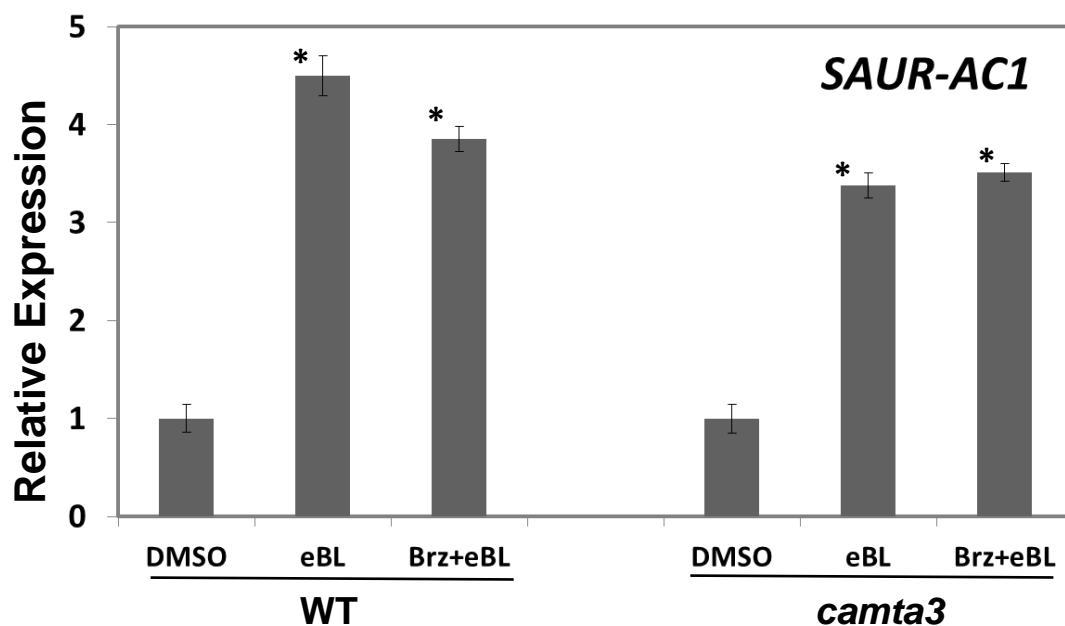
**Figure 42. CAMTA3 involvement in BR induced  $\text{Ca}^{2+}$ -dependent *IAA1* and *BAS1* expression.** *IAA1* (A) and *BAS1* (B) expression are monitored (q-PCR analysis) in wild type (WT) plants and *camta3* treated with 1  $\mu\text{M}$  BR or DMSO (control). DMSO is used as a solvent for BR. Results are shown as means ( $n = 3$ )  $\pm$  SE. ANOVA analysis is used to evaluate means separation; an asterisk above the bar representing the BR treatment indicates it is significantly different (at  $P < 0.05$ ) from the control (DMSO) treatment for that genotype.



**Figure 43. Gene expression data obtained by qPCR presents BR effect on *SAUR-AC1* in WT and *camta3* Arabidopsis seedlings.** *SAUR-AC1* expression is monitored in WT and *camta3* seedlings treated with 1  $\mu$ M BR or DMSO (control). Results are shown as means ( $n = 3$ )  $\pm$  SE. ANOVA analysis is used to evaluate means separation; an asterisk above the bar representing the BR treatment indicates it is significantly different (at  $P < 0.05$ ) from the control treatment.



**Figure 44. Brz pretreatment does not affect exogenous BR induced *BAS1* expression in both WT and *camta3* Arabidopsis seedlings.** Relative expression (qPCR analysis) of *BAS1* in WT plants treated with DMSO (control), 1  $\mu$ M eBL alone or 2  $\mu$ M Brz and 1  $\mu$ M eBL are shown as means ( $n = 4$ )  $\pm$  SE normalized to the transcript level of control. ANOVA analysis is used to evaluate means separation; an asterisk above the bar representing the eBL alone or Brz and eBL treatment indicates it is significantly different (at  $P < 0.05$ ) from the control treatment.

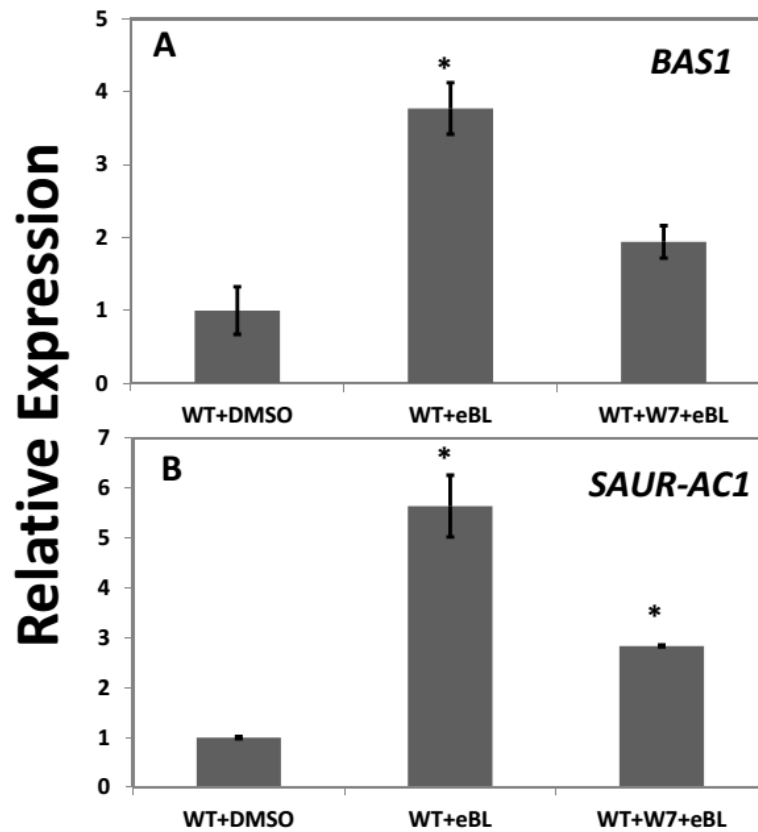


**Figure 45. Brz pretreatment does not affect exogenous BR induced *SAUR-AC1* expression in both WT and *camta3* Arabidopsis seedlings.** Relative expression (qPCR analysis) of *SAUR-AC1* in WT plants treated with DMSO (control), 1  $\mu$ M eBL alone or 2  $\mu$ M Brz and 1  $\mu$ M eBL are shown as means ( $n = 4$ )  $\pm$  SE normalized to the transcript level of control. ANOVA analysis is used to evaluate means separation; an asterisk above the bar representing the eBL alone or Brz and eBL treatment indicates it is significantly different (at  $P < 0.05$ ) from the control treatment.

synthesis does not affect the extent of exogenous eBL regulated gene expression. The results in *camta3* mutant reassure CAMTA3 is involved in decoding and transmitting the  $\text{Ca}^{2+}$  signal into the nucleus. Moreover, it indicates that in our experiment, exogenous but not endogenous BR is the cause of initiating the signal transduction.

### **CaM antagonist impairs BR induced $\text{Ca}^{2+}$ -dependent gene expression**

Aside from showing genetic evidence, we also used a pharmacological approach to further examine the novel finding of CaM involvement (through CAMTA3 acting as a CaM-dependent transcription activator) in the BR induced,  $\text{Ca}^{2+}$ -dependent signaling pathway. N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7, Enzo Life Sciences) is a CaM antagonist. The rationale underlying this work is as follows. W7 binds to CaM hydrophobic pockets so that CaM cannot bind to proteins that usually interact with CaM (Hidaka et al., 1981). According to Om et al. (2012), W7 binds to BRI1 receptor to prevent BRI1 autophosphorylation and phosphorylation of other proteins in the heterologous system of *E. coli* when  $\text{Ca}^{2+}$  is present. In addition, the competition between W7 and  $\text{Ca}^{2+}$  binding to CaM will also prevent CaM binding to CAMTAs. qPCR results presented in Figure 46A show that, in comparison to control (DMSO), BR induced expression of *BASI* in Arabidopsis seedlings is impaired by W7 (50  $\mu\text{M}$  final concentration) application 5 min prior to eBL treatment. BR induced *SAUR-AC1* expression of Arabidopsis treated with W7 prior to eBL is slightly impaired, but still show significant increase when compared to control (Figure 46B). Nevertheless, W7 itself has an effect on BR induced *SAUR-AC1* expression. We acknowledge we do not understand the system entirely, and that there might be unknown effects of W7. Therefore, we cannot conclude that the effects on *BASI* are only due to W7 mimicking *camta3*. Nonetheless, these results are consistent with the effects of *camta3*,



**Figure 46.** CaM antagonist W7 effects on *BAS1* and *SAUR-AC1* on WT Arabidopsis seedlings. BR dependent *BAS1* (A) expression requires  $\text{Ca}^{2+}$  whereas *SAUR-AC1* (B) does not. Relative expression of *BAS1* and *SAUR-AC1* in WT plants treated with DMSO (control) or 50  $\mu\text{M}$  W7 and 1  $\mu\text{M}$  BL are shown as means ( $n = 4$  in *BAS1*,  $n = 3$  in *SAUR-AC1*)  $\pm$  SE normalized to the transcript level of control. ANOVA analysis is used to evaluate means separation; an asterisk above the bar representing the W7 and BR treatment indicates it is significantly different (at  $P < 0.05$ ) from the control treatment.

supporting CAMTA3 as a molecular step involved in the BR induced,  $\text{Ca}^{2+}$ -dependent signal transduction pathway.

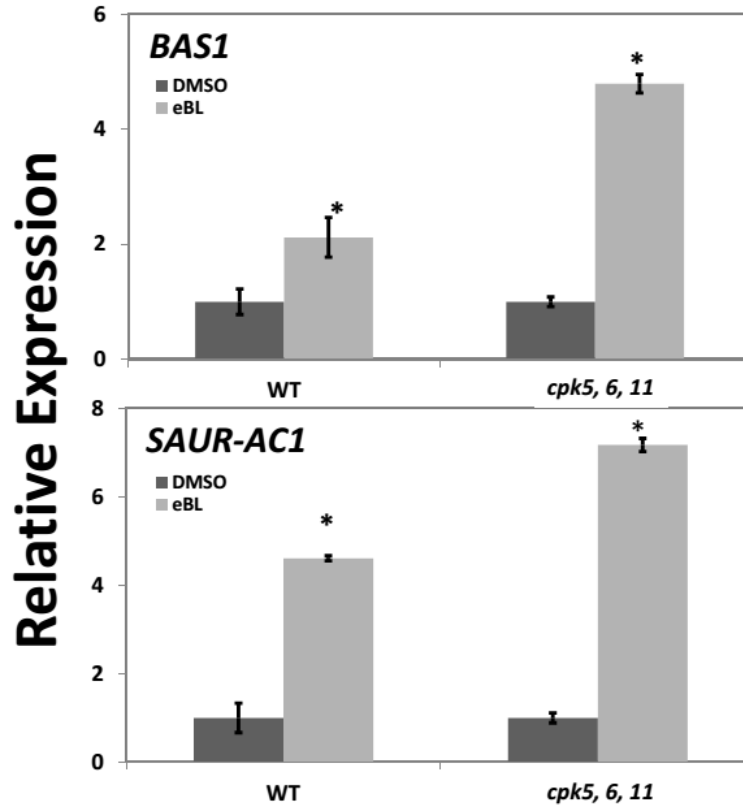
**Calcium-Dependent Protein Kinases may not be involved in the molecular steps that decode and transmit BR induced  $\text{Ca}^{2+}$  signals to affect BR responsive gene expressions**

As reported above, CAMTA3 is involved in decoding  $\text{Ca}^{2+}$  signals to modulate downstream gene expression. Calcium-Dependent Protein Kinases (CPKs) are also involved in transmitting the early  $\text{Ca}^{2+}$  signals to downstream steps. Up until now, there are 34 CPKs identified in Arabidopsis. The 34 CPKs are divided into four subgroups. CPK5, 6, 11 are all in subgroup I, and the *cpk5, 6, 11* triple mutant happened to show the strongest reduction of flg22 (a 22-amino acid peptide of flagellin) responsive gene expression and pathogen resistance (Boudsocq and sheen, 2013). Besides pathogen, Ma et al. (2013) also show CPK5, 6, 11 are involved in transmitting pep3 (a peptide in Arabidopsis) dependent  $\text{Ca}^{2+}$  signal to modulate defense gene expression. This suggests that CPK5, 6, 11, which is involved downstream from  $\text{Ca}^{2+}$  in one signaling pathway, may also be involved in other signaling pathways. Therefore, *cpk5, 6, 11* is another candidate to test whether or not it is involved in decoding BR induced  $\text{Ca}^{2+}$  signals and transmitting the signal into the nucleus to modulate gene expression. We applied eBL to *cpk5, 6, 11* triple null mutants and observed no impaired expression of *BAS1* and *SAUR-AC1* (Figure 47A and B). These results suggest at least CPK5, 6, 11 are not the molecular steps mediating  $\text{Ca}^{2+}$ -dependent BR responsive gene expression.

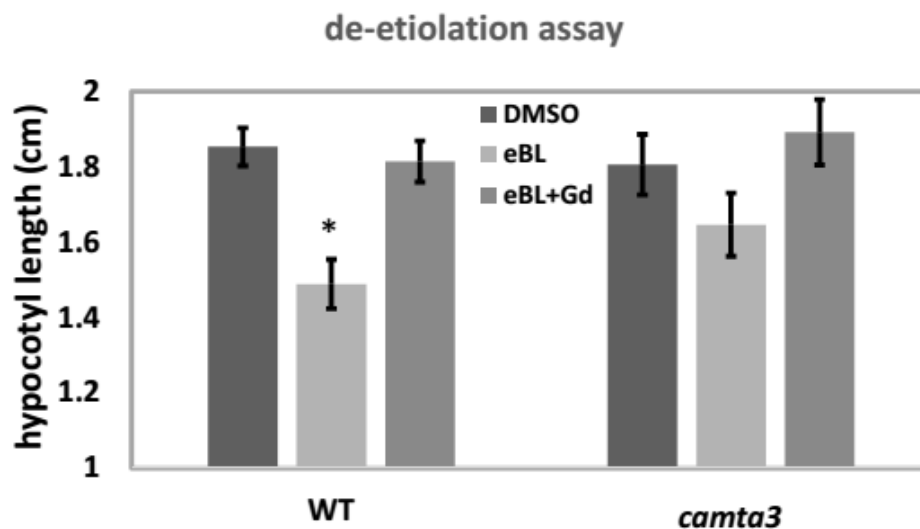
**CAMTA3 involvement in BR induced de-etiolated hypocotyl phenotype**

Growing Arabidopsis seedlings in the dark can cause an etiolated hypocotyl phenotype. Exogenous BR (epibrassinolide; eBL) application has been found to be able to alter the phenotype by reducing the hypocotyl length (Xu et al., 2008; Gou et al., 2012). Zhao et al. (2013)

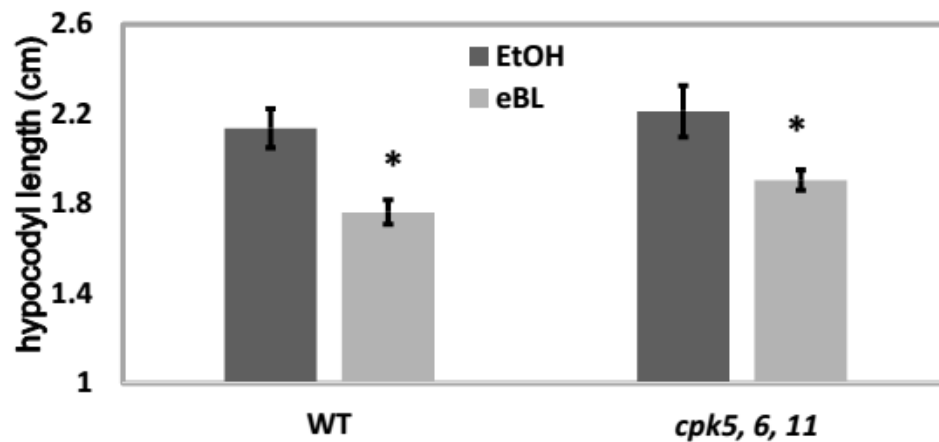




**Figure 47. CPKs may not be involved in the molecular steps that decode and transmit BR induced  $\text{Ca}^{2+}$  signals to affect BR responsive gene expressions.** *cpk5, 6, 11* triple mutant is used to determine whether it plays a part in  $\text{Ca}^{2+}$ -dependent BR responsive gene expression. qPCR results show when given BR (1  $\mu\text{M}$ ), both *BAS1* (A) and *SAUR-AC1* (B) expression are not affected in *cpk5, 6, 11* triple mutant. Results are shown as means ( $n = 3$  in *BAS1*,  $n = 4$  in *SAUR-AC1*)  $\pm$  SE. ANOVA analysis is used to evaluate means separation; an asterisk above the bar representing the BR treatment indicates it is significantly different (at  $P < 0.05$ ) from the control treatment.



**Figure 48. CAMTA3 is involved in the effect of  $\text{Ca}^{2+}$ -dependent BR on etiolated hypocotyl length of dark-grown *Arabidopsis* seedlings.** WT (Col) and *camta3* seedlings were grown on solid medium with DMSO (Control), BR, or BR and  $\text{GdCl}_3$  added. Results are shown as means ( $n > 10$ )  $\pm$  SE. ANOVA analysis is used to evaluate means separation; an asterisk above the bar representing the BR treatment indicates it is significantly different (at  $P < 0.05$ ) from the control treatment.



**Figure 49. CPK5, 6, 11 is not involved in the effect of  $\text{Ca}^{2+}$ -dependent BR on etiolated hypocotyl length of dark-grown *Arabidopsis* seedlings.** WT (Col) and *cpk5, 6, 11* seedlings were grown on solid medium with EtOH (Control) or BR added. Results are shown as means (  $n > 10$ )  $\pm$  SE. ANOVA analysis is used to evaluate means separation; an asterisk above the bar representing the BR treatment indicates it is significantly different (at  $P < 0.05$ ) from the control treatment.

suggests that this BR effect of reducing hypocotyl length of dark-grown Arabidopsis seedlings is  $\text{Ca}^{2+}$ -dependent. Measuring the hypocotyl length of dark grown Arabidopsis seedling is a quantitative assay to study  $\text{Ca}^{2+}$  involvement in signaling pathways affected by the BR hormone. As mentioned above, *CAMTA3* is suggested to be involved in the molecular steps decoding BR induced  $\text{Ca}^{2+}$  signals and transmitting it into the nucleus to modulate gene expression. Therefore we can ask the question whether *CAMTA3* also plays a role in the  $\text{Ca}^{2+}$ -dependent, BR induced de-etiolated hypocotyl phenotype. In order to test the hypothesis, WT and *camta3* Arabidopsis seedlings are grown on 1/2 strength Murashige and Skoog (MS) plates with and without eBL in the dark for 14 d. Zhao et al. (2013) show that application of a  $\text{Ca}^{2+}$  channel blocker along with eBL can reverse the BR-dependent phenotype in Arabidopsis seedlings. Therefore, seedlings grown on 1/2 MS agar plates containing a  $\text{Ca}^{2+}$  blocker along with eBL is used as a control. The Results in Figure 48 show that eBL no longer significantly impairs the hypocotyl length in *camta3* seedlings. We notice that there is still a reduction of hypocotyl length on *camta3* seedlings grown on plates with eBL addition. We suspect that there might be other factors associated with *CAMTA3* in the BR induced de-etiolated hypocotyl phenotype. Additional studies are undertaken with *cpk5,6,11* seedlings. In this case, BR still has an effect on reducing the hypocotyl length of *cpk5,6,11* seedlings (Figure 49). This is consistent with the qPCR results, that *CPK5,6,11* is likely not involved in the BR induced,  $\text{Ca}^{2+}$ -dependent signaling pathway. The experimental results shown in Figures 47 and 48 provide genetic evidence and link *CAMTA3* to the BR related,  $\text{Ca}^{2+}$  mediated phenotype.

## Discussion

Although a great number of studies describe the BR phosphorelay signaling cascade, more evidence suggests the involvement of  $\text{Ca}^{2+}$  in the BR signaling pathway (Du and Poovaiah, 2005;

Singla et al., 2006; Oh et al., 2012; Zhao et al., 2013). According to Zhao et al. (2013), the BR induced  $\text{Ca}^{2+}$ -dependent signaling pathway is activated via the GC domain from its receptor BRI1. However, a recent publication shows results of the crystal structure of BRI1 and maps the previously identified phosphorylation sites onto the structure (Bojar et al., 2014). The paper provides a detailed insight into the BRI1 kinase mechanism, and the analyses are inconsistent with the proposed GC activity. Bojar et al. (2014) mapped the putative GC domain suggested for BRI1 onto the structure of BRI1<sup>865-1160</sup> and the suggested GC catalytic core was buried deep inside the hydrophobic core of the kinase domain. The analysis suggests that it is unlikely that a catalytically competent GC domain can form in BRI1. Bojar et al. (2014) also find that BRI1<sup>865-1160</sup> hydrolyses GTP to GDP and GMP, but cannot form cGMP in HPLC-based activity *in vitro* assays. We acknowledge that the cGMP increase prior work from this lab demonstrated to occur in response to exogenous BR (eBL) application may not be necessarily generated by GC activity of the BRI1 receptor. It is possible that the BR dependent cGMP generation may come from other unknown sources in plants to activate CNGC channels. Nevertheless, evidence from Zhao et al. (2013) and Oh et al. (2012) show support of the involvement of  $\text{Ca}^{2+}$  in the BR signaling pathway. Therefore, it is important to study what molecules are involved in decoding the  $\text{Ca}^{2+}$  signal and transmitting it to the nucleus to modulate BR induced gene expression.

CAMTAs are by far the most well studied transcription factors that are associated with CaM. CAMTA3 has been shown to be involved in insect resistance, pathogen defense response and cold/freezing tolerance (Galon et al., 2008; Doherty et al., 2009; Qiu et al., 2012). Here we show that CAMTA3 is involved in BR hormone signaling. Our results suggest that CAMTA3 may decode BR-induced  $\text{Ca}^{2+}$  elevations in the cytosol and transmit this signal to the nucleus to modulate the expression of at least some (*IAA1* and *BAS1* but not *SAUR-AC1*) BR responsive

genes and control some BR-dependent phenotypes. This work further characterizes the generation of a  $\text{Ca}^{2+}$  signal and the transmission of this signal to the nucleus as a step in at least one component of the BR signal transduction cascade.

A very recent paper (Yan et al., 2015) shows evidence of calcium/calmodulin-dependent protein kinase (CCaMK) involvement in BR induced antioxidant defense in *Zea mays*. Yan et al (2015) showed that BR treatment can lead to cytosolic  $\text{Ca}^{2+}$  increase in protoplasts made from maize leaf mesophyll and that BR has an effect on ZmCCaMK gene expression and enzyme activity in maize leaves. Moreover, BR treatment could not induce antioxidant defense in maize protoplasts where ZmCCaMK is transiently silenced. CCaMK genes have been identified in several plant species including *Zea mays*, *Oryza sativa*, *Lotus japonicus* and *Medicago truncatula* (Pandey and Sopory, 1998; L  vy et al., 2004; Tirichine et al., 2006; Ikeda et al., 2011). Although there are no CCaMKs identified in Arabidopsis plants, this work is consistent with our model, that  $\text{Ca}^{2+}$  is involved in the BR signal transduction pathway.

With the finding of  $\text{Ca}^{2+}$  involved as an alternative pathway in the BR signaling cascade and the molecular steps that link  $\text{Ca}^{2+}$  to modulate BR-induced gene expression, it is of interest to identify more genes that are regulated by the  $\text{Ca}^{2+}$ -dependent BR signaling cascade. Analyzing whole-genome data, e.g. RNA-seq data of BR treated WT and *camta3* Arabidopsis seedlings will provide us more genes that are involved in the  $\text{Ca}^{2+}$ -dependent, BR signaling pathway. Since BR plays a role in several physiological activities, the RNA-seq data will help us have a better standing of what genes in these physiological activities (whether development, growth or immunity) are mediated by the  $\text{Ca}^{2+}$  signaling pathway.

## **Materials and Methods**

### **Plants material**

All *Arabidopsis* lines used in the reported work are in the Columbia (Col) background, and Col plants were used as controls as appropriate. The allele of *camta3* mutant used in the experiments is *camta3-1*. *Arabidopsis* seeds were surface-sterilized by first washing the seeds in 70% (v/v) ethanol, 20% (v/v) bleach and 0.02% (v/v) Triton X-100, shaking at 300 rpm for 10 min, and then rinsing with 95% (v/v) ethanol 3 to 4 times.

For all measurements, seeds were planted on ½ strength Murashige and Skoog (MS) medium (Caisson Labs, Logan, UT) supplemented with 1% (w/v) suc and solidified with 1% (w/v) agar. Seeds were stratified (4 °C) for 2 to 3 d after plating to break dormancy. Plants used for qPCR measurement of gene expression were grown on solid medium for 9 d and transferred to liquid ½ MS medium in a 24 well plate (1.5 mL medium for 1 seedling) for 5 d for additional treatments.

### **qPCR analysis**

Treatment and genotype effects on the expression level of BR-responsive genes were evaluated using q-PCR analysis. *Arabidopsis* seedlings were grown on solid ½ strength MS medium first then transferred to ½ strength MS liquid medium on a shaker (180 rpm) with 24 h illumination ( $\sim 90 \text{ mol m}^{-2} \text{ s}^{-1}$ ) at 22 °C for further growth and were used after a total of 14 d. Over 60 different BR compounds have been identified in plants. Brassinolide and epibrassinolide (eBL) are the most biologically active of these compounds and are typically used in tissue culture applications to study BR effects on plants (Ferrie et al., 2005). The eBL (Sigma-Aldrich, St Louis, MO) (at 1  $\mu\text{M}$  final concentration) was added to the liquid medium and the seedlings were incubated for 8 h. For experiments involving addition of inhibitors, W7 (at 50  $\mu\text{M}$  final concentration) (Enzo Life Sciences; for use of W7, see Ali et al., 2007) was added to the medium containing plants 5 min prior to addition of BR. Total RNA was isolated from the seedlings of

the 14-day-old Arabidopsis as follows. After immediately killing seedlings in liquid nitrogen, tissue was stored at -80 °C until use. RNA was extracted following the manufacturer's protocol (Macherey-Nagel; Bethlehem, PA). During the RNA extraction process, tissue was treated with rDNase (Macherey-Nagel) to remove potential genomic DNA from samples.

After extraction, 500 ng of total RNA was used for reverse transcription by using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems (ABI); Carlsbad, CA). The synthesized cDNA was diluted 1:4 (v/v) in water, and 1 µL of the diluted cDNA was used for each qPCR reaction. qPCR assays were performed using the ABI 7900 HT Real-time PCR system with the SYBR Green gene expression assay (ABI). Treatment effects on the expression level of *IAA1*, *BAS1* and *SUAR-AC1* were examined. *Tubulin* was used as an endogenous control. The primers used for these analyses were as follows: *IAA1* (Forward) 5'-AGTCACCAATGGGCTTAACC-3', *IAA1* (Reverse) 5'-CTGTTGAGTCGTTGTTCT TGC-3'; *BAS1* (Forward) 5'-CCCGTTGGCTTCATACCGT-3', *BAS1* (Reverse) 5'-TTACAGCGAGTGTC AATTTGGC-3'; *SAUR-AC1* (Forward) 5'-AGGAGTTTCTTGGGT GCTAAG-3', *SAUR-AC1* (Reverse) 5'- CATAGACCGCCATGAATCCTC-3'; and *Tubulin* (Forward) 5'- GAGCCTTACAACGCTACTCTGTCTGTC -3', *Tubulin* (Reverse) 5'- ACA CCAGACATAGTAGCAGAAATCAAG -3'.

For each analysis, 3 mechanical replications were tested on one plate, and each treatment mean was generated from analysis of at least 3 biological replications from separate RNA isolated from different individual seedling. ANOVA of corresponding threshold cycle (CT) values was used for evaluation of means separation amongst treatments in an experiment (as noted in figure legends), and to generate standard errors of the means for control treatments (Schmittgen et al., 2008). In many cases, experiments involving qPCR analysis of gene



expression involved the evaluation of BR effects on different genotypes. In these cases, gene expression in the absence of exogenous BR was normalized to '1' for each genotype in a given experiment (as noted in figures). In these cases, the relative amount of gene expression in the absence of BR for each mutant line used in an experiment (i.e. in a given figure) is compared to the expression level in WT plants for that experiment. This information is provided in the corresponding figure legend.

### **Measurement of etiolated hypocotyl length**

This assay allows direct measurement of BR effect on Arabidopsis seedlings phenotype for quantitative evaluation. The assay was adopted from Xu et al (Xu et al., 2008; Müssig et al., 2002). Different treatments (0.001% (v/v) DMSO or EtOH as control, 100 nM eBL and/or 150  $\mu$ M GdCl<sub>3</sub>) were added to the solid ½ strength Murashige and Skoog (MS) medium containing 1% (w/v) agar before pouring into square plates. At least 10 seeds of each genotype were planted on ½ MS media in each treatment. The square plates were covered with foil paper and put in a box for 14 d. Arabidopsis seedlings were grown vertically at 22 °C in the dark. The hypocotyl length of seedlings was measured after 14 d.

## Chapter 6 Overall Conclusion

The research objective in this dissertation is to have a better understanding about secondary messengers, especially cyclic nucleotides and  $\text{Ca}^{2+}$ , and how they are involved in different signaling pathways to affect Arabidopsis physiology. This dissertation provides some new insights in secondary messengers signal transduction in plants and the importance is as followed. (1) We provide new insights on SAM development by suggesting  $\text{Ca}^{2+}$  is involved in mediating peptide/receptor regulated gene expression to control the SAM. Despite the recent publication providing evidence against the GC catalytic activity (Bojar et al., 2014), our results suggest CNGC-dependent  $\text{Ca}^{2+}$  signaling in SAM development. (2) We developed a biosensor which we can measure cAMP level changes in a non-invasive, real time manner. (3) We show evidence that CAMTA3 is a molecular step involved downstream from  $\text{Ca}^{2+}$  to decode the signal and transmit it into the nucleus to modulate gene expression in the BR signaling pathway.

As mentioned in previous chapters, evidence for the presence and physiological roles of cyclic nucleotide based signaling in plants has been accumulating over the past few decades. Cyclic nucleotide-gated ion channels (CNGCs) have been elucidated in plants and studies suggest that cyclic nucleotides activate CNGCs to affect downstream signaling. It is important for us to find out which cyclic nucleotide (whether in its general form or isoform) activates which CNGC and how the CNGC structure changes upon cyclic nucleotide activation. It is more importantly to understand the physiological role each CNGCs play in plants, whether it is in development, biotic or abiotic stress response, etc. Aside from the activation of CNGCs, cyclic nucleotides are also involved in the regulation of transcription of many cyclic nucleotides responsive genes (Bastian et al., 2010; Ohno et al., 2012; Zahra et al., 2014). Although there is only little evidence about cyclic nucleotide dependent protein kinases (PKA and PKG) in plants,

they cannot be completely dismissed (Janistyn 1989; Kato et al. 1983; Komatsu and Hirano 1993; Dubovskaya et al. 2002; Szmidt-Jaworska et al. 2003; Newton and Smith 2004). Understanding the phosphatase function, which is suggested to be the key activity of several candidate cyclic nucleotide dependent phosphatase/kinases, may open new perspectives on cyclic nucleotide dependent phosphorylation events in plants (Martinez-Atienza et al., 2007).

To understand the role of cyclic nucleotides and nucleotide cyclases (NCs) in plant functions, it will be necessary to perform analysis in all aspects, including physiological, biochemical, and metabolic data obtained from wild-type, mutant, and knockout lines. The key challenge to study NCs is to (1) identify more NCs, (2) elucidate NC activity of the untested NCs *in vitro* and *in vivo* to establish the roles of cNMP in downstream signaling. This will yield important new insights into this family of plant proteins. However, identification and functional characterization of (signaling) proteins that bind cAMP and cGMP in plants are as important as identifying new NCs.

$\text{Ca}^{2+}$  is a universal secondary messengers found in all cells. It is amazing that such a simple ion can act specifically and appropriately to physiological response. Almost every stress or developmental process requires  $\text{Ca}^{2+}$  fluxes in the cell. Therefore, it is reasonable to search for conserved  $\text{Ca}^{2+}$  signaling processes between bacteria and eukaryotes. In Arabidopsis, there are potentially more than 67  $\text{Ca}^{2+}$  regulated protein kinases (Harper et al., 2004). However, little is known about the regulatory mechanisms that code and decode  $\text{Ca}^{2+}$  signals in plants. One of the future studies of  $\text{Ca}^{2+}$  will be to understand the biochemical and biological functions of different kinase genes. It is also important to understand the downstream mechanisms that convert the signals arising from activation of these proteins into alterations in gene expression or enzyme activity. It is also crucial to study whether these  $\text{Ca}^{2+}$  regulated protein kinases are species-

specific, since understanding the role of this variation may provide modified signaling pathways of important commercial traits for engineering crops. Last but not least, the functional interconnections between the systems that decode  $\text{Ca}^{2+}$  signals and other cell signaling systems such as receptor-like kinases and MAPK modules also need to be elucidated.

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