DISSERTATION

CHARACTERIZATION OF A SYNTHETIC SIGNAL TRANSDUCTION SYSTEM

Submitted by

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ABSTRACT

CHARACTERIZATION OF A SYNTHETIC SIGNAL TRANSDUCTION SYSTEM

The Medford laboratory has developed a synthetic signal transduction system linking exogenous perception of a particular ligand to a transcriptional response. One application of this system is to produce plants that sense and respond to a specific ligand. The system was designed based on evolutionary conservation of histidine kinase signaling and uses bacterial components adapted to function in plants. The synthetic signaling system is responsive to extracellular ligand perception by a wild-type or modified ribose binding protein (RBP) scaffold. Upon ligand binding, RBP binds and activates a synthetic fusion histidine kinase made from the extracellular portion of the bacterial chemotactic receptor Trg and the cytoplasmic portion of the bacterial phosphate sensor PhoR. Activated Trg-PhoR transmits a phosphate signal to the bacterial response regulator PhoB. Upon phosphorylation PhoB translocates into the nucleus of a plant cell and activates transcription of the response gene(s).

In addition to receiving a phosphate signal from Trg-PhoR, PhoB can be activated by exogenous cytokinin application suggesting that components of the cytokinin signaling pathway can interact with PhoB. Elimination or reduction of the interaction with cytokinin signaling components allows production of a more reliable signaling system. One goal of the following work was to reduce the interaction of PhoB with endogenous cytokinin signaling components. I attempted to identify a mutant form of PhoB that does not interact with cytokinin signaling components yet maintains function with the synthetic signaling system. I screened six different rationally selected PhoB mutants in plants for reduced response to exogenous cytokinin application. I

concluded that a different approach will be needed to successfully reduce interaction with cytokinin signaling components.

Another goal of this work was the identification cytokinin signaling components that interact with PhoB, possibly revealing a means to eliminate the interaction. I attempted to functionally express selected cytokinin signaling components in a bacterial testing system. After several failed cloning strategies, I conclude that the cytokinin sensor histidine kinase, AHK4, may be toxic and/or unstable in bacteria and expression of alternative genes will be needed to identify cytokinin signaling components that interact with PhoB.

Additional work described here includes the independent testing of two computationally designed RBPs; one reported to bind the environmental pollutant methyl tert-butyl ether and the other reported to bind the explosive trinitrotoluene, for ligand dependent activation of the synthetic signaling system. These results show that the computationally designed RBPs do not function in a reliable manner and lead to the production of a detector plant using wild-type RBP to activate the synthetic signaling system that enables further analysis of the system components in plants.

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Chapter One

Introduction to Synthetic Signal Transduction

I. Introduction to Synthetic Biology

Synthetic biology is the development of systems to enable novel functions in living organisms and gain a better understanding of how natural systems function. Building of standard transgenic technology, synthetic biology assembles several transgenic parts into a functional system. Synthetic biology uses concepts from engineering such as standardization of parts, decoupling and abstraction, along with mathematical modeling to design systems for a specific purpose (Bowen et al., 2008). To date, most work on synthetic biology has been accomplished with microorganisms. One of the first examples of synthetic biology was the construction of a genetic toggle switch, a bi-stable gene regulatory network in *Escherichia coli* from well characterized genetic components (Gardner et al., 2000). The bi-stable gene regulatory network enables switching between two stable states using some extracellular force. Other accomplishments include the design of an artificial oscillatory gene regulation network allowing for oscillation between states without an external force (Elowitz and Leibler, 2000), construction of synthetic cell-cell communication resulting in programmed pattern formation (Basu et al., 2005), and production of a bacterial "camera" that produces a chemical image in response to light patterns (Levskaya et al., 2005). More recently synthetic gene regulatory networks have been used in microorganisms engineered to treat bacterial infections (Lu and Collins, 2007; Duan and March, 2010) and invade cancer cells (Anderson et al., 2006), demonstrating the potential clinical applications of

synthetic biology. Several companies (Algenol, Solarvest BioEnergy, Synthetic Genomics and others) are developing synthetic gene regulatory networks in photosynthetic microorganisms to optimize synthesis of renewable biofuels and other useful products (Waltz, 2009). From a basic research perspective, designing and modeling synthetic systems helps us to better quantify natural systems and understand how they operate (Mukherji and van Oudenaarden, 2009).

An important aspect in the development of synthetic systems is the characterization and standardization of biological parts from the wealth of information provided by systems biology (Bowen et al., 2008). Characterization of components working in natural systems allows the identification of modular genetic regulatory elements with known function. In 2003, a group at MIT founded the web-based Registry of Standard Biological Parts (partsregistry.org), a continually growing searchable catalog of genetic parts for building synthetic systems. Another advancement that has enabled the development of synthetic systems is the ability to synthesize large fragments of DNA and the increased affordability of this service. This technology allows genes to be codon optimized for expression in a target organism, restriction sites can be added or removed for ease of cloning, and regulatory elements that would be difficult to incorporate with traditional cloning methods can be added to a synthetic gene or fragment. Finally, the contributions of computational biology have advanced the field of synthetic biology dramatically. The predictive power offered by a computational model of a synthetic system allows identification of system components and regulatory elements that require adjustments to produce the desired system function (Bowen et al., 2008; Purnick and Weiss, 2009). Using these resources synthetic biology expands our

understanding of natural biological systems and allows engineering of novel functions in living organisms for a useful purpose. The potential applications of synthetic biology are vast and we are only beginning to explore the possibilities.

One application of synthetic biology is the production of biosensors. Research in the Medford laboratory focuses on the development and optimization of synthetic systems that enable plants to sense a specific substance and respond in an easily detectable manner. The development of detector plants requires a means by which an external stimulus can be converted into an intracellular response. The Medford lab used the vast amount of information provided by systems biology to identify components with which a synthetic signal transduction system might be constructed.

II. Introduction to Natural Signal Transduction

Living organisms have evolved a variety of signal transduction pathways allowing them to sense and respond to environmental factors and internal conditions. Phospho-transfer between proteins in a pathway is a key mechanism regulating signal transduction (Schaller, 2008). In prokaryotes, phospho-transfer typically occurs between conserved histidine and aspartate residues of signaling proteins in two-component signaling systems, also referred to histidine kinase (HK) signaling systems. However, in eukaryotes phospho-transfer is usually more complex and can occur between serine, threonine, and tyrosine residues as well as between histidine and aspartate residues of signaling proteins (Kieber and To, 2008).

A. Histidine Kinase Signaling

Bacteria, fungi, and plants use HK systems to sense environmental factors, such as the presence of ligands, osmotic and oxidative conditions, or pathogenic factors (Stock et al., 2000; Mizuno, 2005; Nemecek et al., 2006). In the simplest form, a HK or two-component signaling system uses a sensor histidine kinase protein that uses ATP to trans-phosphorylate a conserved histidine residue (autophosphorylate) on the adjacent subunit of a given HK dimer, in response to a particular stimulus (Mizuno, 2005). The phosphate is then transferred to a conserved aspartate residue on the receiver domain of a response regulator (RR). The RR then mediates downstream signaling responses such as transcriptional regulation or phospho-transfer to other regulatory proteins (Schaller, 2008). This simple signaling system allows organisms to perceive a particular stimulus and respond with a change in transcriptional regulation using only two proteins. Some more complex systems use an intermediate molecule, referred to as a histidine phospho-transfer protein (Hpt) to transmit a signal, in addition to the sensor HK and RR.

B. Bacterial Histidine Kinase Signaling Systems

One HK signaling system in bacteria regulates phosphate uptake and metabolism. This signal transduction system consists of a transmembrane sensor HK, PhoR, and a RR, PhoB (Hsieh and Wanner, 2010). Under phosphorus starvation conditions, PhoR is autophosphorylated at a conserved histidine residue and transfers a phosphate signal to PhoB. Upon phosphorylation PhoB regulates transcription of

downstream phosphorus starvation response genes (Hsieh and Wanner, 2010) (Figure 1.1).

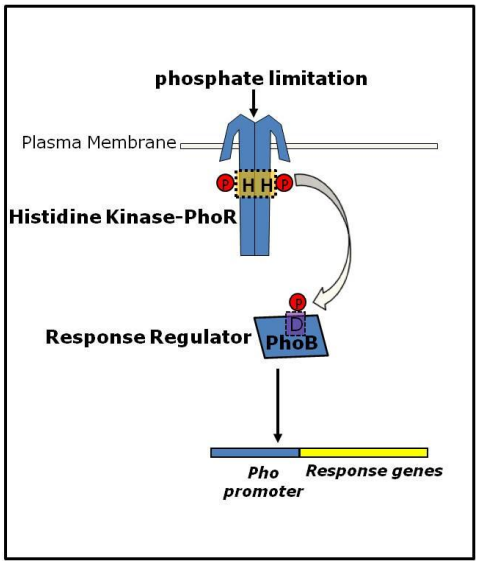

Figure 1.1 Diagram of phosphate signal transduction pathway. H: histidine; D: aspartate; P: phosphate signal

PhoR is a member of the class I or the, well-studied, EnvZ family of histidine kinases (Dutta et al., 1999). PhoR is localized to the plasma membrane containing two hydrophobic membrane-spanning regions (Scholten and Tommassen, 1993) followed by a highly helical region referred to as the charged region that may play a role in stabilizing membrane interactions. PhoR also contains a PAS domain, thought to mediate signal transduction through phosphate sensing and protein-protein interaction (Taylor and Zhulin, 1999). The exact mechanism by which PhoR senses low phosphate levels is unknown. However recent work found that the PAS (Period circadian Aryl hydrocarbon receptor Single-minded) domain of PhoR interacts with the PstB transporter and/or PhoU to regulate the downstream phosphorylation state of PhoB (Hsieh and Wanner, 2010). Similar to EnvZ, PhoR contains a catalytic and ATP-binding domain (CA) with the dimerization and histidine phosphorylation domain (DHp) to follow

(Carmany et al., 2003). The DHp domain contains the conserved histidine residue that is autophosphorylated upon activation of PhoR under phosphate limiting conditions.

PhoB is a member of the largest family of bacterial response regulators, the OmpR family (Lamarche et al., 2008). PhoB is made up of two functional domains, the receiver and effector domain. The N-terminal receiver domain accepts a phosphate from PhoR at the canonical Aspartate 53 (Ellison and McCleary, 2000). Upon phosphorylation PhoB undergoes a conformational change to reveal the C-terminal effector domain (Ellison and McCleary, 2000). The effector domain has a high affinity for a specific sequence of DNA, the *pho box*, and upon binding the *pho box* recruits RNA polymerase to activate transcription (Blanco et al., 2002). This simple two component system regulates the expression of 137 genes involved in phosphate transport and metabolism in *E.coli* (Vershinina and Znamenskaya, 2002).

C. Plant Histidine Kinase Signaling Systems

In plants, cytokinin is perceived through a HK phospho-relay pathway that regulates transcription of cytokinin response genes. At least three cytokinin receptors have been identified, Arabidopsis Histidine Kinase (AHK) 2, 3, and 4 (also known as CreI) (Kakimoto et al., 2001; Ueguchi et al., 2001b; Ueguchi et al., 2001c; Ueguchi et al., 2004). Previously the cytokinin receptors were thought to be localized to the plasma membrane, based on bioinformatic analyses and analogy with sensor HK localization in bacteria and yeast (Kakimoto et al., 2001; Ueguchi et al., 2001b). This was experimentally supported by the localization of an AHK3-GFP fusion protein overexpressed in Arabidopsis protoplasts (Kim et al., 2006) however, endomembrane localization was not excluded. Biochemical studies of cytokinin receptors shows that

maximal cytokinin-binding occurs at neutral or weakly alkaline pH (Romanov et al., 2006), which is characteristic for the endoplasmic reticulum (Tian et al., 1995) but not the apoplast. Recent work that directly examined the localization of the cytokinin receptors used expression of fluorescent fusion proteins and biochemical fractionation assays to show localization at the endoplasmic reticulum, yet did not rule out plasma membrane localization (Wulfetange et al., 2011).

The cytokinin receptors contain a CHASE (cyclase/Histidine kinase-associated sensing extracellular) domain that directly binds cytokinin followed by autophosphorylation of a conserved histidine residue on the cytoplasmic HK domain (Kieber and To, 2008). The phosphate signal is transduced to a conserved aspartate on the receiver domain of the cytokinin receptor, then to a conserved histidine on an Arabidopsis Histidine Phosphotransferase (AHP) (Schmulling and Heyl, 2003). There are five AHPs that are known to contain the conserved histidine residue required for phospho-transfer from AHKs. However, AHP4 has not been shown to be phosphorylated by any of the known cytokinin receptors while the other four AHPs have been shown to be redundant positive regulators of cytokinin signaling (Kieber et al., 2006). Upon phosphorylation AHPs transmit the signal to Arabidopsis Response Regulators (ARRs) which mediate downstream cytokinin responses. The mechanism by which the phosphate signal is transmitted to the nucleus is unclear. The previously accepted model of cytokinin signal transduction suggested bulk movement of phosphorylated AHPs to the nucleus in response to cytokinin. The work supporting this model examined the subcellular localization of AHPs using protoplasts (Sheen and Hwang, 2001) and tissue cultured cells (Mizuno et al., 2004). However, more recent

work examining the subcellular localization of AHPs in intact plants contradicts the idea that AHPs translocate to the nucleus in response to cytokinin, and shows that AHPs undergo constant, active, bidirectional nuclear-cytosolic transport independent of the phosphorylation state (Kieber et al., 2010). Based on these results the current model of cytokinin signal transduction proposes that active transport of AHPs into and out of the nucleus is regulated to move phosphoryl groups into the nucleus and mediate the phosphorylation state of ARRs (Kieber et al., 2010). The mechanism that regulates AHP nuclear-cytosolic transport is yet to be elucidated.

The predominantly nuclear localized type-B ARRs contain a receiver domain that accepts a phosphate signal from AHPs at a conserved aspartate residue and a C-terminal DNA binding domain that binds a specific sequence of DNA and activates transcription of target cytokinin response genes (Tajima et al., 2004; Yokoyama et al., 2007). Type-A ARRs are up-regulated in response to cytokinin by type-B ARRs and many type-A ARRs have been found to serve as negative regulators of cytokinin signaling (To et al., 2004). Other proteins reported to regulate cytokinin response genes in an AHK/AHP dependent manner are Cytokinin Response Factors (CRF). There are six known CRFs each containing a plant-specific AP2 DNA binding domain (Magnani et al., 2004) with the CRFs acting as transcription factors. CRFs were found to translocate to the nucleus and activate transcription of cytokinin response genes in response to cytokinin (Rashotte et al., 2006). The mechanism by which CRFs are induced by phospho-AHPs and translocate to the nucleus is unknown. Many cytokinin response genes regulated by CRFs are also regulated by type-B ARRs (Rashotte et al., 2006). In addition to the role CRFs play in cytokinin signaling, CRFs are thought to

receive input from other non-cytokinin dependent signaling pathways (Rashotte et al., 2006). Together components of the above described system interact to regulate important processes in plants including shoot and root development, de-etiolation, leaf expansion, root vascular differentiation, and senescence (Kieber and To, 2008) (Figure1.2).

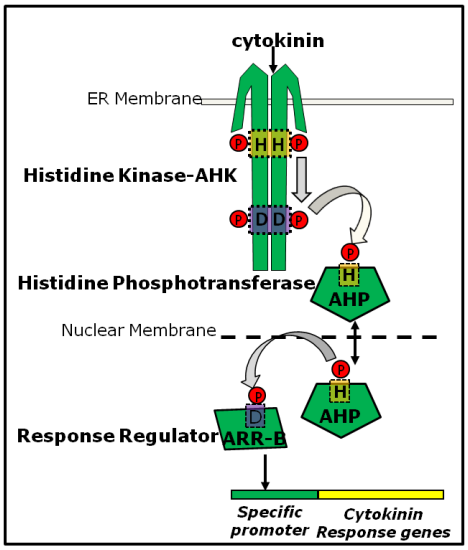

Figure 1.2 Diagram of cytokinin signaling pathway. H: histidine; A: aspartate; P: phosphate

III. Development of a Synthetic Signaling System for use in a Detector Plant

The production of biosensors requires the design of signaling systems that enable organisms to sense and respond to substances of interest. Plants have natural detection abilities and are ubiquitous in most human environments. These properties, along with their ability to cover wide areas, means plants could serve as inexpensive detectors provided they can be made to sense specific substances with high sensitivity and respond in an easily observable manner (Bowen et al., 2008). The Medford lab has developed a synthetic signal transduction system that links extracellular perception of a specific ligand to a transcriptional response that is easily observable in plants (Morey et al., 2011). Development of this synthetic signaling system required identification of

individual components that fill distinct functional roles in the system. Construction of a synthetic signaling system specifically for application to a detector plant required a system that is responsive to perception of a specific extracellular ligand. In doing so, we needed to identify components that serve three distinct functions; transmit the extracellular signal across the plasma membrane to the inside of the cell, translocate the signal across the nuclear membrane and activate transcription of a defined set of response genes. To fill these functional roles the Medford laboratory assembled characterized bacterial proteins from HK signaling systems that were specifically adapted to function *in planta,* to link extracellular perception of a specific ligand to a transcriptional response (Antunes et al., 2011; Morey et al., 2011).

A. The Partial Synthetic Signal Transduction System

First our laboratory developed a partial synthetic signaling system in plants based on conserved histidine kinase (HK) signal transduction systems. HK signal transduction systems exhibit relatively modular architecture built from a limited number of protein domains, with individual domains often conserved across pathways and species (Koretke et al., 2000; Stock et al., 2000; Ferreira and Kieber, 2005; Mizuno, 2005; Zhang and Shi, 2005). The *in planta* partial synthetic signaling system developed by our laboratory uses input from the endogenous cytokinin HK signaling pathway to phosphorylate the bacterial response regulator (RR) PhoB. As previously described, PhoB is a member of the two-component phosphate sensing pathway and is phosphorylated under phosphate limiting conditions by the bacterial phosphate sensor PhoR (Hsieh and Wanner, 2010). The development of a synthetic signaling system in

plants based on bacterial components requires the ability to transmit a signal across the nuclear membrane. To determine if the bacterial RRs PhoB and OmpR can translocate to the nucleus *in planta* in a signal dependent manner, RR-green fluorescent protein (GFP) fusions were produced and tested. Analyses (see Chapter 2) of transgenic Arabidopsis roots shows active, cytokinin dependent, nuclear translocation of PhoB-GFP and OmpR-GFP, with PhoB-GFP showing more robust nuclear translocation (Antunes et al., 2009). This work showed that the bacterial RR PhoB can translocate to the nucleus of a plant cell in a signal dependent manner.

The development of a synthetic signaling system *in planta* using bacterial signaling components also requires the ability to recruit eukaryotic transcriptional machinery to activate a transcriptional response. To test the bacterial RR for signal dependent transcriptional activation, PhoB was C-terminally fused to four copies of the eukaryotic transcriptional activator VP16, generating PhoB-VP64 (Antunes et al., 2009). To produce a PhoB-VP64 mediated transcriptional response, a synthetic *PlantPho* promoter was designed using four copies of the *Pho box* sequence (Blanco et al., 2002) upstream of a minimal plant promoter, *-46 CaMV35S*. Cytokinin dependent transcriptional activation via PhoB-VP64 was quantified using the *β-glucoronidase* (GUS) reporter gene under control of the synthetic *PlantPho* promoter. Transgenic Arabidopsis plants expressing the partial synthetic signaling system show an increase in GUS activity in response to exogenous cytokinin application (Antunes et al., 2009). This work demonstrates that PhoB-VP64 together with the synthetic *PlantPho promoter* can activate transcription *in planta* in a signal dependent manner. These data suggest that PhoB-VP64 could fulfill the biological function of signal dependent nuclear

translocation followed by transcriptional activation of defined response genes, as a member of a complete synthetic signaling system in plants.

The above study suggested that PhoB is phosphorylated by cytokinin signaling components endogenous to plants. As previously described, cytokinin is perceived by AHKs 2, 3, and 4 initiating a histidine to aspartate phospho-relay from the conserved histidine internally to the conserved N-terminal aspartate, before transfer to a conserved histidine on AHPs 1, 2, 3 or 5. Active bidirectional nuclear-cytosolic transport of AHPs, transmit the signal to the nucleus, by an unknown mechanism, where type-B ARRs activate transcription of cytokinin response genes (Ferreira and Kieber, 2005), including genes encoding CRFs. CRFs are also known to transmit a phosphate signal to the nucleus, however this signal is dependent on cytokinin induced AHK/AHP phosphorylation (Rashotte et al., 2006). Based on results showing that plants containing the partial synthetic signaling system respond to exogenous cytokinin application (Antunes et al., 2009), I hypothesize that PhoB can interact with up-stream cytokinin signaling components AHKs and/or AHPs (Figure 1.3).

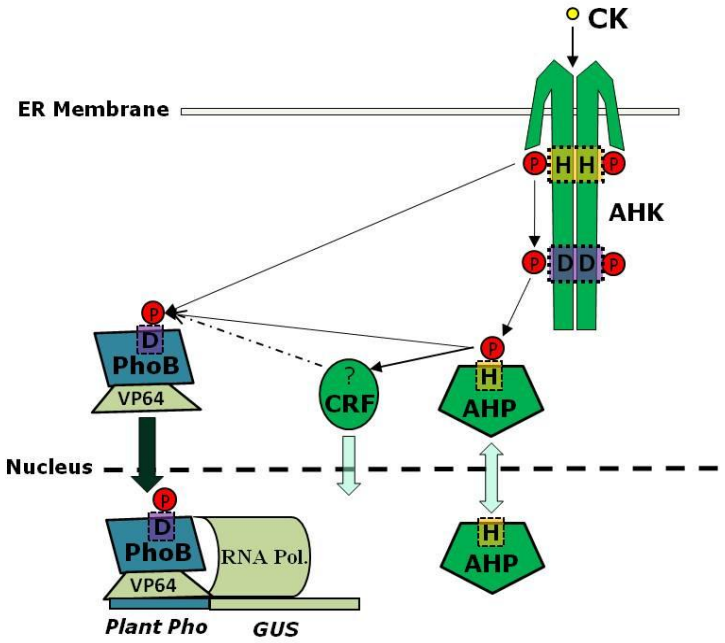

Figure 1.3 Model of possible phospho-transfer from cytokinin signaling components to PhoB. Possible mechanisms for cytokinin dependent transcriptional response in the partial synthetic signaling system follow: Cytokinin is perceived by the transmembrane sensor HK, AHK2, 3, or 4, that initiates a histidine (H) to aspartate (D) phospho-relay. One possible path is the high energy phosphate is transferred internally between H and D on the AHK, to a H on AHPs 1, 2, 3 or 5, to an D on PhoB-VP64, then PhoB-VP64 translocates to the nucleus, binds DNA at the *PlantPho promoter* and activates transcription of the GUS reporter gene; or another possible path is the phosphate is transferred from the H on the AHK directly to the D on PhoB-VP64, bi-passing the internal phospho-relay and AHPs; or another less probable path involves phospho-transfer from the AHP to a CRF and to the D on PhoB-VP64. P: phosphate signal; Block arrows represent whole protein movement; Line arrows represent phospho-transfer between proteins.

PhoB could interact with other proteins of the cytokinin signaling system such as CRFs or cytosolic localized ARRs, however these interactions seem improbable due to the phospho-relay characteristics of HK signaling components. For example, phospho-relay between HK signaling components typically occurs between histidine and aspartate residues and phospho-relay between aspartate residues of two proteins is not known to occur. A pathway involving phospho-transfer interaction between PhoB and ARRs would involve movement of the phosphate from aspartate to aspartate among proteins. Additionally, if the AHP-CRF interaction is typical of HK signal transduction pathways, phospho-relay would occur between the histidine on an AHP to an aspartate

on a CRF, followed by a novel phospho-transfer to an aspartate on PhoB (Figure1.1). Another reason that phospho-transfer interactions between PhoB and CRFs are unlikely, is that both proteins contain DNA binding domains acting as transcription factors in their natural systems, and neither protein is known to transfer a phosphate signal to other downstream signaling proteins. These characteristics of PhoB, CRFs, and ARRs do not preclude possible novel phospho-transfer interactions but I consider the hypothesized interaction between PhoB and AHPs and /or AHKs more likely (Figure 1.3).

B. The Complete Synthetic Signal Transduction System

Building on the partial system, our laboratory developed a complete synthetic signaling system that transmits extracellular perception of a specific ligand to a transcriptional response, and we applied this system to plants that serve as detectors (Antunes et al., 2011; Morey et al., 2011). In the partial synthetic signaling system described above, the bacterial RR PhoB was found to translocate to the nucleus and PhoB-VP64 was found activate transcription of the synthetic PlantPho promoter in a signal dependent manner *in planta.* These data suggest that PhoB could be used as a component of a complete synthetic signaling system to translocate into the nucleus and affect gene expression. Development of the complete synthetic signaling system required one additional component that functions to transmit extracellular perception of the ligand across the plasma membrane to the cytosolic localized PhoB-VP64 to regulate a transcriptional response. Additionally, development of a complete synthetic

signaling system suitable for application to a detector plant needs to be responsive to extracellular perception of the ligand of interest.

To detect a specific ligand of interest outside the cell for use in a detector plant, we used a periplasmic binding protein (PBP) scaffold in which the binding pocket was computationally designed to bind the novel ligand trinitrotoluene (TNT) (Looger et al., 2003; Antunes et al., 2011; Morey et al., 2011). Members of Dr. Homme Hellinga's laboratory at Duke University developed a computational design program that identifies changes to the amino acid sequence of the binding pocket of a bacterial PBP to produce novel binding proteins that bind a different ligand (Looger et al., 2003). PBPs have been computationally designed to produce proteins that bind non-cognate ligands TNT, L-lactate, serotonin (Looger et al., 2003), zinc (Marvin and Hellinga, 2001) and methyl tert-butyl ether (MTBE, unpublished). The complete synthetic signaling system was designed by our laboratory to be responsive to the computationally designed receptor tailored to bind the explosive TNT. The TNT receptor (TNT.R3) was designed using ribose binding protein (RBP) as a scaffold protein and the receptor was shown to have a binding affinity of two nanomolar TNT (Looger et al., 2003).

Heterologous expression of bacterial proteins for use in a synthetic signaling system in plants requires proper protein localization. In bacteria, wild-type RBP is secreted from the cell and localized to the periplasmic space. To facilitate direct contact between the computationally designed TNT receptor and an exogenous ligand, the TNT receptor was targeted to the plant apoplast using the Pex secretory sequence (Baumberger et al., 2003). The N-terminal fusion of the Pex secretory sequence to an RBP-GFP fusion shows apoplastic localization of the protein in transient assays with

onion epidermal cells (Antunes et al., 2011). Once localized to the apoplast, the computationally designed TNT receptor (ssTNT) should freely diffuse in the apoplastic space (Somerville et al., 2004) due to the relatively small size of the protein.

The final function needed to produce a complete synthetic signaling system was a means to transmit extracellular perception of the ligand across the plasma membrane and activate a transcriptional response. Dr. Jeff Smith of Homme Hellinga's laboratory used the vast amount of information provided by systems biology to produce a fusion between two proteins that links extracellular receptor-ligand binding to an intracellular PhoB-VP64 mediated response. The two bacterial proteins used to make the novel fusion protein were the chemotactic receptor Trg (Levit et al., 1998) and the phosphate sensor HK PhoR (Dutta et al., 1999). In natural systems, bacterial PBPs bind sugars then activate chemotactic proteins that modulate flagella movement to direct the bacteria towards food. Wild-type RBP, the scaffold used for the computationally designed TNT receptor, binds ribose and the receptor-ligand complex develops an affinity for the extracellular portion of the transmembrane chemotactic protein Trg which autophosphorylates upon binding (Levit et al., 1998) and modulates downstream responses. As previously described, the sensor HK PhoR transmits a phosphate signal to the RR PhoB under phosphate limiting conditions (Hsieh and Wanner, 2010). To transmit perception of the TNT ligand by the computationally designed TNT receptor to the cytoplasmic localized PhoB, the periplasmic and transmembrane regions of Trg were fused to the cytoplasmic DHp domain of the phosphate sensor HK, PhoR (Antunes et al., 2011; Morey et al., 2011). Multiple rationally designed fusions points were tested in bacteria. The phosphate sensing PAS domain was deleted from PhoR

and fusions were made at both the conserved DHp domain and the CR. Fusions are at position 267 in Trg and link PhoR at successive one amino acid points, to account for helix rotation in the HK dimers. When tested in bacteria, most fusions have a basal signal in the absence of the ligand or no induction in response to the ligand (Antunes et al., 2011). One fusion (DHP8), which fuses the Trg HAMP (present in histidine kinases, adenylyl cyclases, methyl accepting chemotaxis proteins, and phosphatases) domain (Hulko et al., 2006) to position M197of PhoR, showed the best ligand-dependent induction and was chosen for further analysis (Antunes et al., 2011).

The Medford laboratory hypothesized that the synthetic fusion HK Trg-PhoR could be used in plants, as a member of a complete synthetic signaling system serving to transmit a signal across the membrane to PhoB-VP64. If functional, this system would be suitable for application to a detector plant because it is responsive to extracellular perception of a ligand of interest by an RBP scaffold, transmits the signal across the plasma membrane to PhoB-VP64 which translocates to the nucleus and activates transcription of response genes.

Expression of Trg-PhoR, composed of bacterial protein domains, in plants required consideration of proper protein localization. To enable interaction between the apoplastically localized TNT receptor and the synthetic fusion HK, Trg-PhoR was targeted to the plasma membrane using the Arabidopsis flagellin sensor, FLS2 (Gomez-Gomez and Boller, 2000) signal peptide. This produced a plasma membrane targeted, novel fusion HK (Fls-Trg-PhoR) designed to accept input from a ligand bound-RBP scaffold, transmit the signal from the apoplast to the cytoplasm and phosphorylate the bacterial RR PhoB, completing the synthetic signaling system.

To determine whether the complete synthetic signaling system can produce a signal dependent response, transgenic Arabidopsis plants containing the complete system with the TNT receptor were tested for TNT dependent nuclear translocation of PhoB and transcriptional activation of the *PlantPho promoter*. I found that some plants show signal-dependent nuclear translocation of PhoB in response to TNT (data shown and described in Chapter 2) (Antunes et al., 2011). Other members of the lab analyzed transcriptional activation in plants exposed to TNT and found that some plants show a modest level of induction in response to TNT (Antunes et al., 2011). However, some plants show nuclear translocation or high levels of transcriptional activation in tissue that was not exposed to TNT, indicating substantial background signaling in the system. Background signaling may be caused by phosphorylation of PhoB by endogenous cytokinin signaling components. As seen in the partial synthetic signaling system, PhoB responds to exogenous cytokinin application (Antunes et al., 2009), suggesting cross-talk with the cytokinin signaling components. Nevertheless, the *in-planta* complete synthetic signaling system shows some nuclear translocation and transcriptional activation in response to exogenous TNT exposure.

C. TNT Detector Plants

The Medford lab generated TNT detector plants that produce an easily observable visible response to TNT, by placing a synthetic de-greening circuit (Antunes et al., 2006) under transcriptional control of the complete synthetic signaling system (Figure 1.4). The de-greening circuit simultaneously initiates the degradation of chlorophyll and inhibits synthesis of new chlorophyll (Antunes et al., 2006).

Chlorophyllase is thought to play a role in removing the hydrophobic tail (Benedetti and Arruda, 2002) and red-chlorophyll catabolite reductase (RCCR) cleaves the porphyrin ring (Pruzinska et al., 2005) to degrade chlorophyll while an *RNAi* to POR C, the

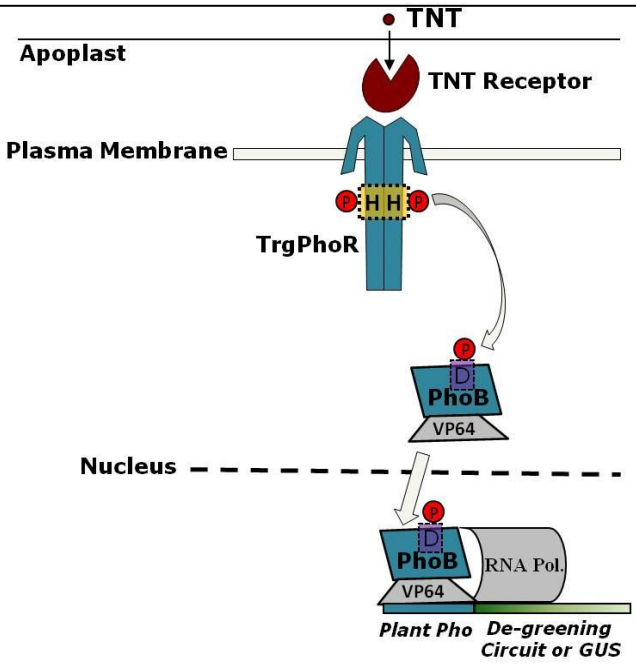

Figure 1.4 Schematic of complete synthetic signaling system in TNT detector plant. The synthetic signaling system links extracellular perception of the TNT ligand to a transcriptional response. The designed mechanism for signal transduction follows: the computationally designed TNT receptor binds TNT and the receptor-TNT complex develops an affinity for the extracellular portion of the synthetic fusion HK Trg-PhoR initiating a histidine (H) to aspartate (D) phospho-relay. The high energy phosphate signal (P) is transferred from the H on PhoR to a conserved D on PhoB-VP64 which translocates to the nucleus and binds the *PlantPho* promoter activating transcription of the de-greening circuit genes or the GUS reporter gene.

rate-limiting enzyme in chlorophyll biosynthesis (Masuda et al., 2003), inhibits synthesis of new chlorophyll. Transgenic Arabidopsis plants that contain the de-greening circuit under control of a hormone inducible promoter, show that activation of the de-greening circuit results in reduction of the photosynthetic efficiency of photosystem II (F_V/F_M). Reduction in photosynthetic efficiency is due to chlorophyll degradation causing the plants to lose their green color 48 hours after exposure to the hormone inducer (Antunes et al., 2006). We hypothesize that reactive oxygen species produced from

photosystem degradation caused by activation of the de-greening circuit, play a major role in driving the total loss of all pigments making the plants turn white instead of yellow or brown.

The Medford lab produced TNT detector plants by expressing the complete TNT responsive synthetic signaling system and the synthetic de-greening circuit under control of the *PlantPho* promoter. These plants show a loss of chlorophyll in response to TNT in the growth medium (Antunes et al., 2011). These transgenic plants are one of the first examples of detector plants and the first fully synthetic signaling system in a higher eukaryotic organism. The synthetic signaling system was built using a modular assembly of bacterial and plant protein domains to produce components with specific biological functions. Furthermore, this modular assembly enables modification and testing of individual components within the system to change overall system behavior. With refinement of signal transduction components, stronger and more specific signaling systems may be possible (Antunes et al., 2011).

This research explores cross-talk between cytokinin signaling components and the synthetic signaling system. Because cross-talk with components of other signaling pathways is an undesirable characteristic of the synthetic signaling system, I first addressed the question of whether single amino acid mutations in the RR PhoB can be identified that reduce interaction with cytokinin signaling components yet maintain function in the synthetic signaling system (Chapter Two). To further investigate cross-talk, I address the hypothesis that PhoB interacts specifically with AHPs and/or AHKs of the cytokinin signaling pathway (Chapter Three). To experimentally determine the interactions of PhoB with AHPs and/or AHKs without interference from other signaling

components endogenous to plants, the components of interest were examined using a simple bacterial testing system.

In addition to exploring cross-talk, this research investigated the functionality of the computationally designed receptors in providing input to the complete synthetic signaling system. A receptor, designed to bind the environmental pollutant methyl tert-butyl ether (MTBE), was tested in plants to determine the feasibility of producing an MTBE detector plant (Chapter Four). Also the MTBE receptor, along with the TNT receptor described above, were examined in bacteria to determine if the receptors function as reported, with our synthetic signaling system (Chapter Five).

Chapter Two

Functional Analysis of PhoB *In Planta*

I. Introduction

A. Cross-talk among Natural Signaling Systems

Cross-talk is a term used to describe signaling interactions between non-partner proteins (Wanner, 1992). These interactions are known to occur frequently in two-component signaling systems and can have a significant effect on signaling output and regulation of gene expression (Noriega et al., 2010). In some cases, non-specific interaction among signaling components can lead to the integration of two signaling systems that conveys a selective advantage. Some hypothesize that molecular cross-talk can act as a means for the evolution of new signaling systems resulting in improvement of an organism's ability to respond to their environment (Wanner, 1992).

Cross-talk among signaling components of different systems is common and is thought to occur in all organisms with known examples in bacteria, fungi, plants, and mammalian cells. The first example of molecular cross-talk was shown between components of the chemotactic and nitrogen response two-component signaling systems (Ninfa et al., 1988). Because two different signaling systems can exchange a functional phospho-relay, suggests that the signaling mechanisms are functionally conserved. Some cross-talk results in a functionally relevant system that conveys a selective advantage to the organism. This specific type of cross-talk is referred to as cross regulation (Wanner, 1992). One example of cross regulation in bacteria is the interaction between the nitrite sensor HK NarQ that normally interacts with NarP, and

the RR NarL that normally interacts with the nitrate sensor HK NarX (Noriega et al., 2010) resulting in dual regulation of the RR, NarL, by two distinct sensor HKs. Examples of non-specific cross-talk among bacterial signaling components are relatively common in *in vitro* experiments. Because specificity among HK signaling systems is important for proper regulation of gene expression in response to distinct stimuli, bacteria employ a variety of mechanisms to control non-specific interactions of signaling components *in vivo.* For example, in certain mutant *E.coli* lines where the phosphate sensor HK PhoR is not expressed, the RR PhoB is responsive to non-partner sensor HKs EnvZ and CreC (Kim et al., 1996; Lee et al., 2007). These non-partner interactions with PhoB are only observed in the absence of the partner sensor HK PhoR. In wild-type lines, PhoR has been shown to have phosphatase activity with PhoB when not stimulated by phosphate starvation (Carmany et al., 2003) serving to reconcile non-specific phosphorylation of PhoB by non-partner HKs. Phosphatase activity and specificity of recognition residues among partner proteins provides mediation of cross-talk allowing physiologically relevant signal transduction (Hoch and Varughese, 2001).

Unlike in bacteria, cross-talk and cross regulation among hormone signaling systems *in planta* is quite prevalent and serves to integrate hormone responses producing a network of signaling systems rather than a collection of distinct linear pathways. For example, the signaling systems of phytohormones cytokinin and auxin interact antagonistically to regulate cell elongation and differentiation in developing roots (Zheng et al., 2011). The cytokinin responsive TypeB ARR1 regulates transcription of the SHY2 gene that down-regulates auxin transport genes, limiting cell division and stimulating differentiation of root cells (Dello Ioio et al., 2008). Auxin promotes the

degradation of SHY2, reversing the response to stimulate auxin transport and subsequent cell division. Balanced interaction between these systems results in proper root development and morphology. Cross-talk is known to occur among the ethylene and jasmonic acid (Onkokesung et al., 2010) and abscisic acid (Anderson et al., 2004) signaling systems in the regulation of herbivory defense responses. *In planta* HK signaling systems are thought to share some downstream components. For example, the cytokinin and ethylene receptor(s) are both known to interact with AHP1 (Schmulling and Heyl, 2003; Scharein and Groth, 2011). Also the cytokinin responsive ARR4 has been shown to interact directly with the photoreceptor phytochrome B (Sweere et al., 2001). There is evidence supporting the existence of some form of cross-talk in all known phytohormone signaling systems.

B. Role of PhoB in the Synthetic Signaling System

The bacterial RR PhoB is a key component of the synthetic signaling system, described in Chapter One, serving the functions of signal dependent nuclear translocation and transcriptional activation of defined response genes. When developing the synthetic signaling system, a point of concern in using a bacterial RR in a plant cell is the ability of the protein to cross the nuclear membrane in a signal-dependent manner. To determine the nuclear translocation ability of the bacterial RR PhoB *in planta*, green fluorescent protein (GFP) was C-terminally fused to PhoB (PhoB-GFP) and analyzed in transgenic Arabidopsis plants. Visualization of roots using epi-fluorescent microscopy shows PhoB-GFP is diffuse throughout the cells in the absence

of cytokinin and localizes in the nucleus following treatment with exogenous cytokinin (Figure 2.1) (Antunes et al., 2009).

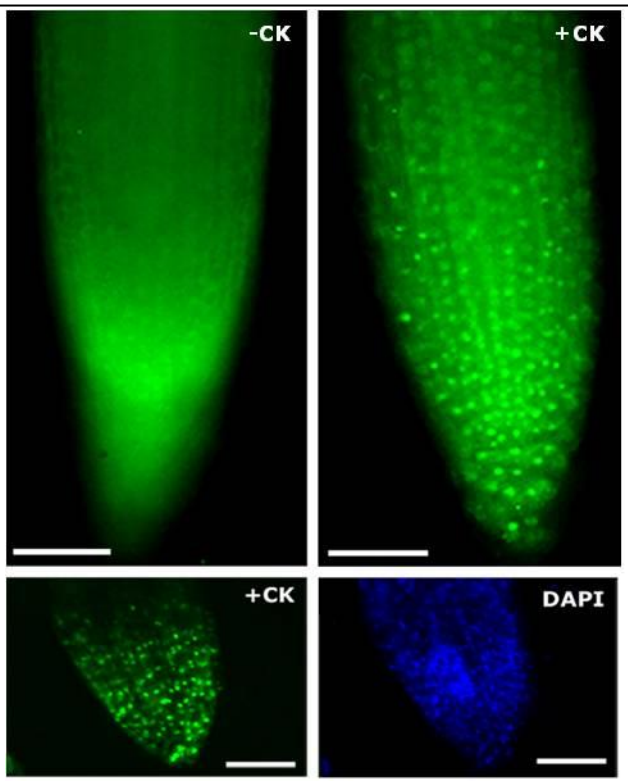

Figure 2.1 Test for cytokinin dependent nuclear translocation of PhoB. Epi-fluorescent images of roots of transgenic Arabidopsis plants expressing PhoB-GFP. Roots were imaged before cytokinin application and after exposure to 1μM t-zeatin (cytokinin). Roots were stained with 1ng μL^{-1} DAPI to confirm nuclear localization of PhoB-GFP. CK: cytokinin; Scale bars = 50μm (Antunes et al., 2009).

Subcellular localization of PhoB-GFP was examined in leaf and crown tissues and show cytokinin dependent nuclear translocation similar to that seen in roots (Antunes et al., 2009). To determine whether PhoB actually moves into the nucleus or accumulates at the nuclear membrane, fluorescence patterns were examined in more detail using a confocal microscope (Antunes et al., 2009). Our laboratory also showed that nuclear translocation of PhoB requires active transport. The β-glucuronidase (GUS) coding sequence was added to the PhoB-GFP fusion to produce a protein that is too large to diffuse across the nuclear membrane. Analysis of subcellular localization of PhoB-GFP-

GUS shows that PhoB is actively transported across the nuclear membrane in response to exogenous cytokinin (Antunes et al., 2009).

To determine whether PhoB can activate transcription in a signal dependent manner the eukaryotic transcriptional activator VP64 was C-terminally fused to PhoB. PhoB-VP64 was tested in transgenic Arabidopsis plants for transcriptional activation of the *PlantPho* promoter in response to exogenous cytokinin. The GUS reporter gene was placed downstream of the *PlantPho* promoter to measure transcriptional activation. GUS activity was quantified in tissue of transgenic Arabidopsis in response to cytokinin. As shown in Figure 2.2, GUS activity increases in tissue exposed to increasing concentrations of cytokinin, showing that PhoB-VP64 activates transcription of response genes in a signal dependent manner *in planta* (Antunes et al., 2009). This work suggests that PhoB can be used as a member of a complete synthetic signaling system serving to translocate a signal across the nuclear membrane and activate transcription of defined response genes.

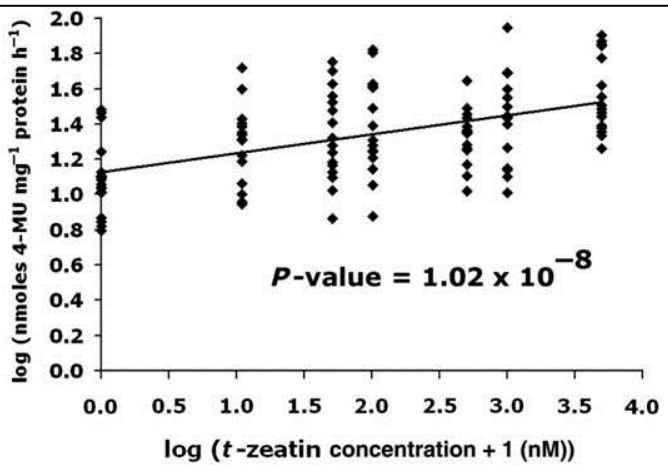

Figure 2.2 Test for cytokinin dependent transcriptional activation of partial signaling system. Linear regression of GUS activity in transgenic Arabidopsis plants expressing PhoB-VP64 and the GUS reporter gene under control of the *PlantPho promoter.* GUS activity (nmoles 4-MU mg^{-1} protein h^{-1}) increases with increasing concentration of t-zeatin. 4-MU: 4-methylumbelliferone (Antunes et al., 2009).

As previously described the complete synthetic signaling system is composed of the synthetic fusion HK Trg-PhoR, the RR PhoB, and the *PlantPho* promoter controlling the response gene(s) (Antunes et al., 2011; Morey et al., 2011). The complete synthetic signaling system was designed to be responsive to ligand-binding by the computationally designed TNT receptor (Looger et al., 2003). This system was designed so that the TNT receptor/TNT complex activates the synthetic fusion HK Trg-PhoR and a histidine to aspartate phospho-relay is initiated. The phosphate signal is transferred from a conserved histidine on PhoR to the canonical phopho-accepting aspartate 53 of PhoB. Upon phosphorylation PhoB translocates to the nucleus and activates transcription of response genes controlled by the *PlantPho* promoter (Figure 1.2) (Antunes et al., 2011).

To determine if PhoB could be used in the complete synthetic signaling system to accept a signal from Trg-PhoR and translocate to the nucleus I analyzed the subcellular distribution of PhoB-GFP in transgenic Arabidopsis plants expressing the complete synthetic signaling system with the computationally designed TNT receptor. I visualized roots of these plants with a compound and confocal microscope before and after exposure to TNT. Some plants show TNT dependent nuclear translocation of PhoB-GFP (Figure 2.10 in the results section) (Antunes et al., 2011), yet several plants show nuclear localized PhoB-GFP prior to induction with TNT. This could be a result of cross-talk or phosphorylation of PhoB by endogenous plant signaling components such as those of the cytokinin signaling system.

Other members of the Medford laboratory determined if PhoB-VP64 can activate transcription as a member of the complete synthetic signaling system. Transgenic

Arabidopsis plants containing the complete synthetic signaling system with the computationally designed TNT receptor and the GUS reporter gene controlled by the *PlantPho* promoter were analyzed for changes in GUS activity in response to TNT exposure. These plants show a significant increase ($P = 3.96 \times 10^{-93}$) in GUS activity in response to the TNT ligand. Statistically, the low R^2 value ($R^2 = 0.09$), indicates noise in the system. The noise is also apparent as GUS activity in the absence of the TNT ligand (Figure 2.3) (Antunes et al., 2011).

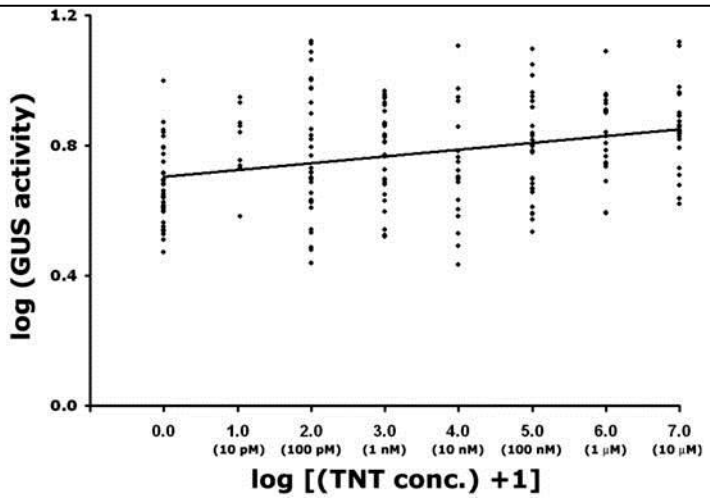

Figure 2.3 Test for TNT dependent transcriptional activation of complete synthetic signaling system. Linear regression of GUS activity in transgenic Arabidopsis plants expressing *ssTNT*, *Fls-Trg-PhoR*, *PhoB-VP64* and the *GUS* reporter gene under control of the *PlantPho promoter.* (Antunes et al., 2011)**.**

C. Cross-talk in the synthetic signaling system

The current synthetic signaling system allows for a TNT inducible response in detector plants. However, as shown by the partial synthetic system, PhoB is responsive to cytokinin, suggesting interaction with cytokinin signaling components (Figure 2.1 and 2.2). The possibility that PhoB can be activated, in response to an endogenous plant hormone, presents complications for the use of PhoB in a reliable detector plant system.

Hence, I aimed to reduce interaction between PhoB and endogenous signaling components while maintaining interaction with the synthetic signaling components, by mutating key amino acids (AAs) in PhoB (Figure 2.4).

The development of a synthetic signaling system that acts independently of endogenous plant signaling systems is important to the production of a functional and reliable detector plant. The goal of the work described in this chapter was to identify amino acids in PhoB that when mutated reduce the response to endogenous cytokinin signaling components but maintain the response to the synthetic signaling components *in planta*. By focusing on amino acids of interest and producing mutated versions of PhoB, the functionality of each mutation can be tested independently in response to cytokinin and TNT. Comparison of the response to cytokinin and the response to TNT, in plants containing each mutated version of PhoB, allows for identification of amino acid residue(s) in PhoB that reduce the response to cytokinin while maintaining the response to TNT. Identification of AAs of PhoB that reduce unwanted interactions may lead to the production of synthetic signaling components that are not responsive to endogenous signaling components, which is important in the development of a reliable detector plant.

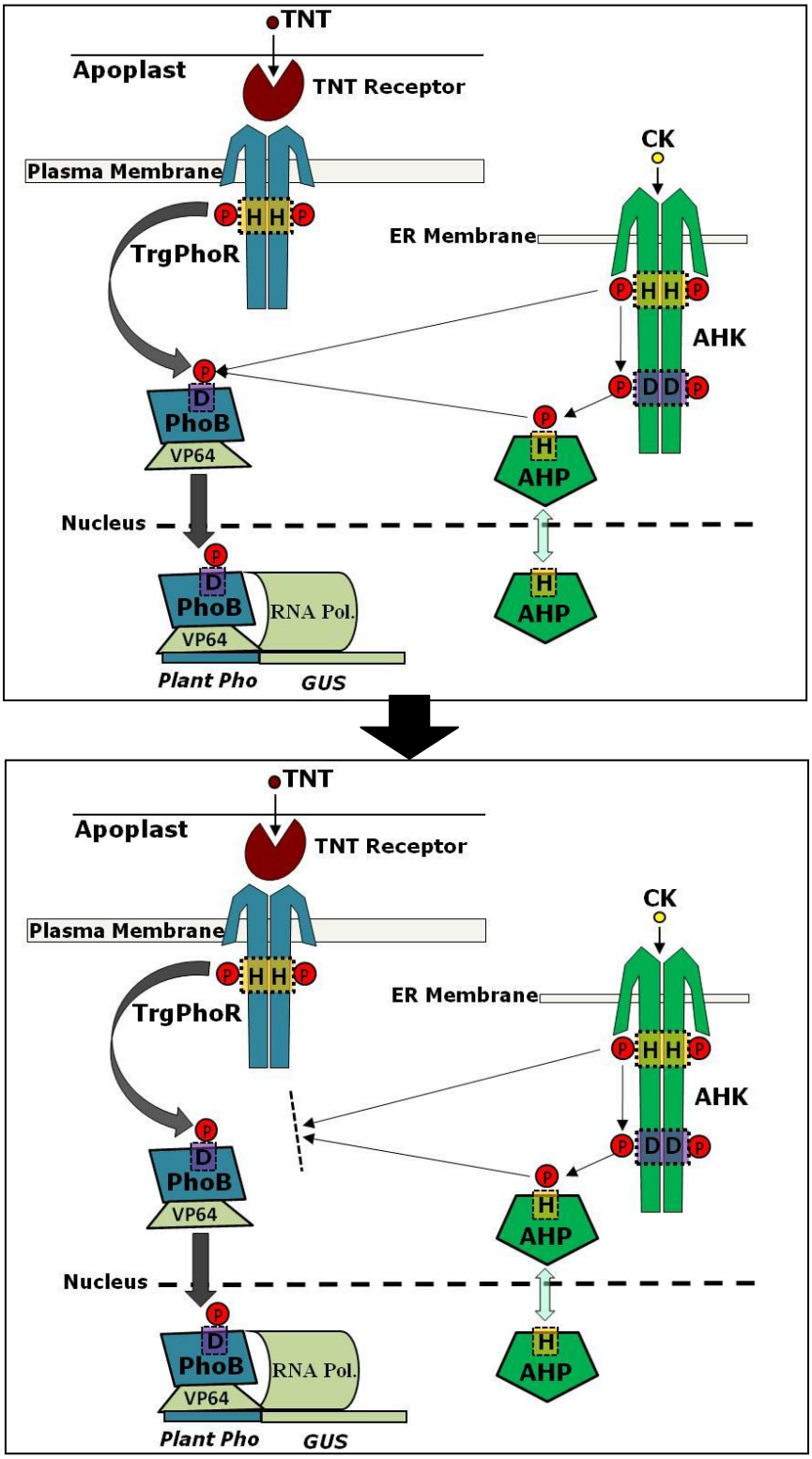

Figure 2.4 Schematic of synthetic signaling system with cytokinin signaling components. The top panel represents the possible phospho-relay interaction between cytokinin signaling components and PhoB. The bottom panel represents reduced interaction between cytokinin signaling components and a mutant version of PhoB that maintains response to the synthetic signaling system.

D. Selection of PhoB Mutations

I first determined whether or not the canonical phospho-accepting aspartate 53 (D53) of PhoB is required to receive input from cytokinin signaling components as well as the synthetic signaling components as is required in the natural bacterial system. To accomplish this, a mutated form of PhoB was generated by site directed mutagenesis, in which D53 was changed to an alanine ($PhoB^{D53A}$). This mutated form of PhoB was tested independently for signal dependent nuclear translocation and transcriptional activation in plants with input from both cytokinin and TNT.

Identification of amino acids that may play a role in the interaction between PhoB and endogenous cytokinin signaling components, including possible alternate phosphorylation sites, was done by selecting conserved amino acids of PhoB that can accept a phosphate. The amino acid sequence of PhoB was aligned with that of another bacterial response regulator, OmpR, and AHP1 and AHP2 (Figure 2.5).

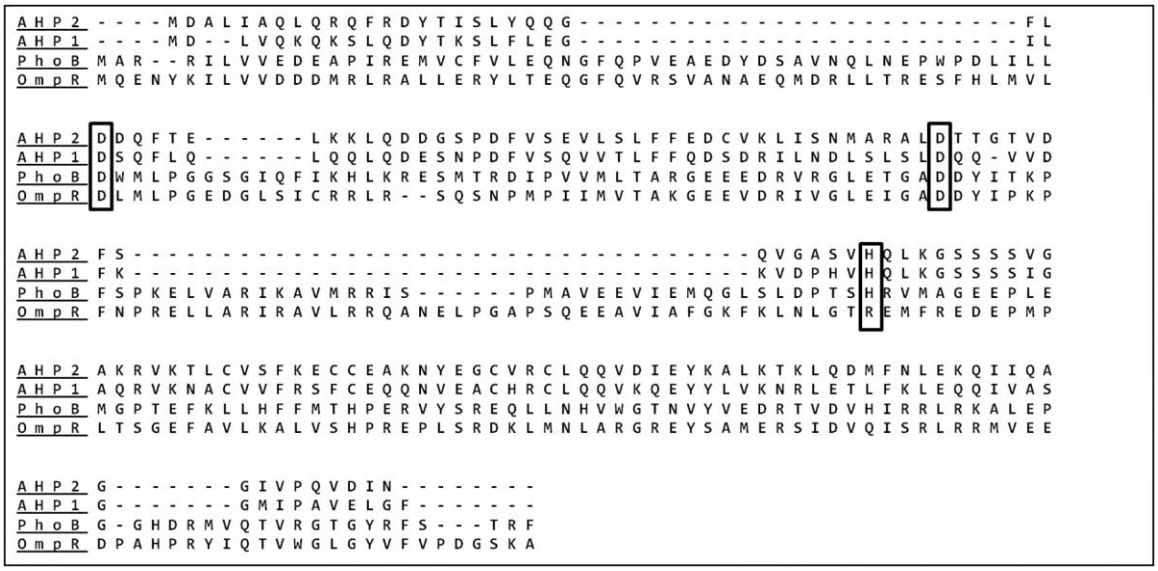

Figure 2.5 Alignment between AHP1, AHP2, PhoB, and OmpR. Conserved amino acids chosen for mutation, D53, D100, and H144, of PhoB are boxed.

This was done by Dr. Neera Tewari-Singh to identify candidate amino acids that could be phosphorylated and seem to be conserved across signaling components. AHPs do not appear to have structural homology to PhoB. However, AHPs are critical components of the cytokinin signaling pathway (Ferreira and Kieber, 2005) and share functional characteristics with heterologously expressed PhoB, including receiving a phosphate signal from the cognate HK and transmitting that signal to the nucleus. Upon identification of amino acids of interest, mutated versions of PhoB were generated in which these residues were changed to an alanine and/or another residue that carries a similar charge as the original.

PhoB amino acids selected for mutation (in addition to D53), included aspartate 76 (D76), aspartate 100 (D100), and histidine 144 (H144). Aspartate 76 does not appear to be conserved across signaling components. However, the crystal structure for the receiver domain (Sola et al., 1999) indicates that D76 is in the same three dimensional plane and is proximal to the canonical phospho-accepting D53. Aspartate 76 was changed to a similarly charged glutamate (D76E), to presumably have minimal impact on the structure of PhoB, yet not able to accept a phosphate from plant signaling components. Aspartate 100 was selected for mutation because it is conserved among the signaling components used in the previously described alignment. Also, D100 is in the same three dimensional plane and is proximal to D53 (Figure 2.6).

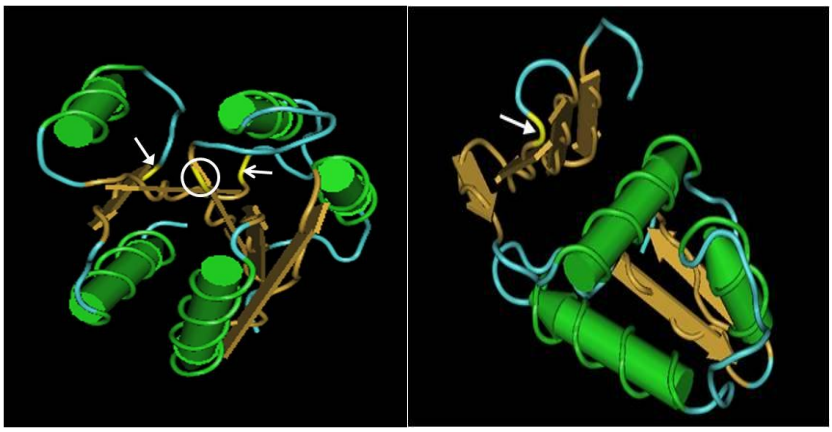

Figure 2.6 Representation of crystal structure of PhoB. The receiver domain is shown in the left panel and the effector domain is shown in the right panel. In the receiver domain, the circle represents the position of D53, closed arrow head represents the position of D100, and the open arrow represents the position of D76. In the effector domain, the closed arrow represents H144. Images generated by Cn3D structure viewer (www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d).

Aspartate 100 was replaced by two different AAs independently; to an alanine (D100A), lacking any significant charge, and to a similarly charged glutamate (D100E). Histidine 144 is located on the effector domain of PhoB (Figure 2.6), and aligns with the phospho-accepting histidine of AHP1 and AHP2 (Figure 2.5). Histidine 144 was also replaced by two different AAs independently; to an alanine (H144A) and to a similarly charge arginine (H144R).

II. Materials and Methods

A. Plasmid Construction

1. PhoB Mutants

All mutant versions of *PhoB-VP64* were generated using QuikChange Site Directed mutagenesis kit from Stratagene, LaJolla, CA. All primers were synthesized by IDT, Coralville, IA. Four of the six mutant versions of PhoB were generated by Dr. Neera Tewari-Singh. I cloned each mutant version of *PhoB-VP64* into an already existing plasmid assembled by Dr. Mauricio Antunes, in which the pCambia2300 binary

vector contains the wild-type *PhoB-VP64* fusion under control of the *FMV* promoter with *NOS* terminator. This plasmid also contains the synthetic *PlantPho* promoter controlling the *β-glucuronidase (GUS)* reporter gene with *NOS* terminator. After insertion of PhoB mutants into the already existing plasmid, each PhoB mutant was sequence verified (Macrogen USA). The pCambia binary vector carries two copies of the *Neomycin phosphotransferase II* gene (*NPTII*), confers resistance to the antibiotic kanamycin sulfate, as a selectable marker. Each copy of the *NPTII* gene is controlled by a distinct promoter to provide kanamycin resistance in plants and bacteria (Figure 2.7).

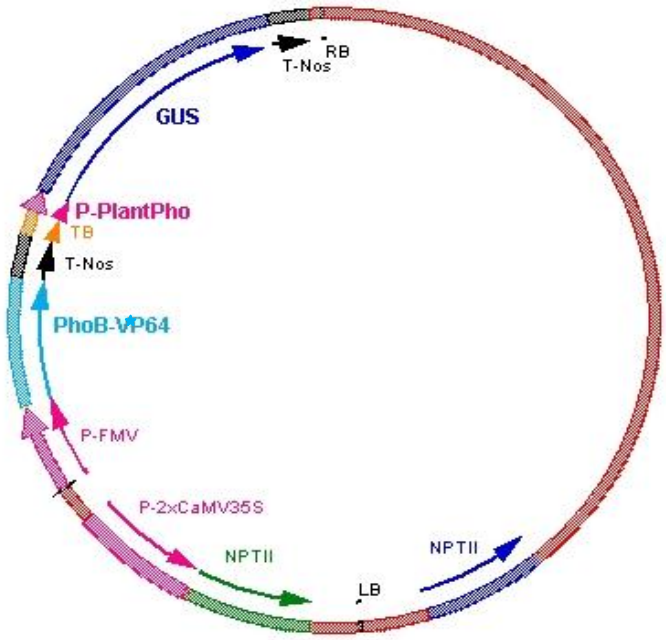

Figure 2.7 Map of plasmid containing a mutated version of *PhoB-VP64* and the *GUS* reporter gene controlled by the *PlantPho* promoter, with plasmid backbone of pCambia 2300. *T-Nos*: *Nopaline Synthetase* terminator; *TB*: *Transcription block*; *P-FMV*: Figwort Mosaic Virus promoter; *P-2xCaMV35S*: enhanced Cauliflower Mosaic Virus *35S promoter*; *NPTII*: selectable marker; *neomycin phosphotransferase II*, provides resistance to the antibiotic kanamycin sulfate; RB: right border; LB: left border. There are two copies of the *NPTII* gene, the green *NPTII* is under control of a plant promoter and the blue *NPTII* is under control of a bacterial promoter.

2. Synthetic signaling components

To test the mutant versions of PhoB with the complete synthetic signaling system, I co-transformed the above described plasmid with a plasmid previously constructed by Dr. Mauricio Antunes. This plasmid was used in the TNT detector plants (Antunes et al., 2011) described in Chapter One in which the synthetic fusion HK *Trg-PhoR* and computationally designed *TNT receptor* were assembled on the binary vector pCB302-3. The *Pex secretory sequence* (ss) was fused to the *TNT.R3* receptor using overlapping extension PCR and the resulting *ssTNT.R3* gene cloned downstream of the *CaMV35S promoter* in pCB302-3. The signal peptide from the Arabidopsis *FLS2* gene (At5g46330) was fused to the start codon of *Trg-PhoR*, and cloned into pCB302-3 with *ssTNT.R3* (Antunes et al., 2011). The vector pCB302-3 carries the *Bar* gene which confers resistance to the herbicide glufosinate ammonium for selection in plants (Figure 2.8).

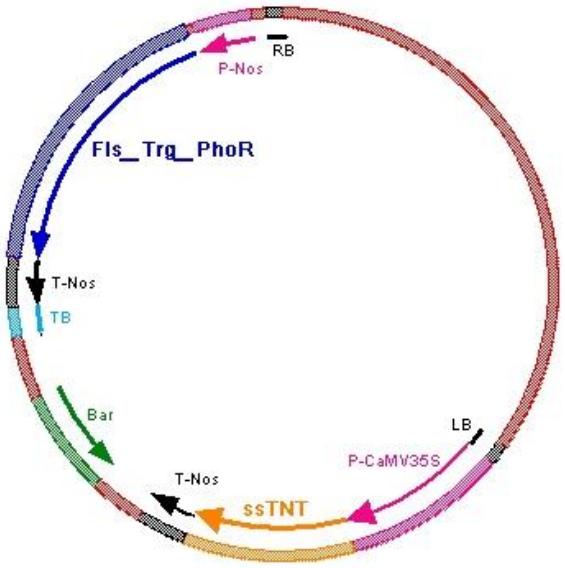

Figure 2.8 Map of plasmid containing synthetic signaling components *Fls-Trg-PhoR* and the computationally designed *TNT receptor*, with plasmid backbone of pCB302-3. *Fls-Trg-PhoR*: *Fls signal peptide* sequence fused to synthetic HK, *Trg-PhoR*; *P-Nos*: *Nopaline Synthetase* promoter; TB: Transcription block; *Bar:* selectable marker, confers resistance to the herbicide glufocinate ammonium; *T-Nos*: *Nopaline Synthetase* terminator; *P-CaMV35S*: Cauliflower Mosaic Virus *35S promoter*; *ssTNT*: *Pex secretory sequence* fused to computationally designed *TNT receptor;* RB: right border; LB: left border.

3. PhoB_GFP

To visualized sub-cellular localization of PhoB^{D53A}, I replaced wild-type PhoB with the mutant version in a previously constructed *PhoB-smGFP* fusion in the binary vector pCB302-3 constructed by Dr. Kevin Morey (Antunes et al., 2009). I cloned the resulting *PhoB^{D53A}-smGFP* fusion into pCambia2300 (Figure 2.9). This was done so that plants containing both the kanamycin resistant T-DNA (pCambia) and the glufocinate ammonium resistant T-DNA (pCB302-3) could be selected, allowing for testing of PhoB^{D53A} with the complete synthetic signaling system for response to TNT.

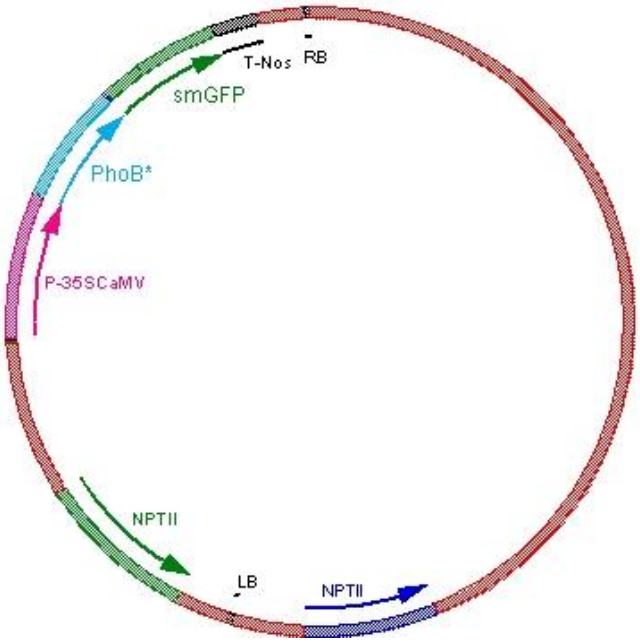

Figure 2.9 Map of plasmid containing PhoB mutant fused to smGFP with pCambia 2300 backbone. *P-35SCaMV*: Cauliflower Mosaic Virus 35S promoter; *T-Nos*: *Nopaline synthetase* terminator; *NPTII*: Selectable marker, conveys resistance to the antibiotic kanamycin sulfate; RB: right border; LB: left border.

B. Plant Material and Transformation

Arabidopsis thaliana, ecotype Columbia (*Col-0*), grown under a 16-h light (70-100 $\mu E.m^{-2}.s^{-1}$ fluorescent light)/8-h dark cycle, at 25° C, was used for experiments. Plants

were transformed with two types of *Agrobacterium* GV3011, each harboring one of the plasmids described above, following standard procedures (Clough and Bent, 1998). The T_0 seeds were sterilized and plated on general plant growth medium, Murashige and Skoog (MS) agar, supplemented with 50mg/l kanamycin sulfate, Sigma-Aldrich, St. Louis, MO, for selection of plants containing the derivative of the pCambia 2300 T-DNA, and 5mg/l Glufosinate ammonium (BASTA), Crescent Chemical Islandia, NY, for selection of the derivative of the pCB302-3 T-DNA. Following assays all plants were transferred to soil and grown to maturity for seed collection.

Transgene copy number has been shown to affect expression levels of transgenes (Matzke et al., 1996) which can affect the activity and interactions of transgene products. To control for T-DNA copy number, seed from the T_0 generation was germinated on selective media and segregation analysis (Appendix A) was performed, using chi-squared calculations, to select lines containing only a single copy of each T-DNA for further analysis.

C. GUS Assays

Leaves one and two from fourteen-day-old plants containing the T-DNA(s) described above were incubated for 14-16 h in water (control), or water plus t-zeatin or TNT with concentrations as specified. Leaves were ground in an extraction buffer and extract was exposed to the β-glucuronidase (GUS) enzyme substrate methylumbelliferyl glucuronide (MUG) then fluorometric measurements of the product, 4-methylumbelliferone (4-MU), were performed on a DynaQuant 200 fluorometer, Hoefer Inc, San Francisco, CA (Gallagher, 1992), kindly provided by the Bush lab. GUS

activity was normalized to the total protein content of each sample and expressed as nmoles 4-MU^{-1} mg $protein^{-1}$ hour. Total protein content of samples was measured with the Bradford reagent, Bio-Rad Laboratories, Hercules, CA.

D. Microscopy

Sub-cellular localization of PhoB_smGFP fusion protein (and the mutant $PhoB^{D53A}$_smGFP) was visualized in roots from six-day-old plants before and after treatment with 1 mM t-zeatin or 10 µM TNT for a minimum of 2 hours, using either a Carl Zeiss LSM 510 META confocal microscope with 20X lens (Plan-Neofluar 0.3NA) in multi-track mode and in-line switching with a scan speed of 5, or an inverted Nikon Diaphot epifluorescence microscope equipped with GFP (excitation 480/20, emission. 510/20) and DAPI (excitation 350/50, emission 460/50) filters. Stacks of 0.5 – 2 µm were taken with Carl Zeiss LSM 510 META confocal microscope using manufacturer-defined filter sets for DAPI and GFP. Nuclei were visualized after staining tissues with 1 ng/µL DAPI in water, Sigma-Aldrich, St.Louis, MO.

III. Results

A. Analysis of sub-cellular distribution of PhoB in the complete synthetic signaling system

Prior to analysis of mutant versions of PhoB, I determined the ability of wild-type PhoB to translocate to the nucleus in a signal dependent manner as a member of the complete synthetic signaling system *in planta*. Transgenic Arabidopsis plants expressing *ssTNT*, *Fls-Trg-PhoR*,and *PhoB-GFP* were generated and the sub-cellular

distribution of PhoB-GFP was examined. Initial transformants were germinated on selective media then selected individuals were transferred to soil and grown for seed. Segregation analysis was performed to select lines containing a single copy of each T-DNA. Roots from six-day-old plants were visualized before and after exposure to TNT. Before exposure to TNT, PhoB is diffuse with some GFP fluorescence seen localized to nuclei. After a minimum two hours of incubation in 10 μM TNT, nuclear localization of PhoB-GFP was observed in many cells (Figure 2.10). Numerous roots from at least seven independent transgenic lines were examined using both the compound and confocal microscopes.

These data show that PhoB can accept a signal from Trg-PhoR and translocate to the nucleus *in planta*. However, the mechanism by which PhoB accepts the signal from PhoR and if the canonical phospho-accepting aspartate 53 is required for signal dependent nuclear translocation was yet to be elucidated.

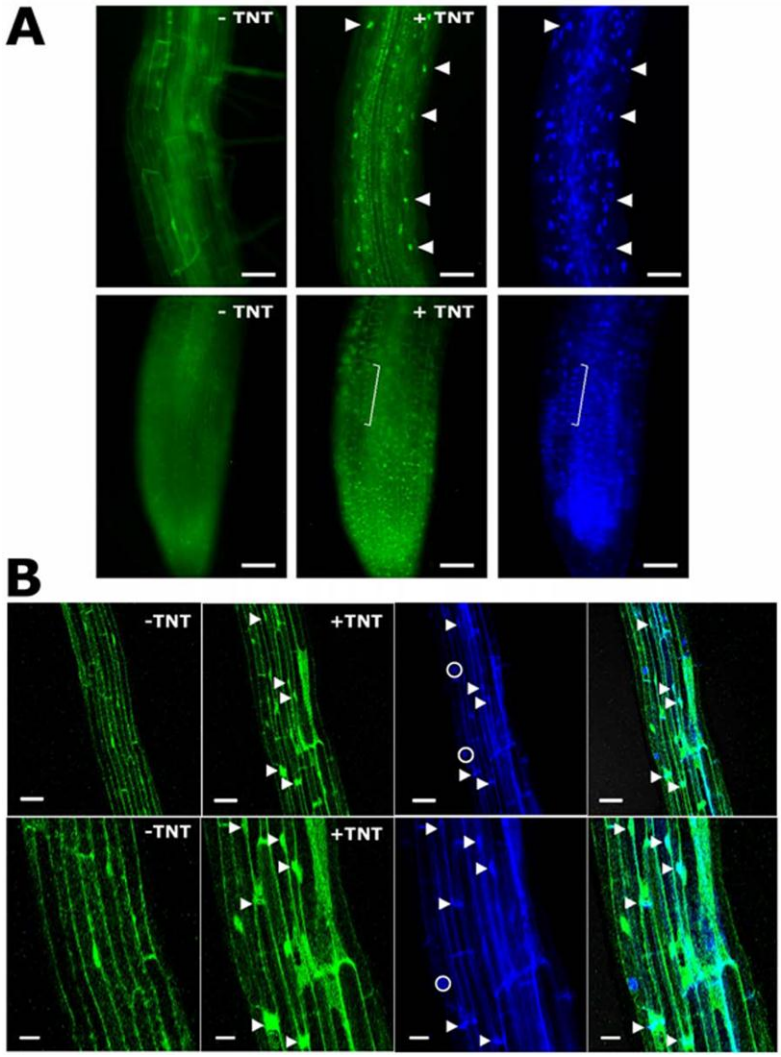

Figure 2.10 Test for TNT dependent nuclear translocation of PhoB in the complete synthetic signaling system. (A) Epi-fluorescence images of transgenic Arabidopsis roots containing ssTNT, Fls-Trg-PhoR and PhoB-GFP. Top Panels show upper portion of root. Bottom panels show root tip. Bracket shows nuclei evident after TNT treatment. Right-most panels show DAPI nuclear staining. Arrowheads indicate nuclei. Scale bar = 25 µm. (B) Confocal images of transgenic Arabidopsis roots. Top Panels show upper portion of root, Scale bar = 50 µm. Bottom Panels,show maginfied image of same root. Scale bar = 20 µm. Panels with blue color show DAPI nuclear staining. Rightmost panels show overlay of GFP and DAPI images. Arrowheads indicate nuclei and circles indicate the base of a root hair (Antunes et al., 2011).

B. Testing of PhoB^{D53A} for response to cytokinin and TNT

1. Results

To determine if PhoB^{D53A} translocates to the nucleus in response to exogenous cytokinin or TNT, a plasmid containing *smGFP* fused to the C-terminal end of *PhoB^{D53A}* was assembled. Transgenic Arabidopsis plants containing this plasmid were produced and segregation analysis was performed to select lines carrying a single copy of each T-DNA. Roots from six-day-old selected Arabidopsis plants were visualized to determine sub-cellular localization of PhoB^{D53A}-GFP before and after exposure to cytokinin or TNT. Before treatment, PhoB^{D53A}-smGFP shows some GFP fluorescence localized to nuclei. After treatment with cytokinin, generally PhoB^{D53A}-smGFP did not exhibit a uniform pattern of nuclear localization. Numerous roots from at least 10 independent transgenic lines were examined and I found that in the presence of exogenous cytokinin, PhoB^{D53A}-smGFP shows highly variable nuclear translocation (Figure 2.11) (Antunes et al., 2009).

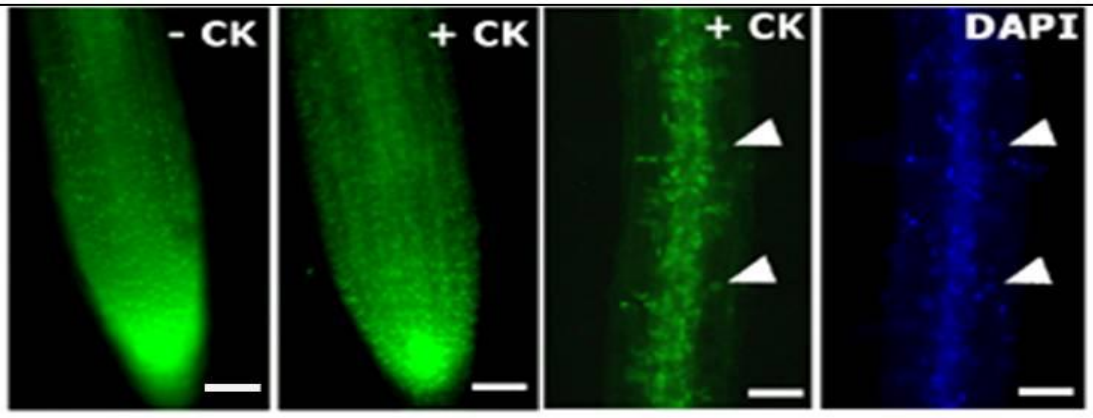

Figure 2.11 Nuclear translocation response to cytokinin in T_1 Arabidopsis Roots containing PhoB^{D53A}-smGFP. Epi-fluorescent images of sub-cellular localization of PhoB^{D53A}-smGFP in roots of transgenic Arabidopsis plants before and after exposure to cytokinin. Left images show a root tip and right two images show the upper portion of a root. Image in blue show the same root stained with 1 ng μL^{-1} DAPI. Arrowheads point to corresponding nuclei. CK: cytokinin, t-zeatin (1mM). Scale bars = 50µm.

Plants containing the complete synthetic signaling system, *ssTNT* and *Fls-Trg-PhoR* with *PhoB*^{D53A}*-smGFP,* were visualized before exposure to 10μM TNT and again following a minimum of 2 hours of incubation in TNT solution. PhoB^{D53A}-smGFP does not accumulate in the nucleus in response to exogenous TNT application (Figure 2.12). The mutant form of PhoB, where the canonical phospho-accepting D53 is mutated, does not translocate to the nucleus in response to the TNT ligand.

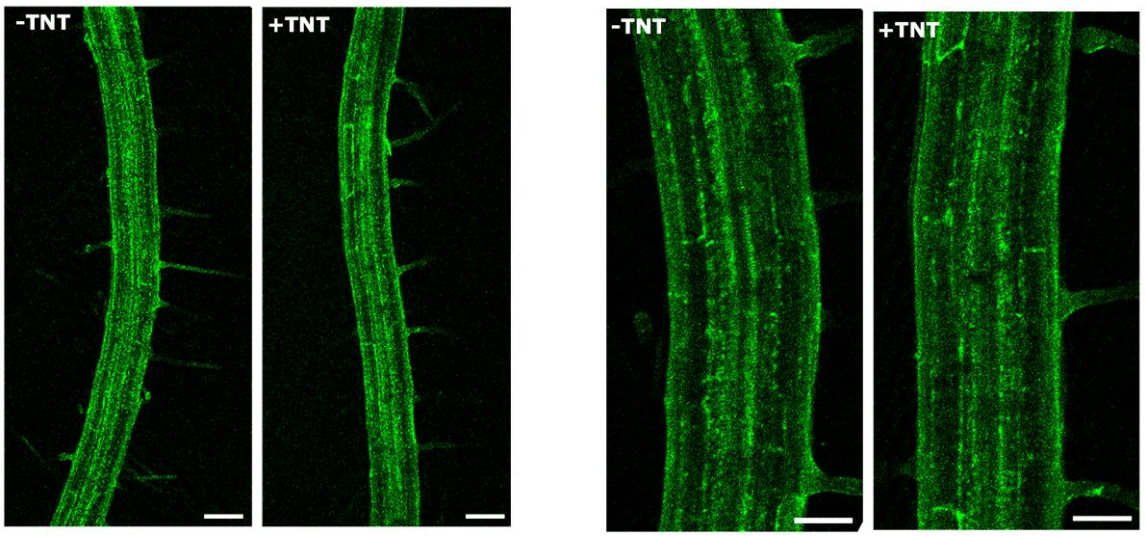

Figure 2.12 Nuclear translocation response to TNT in T_1 Arabidopsis plants containing the complete synthetic signaling system with PhoB^{D53A}-smGFP. Confocal images show the sub-cellular localization of PhoB^{D53A}-GFP in roots of transgenic Arabidopsis plants before and after exposure to 10μM TNT. Plants are expressing ssTNT and Fls-Trg-PhoR in addition to the PhoB mutant. Left two panels, scale bar = 100 μm; right two panels, scale bar = 50 μm.

Primarily transformed (T_0 generation) independent transgenic Arabidopsis plants expressing PhoB^{D53A} and the GUS reporter gene under control of the *PlantPho* promoter (*PhoB*^{D53A}*-VP64* and *PlantPho*::*GUS*) were analyzed for GUS activity in response to exogenous cytokinin application. GUS activity for these plants is variable between individual transformed plants and show inconsistent patterns of ligand induced GUS expression (Figure 2.13) ($t(49)=0.31$, $p=0.383$).

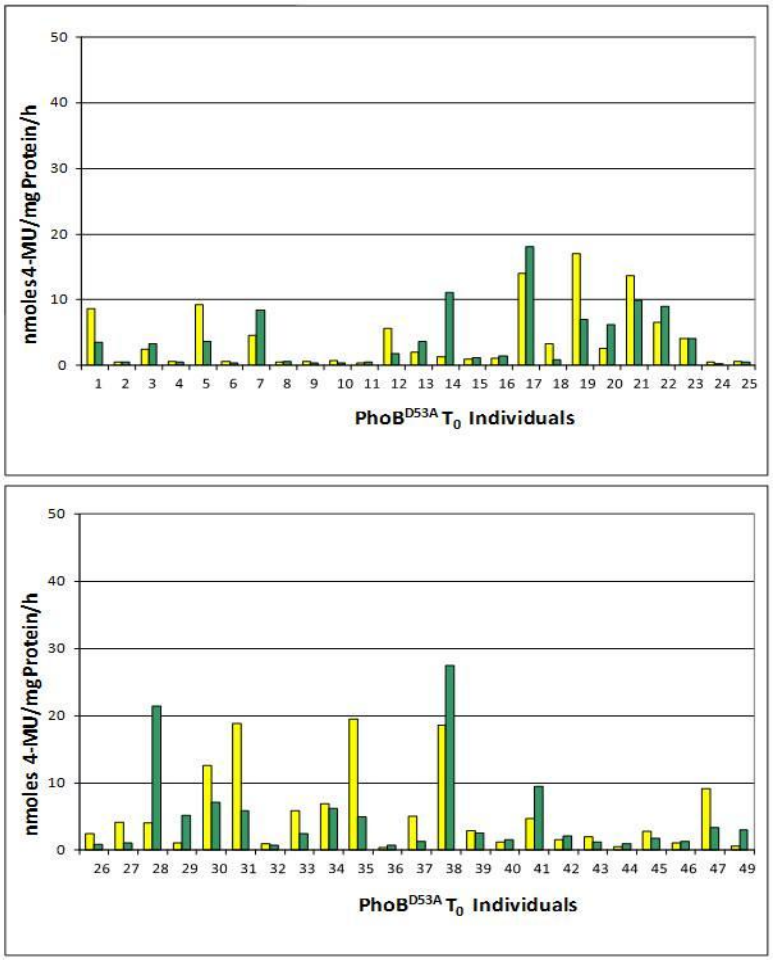

Figure 2.13 Transcriptional response to cytokinin in T_0 Arabidopsis plants containing PhoB^{D53A} with the partial synthetic signaling system. GUS activity (nmoles 4-MU mg protein^{-1} hour^{-1}) in leaves of T_0 Arabidopsis expressing the partial signaling system *PhoB^{D53A}-VP64* and *GUS* controlled by the *PlantPho promoter.* Yellow bars represent GUS activity of leaves incubated in water and the corresponding green bars represent a leaf from the same plant incubated in 1 mM t-zeatin.

Individual plants were placed into one of four categories based on the amount of GUS activity in the extract of the leaf incubated with the experimental ligand, relative to that of the corresponding control leaf incubated in water. Individual plants were considered to have significant GUS activity if the amount of 4-MU was greater than or equal to 10 nmoles 4-MU^{-1} mg protein^{-1} hour. This value was determined, from the experimental data using wild-type PhoB fused to VP64, to be the threshold for background GUS activity. Plants with leaves exposed to the experimental ligand having GUS activity 1.4 fold higher or more above that of control leaves are defined as showing

induction. Plants with leaves exposed to the experimental ligand having GUS activity 0.6 or less of that of the control leaves are defined as showing repression. Plants with both experimental and control leaves having GUS activity above 10 nmoles 4-MU^{-1} mg $protein^{-1}$ hour but, not fitting into the repression or induction group, are defined as showing some GUS activity that is relatively equivalent between the leaves. Plants with both experimental and control leaves having values below 10 nmoles 4-MU^{-1} mg $protein^{-1}$ hour, are defined as having no significant GUS activity.

Of all T_0 plants assayed, 28 individuals showed little to no GUS activity in either leaf; 9 plants show induction and 11 plants show repression. All of the T_0 plants were transferred to soil and grown to maturity for seed. Seed from each plant was germinated on selective media to perform segregation analysis and identify lines that contain a single copy of the T-DNA (Appendix A). Progeny from eight T_0 lines containing a single copy of the T-DNA were assayed for GUS activity. Of those eight lines, five show little to no GUS activity (Figure 2.14 B ($t(9)=0.01$, $p=0.5$), C ($t(9)=0.07$, $p=0.461$), D ($t(9)=0.32$, $p=0.386$), G ($t(9)=0.23$, $p=0.423$), H ($t(9)=0.13$, $p=0.461$) and the other three show variable activity. The three lines with variable GUS activity (20 plants assayed/line) include ten individuals showing repression, six individuals showing induction, 28 with no significant GUS activity and 16 show some GUS activity that is relatively equivalent between leaves exposed to water and cytokinin (Figure 2.14A ($t(19)=0.52$, $p=0.311$), E ($t(19)=0.38$, $p=0.347$), F ($t(19)=0.001$, $p=0.5$)).

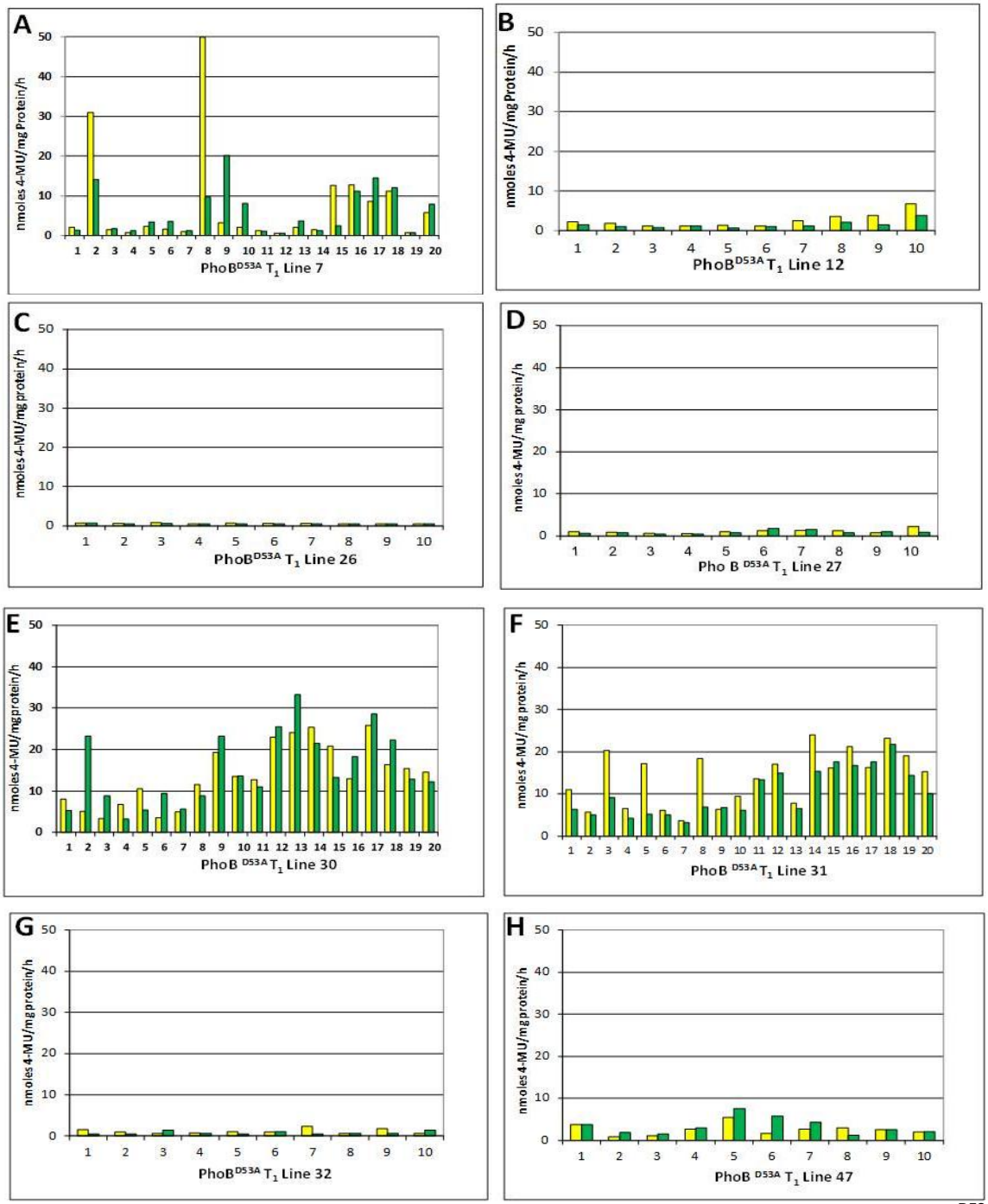

Figure 2.14 Transcriptional response to cytokinin in T_1 Arabidopsis plants containing PhoB^{D53A} with the partial synthetic signaling system. GUS activity of T_1 lines of Arabidopsis containing a single copy of *PhoB^{D53A}-VP64* with the partial signaling system. Each panel contains data from individual plants of eight T_1 lines. Yellow bars represent GUS activity of leaves incubated in water and green represent leaves incubated in 1 mM cytokinin.

Transgenic Arabidopsis plants expressing the previously described *PhoB^{D53A}* plasmid and another plasmid expressing the complete synthetic signaling system were

analyzed in the T_0 generation for GUS activity in response to exogenous TNT (10μM) application. As described in Chapter One, the complete synthetic signaling system includes the computationally designed TNT receptor and the synthetic fusion HK, Fls-Trg-PhoR. The synthetic fusion HK, Fls-Trg-PhoR is thought to be activated by the receptor-ligand complex, initiating a histidine to aspartate phospho-relay to wild-type PhoB-VP64 that translocates to the nucleus and activates transcription of the reporter gene controlled by the *PlantPho promoter*. GUS activity for all 20 T_0 plants assayed is consistently negligible (Figure 2.15; t(19)=0.99, p=0.165).

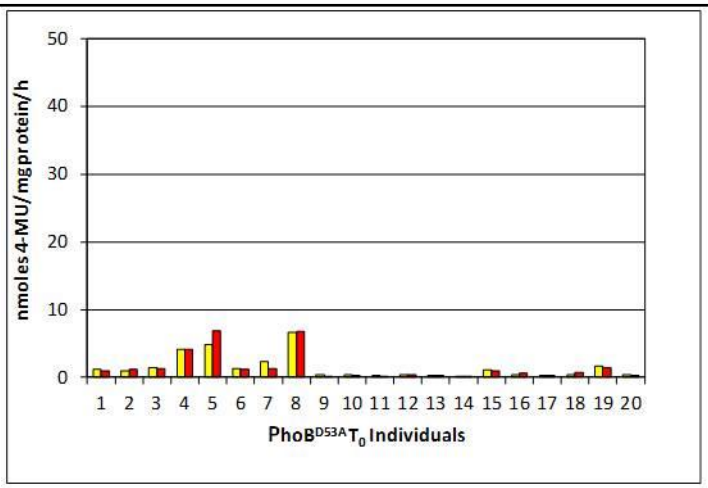

Figure 2.15 Transcriptional response to TNT in T_0 Arabidopsis plants containing PhoB^{D53A} with the complete synthetic signaling system. GUS activity in leaves of T_0 Arabidopsis expressing *PhoB^{D53A}* in the complete synthetic signaling system. Yellow bars represent GUS activity of leaves incubated in water and the corresponding red bars represent that of a leaf from the same plant incubated in 10μM TNT.

All T_0 plants were transferred to soil and grown to maturity. Again seed was collected and segregation analysis was performed to select lines that contain a single copy of each T-DNA (Appendix A). Progeny from two T_0 lines were assayed for GUS activity in response to exposure to TNT. All 20 individuals showed very little or no GUS activity in both leaves incubated in water and TNT (Figure 2.16 (t(9)=0.24, p=0.423; t(9)=0.97, p=0.172)).

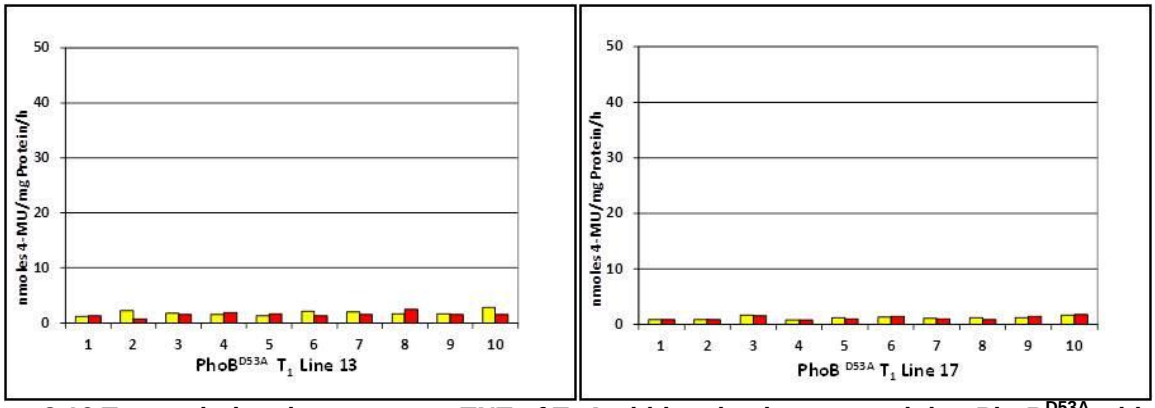

Figure 2.16 Transcriptional response to TNT of T_1 Arabidopsis plants containing PhoB^{D53A} with the complete synthetic signaling system. GUS activity of T_1 lines of Arabidopsis containing a single copy of *PhoB^{D53A}-VP64*, *PlantPho::GUS, ssTNT*, and *Fls-Trg-PhoR*. Each panel contains data from individual plants of two T_1 lines. Yellow bars represent GUS activity of leaves incubated in water and red bars represent that of leaves incubated in 10μM TNT.

The first set of data taken from plants expressing the plasmid containing *PhoB^{D53A}-VP64* and *PlantPho*::*GUS* showed reduced GUS activity compared to that of plants expressing wild-type *PhoB* in the partial signaling system (Antunes et al., 2009). However, these plants do show some significant GUS activity, suggesting that endogenous cytokinin signaling components have another means to activate PhoB other than the canonical Phospho-accepting D53. The second set of data taken from plants expressing the complete synthetic signaling system, containing the same *PhoB^{D53A}-VP64* and *PlantPho*::*GUS* described above and another plasmid containing *Fls-Trg-PhoR* and *ssTNT*, showed little to no GUS activity in all plants assayed. This suggests that the canonical phospho-accepting D53 of PhoB is required for the function of the synthetic signaling system in plants.

An additional observation is made from the comparison of these two sets of data. The GUS activity of leaves exposed to only water represents the background level of signaling for each of the systems tested. This background signaling is clearly very different between the plants that express the single T-DNA, with *PhoB^{D53A}-VP64* and

PlantPho::*GUS*, and the plants also expressing *Fls-Trg-PhoR* and *ssTNT* on a separate T-DNA. The plants expressing both T-DNAs have almost no GUS activity while the plants expressing the single T-DNA have variable GUS activity and background signaling. This suggests that the presence of either the fusion HK, Fls-Trg-PhoR, or the computationally designed TNT receptor, ssTNT, reduces background signaling levels in the system. In bacteria the wild-type HK PhoR has been shown to have phosphatase activity with PhoB (Carmany et al., 2003). Perhaps the PhoR portion of Fls-Trg-PhoR is reducing background signaling levels in plants through phosphatase activity. To collect further evidence to test this hypothesis, the same T_1 lines of plants containing *PhoB*^{D53A}-*VP64*, *PlantPho*::*GUS*, *ssTNT*, and *Fls-Trg-PhoR* previously tested with TNT, were induced with cytokinin in the same manner described above. GUS activity in leaves of these plants is minimal after incubation in both water and cytokinin (Figure 2.17 (t(9)=0.82, p=0.222; t(9)=0.77, p=0.222)). These results further support the hypothesis that the presence of the HK portion of PhoR reduces both input from cytokinin components to PhoB^{D53A} and background signaling.

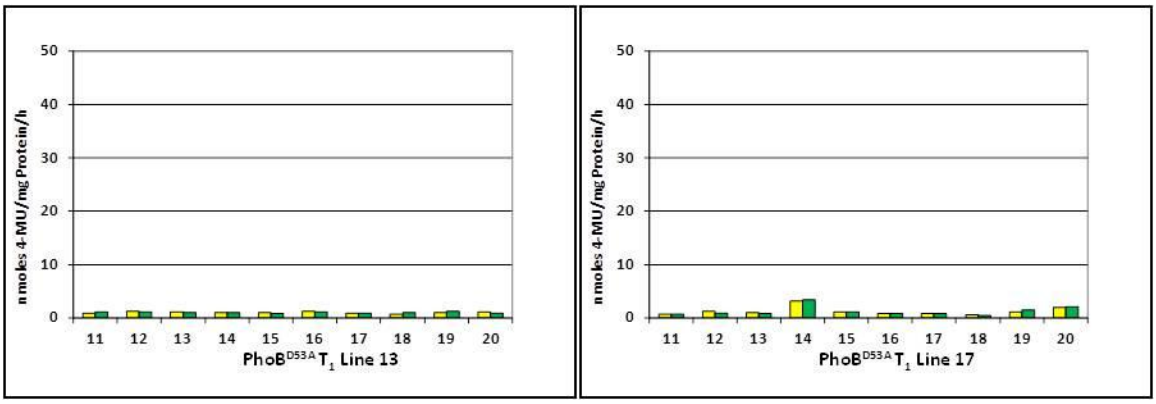

Figure 2.17 Transcriptional response to cytokinin in T_1 Arabidopsis plants containing the complete synthetic signaling system with PhoB^{D53A}. GUS activity of T_1 lines of Arabidopsis containing a single copy of *PhoB*^{D53A}*-VP64*, *PlantPho::GUS, ssTNT*, and *Fls-Trg-PhoR*. Each panel contains data from individual plants of two T_1 lines. Yellow bars represent GUS activity of leaves incubated in water and green bars represent that of leaves incubated in 1 mM cytokinin.

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2. Conclusions

Here I tested a mutant version of PhoB in which the canonical phospho-accepting aspartate was mutated to an alanine. $PhoB^{D53A}$ was tested with the partial synthetic signaling system for a response to cytokinin and with the complete synthetic signaling system for a response to TNT. Both nuclear translocation and transcriptional activation were analyzed to determine whether the canonical phospho-accepting aspartate is required for function in each system.

In T_1 plants assayed for GUS activity in response to exogenous cytokinin application highly variable GUS activity is observed. Six individuals show induction, ten show repression, and 16 show GUS activity that is relatively equivalent between leaves exposed to cytokinin and those exposed to water (Figure 2.14). These data suggest that $PhoB^{D53A}$ is a functional form of PhoB and can activate transcription in some cases. The mechanism by which transcription is activated is unknown, and whether the resulting GUS activity observed is due to cytokinin exposure is also unknown.

In contrast, all plants containing the complete synthetic signaling system with $PhoB^{D53A}$ showed little to no GUS activity in both leaves exposed to TNT and those exposed to water (Figure 2.16). This lack of response in plants with $PhoB^{D53A}$ contrasts the response seen in plants expressing the non-mutated version of PhoB in the complete synthetic signaling system. These results suggest that the canonical phospho-accepting D53 of PhoB is required for function in the complete synthetic signaling system.

As previously described, plants tested for a cytokinin response contain a partial signaling system: *$PhoB^{D53A}$-VP64* and *PlantPho*::GUS (lacking the synthetic fusion HK).

The plants tested for a TNT response contain the complete synthetic signaling system: *PhoB*^{D53A}*-VP64*, *PlantPho*::*GUS*, *Fls-Trg-PhoR*, and *ssTNT*. Plants containing the partial system showed variable GUS activity (un-induced average GUS activity = 7.2 nmoles 4-MU^{-1} mg protein^{-1} hour;standard deviation = 8.7) (Figure 2.14) while plants with the complete system showed virtually no GUS activity (un-induced average GUS activity = 1.1 nmoles 4-MU^{-1} mg protein^{-1} hour; standard deviation = 0.5) (Figure 2.16). Comparison of the un-induced or background GUS activity of these two sets of plants suggests that the presence of components in the complete system reduce background signaling. This reduction in background signaling could be due to phosphatase activity in the PhoR portion of Fls-Trg-PhoR with PhoB. Further experimentation showed that cytokinin could not produce a response in plants containing the complete synthetic signaling system with mutant PhoB (Figure 2.17), where-as plants containing the partial system with mutant PhoB did show some variable GUS activity in response to cytokinin. This suggests that presence of components in the complete system (likely Fls-Trg-PhoR) might reduce background signaling in the system with PhoB^{D53A}.

C. Response of PhoB^{D100} mutants to cytokinin and TNT

1. Results

a. PhoB^{D100A}

To determine the cytokinin mediated transcriptional response of PhoB^{D100A}, transgenic Arabidopsis plants expressing the mutant in the partial synthetic signaling system were analyzed for GUS activity in response to exogenous cytokinin. Of the primarily transformed independent transgenic lines or T_0 plants assayed, 11 show little

to no GUS activity, one shows induction, and three show repression. All of the T_0 plants were transferred to soil and grown for seed. Seed from each plant was germinated on selective media for segregation analysis to identify lines that contain a single copy of the T-DNA (Appendix A). Progeny of six T_0 lines containing a single copy of the T-DNA were assayed for GUS activity. Of those six lines, five lines show little to no GUS activity and one T_1 line shows variable GUS activity (Figure 2.18 A $t(9)=0.29$, $p=0.386$; B $t(9)=0.27$, $p=0.386$; C $t(9)=0.44$, $p=0.5$; D $t(7)=0.41$, $p=0.351$; E $t(9)=0.61$, $p=0.282$; F $t(9)=0.03$, $p=0.5$)). The one line with variable GUS activity included one individual plant showing induction and two individuals showing repression (Figure 2.18 B).

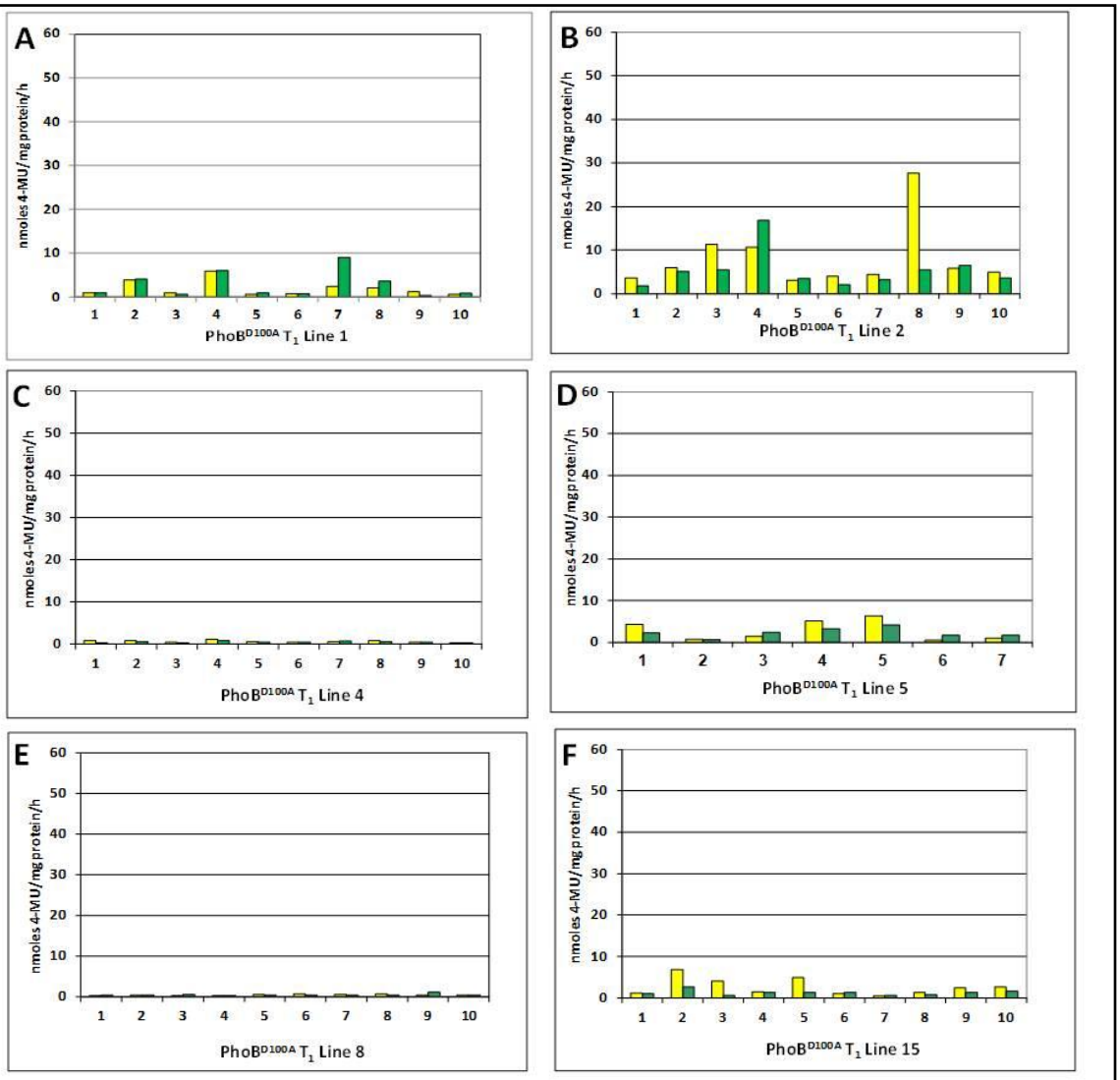

Figure 2.18 Transcriptional response to cytokinin in T_1 Arabidopsis plants containing PhoB^{D100A} with the partial signaling system. GUS activity of T_1 lines of Arabidopsis containing a single copy of *PhoB^{D100A}-VP64 and PlantPho::GUS*. Each panel contains data from individual plants of six T_1 lines. Yellow bars represent GUS activity of leaves incubated in water and green bars represent leaves incubated in 1 mM cytokinin.

Transgenic Arabidopsis plants expressing the complete synthetic signaling system with *PhoB^{D100A}-VP64* were analyzed in the T_0 generation for GUS activity in response to exogenous TNT application. Of the 20 T_0 plants assayed, one plant shows GUS activity in both leaves exposed to water and TNT that was approximately equivalent. Again seed was collected from all T_0 plants and

segregation analysis was performed to select lines that contain a single copy of each T-DNA (Appendix A). Progeny of three T_0 plants were assayed for GUS activity in response to exposure to TNT. One line shows little to no GUS activity, another shows relatively low levels of GUS activity with two of ten individuals showing induction, and the final T_1 line shows highly variable GUS activity (Figure 2.19 A $t(9)=0.02$, $p=0.5$; B $t(9)=0.47$, $p=0.315$). Line 4, showing highly variable activity was tested more extensively than the other two lines, with five individuals that show little GUS activity, eight show induction, eight show repression, and the other 42 individuals show GUS activity that is relatively equivalent between leaves (Figure 2.19 C, D, E $t(63)=0.33$, $p=0.383$).

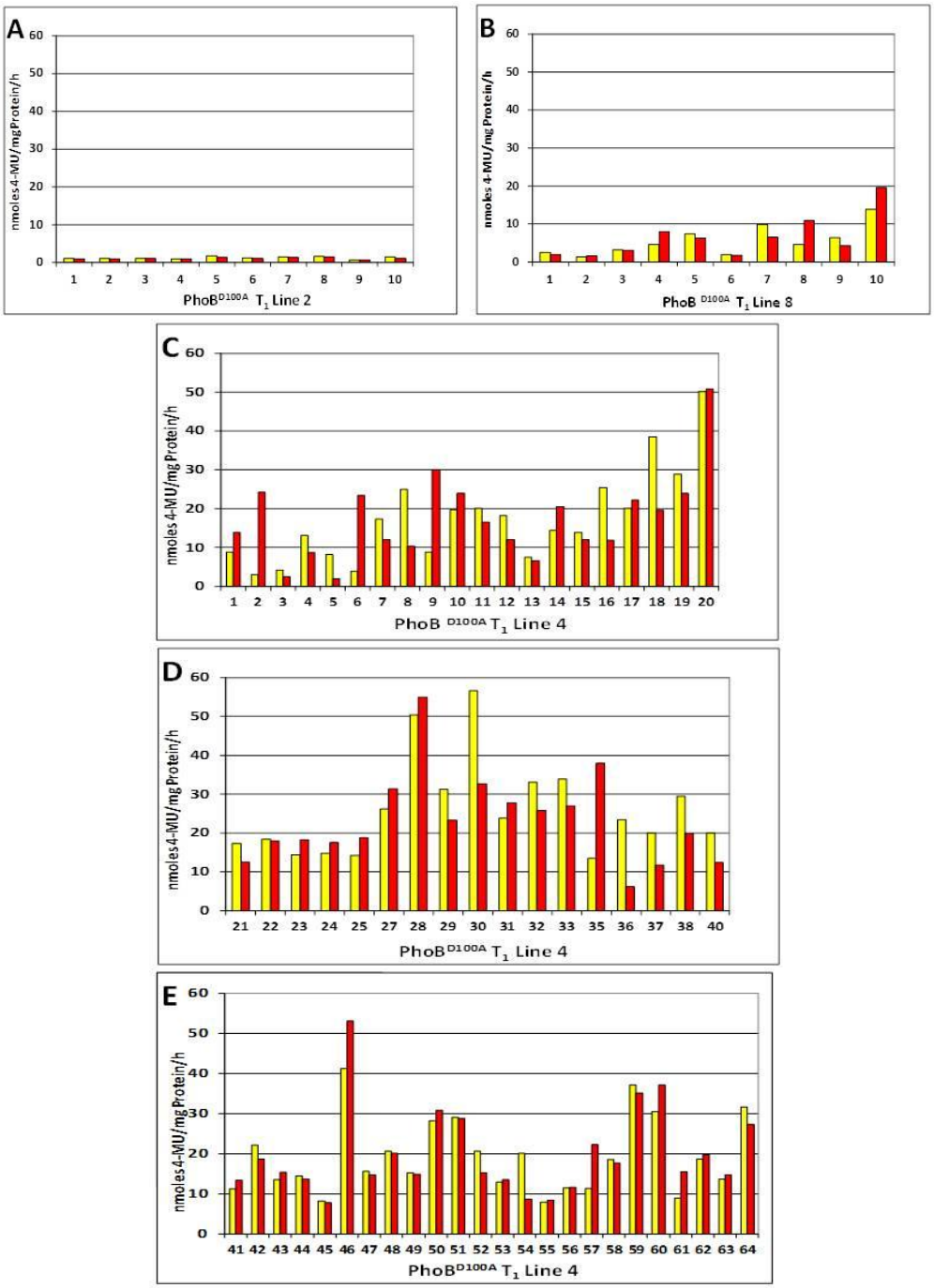

Figure 2.19 Transcriptional response to TNT in T_1 Arabidopsis plants containing PhoB^{D100A} with the complete synthetic signaling system. GUS activity of T_1 lines of Arabidopsis containing a single copy of *PhoB^{D100A}-VP64*, *PlantPho::GUS, ssTNT*, and *Fls-Trg-PhoR*. Each panel contains data from individual plants of three T_1 lines. Yellow bars represent GUS activity of leaves incubated in water and red bars represent that of leaves incubated in 10μM TNT.

b. PhoB^{D100E}

To determine the cytokinin mediated transcriptional response of PhoB^{D100E}, transgenic Arabidopsis plants expressing the mutant in the partial synthetic signaling system were analyzed for GUS activity in response to exogenous cytokinin. Of the T_0 plants assayed, 12 show little to no GUS activity and two show induction. All of the T_0 plants were transferred to soil and grown for seed. Following segregation analysis (Appendix A) three T_0 lines containing a single copy of theT-DNA were assayed for GUS activity (Figure 2.20 A t(9)=0.06, p=0.461; B t(9)=0.75, p=0.222; C t(9)=0.15, p=0.423). Of those three lines, two show negligible GUS activity and the other line includes one individual showing repression (Figure 2.20 A).

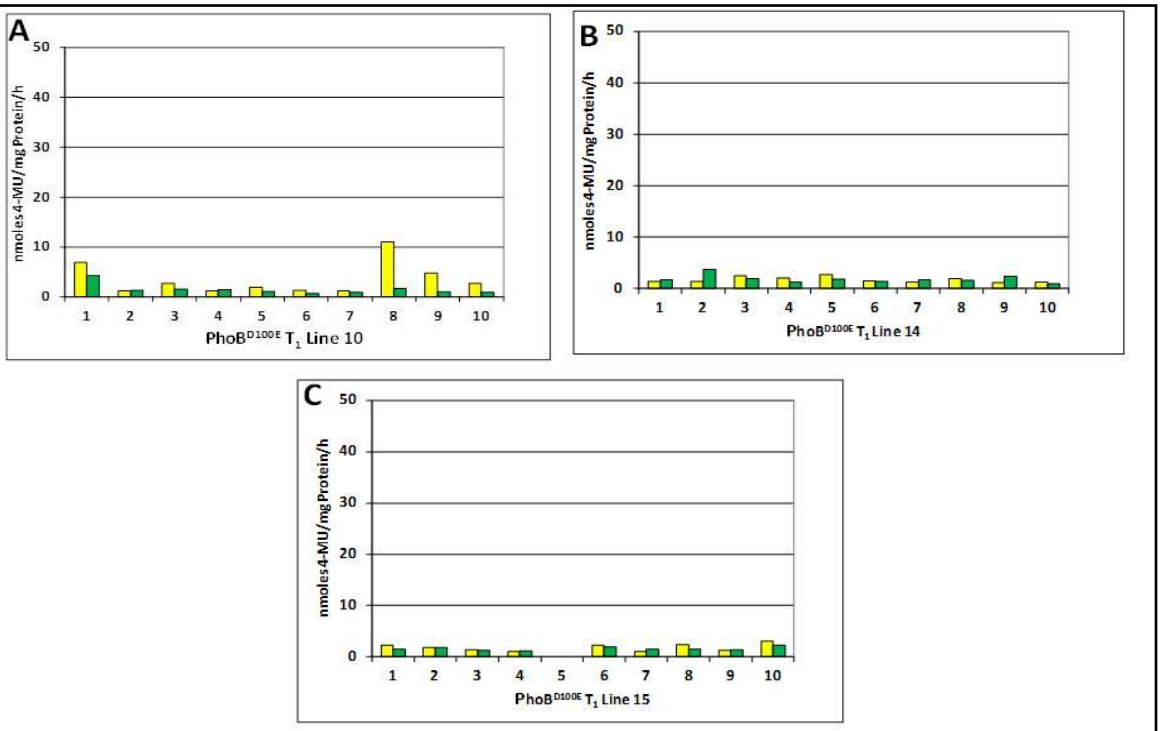

Figure 2.20 Transcriptional response to cytokinin in T_1 Arabidopsis plants containing PhoB^{D100E} with the partial signaling system. GUS activity of T_1 lines of Arabidopsis containing a single copy of *PhoB^{D100E}-VP64* and *PlantPho::GUS*. Each panel contains data from individual plants of three T_1 lines. Yellow bars represent GUS activity of leaves incubated in water and green bars represent leaves incubated in 1 mM cytokinin.

Transgenic Arabidopsis plants expressing the complete synthetic signaling system with *PhoB*^{D100E}*-VP64* were analyzed in the T_0 generation for GUS activity in response to exogenous TNT application. All 20 of the T_0 plants assayed show little to no GUS activity. Again plants were grown to maturity, seed was collected, and segregation analysis was performed (Appendix A). Progeny from two T_0 lines containing a single copy of each T-DNA were analyzed in the T_1 generation. Twenty-nine T_1 plants assayed show little to no GUS activity with one individual showing repression (Figure 2.21 t(19)=0.57, p=0.278; t(9)=0.73, p=0.251).

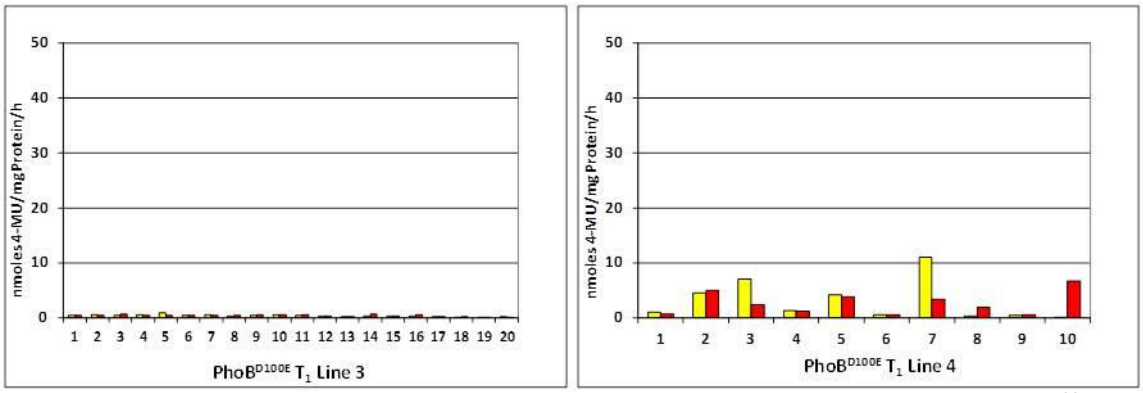

Figure 2.21 Transcriptional response to TNT in T_1 Arabidopsis plants containing PhoB^{D100E} with the complete synthetic signaling system. GUS activity of T_1 lines of Arabidopsis containing a single copy of *PhoB*^{D100E}*-VP64*, *PlantPho::GUS, ssTNT*, and *Fls-Trg-PhoR*. Each panel contains data from individual plants of two T_1 lines. Yellow bars represent GUS activity of leaves incubated in water and red bars represent that of leaves incubated in 10μM TNT.

c. PhoB$^{D53A+D100A}$

The cytokinin mediated response of the double PhoB mutant was determined in which both the canonical phospho-accepting aspartate 53 and the aspartate 100 residues are mutated to alanine. Both mutant versions of PhoB show a reduced response to cytokinin. Transgenic Arabidopsis plants expressing *PhoB*$^{D53A+D100A}$ with the partial synthetic signaling system were analyzed for GUS activity in response to exogenous cytokinin application. All 20 T_0 plants assayed show little to no GUS activity

(Figure 2.22 t(19)=0.41, p=0.347). Due to minimal GUS activity in the majority of T_0 plants assayed, progeny of these individuals were not analyzed.

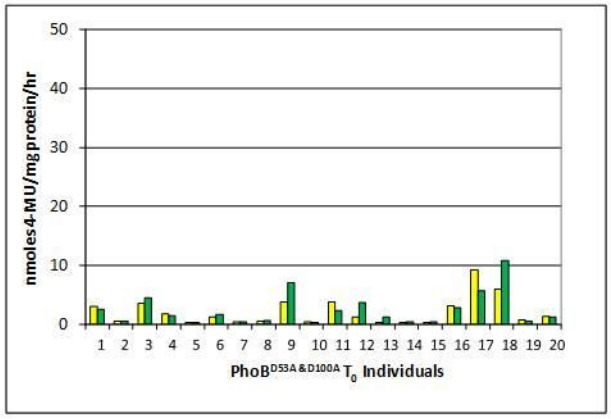

Figure 2.22 Transcriptional response to cytokinin in T_0 Arabidopsis plants containing PhoB$^{D53A+D100A}$ with the partial signaling system. GUS activity in leaves of T_0 Arabidopsis expressing *PhoB$^{D53A+D100A}$-VP64* and *PlantPho::GUS*. Yellow bars represent GUS activity of leaves incubated in water and the corresponding green bars represent that of a leaf from the same plant incubated in 1mM cytokinin.

2. Conclusions

Here I analyzed the transcriptional response of aspartate 100 mutations of PhoB with the partial and complete synthetic signaling system to determine if this residue affects response to cytokinin or TNT. GUS activity in transgenic Arabidopsis plants containing the PhoB mutants PhoB^{D100A}, PhoB^{D100E} as well as a double PhoB mutant PhoB$^{D53A+D100A}$ was measured in leaves exposed to cytokinin or TNT. The mutant form PhoB^{D100A} appears to have reduced a transcriptional response to cytokinin yet maintains some function with the components of the synthetic signaling system. As described above, plants containing PhoB^{D100A} with the partial signaling system show very little GUS activity in response to exogenous cytokinin application (Figure 2.18). This mutation does not eliminate input from cytokinin components but reduces the transcriptional response compared to that of wild-type PhoB. Furthermore, plants

containing the complete signaling system with PhoB^{D100A} maintain some GUS activity in response to exogenous TNT application (Figure 2.19). Most of these individual plants show high un-induced GUS activity levels in leaves exposed to water, and only a few plants show higher GUS activity in response to TNT and GUS activity of induced leaves is not significantly different than that of uninduced leaves. However, the complete synthetic signaling system with wild-type PhoB also shows some high un-induced GUS activity levels, and variable amounts of induction (Antunes et al., 2011). The data suggest that this mutated form of PhoB maintains some function in the complete synthetic signaling system, while reducing interaction with endogenous cytokinin signaling components.

D. Response of PhoB^{H144} and PhoB^{D76} mutants to cytokinin and TNT

1. Results

a. PhoB^{H144A}

To test the transcriptional response of the PhoB mutant transgenic Arabidopsis plants expressing PhoB^{H144A} with the partial synthetic signaling system were analyzed for changes in GUS activity in response to exogenous cytokinin application. Of the 25 independent transgenic T_0 plants assayed all showed minimal GUS activity. T_0 plants were transferred to soil and grown for seed. Following segregation analysis (Appendix A) seven T_1 lines containing a single copy of the T-DNA were assayed for GUS activity. Of those seven lines, ten individual per line assayed, all showed negligible GUS activity (Figure 2.23 A $t(9)=0.94$, $p=0.196$; B $t(9)=0.04$, $p=0.5$; C $t(9)=0.004$, $p=0.5$; D $t(9)=0.98$, $p=0.172$; E $t(9)=0.005$, $p=0.5$; F $t(9)=0.09$, $p=0.461$; G $t(9)=0.05$, $p=0.461$).

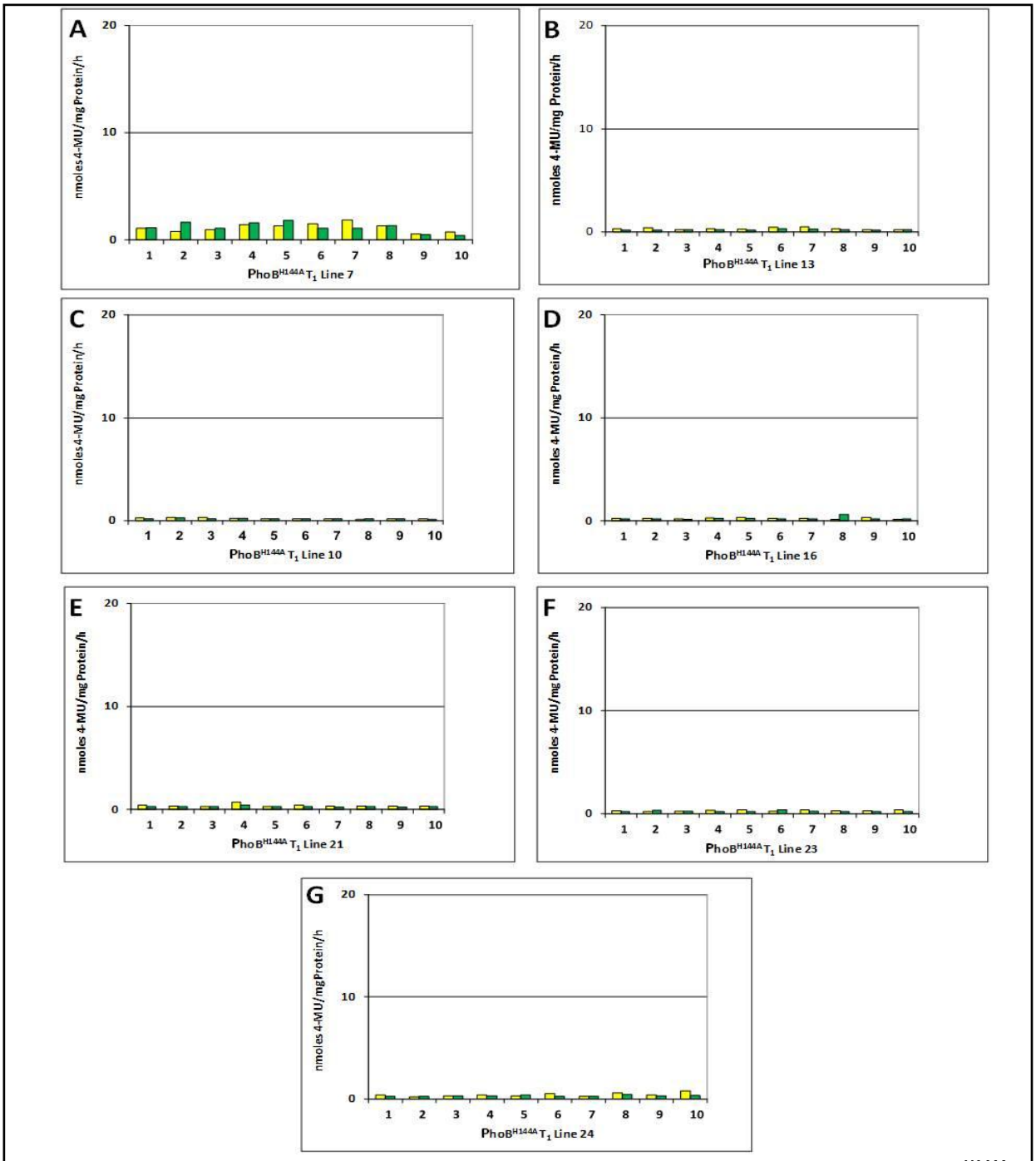

Figure 2.23 Transcriptional response to cytokinin in T_1 Arabidopsis plants containing PhoB^{H144A} with the partial signaling system. GUS activity of T_1 lines of Arabidopsis containing a single copy of *PhoB^{H144A}-VP64* and *PlantPho::GUS*. Each panel contains data from individual plants of seven T_1 lines. Yellow bars represent GUS activity of leaves incubated in water and green represent leaves incubated in 1 mM cytokinin.

Transgenic Arabidopsis plants expressing the complete synthetic signaling system with PhoB^{H144A} were analyzed in the T_0 generation for GUS activity in response

to exogenous TNT application. All 17 of the T_0 plants assayed show little to no GUS activity. Because none of the T_0 plants show significant GUS activity limited analysis was performed on the progeny. Following segregation analysis (Appendix A) one T_1 line containing a single copy of each T-DNA was assayed for GUS activity. All ten individuals show negligible GUS activity (Figure 2.24 t(9)=0.29, p=0.386).

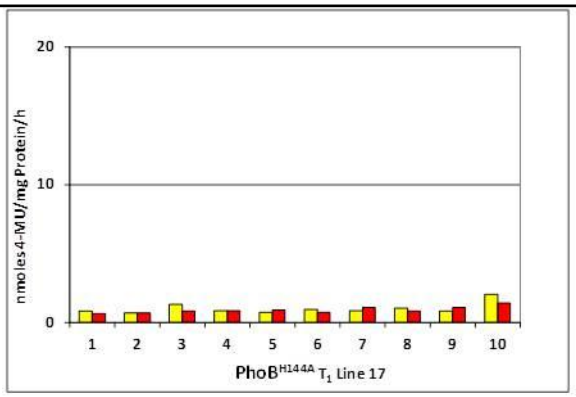

Figure 2.24 Transcriptional response to TNT in T_1 Arabidopsis plants containing $PhoB^{H144A}$ with the complete synthetic signaling system. GUS activity in individuals of a T_1 line of Arabidopsis containing a single copy of *$PhoB^{H144A}$-VP64*, *PlantPho::GUS, ssTNT*, and *Fls-Trg-PhoR*. Yellow bars represent GUS activity of leaves incubated in water and red bars represent that of leaves incubated in 10μM TNT.

b. $PhoB^{H144R}$

To test the transcriptional response of the PhoB mutant transgenic Arabidopsis plants expressing *$PhoB^{H144R}$* with the partial synthetic signaling system were analyzed for GUS activity in response to exogenous cytokinin application. Of the T_0 plants assayed, 12 show little to no GUS activity, four show induction, one shows repression, and three individuals show some GUS activity that is relatively equivalent between leaves exposed to water and cytokinin (Figure 2.25 t(19)=0.46, p=0.311). Progeny of these lines were not analyzed because a few individual plants show a transcriptional

response to cytokinin, suggesting this PhoB mutant is responsive to endogenous cytokinin signaling components.

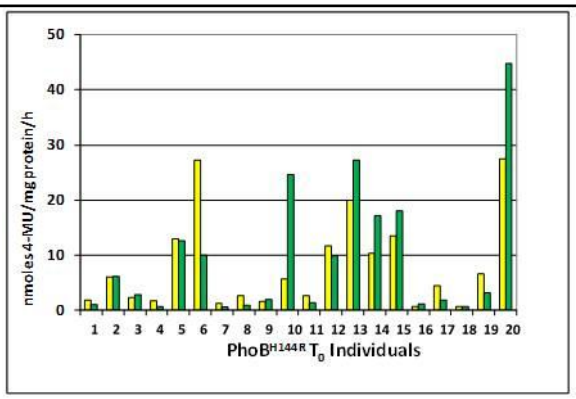

Figure 2.25 Transcriptional response to cytokinin in T_0 Arabidopsis plants containing PhoB^{H144R} with the partial signaling system. GUS activity in leaves of T_0 Arabidopsis plants expressing *PhoB^{H144R}-VP64* and *PlantPho::GUS*. Yellow bars represent GUS activity of leaves incubated in water and the corresponding green bars represent that of a leaf from the same plant incubated in 1mM cytokinin.

Transgenic Arabidopsis plants expressing the complete synthetic signaling system with *PhoB^{H144R}* were analyzed in the T_0 generation for GUS activity in response to exogenous TNT application. Eleven of the T_0 plants assayed show little to no GUS activity and one plant shows repression (Figure 2.26 $t(19)=0.38$, $p=0.347$). Progeny of these lines were not analyzed because nearly all T_0 plants assayed do not show a response to TNT, suggesting this mutant version of PhoB is not functional in the complete synthetic signaling system.

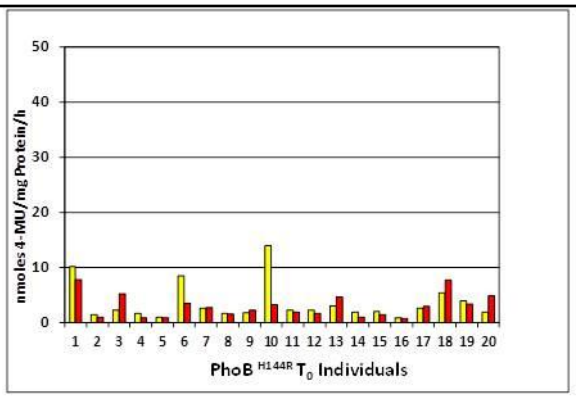

Figure 2.26 Transcriptional response to TNT in T_0 Arabidopsis plants containing PhoB^{H144R} with the complete synthetic signaling system. GUS activity in leaves of T_0 Arabidopsis plants expressing *PhoB^{H144R}-VP64, PlantPho::GUS, ssTNT, Fls-Trg-PhoR*. Yellow bars represent GUS activity of leaves incubated in water and the corresponding red bars represent that of a leaf from the same plant incubated in 10μM TNT.

c. PhoB^{D76E}

To test the transcriptional response of the PhoB mutant transgenic Arabidopsis plants expressing PhoB^{D76E} with the partial synthetic signaling system were analyzed for GUS activity in response to exogenous cytokinin application. Of the T_0 plants assayed, 24 show little to no GUS activity, one shows induction, one shows repression, and four plants show some GUS activity that is relatively equivalent between leaves exposed to water and cytokinin (Figure 2.27 $t(29)=0.81$, $p=0.215$). Progeny of these plants were not analyzed because some of the T_0 plants assayed show GUS activity suggesting this mutant version of PhoB is responsive to endogenous cytokinin signaling components.

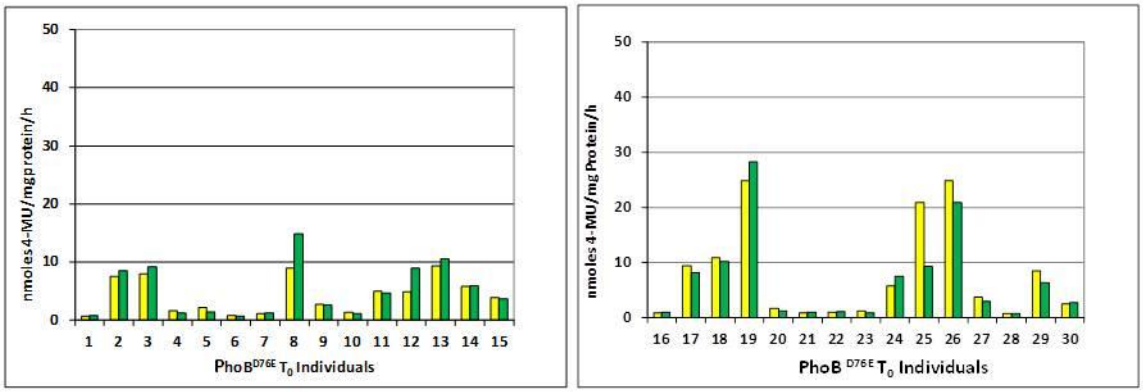

Figure 2.27 Transcriptional response to cytokinin in T_0 Arabidopsis plants containing PhoB^{D76E} with the partial signaling system. GUS activity in leaves of T_0 Arabidopsis plants expressing *PhoB^{D76E}-VP64* and *PlantPho::GUS*. Yellow bars represent GUS activity of leaves incubated in water and the corresponding green bars represent that of a leaf from the same plant incubated in 1mM cytokinin.

Transgenic Arabidopsis plants expressing the complete synthetic signaling system with PhoB^{D76E} were analyzed in the T_0 generation for GUS activity in response to exogenous TNT application. Of the T_0 plants assayed, 14 show little to no GUS activity, one shows induction, and two plants show some GUS activity that is relatively equivalent between leaves exposed to water and TNT (Figure 2.28 t(16)=0.08, p=0.461). Progeny of these lines were not analyzed because this mutant version of PhoB is responsive to endogenous cytokinin signaling components.

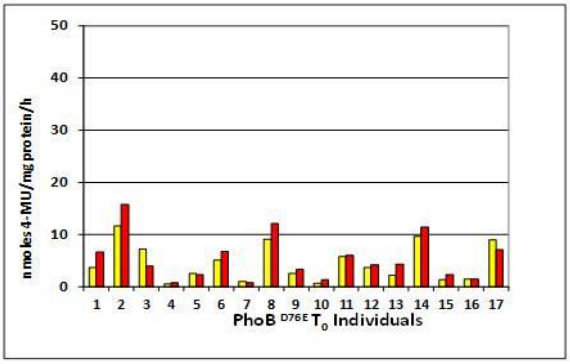

Figure 2.28 Transcriptional response to TNT in T_0 Arabidopsis plants containing PhoB^{D76E} with the complete synthetic signaling system. GUS activity in leaves of T_0 Arabidopsis plants expressing *PhoB^{D76E}-VP64* and *PlantPho::GUS*. Yellow bars represent GUS activity of leaves incubated in water and the corresponding red bars represent that of a leaf from the same plant incubated in 10 µM TNT.

2. Conclusions

None of the above described mutations in PhoB show a reduced response to cytokinin while maintaining response to TNT in the complete synthetic signaling system. $PhoB^{H144A}$ does not respond to either cytokinin or TNT. One explanation for this may be that the mutation causes a structural disruption of PhoB generating a non-functional form. $PhoB^{H144R}$ responds to cytokinin but not to TNT suggesting that this form of PhoB is functional but is not activated by the fusion HK, Trg-PhoR. $PhoB^{D76E}$ appears to be a functional form of PhoB, in that some GUS activity was measured, however this form sometimes responds to cytokinin as well as TNT. Therefore, PhoB mutations H144A, H144R, and D76E do not reduce input from cytokinin signaling components while maintaining interaction with the synthetic signaling components.

IV. Conclusions and Discussion

A. Summary of Conclusions

The goal of the experiments described above was to determine if mutant versions of PhoB could be identified that do not respond to cytokinin mediated signaling yet maintain response to TNT mediated synthetic signaling *in planta.* To accomplish this goal I tested several mutant versions of PhoB for transcriptional response to cytokinin in the partial synthetic signaling system and to TNT in the complete synthetic signaling system. Specific histidine and aspartate residues in PhoB were selected for mutation based on three-dimensional proximity to the canonical phospho-accepting aspartate 53 and/or alignment with histidines and aspartates in AHPs. I also tested a mutant version of PhoB in which the canonical phospho-accepting aspartate 53 was

mutated to an alanine, to determine whether this residue is needed for nuclear translocation and transcriptional activation in both the partial and synthetic signaling systems.

Results for testing nuclear translocation (Figure 2.11) and transcriptional activation (Figure 2.13, Figure 2.14) of the mutant version of PhoB, in which the canonical phospho-accepting aspartate was changed to an alanine, in the partial synthetic signaling system show $PhoB^{D53A}$ responds to cytokinin. These data suggest that endogenous cytokinin signaling components have a means of activating PhoB other than the canonical phospho-accepting aspartate 53.

Results for testing nuclear translocation (Figure 2.12) and transcriptional activation (Figure 2.15, Figure 2.16) of $PhoB^{D53A}$ in the complete synthetic signaling system show no response to TNT. These data suggest that the canonical phospho-accepting aspartate 53 is required for ligand mediated response in the synthetic signaling system.

The aspartate 100 of PhoB was identified by Dr. Neera Tewari-Singh as a residue that aligns with aspartates of AHP2, AHP1, and OmpR. Furthermore, this residue has the same exposure and is proximal to the canonical phospho-accepting aspartate 53 of PhoB. Mutant versions of PhoB in which aspartate 100 was changed to an alanine were tested for transcriptional activation in the partial and complete synthetic signaling systems. Results for testing $PhoB^{D100A}$ in the partial synthetic signaling system show a decreased transcriptional response to cytokinin having three of the 57 T_1 plants assayed show some GUS activity (Figure 2.18). Results for testing $PhoB^{D100A}$ in the complete synthetic signaling system show some GUS activity in response to TNT

(Figure 2.19), suggesting that PhoB^{D100A} is functional in the complete synthetic signaling system. These data show that PhoB^{D100A} has a reduced response to endogenous cytokinin signaling components yet maintains TNT mediated response in the synthetic signaling system.

Other mutant versions of PhoB were tested for transcriptional response in the partial and complete synthetic signaling systems. PhoB^{D100E}, PhoB^{H144A}, PhoB^{H144R} and PhoB^{D76E} do not show a reduced response to cytokinin mediated signaling while maintaining response to TNT mediated synthetic signaling.

Table 2.1 Summary of PhoB Mutant Results and Conclusions

PhoB Mutation	Cytokinin	TNT	Conclusions
D53A Canonical Phospho-accepting Aspartate	↓ X	X	•Suggests other residues might be phosphorylated by cytokinin components occasionally. •Typical phosphorylation site for PhoR in bacteria •Trg_PhoR reduces background
D100A	↓	—	•Reduced activity with CK and some induction with TNT
D100E	↓	↓	•No significant GUS activity with CK or TNT
H144A	X	X	•No GUS activity with CK or TNT •Suggests possible structural disruption of PhoB
H144R	—	↓	•Shows some activity with CK and little activity with TNT
D76E	↓	↓	•No significant GUS activity with CK or TNT

B. Phosphatase activity of Trg-PhoR

Comparison of results from testing the transcriptional response of $PhoB^{D53A}$ to cytokinin (Figure 2.14) and TNT (Figure 2.16) led to an additional observation. The background or un-induced levels of GUS activity are minimal for $PhoB^{D53A}$ in the complete synthetic signaling system yet GUS activity is variable and generally higher for $PhoB^{D53A}$ in the partial synthetic signaling system. These results suggest that the presence of a component in the complete synthetic signaling system reduces background signaling and/or interaction with endogenous plant signaling components. The presence of ssTNT is unlikely to cause a reduction in interaction with plant components because of its extracellular localization. The TNT receptor does not have direct interaction with PhoB. In the proposed mechanism for signaling in the complete synthetic signaling system, the synthetic fusion HK Trg-PhoR, has direct interaction with PhoB-VP64. The wild-type HK PhoR has been shown, in bacteria, to have phosphatase activity with PhoB (Carmany et al., 2003). Perhaps the PhoR portion of the synthetic fusion HK Fls-Trg-PhoR has phosphatase activity with PhoB in our synthetic system, reducing cross-talk and background signaling.

C. Use of $PhoB^{D100A}$ in subsequent versions of synthetic signaling system

The goal of these experiments was to identify mutations in PhoB that reduce cytokinin mediated signaling in the partial synthetic signaling system while maintaining function in the complete synthetic signaling system. The PhoB mutation D100A has a reduced response to cytokinin (Figure 2.18), compared that of wild-type PhoB. In contrast, $PhoB^{D100A}$ with the complete synthetic system responds to TNT but the

response is variable and not reliable (Figure 2.19). Also, there may be background signaling and/or input from endogenous plant signaling pathways other than cytokinin in both the partial system and the complete synthetic signaling system that may interfere with clear interpretation. This mutation alone will not solve the cross-talk problem; however it does reduce input from cytokinin signaling components.

The double mutant PhoB$^{D53A+D100A}$ shows minimal response to cytokinin and little background signaling (Figure 2.22). This suggests that the primary means for cytokinin signaling components to interact with PhoB are absent. Recall the single mutants PhoB^{D53A} and PhoB^{D100A} show only a reduced response to cytokinin and substantial background signaling (Figure 2.14 and Figure 2.18). PhoB^{D100A} still has the canonical phospho-accepting D53 that is likely the primary means of interaction with endogenous signaling components. In addition, D100 is likely not an alternate phosphorylation site for cytokinin signaling components. Aspartate 100 was changed to an alanine and a glutamate, and each mutation produced different transcriptional responses (Figure 2.19 and Figure 2.21) suggesting a difference between PhoB^{D100A} and PhoB^{D100E} that is not related to phospho-relay as neither alanine nor glutamate are known to accept a phosphate signal in HK signaling pathways.

In contrast to the variable response to TNT, the D100A mutation of PhoB reduces the response to cytokinin in the partial synthetic signaling system suggesting reduced interaction with endogenous cytokinin signaling components. Perhaps this mutated form of PhoB could be used in subsequent version of the synthetic signaling system to reduce cross-talk. As seen in many of the PhoB mutants, high background and un-induced signaling occur in both the partial and complete synthetic signaling

systems. This suggests that cross-talk occurs with multiple plant signaling pathways and additional methods will be needed to make PhoB responsive to a signal that comes exclusively from the synthetic fusion HK Trg-PhoR.

D. Evaluation of approach

The synthetic signaling system that is being analyzed in this work is novel and at the cutting edge of plant synthetic biology. Therefore, an evaluation of the approach taken to reduce cross-talk in this system is warranted.

The reporter gene, GUS, used in these experiments may not have been an ideal choice for this particular system. The GUS protein is very stable and can persist in plant cells for days (Jefferson et al., 1987). The stability of GUS could mean that a small amount of expression cause by leaking of the promoter or background signaling can lead to a build-up of the protein within the cell. This could make it difficult to differentiate between a small amount of induction and a large amount of background. A less stable reporter would likely provide more accurate information about system activity. We have recently found that the use of luciferase (Thompson et al., 1991; de Ruijter et al., 2003) as a reporter gene in this system produces less background (Morey *et al*, unpublished).

Another issue that complicates the work presented here is the use of the computationally re-designed TNT receptor. This receptor has not provided a reliable signal for detailed comparison of various PhoB mutations. Therefore it is difficult to determine the transcriptional response of PhoB mutants when the signal produced using the non-mutated version of PhoB is not obvious. Furthermore effectively

exposing the apoplastically localized TNT receptor to the ligand is challenging because TNT has low solubility in water, can be metabolized and conjugated in plants (Kurumata et al., 2005), and oxidizes readily upon exposure to air. These characteristics of TNT make it difficult to be certain that the plant has been effectively exposed to TNT and at what concentration. One means to partially circumvent this challenge is to analyze the accumulated TNT in the plant. Also, TNT (22 μM, approximately twice the levels used here) has been found to have an effect on the transcriptome of Arabidopsis causing up-regulation of a variety of genes including detoxification and cell wall modification genes (Vanek et al., 2010). Finally, questions about the stability of the TNT receptor in *in vitro* assays, have been raised and some versions of the computationally designed receptors were found to be unstable and prone to aggregation (Schreier et al., 2009). These experiments were not done *in vivo* and possibly the designed receptors behave differently in a cell due to continuous production and the different environment. The stability and function of the computationally designed receptors *in planta* has not been investigated. These complications stimulate the question as to what is causing the expression of the reporter gene, background signaling, cross-talk, and/or response to TNT.

Experiments to understand the behavior of the various PhoB mutants were performed *in planta* because one application for the complete synthetic signaling system is to produce detector plants. However, use of plants as a testing system in this case presents some complications and variables. Plants contain multiple HK signaling components that could possibly affect the activation state of PhoB, directly or indirectly. The cytokinin signaling pathway is the only plant HK pathway known to affect PhoB

(Antunes et al., 2009). Yet other HK pathways exist in plants, such as the ethylene and phytochrome signaling pathways (Schaller et al., 2011) that have not been analyzed to determine possible effects on our synthetic signaling components. Furthermore, the activation of the synthetic signaling system relies on direct contact between the apoplastically localized TNT receptor and externally applied TNT. Leaves in the above described experiments were submerged in a solution containing the ligand and briefly exposed to a vacuum. This is thought to pull the ligand into the apoplast but likely damages membranes and ruptures some cells causing stress and possibly ethylene production. The effect of ethylene on the activation state of PhoB has not been investigated thoroughly. Also, the ethylene receptor has been shown to interact with components involved in cytokinin signaling (Yamaguchi-Shinozaki et al., 2000; Groth et al., 2008; Scharein and Groth, 2011), and cytokinin signaling has been shown to affect PhoB mediated transcriptional response (Antunes et al., 2009). Therefore we cannot rule out the possibility that stress caused by the excised leaf assay used in these experiments may produce artifacts.

The success of the approach taken in this work depended on rationally choosing mutations in PhoB that affect response to cytokinin. However, we do not know what components of the cytokinin signaling pathway PhoB can interact with or what portion of the protein might be interacting. Understanding which components of the cytokinin signaling pathway affect the activation state of PhoB may help in determination of how to reduce cross-talk with the synthetic signaling system. Upon identification of components that interact with PhoB, a selection system can be developed to identify versions of the synthetic components that have desirable characteristics. Different

mutated versions of the synthetic signaling components can be generated and tested in the selection system. Using bacteria as a host for these experiments will allow for high through-put screening of component libraries and will eliminate some of the variables presented by using plants as a host. For example we can test the cytokinin signaling components and not need to consider input from other plant signaling pathways as an additional variable. This approach should be very powerful in that it screens a large number of randomly generated possibilities as opposed to the rational design approach taken here. Testing of cytokinin signaling components for interaction with PhoB in bacteria will be explored in the next chapter.

Chapter Three
Towards Identification of Cytokinin Signaling Components That Can Activate PhoB-mediated Transcriptional Response

I. Introduction

A. Brief Review of Cytokinin Signaling

The Medford Laboratory produced a partial synthetic signal transduction pathway in plants (Antunes et al., 2009). This system is composed of the bacterial response regulator (RR) fused to a eukaryotic transcriptional activator, PhoB-VP64, and the β-glucuronidase (GUS) reporter gene under transcriptional control of the *PlantPho promoter*. Our laboratory showed that PhoB translocates to the nucleus and PhoB-VP64 activates transcription in response to exogenous cytokinin application (Antunes et al., 2009). These results suggest that components of the cytokinin signaling pathway can interact with PhoB.

In plants cytokinin is perceived through a histidine kinase (HK) phospho-relay pathway that regulates transcription of cytokinin response genes. At least three cytokinin receptors have been identified, Arabidopsis Histidine Kinase (AHK) 2, 3, and 4 (also known as CreI) (Kakimoto et al., 2001; Ueguchi et al., 2001b; Ueguchi et al., 2001c; Ueguchi et al., 2004) that bind cytokinin then autophosphorylate the intracellular conserved histidine residue on the HK domain (Kieber and To, 2008). The phosphate signal is transduced to a conserved aspartate on the receiver domain of the cytokinin receptor, then to a conserved histidine on an Arabidopsis Histidine Phosphotransferase

(AHP) (Schmulling and Heyl, 2003). Upon phosphorylation AHPs transmit the signal to Arabidopsis Response Regulators (ARR). The predominantly nuclear localized type-B ARRs activate transcription of target cytokinin response genes (Tajima et al., 2004; Yokoyama et al., 2007). Type-A ARRs are up-regulated in response to cytokinin by type-B ARRs and serve as negative regulators of cytokinin signaling (To et al., 2004). Other proteins reported to regulate cytokinin response genes in an AHK/AHP dependent manner are Cytokinin Response Factors (CRF). Together components of the above described system interact to regulate important processes including shoot and root development, de-etiolation, leaf expansion, root vascular differentiation, and senescence (Kieber and To, 2008).

B. Crosstalk with Synthetic Signaling System

As previously shown, the bacterial response regulator used in our synthetic signal transduction system, PhoB, localizes to the nucleus and activates transcription *in planta*, in response to exogenous cytokinin application (Antunes et al., 2009). The cytokinin signaling pathway is similar to bacterial two-component signaling systems, such as the PhoR/PhoB system, in that the phosphate signal is transmitted between histidine and aspartate residues on signaling components. However, the cytokinin signaling pathway is more complex than bacterial two-component systems containing more signaling components with more phopho-transfer steps. The following work aimed to determine whether cytokinin signaling components, a cytokinin receptor histidine kinase and/or histidine phospho-transferase, can interact with PhoB to better understand possible cross-talk that may occur with our synthetic signaling system.

Cytokinin signaling components chosen for testing are the cytokinin receptor AHK4, and the next components to receive the signal in the pathway, AHPs 1, 2, 3, and 5. By testing AHK4 first for activation of PhoB mediated transcriptional response, I can determine whether there is an interaction between PhoB and AHK4. Then AHPs can be added to the system individually, to determine if any of the AHPs known to receive a signal from AHK4 activate PhoB mediated transcriptional response. Because AHK4 is needed to phosphorylate AHPs in a cytokinin dependent manner, I must first establish the level of PhoB mediated transcriptional response from AHK4 before testing whether or not AHPs have an effect on the system.

The cytokinin receptor AHK4 was chosen to test the interaction between cytokinin receptor histidine kinases and PhoB, because AHK4 was reported to be functionally expressed in bacteria by others in the past (Suzuki et al., 2001; Yamada et al., 2001). Suzuki *et al.* showed that AHK4 is a cytokinin receptor by expressing the plant gene in bacteria. They used the SRC122 bacterial strain that contains a deletion in the sensor HK, *RcsC*. The Rcs phospho-relay system contains the sensor HK RcsC, the histidine phosphotransferase (Hpt) YojN, and the RR rcsB that activates transcription of capsular polysaccharide synthesis genes controlled by the *cps promoter* (Roberts, 1996). The SRC122 bacterial strain also contains the *LacZ* (β-galactosidase reporter gene) controlled by the *cps promoter* allowing for measurement of YojN/rcsB mediated transcriptional response. This system relies on phospho-relay between AHK4 and the bacterial Hpt YojN in the absence of the cognate HK RcsC (Figure 3.1).

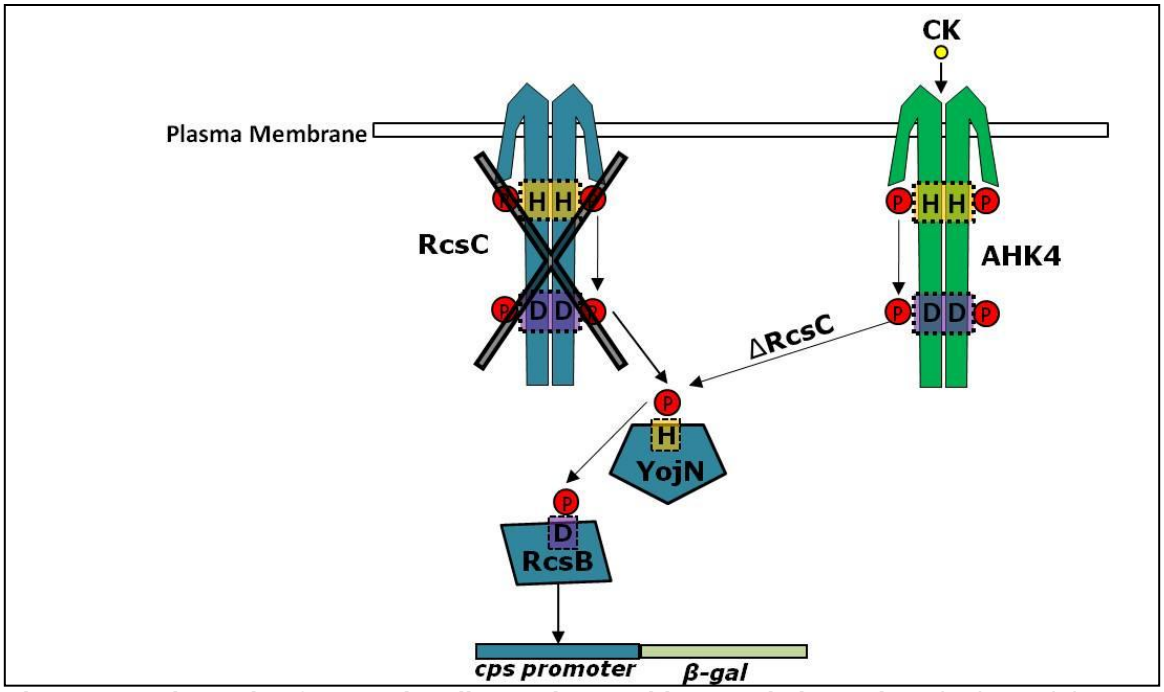

Figure 3.1 Schematic of RcsC signaling pathway with AHK4 in bacteria. The bacterial osmosensor HK, RcsC, initiates a phospho-relay to the Hpt, YojN, that transfers the phosphate signal to the RR RcsB that activates transcription at the *cps promoter.* The Mizuno laboratory reported that in the absence of the HK, RcsC, the plant cytokinin receptor AHK4 activates YojN/RcsB mediated transcription in response to cytokinin (Suzuki et al., 2001).

Suzuki *et al.* reported that the SRC122 cell line expressing *AHK4* shows an increase in β-galactosidase activity in response to cytokinin exposure (Suzuki et al., 2001) while AHKs 2 and 3 showed no response in this system. Additionally, they added AHPs 1, 2, 3 and 5 to the system and reported phospho-transfer between AHK4 and AHPs 2, 5, and 3 (Suzuki et al., 2001). These data suggest that AHK4, and AHPs 2, 3, and 5 can be functionally expressed in *E.coli*. Therefore, these components were chosen to be tested for interaction with PhoB in our bacterial testing system described below.

C. Bacterial Testing System

By testing the cytokinin signaling components using a bacteria testing system I could circumvent the complexities of testing in plants. As discussed above the cytokinin signaling pathway is made up of many different proteins, AHKs, AHPs, CRF's, and ARR's with several members of each type of signaling element (Kieber and To, 2008). The multitude of elements and interactions involved in the pathway could result in multiple unknown interactions with the bacterial RR, PhoB, in plants. By using a bacterial system, I could isolate the signaling components being tested from all other endogenous plant components that could interact and complicate interpretation of my data.

The *E.coli* cell line BW23423 was used to express the synthetic signaling components and cytokinin signaling component(s). This cell line is described in greater detail in Chapter Five; however, the BW23423 cell line contains the *β-galactoside* reporter gene under control of a *Pho promoter* allowing for measurement of PhoB mediated transcriptional response. By following changes in β-galactoside activity in bacteria, I could determine whether cytokinin signaling components activate PhoB mediated transcription in response to cytokinin exposure (Figure 3.2).

In this chapter, I attempted to express cytokinin signaling components AHK4 and AHPs 1, 2, 3, and 5 in bacteria with the goal of testing these components for PhoB mediated transcriptional response. Success in this approach would have identified components that can interact with PhoB and produced a bacterial screening system for testing different versions of PhoB.

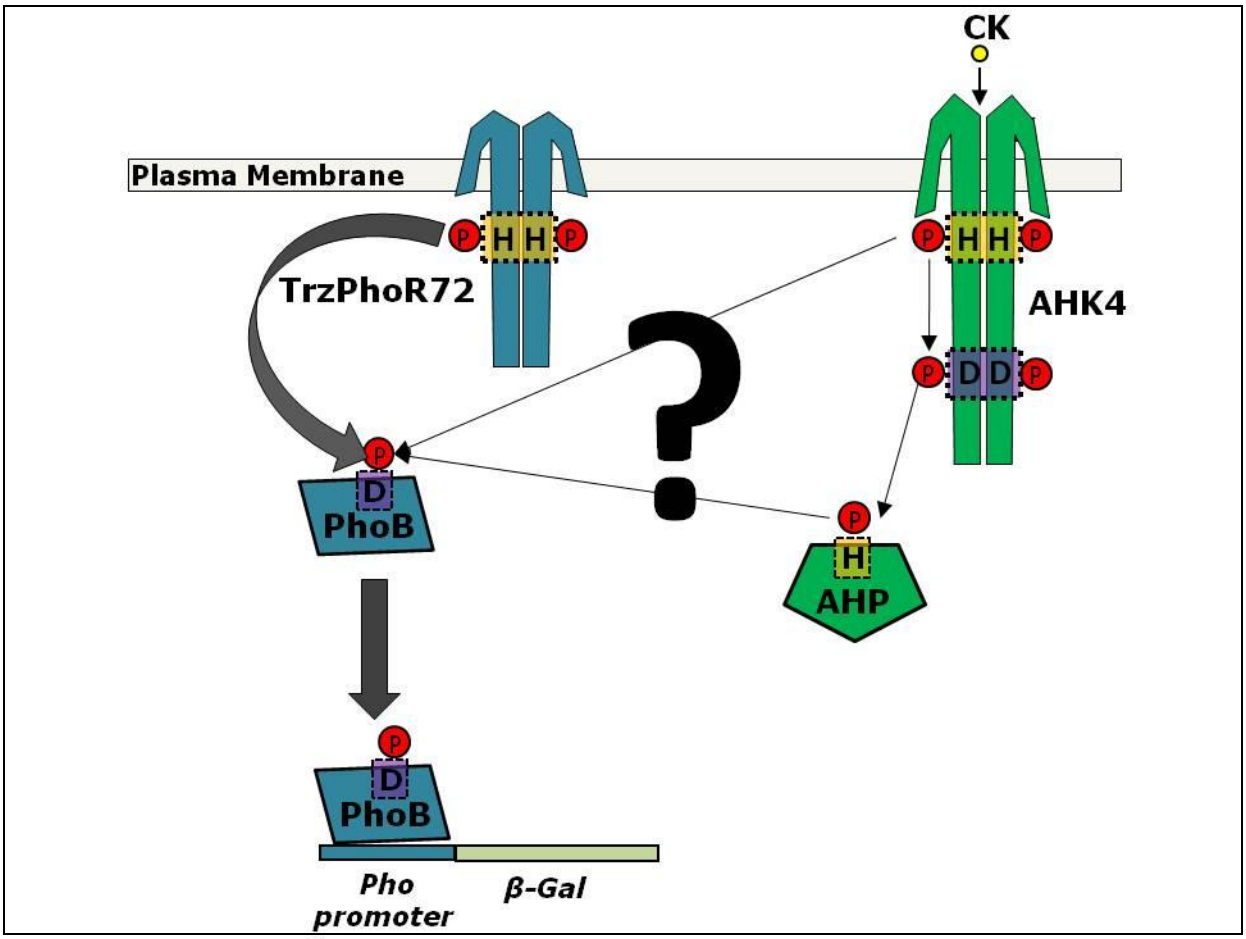

Figure 3.2 Schematic of Bacterial testing system with cytokinin components
Bacteria expressing the synthetic signaling components with AHK4 and separately with each AHP, were tested for changes in β-galactosidase activity in response to exogenous cytokinin. CK: cytokinin; H: histidine; D: aspartate; P: phosphate; β-gal: β-galactosidase reporter gene; TrzPhoR72: version of synthetic HK fusion between chemotactic receptor Trg and the phosphate sensor PhoR (Dr. Kevin Morey, unpublished, described in Chapter 4).

II. Cloning Strategies and Results for Expressing Cytokinin Signaling Components in Bacteria

A. Arabidopsis Histidine Phosphotransferases (AHPs)

Arabidopsis histidine kinases mediate their phospho-relay through AHPs. To test if the AHK-AHP phospho-relay can transmit a signal to PhoB, the coding sequence for each AHP was individually cloned into a previously constructed plasmid constitutively

expressing the synthetic signaling components; the synthetic fusion HK *Trz-PhoR72*, the RR *PhoB*, and the ligand receptive PBP, in this case, ribose binding protein (RBP) (Figure 5.2). The coding sequence for each AHP was PCR amplified with the *XhoI* and *EcoRI* restriction sites (Appendix B-10), from cDNA stocks supplied by The Arabidopsis Information Resource (TAIR, www.arabidopsis.org). Each AHP 1, 2, 3 and 5 was individually cloned in place of *RBP*, producing four separate plasmids, referred to as the AHP plasmids. In addition to expressing each wild-type version of the four AHPs, versions codon optimized for expression in *E.coli* (Appendix B-1) were synthesized by GenScript and cloned in the same manner as described above (Figure 3.3).

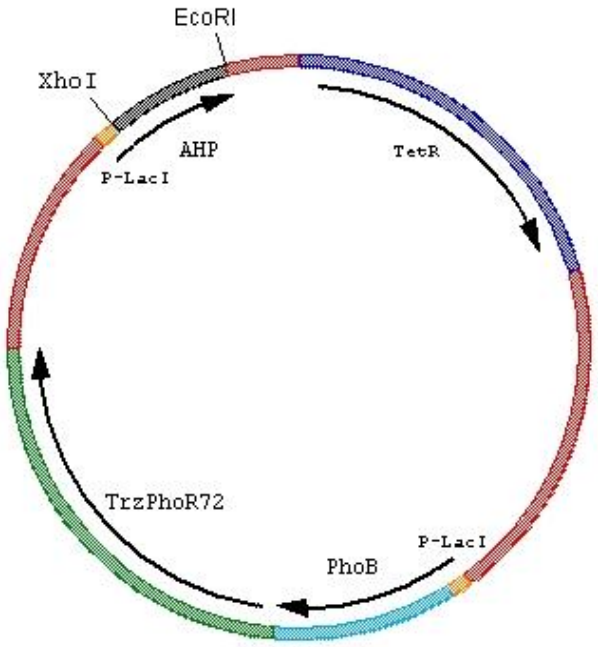

Figure 3.3 Map of AHP Plasmid. Plasmid contains the synthetic signaling components PhoB and fusion HK,Trz-PhoR72, constitutively expressed in an operon. Each of the AHPs was placed downstream of the LacI promoter in a separate position on the pACYC177 backbone (Chang and Cohen, 1978). *P-LacI*: LacI promoter; *TetR*: *tet* gene selectable marker, confers resistance to the antibiotic tetracycline. Plasmid is approximately 7.5 kb depending on which of the four AHPs is encoded.

B. Initial Attempt to Express AHK4 in Bacteria

To test if the cytokinin receptor AHK4 can transmit a signal to PhoB, initially I attempted to constitutively express *AHK4* under control of the *LacI promoter* in the pBR322 plasmid (Bolivar et al., 1977). Due to the relatively large size of the *AHK4* gene (3.2 kb) and an internal *BglII* restriction site (providing a convenient location for splitting the gene), the gene was cloned in two pieces. Each piece was PCR amplified, adding the appropriate restriction sites (Appendix B-10), from an AHK4 clone kindly provided by Tatsu Kakimoto (Higuchi et al., 2004). First the 2.1 kb 3' end of *AHK4* was inserted into the pBR322 plasmid followed by insertion of a 1.2 kb 5' fragment that included the *LacI promoter* (Figure 3.4).

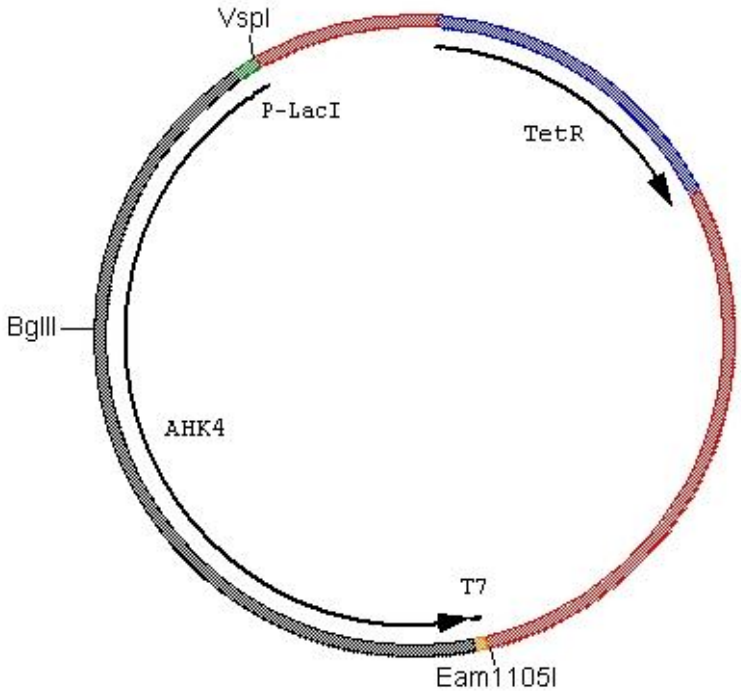

Figure 3.4 Map of Plasmid to Express AHK4 in Bacteria. Plasmid constitutively expresses *AHK4* on the pBR322 backbone (8.2kb). The *BglII* restriction site was used to split the gene into two pieces for cloning. T7: *T7 terminator*; P-Lac: *LacI promoter*; TetR: tet gene, selectable marker, confers resistance to the antibiotic tetracycline.

After several failed attempts at cloning the 5' end of *AHK4* into the pBR322 plasmid containing the 3' end of *AHK4*, examination of the complete *AHK4* sequence revealed 39 instances of arginine codons that are the least frequently used in *E.coli* (Nakamura et al., 2000). Expression of genes containing rare codons in *E.coli* can lead to translational stalling, frame shifting and mis-incorporation of amino acids (Dong et al., 1996). To diminish expression issues due to rare codon usage, *AHK4* ligations were transformed into a commercial cell line that over-expresses rare tRNAs on a plasmid (Rosetta DE3, Novagen). After eleven attempted ligations using the Rosetta cell line, 135 selected colonies growing on media with the antibiotic tetracycline were PCR screened using primers specific to the 5' end of *AHK4*. Ten putative clones were sequenced (Macrogen, USA) to verify the presence of the correct *AHK4* gene. However, sequencing results for the ten putative clones show deletions, point mutations, re-arrangements, and duplications in the coding sequence of *AHK4*. The sequence of the final putative clone is shown with incorrect nucleotides annotated in Appendix B-2.

C. Inducible Expression of *AHK4*

As previously described, others have reported successful expression of AHK4 in *E.coli* (Suzuki et al., 2001; Yamada et al., 2001). Suzuki *et al*. used the pIN-III-OmpA3 bacterial expression vector (Duffaud et al., 1987) to provide inducible expression of the *AHK4* gene. This vector has features to aid in proper localization and expression of foreign proteins. In the pIN-III-OmpA3 bacterial expression vector the constitutive lipoprotein gene promoter (*lpp promoter*) upstream of the *lac operator* (*LacOP*)

sequence controls expression of the gene of interest and the plasmid constitutively expresses the *lac repressor (LacI)*. This combination of regulatory elements provides expression in the presence of a lac inducer such as isopropyl β-D-1-thiogalactopyranoside (IPTG) (Figure 3.5). The pIN-III-OmpA3 bacterial expression vector also uses the OmpA3 signal peptide to direct the protein being expressed to the outer membrane.

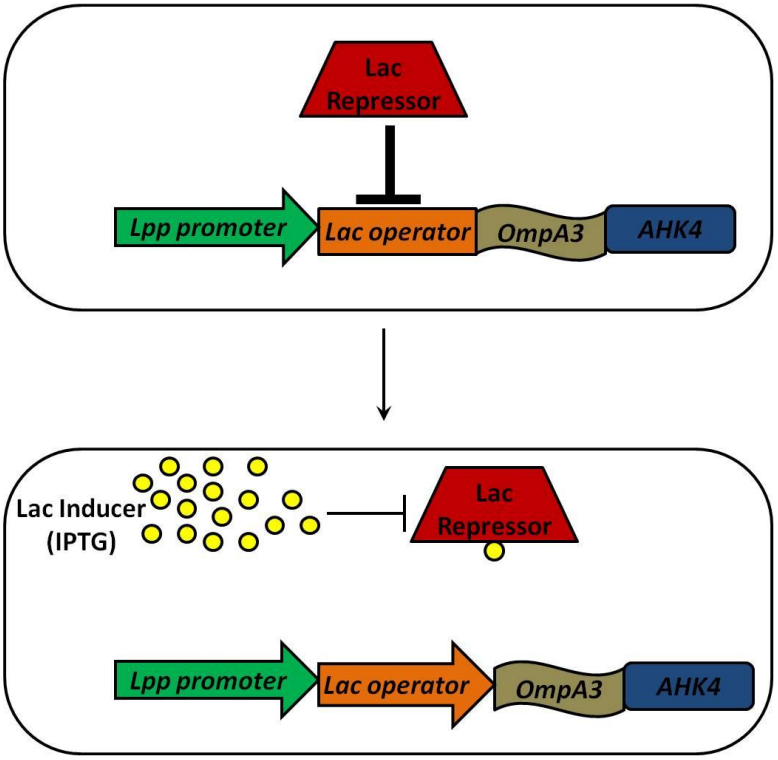

Figure 3.5 Diagram of inducible expression provided by the pIN-III-OmpA3 vector. In the top panel the Lac repressor element (LacI) binds the *Lac operator* sequence and represses transcription of downstream genes. When a Lac inducer, in this case IPTG, is added to the system (bottom panel), IPTG binds the Lac Repressor releasing repression of the *Lac operator* and allowing the *Lpp promoter* to initiate transcription of downstream genes. The pIN-III-OmpA3 bacterial expression vector targets the gene product to the outer membrane using the OmpA3 signal peptide sequence.

The Mizuno laboratory used pIN-III-OmpA3 to express *AHK4* in bacterial strain SRC122 and reported that AHK4 serves as a cytokinin receptor (Suzuki et al., 2001). We

obtained the vector from the Mizuno laboratory (Figure 3.6) which was to provide inducible expression of *AHK4* in our bacterial testing system.

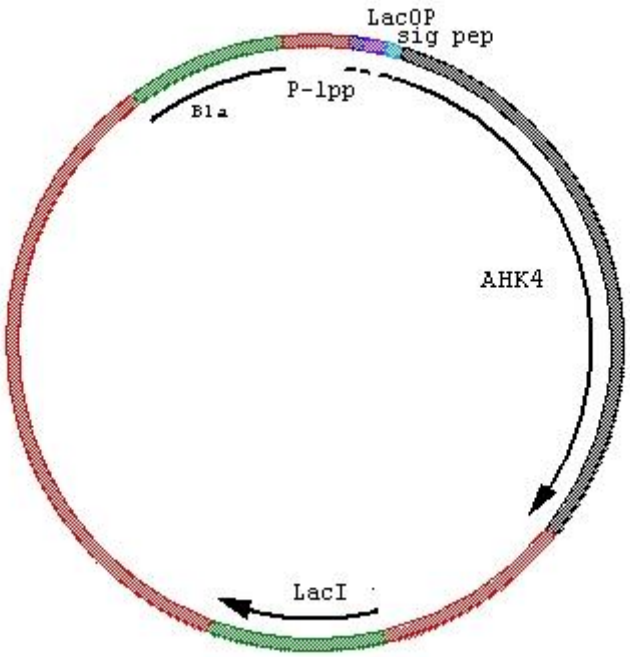

Figure 3.6 Map of pIN-III-OmpA3 plasmid with AHK4. Plasmid map of the pIN-III-OmpA3 plasmid with inducible expression of *AHK4*, using the *lipoprotein promoter* (*P-lpp*) upstream of the *Lac operator* sequence (*LacOP*). The plasmid constitutively expresses the *Lac repressor* (*LacI*) for tight repression of *AHK4*. AHK4 is targeted to the membrane using the OmpA3 signal peptide (*sig pep*). *Bla*: β-lactamase gene, selectable marker, confers resistance to the ampicillin family of antibiotics.

Before proceeding with PhoB studies using AHK4 in our bacterial testing system, I attempted to experimentally verify the pIN-III-OmpA3 vector that contains *AHK4* by reproducing the results obtained by the Mizuno laboratory in the SRC122 cell line. After 18 failed attempts to replicate the reported results, I had *AHK4* in the pIN-III-OmpA3 vector sequenced. Plasmid samples isolated from an experimental plate in the SRC122 cell line and from the glycerol stock cultured with the original bacterial stab sent by Mizuno were sequenced (Macrogen USA). Sequencing results show large deletions in the *AHK4* gene from both the experimental and original samples (Appendix B-3); however the deleted portions were not identical. One possible explanation of these

results could be that additional culturing of bacteria harboring the plasmid causes changes in the AHK4 sequence. It is not known if the gene was already mutated when we received the clone or if I caused mutation when initially culturing the bacteria. However, the bacteria for the glycerol stock were grown immediately upon arrival using standard methods as described by Suzuki *et al.*

D. AHK4 Codon Optimized for *E.coli*

Constitutive and inducible expression of the plant *AHK4* gene in bacteria was not successful. Furthermore, use of the Rosetta DE3 plasmid over-expressing rare *E.coli* tRNAs did not mediate expression issues of *AHK4* in bacteria. To eliminate expression problems caused by codon usage, the sequence for *AHK4* was codon optimized for *E.coli* (*EcAHK4*) and synthesized (GenScript) with the *OmpA3* signal peptide sequence (Appendix B-4), as described by Duffaud *et al.,* 1987, in front of *EcAHK4* (Appendix B-5) to target the protein to the membrane. Upon arrival *OmpA3-EcAHK4* was PCR amplified adding the *LacI promoter* to the 5' end for constitutive expression and the GlmS transcriptional terminator (Gay et al., 1986) to the 3' end (Appendix B-10). The resulting PCR product, *P-LacI-OmpA3-EcAHK4-T-Glms*, was directly cloned into the pBR322 plasmid using the unique *BAMHI* and *SalI* restriction sites (Figure 3.7).

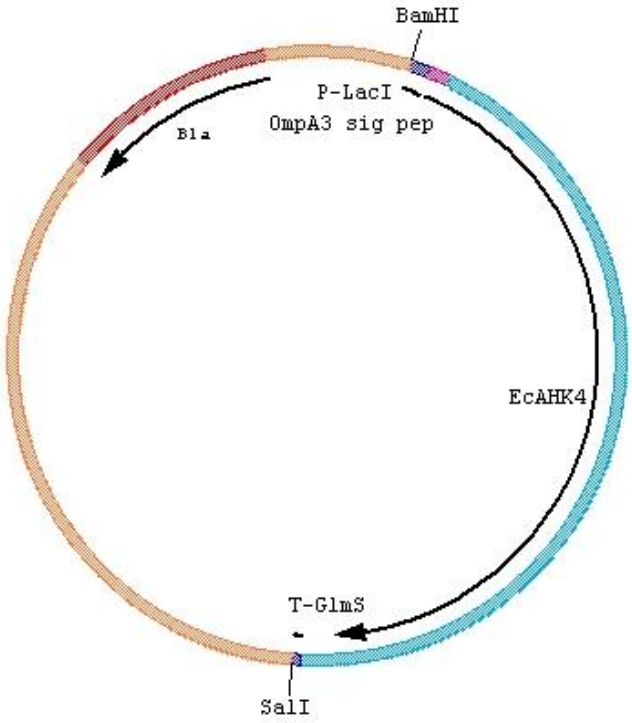

Figure 3.7 Map of plasmid to constitutively express EcAHK4. Map of pBR322 plasmid constitutively expressing *EcAHK4* (codon optimized for *E.coli*) using the *LacI promoter* (*P-LacI*) (7.5 kb). *EcAHK4* was synthesized with the *OmpA3 signal peptide* sequence on the 5' end of the gene for membrane localization. *T-GlmS*: GlmS terminator; *Bla*: β-lactamase gene, selectable marker, confers resistance to the ampicillin family of antibiotics.

After three attempted ligations, 69 colonies growing on media containing the antibiotic carbenicillin were PCR screened using primers specific to *EcAHK4*. Of the 69 colonies PCR screened for the presence of *EcAHK4,* eight putative clones were screened for proper insertion of the entire *P-LacI-OmpA3-EcAHK4-T-GlmS* fragment using restriction digests. Restriction enzymes, BamHI and SalI, used for the digest screen should have produced two distinct fragments if the cloning were successful, however digested DNA ran on agarose gel shows multiple bands of incorrect size (Appendix B-6) suggesting that the *P-LacI-OmpA3-EcAHK4-T-GlmS* fragment is not correct within the pBR322 plasmid.

This version of AHK4 has been codon optimized for *E.coli* and does not contain the rare codons that can cause translational stalling, frame shifting, and mis-incorporation of amino acids. However, codon optimization of *AHK4* did not alleviate cloning and expression issues, leading to the question of whether the presence of rare codons is the only difficulty of expressing AHK4 in bacteria.

E. Construction of bacterial expression vector

Next, I designed and constructed a bacterial expression vector containing features to tightly regulate inducible expression of *EcAHK4*. The pBR322 plasmid was chosen as a starting material to produce the bacterial expression vector because it contains the pMB1 origin of replication which differs from that of the pACYC plasmid used to express the synthetic signaling components and AHPs, ensuring compatibility of both plasmids. Also, the pBR322 plasmid contains the *β-lactamase* gene that confers resistance to the antibiotic carbenicillin while the pACYC plasmid, carries the *tet* gene that confers resistance to the antibiotic tetracycline. Having compatible origin of replications and distinct antibiotic resistances ensures that both the pBR322 plasmid and the pACYC plasmid can be selected for, and maintained, *in vivo.* The pBR322 plasmid expresses the Rop gene product, which regulates plasmid replication and maintains a copy number at approximately 20 per cell (Cesareni et al., 1984). I used the *Lac promoter/operator* (Lac^{PO}) sequence to control transcription of *EcAHK4*, so that transcription is repressed unless a Lac inducer is present. To ensure repression of *EcAHK4*, the Lac repressor gene, *LacI*, was inserted into pBR322 with the constitutive *LacI promoter (P-LacI).* To prevent read-through from other promoters and leaky

expression of *EcAHK4*, the strong *rrnBT1T2 transcriptional terminator* (Guzman et al., 1995; Anthony et al., 2004) was placed up-stream of the *Lac*^{PO} regulatory sequence (Figure 3.8).

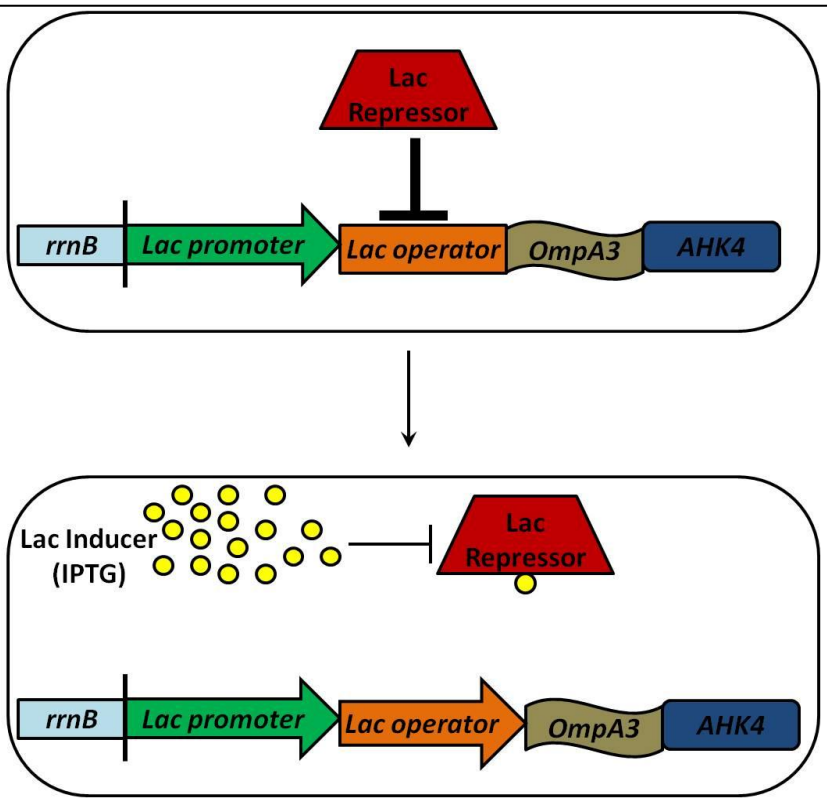

Figure 3.8 Diagram of inducible expression provided by the bacterial expression vector design. In the top panel the Lac repressor element (LacI) binds the *Lac operator* sequence and represses transcription of downstream genes. When a Lac inducer, in this case IPTG, is added to the system (bottom panel), IPTG binds the Lac Repressor releasing repression of the *Lac operator* and allowing the *Lac promoter* to initiate transcription of downstream genes. The gene product is targeted to the outer membrane using the OmpA3 signal peptide sequence. The rrnB transcriptional terminator sequence prevents any uninduced transcription of the downstream AHK4 gene.

Together these elements have been shown to provide tight regulation of genes toxic to bacteria (Anthony et al., 2004) and the resulting plasmid should tightly express *AHK4* in *E.coli* (Figure 3.9).

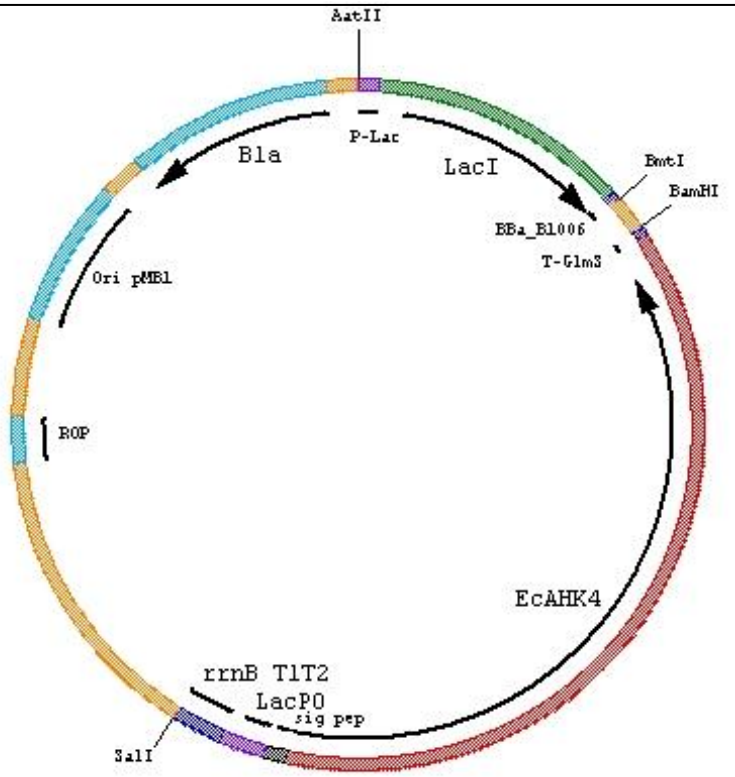

Figure 3.9 Map of bacterial expression vector with EcAHK4. Map of plasmid for inducible expression of *EcAHK4* (codon optimized for *E.coli*) using the *Lac promoter operator* sequence (*LacPO*). The *rrnB T1T2 terminator* sequence (*rrnB T1T2*) was synthesized upstream of *LacPO* to reduce potential leaky expression of *EcAHK4.* The bacterial expression vector (8.8 kb) constitutively expresses the Lac repressor (LacI) for tight repression of *EcAHK4*. *ROP*: gene product regulates plasmid replication; *Ori pMB1*: origin of replication; *Bla*: β-lactamase, selectable marker, confers resistance to the antibiotic carbenicillin; *P-Lac*: *LacI promoter*; *BBa_B1006*: BioBricks terminator sequence; *T-GlmS*: *GlmS terminator.*

The Lac repressor (*P-LacI-LacI)* fragment was PCR amplified from the pET28 plasmid (Novagen) adding the *AatII* restriction site to the 5' end and the *BmtI* restriction site to the 3' end of the fragment for cloning into pBR322. As previously mentioned in Chapter One, the Registry for Standard Biological Parts provides a catalog of standard parts for building synthetic systems. I chose the BioBricks *B1006* transcriptional terminator sequence (www.partsregistry.org/ Terminators/Catalog) to terminate transcription of the *LacI* gene and added the sequence to the 3' end of the *LacI* gene

with PCR. The *P-LacI-LacI-T-B1006* fragment was successfully cloned into pBR322 and sequence verified (Macrogen, USA).

The elements regulating transcription of *EcAHK4,* the transcriptional terminator sequence and Lac promoter/operator, *rrnBT1T2-Lac^{PO},* were synthesized as a single fragment by Integrated DNA Technologies (Appendix B-7). I placed the *rrnBT1T2-Lac^{PO}* fragment up-stream of the *OmpA3* signal peptide sequence at the 5' end of *EcAHK4* using overlapping PCR (Appendix B-10). The first two PCR reactions placed 15 base pairs (bp) of overlap on each fragment; adding 15 bp of the *OmpA3 signal peptide* sequence to the 3' end of the *rrnBT1T2-Lac^{PO}* fragment and adding 15 bp of the *Lac^{PO}* sequence to the 5' end of the *OmpA3-EcAHK4* fragment. The final PCR reaction amplified the entire *rrnBT1T2-Lac^{PO}-OmpA3-EcAHK4* fragment adding a *SalI* restriction site to the 5' end and the *GlmS* transcriptional terminator with a *BAMHI* restriction site to the 3' end for cloning into the pBR322 bacterial expression vector (diagrammed in Appendix B-8). After five attempted ligations, 12 colonies were PCR screened for the presence of *EcAHK4* in pBR322. Four putative clones were sequenced (Macrogen, USA). The resulting sequences show deletions in various parts of the promoter region extending into *EcAHK4* (Appendix B-9). The deleted portions of the inserted fragment were not the same in each of the four clones that were sequenced, suggesting that the deletions occurred after the ligation independently in each of the four clones.

III. Discussion

A. *AHK4* is not properly maintained in *E,coli* using the above described approaches

I attempted five different approaches to clone and/or express *AHK4* in *E.coli*, each of which was unsuccessful. I tried constitutive expression of AHK4 with and without the help of the Rosetta plasmid to reduce expression problems caused by several rare codons in the gene. I obtained the bacterial expression vector pIN-III-OmpA3 with *AHK4* that was reported to produce a functional AHK4 protein in bacteria (Suzuki et al., 2001). I obtained a version of AHK4 codon optimized for *E.coli* and attempted to constitutively express *EcAHK4* using the low-copy pBR322 plasmid. Finally, I produced a bacterial expression vector designed to tightly regulate inducible expression of *EcAHK4*. In all cases sequences of clones show deletions and mutations in the regulatory and/or coding region of *AHK4* and *EcAHK4;* or digest screens of putative clones indicate the gene is not correctly inserted in the vector. These results show that the *AHK4* and *EcAHK4* coding sequences are not properly maintained in the bacterial strains we are using, suggesting there may be selective pressure against expressing this gene in *E.coli*.

Perhaps *AHK4* could be successfully expressed in *E.coli* using different regulatory elements to provide inducible expression. I tried to inducibly express *AHK4* using the pIN-III-OmpA3 vector and the bacterial expression vector I designed. Both of these vectors provide inducible expression using the *Lac operator* sequence. Perhaps the Lac regulatory system is not appropriate for maintaining and expressing this particular gene and another inducible system could successfully express *AHK4* in bacteria. The Chory laboratory was recently able to successfully express then purify the

sensor portion of the AHK4 gene from bacteria using a cold inducible system (Hothorn et al., 2011). Additionally they have provided detailed methods regarding the growth conditions, expression vector and media used in their experiments (Hothorn et al., 2011), whereas the Mizuno laboratory has not provided these details for their work with bacterial expression of AHK4 (Suzuki et al., 2001). Perhaps the cold inducible expression system using the described methods is more appropriate for expression of the AHK4 gene and could be used in future experiments by the Medford laboratory.

Despite successful cloning of all four AHPs for expression in the bacterial testing system I cannot complete the proposed experiments because I was not able to produce a stable bacterial clone of AHK4 allowing expression of the protein, nor show that the coding sequence is maintained in an existing vector. AHK4 is required for cytokinin dependent phosphorylation of AHPs in the bacterial testing system. Therefore, the proposed experiments to test cytokinin signaling components for activation of PhoB mediated transcriptional response are not possible at this time.

B. Other approaches to identify AHPs that may interact with PhoB

1. Other Cytokinin Receptors

Cytokinin is perceived in plants not only by AHK4 but also by AHK2 and AHK3. The above work focused on the AHK4 cytokinin receptor because it was reported to be functional as a cytokinin receptor in bacteria via interaction with the endogenous YojN/rcsB signaling pathway (Suzuki et al., 2001). AHK2 and AHK3 were also examined in the same study and were not reported to be functional in bacteria. However, later work by the same group that examines the function of mutant AHKs and

reported functional AHK2 and AHK3 proteins in bacteria (Yamashino et al., 2007). Furthermore, AHK2 and AHK3 have been reported to interact with each of the four AHPs that are positive regulators of cytokinin signaling (Heyl et al., 2006). Perhaps AHK2 or AHK3 could be expressed in our bacterial testing system to determine whether a cytokinin dependent transcriptional response is observed. Also, because they activate downstream AHPs they could be expressed with the AHPs 1,2,3, and 5 in our bacterial testing system to test for cytokinin dependent response with each of the AHPs individually. However, similarly to AHK4 some have reported instability of AHK2 in bacteria and were only able to express the CHASE domain and associated transmembrane portions of the protein (Schmulling et al., 2011). Yet AHK3 has not been reported to be unstable when expressed in bacteria, so perhaps expression of AHK3 in our bacterial testing system is worth trying.

2. The Ethylene Receptor ETR1 Interacts with AHPs

One of the known ethylene receptors in Arabidopsis, ETR1, is a sensor HK which has been reported to interact with AHPs 1,2 and 3 in pair-wise yeast-two-hybrid studies; AHP5 was not tested (Yamaguchi-Shinozaki et al., 2000). More recently, *in vitro* fluorescence polarization studies have shown a tight complex formation between AHP1 and ETR1 (Groth et al., 2008) that is dependent on the phosphorylation state of the proteins (Scharein and Groth, 2011). Also Hass *et al* used Arabidopsis mutant lines to show that the type-B response regulator ARR2 plays a role in downstream ETR1 signal transduction (Hass et al., 2004). Additionally, ARR2 has been shown to interact with all of the AHPs 1-5 in yeast-two-hybrid analyses (Ueguchi et al., 2001a). Together

these data suggest the possibility of an ethylene responsive signal transduction pathway that uses components known to act in the cytokinin signaling pathway. I have hypothesized that the seemingly promiscuous AHPs also interact with our synthetic signaling system. Perhaps AHPs are the mechanism by which cross-talk occurs with not only the cytokinin signaling pathway, but also the ethylene signaling pathway that has not been tested for cross-talk with the synthetic signaling system. Because ETR1 has been expressed in bacteria (Voet-van-Vormizeele and Groth, 2003), perhaps we could use ETR1 to test AHPs 1, 2 and 3 individually in our bacterial testing system with PhoB to determine the response to ethylene exposure. This would identify AHPs likely to interact with PhoB and identify the mechanism by which the cytokinin signaling pathway could cross-talk with the synthetic signaling system. However, there are several unknown factors that may preclude use of ETR1 to phosphorylate AHPs in bacteria. First, it is not known whether the protein is functional in bacteria as previous experiments expressing ETR1 in *E.coli* did not examine the function of the protein *in vivo* (Voet-van-Vormizeele and Groth, 2003). Also, the possible interaction between ETR1 and AHP5 has not been examined therefore it is not known whether ETR1 interacts with one of the four AHPs that positively regulate cytokinin responses. Finally, exposing bacteria to a known concentration of ethylene could be challenging, which would limit data collection to only qualitative results. At the very least, several preliminary experiments would need to be done to resolve unknown factors in using ETR1 to phosphorylate AHPs in bacteria.

3. Other approaches that might be appropriate to identify cytokinin signaling components that interact with PhoB include pair-wise yeast two-hybrid assays and *in planta* FRET based protein interaction studies. However, there are issues with using these approaches as well. Additionally, one of the advantages of testing cytokinin signaling components in bacteria is that the assays would result in a bacterial screening system that could be used to screen large numbers of PhoB mutants for interaction with cytokinin signaling components that phosphorylate wild-type PhoB. However, if the above described approaches of testing cytokinin signaling components for PhoB mediated transcriptional activation in bacteria cannot be completed, then protein interaction studies in yeast and/or plants could be pursued.

Chapter Four

Towards Detection of Methyl tert-butyl ether in Plants

I. Introduction

A. Description of MTBE

1. Chemical Characteristics

Methyl tert-butyl ether (MTBE) is an alkyl ether that is used as a fuel oxygenate and component of reformulated gasoline. The 1990 Clean Air Act Amendments require several metropolitan areas to use oxygenated fuel in the winter while others must use reformulated gasoline year-round as a means to reduce carbon emissions (Squillace, 1998). MTBE was originally added to gasoline as an octane booster since the elimination of tetra-alkyl leaded gasoline in 1988 (Hunkeler et al., 2001). The chemical properties of MTBE are optimal for use as a fuel oxygenate. MTBE is cost effective, easily produced from *iso*-butylene and blends into gasoline without separating (Squillace, 1998). The same chemical properties that make MTBE a good fuel additive also make this compound a persistent and wide-spread water contaminant. MTBE has been found in major aquifers, ground water of both urban and agricultural areas, and in storm water run-off (Squillace, 1998; Moran et al., 2005). This gasoline additive is highly water soluble, up to 50 g L^{-1}, much more so than other components of gasoline. MTBE sorbs weakly to solids, K_{oc} = 12.3 (measure of a material's tendency to absorb to solid soil particles), and partitions strongly from air to water. Furthermore, this highly hindered ether is not easily biodegraded and very few bacteria have been identified that are able to metabolize it (Davis and Erickson, 2004). These characteristics make MTBE especially prone to spread and persist in the environment. For example, it has been

shown to have a half-life of about one year in an underground aquifer in Canada (Schirmer and Barker, 1998). Furthermore, because of the high water solubility of MTBE, it tends to travel with water. This means that the distance that a plume of MTBE can travel in water is relatively equal to the distance that the water itself travels. Distances that a contaminated plume of surface water has travelled range from 0.8 km to over 900 km, depending on the depth and aeration level of the stream or river (Pankow et al., 1996). The unique chemical properties of MTBE make it predisposed to contaminate and persist in water.

2. Toxicity of MTBE

Toxicity experiments have been done with a variety of freshwater organisms to determine acceptable levels of MTBE in water and soil (Werner et al., 2001; Lee, 2008). Methyl-*tert*-butyl ether is lethal to 14 freshwater invertebrates, amphibia, and fish when exposed to high concentrations ranging from 57 – 2500 mg L^{-1}. Freshwater bacteria are affected at concentrations as low as 7.4 mg L^{-1}, while some fish and an invertebrate tested showed no observable affects caused by MTBE exposure (Werner et al., 2001). Earthworms exposed to MTBE in soil and on filter paper had a reduced survival rate at 2656 mg kg^{-1} and 0.2 mg/cm^2 respectively (Lee, 2008).

Several studies which measure the toxicity of MTBE to humans have been conducted (Ahmed, 2001; McGregor, 2006; Phillips et al., 2008). However, each of these studies has inherent limitations because results include human health complaints which can be non-specific and subjective. Humans can be exposed to MTBE through inhalation of vapors and through consumption of contaminated drinking water. It has been found that levels of MTBE in the air and water are positively correlated with the

amount of *tertiary*-butyl alcohol (TBA) in blood of humans exposed to MTBE. TBA is produced in the liver of exposed humans and is the primary break-down product of MTBE. Other break-down products include 2-hydroxyisoutyrate and 2-methyl-1,2-propanediol (Mumtaz et al., 1996). In general, health complaints associated with MTBE exposure include headache, cough, nausea, mucosal irritation, dizziness and disorientation. A study found that subjects with the highest levels of MTBE in their blood had a significant increase in occurrence of health complaints (White et al., 1995). Several other studies have reported similar results (Mohr, 1994; Anderson, 1995; Cain et al., 1996). One study examined an objective health effect, nasal blockage index, among subjects exposed to MTBE vapors and found a significant positive correlation with exposure (Nihlen et al., 1998). The mechanism of toxicity and extent of damage to mammalian cells has not been investigated. The available data suggests that MTBE has transient yet acute health effects on humans. Information about consequences of long-term exposure is lacking.

Examination of the long-term effects of MTBE exposure has been conducted in mice and rats. Both animals develop tumors in response to MTBE exposure (250-1000 mg kg body weight^{-1}) over their lifetime and several studies also reported decreased lifespan (Belpoggi et al., 1997; Bird et al., 1997; Williams et al., 2000). Consequently the U.S. Environmental Protection Agency has classified MTBE as a possible human carcinogen, and issued a drinking water advisory of 20-40 µg L^{-1} (Squillace, 1998). The possibility of adverse health effects caused by contaminated drinking water presents a need to examine the extent of contamination of ground and surface water by MTBE.

3. Occurrence of MTBE

Information about the occurrence of MTBE in ground water around the U.S. has been collected for the last two decades. A study performed over a ten- year period analyzed 3964 water samples from across the U.S. for presence of MTBE and other gasoline constituents (Moran et al., 2005). Three hundred samples contained detectable levels of MTBE occurring in 28 states (Figure 4.1).

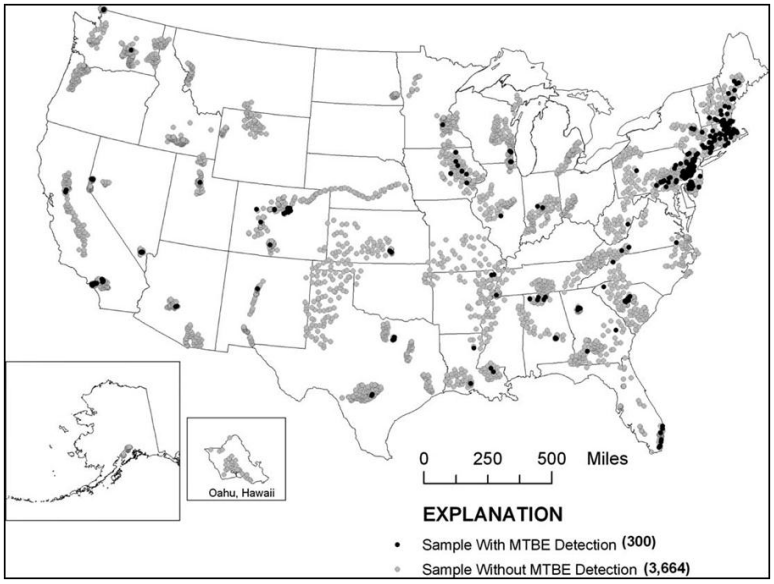

Figure 4.1 Location of samples from ground water quality studies that were analyzed for MTBE and the locations of samples with detections of MTBE (Moran et al., 2005).

Detection frequency was lowest near agricultural lands while being significantly higher in urban areas, especially in the northeast. Only 13 of the samples collected had MTBE concentrations that exceed the lower limit of the EPA drinking water advisory (20 μg L^{-1}) and 113 of the samples reported less that 0.2 μg L^{-1}. However, of the 13 samples exceeding the EPA advisory, 12 were from urban areas and one was from a major aquifer, which supplies millions of people with drinking water (Moran et al., 2005). Despite the EPA drinking water advisory, there are no federal standards for MTBE and 38 states have implemented their own action levels and drinking water standards. For

example, New Hampshire and California have a drinking water standard of 13 μg L^{-1} (Davis and Erickson, 2004; Ayotte et al., 2008). Also, several states have banned the use of MTBE in gasoline hoping to avoid further contamination of water. Collectively these studies suggest that there is a prominent need for continuous monitoring of water over widespread areas to detect MTBE and other potentially harmful contaminants.

Plants could serve as monitors of water and soil if they could be made to detect harmful substances such as MTBE. The limited information available regarding phytotoxicity of MTBE indicates that some plants are relatively tolerant to MTBE compared to many other organisms (An et al., 2002; Yu and Gu, 2006). Phytotoxicity tests suggest that leafy plants, such as lettuce, are more sensitive to MTBE than grasses or trees, and leaves are more sensitive to MTBE exposure than roots or stems. It has been hypothesized that the site of the toxic action of MTBE in plants is likely in the leaves where transpiration and photosynthesis occur (An et al., 2002). Several plants species have been shown to take up large amounts of MTBE through the roots from the soil or water in which they are grown (Hong et al., 2001; Zhang et al., 2001; Yu and Gu, 2006; Arnold et al., 2007). Once taken up by the roots, the majority of MTBE moves with water though the xylem to the leaves where MTBE has been shown to transpire out of the stomata unchanged. Some MTBE is lost to radial movement through the stem but, no breakdown products of MTBE were found in any plant tissue (Hong et al., 2001; Yu and Gu, 2006). Most of this information was gathered to assess the viability of phytoremediation of MTBE, however the same characteristics make MTBE an ideal candidate for detection by plants.

B. Description of MTBE-PBP

1. MTBE-RBP Design

The computational design method used to alter the binding pocket of ribose binding protein to bind TNT instead of ribose was used to produce a protein designed to bind MTBE, again by Dr. Hellinga's laboratory at Duke University. The MTBE receptor was tested in the Hellinga laboratory for functionality in bacteria using a Trz-OmpR signaling system, described in Chapter Five. The bacteria containing this system were reported to have increased GFP expression in response to MTBE in agar (Figure 4.2). The Hellinga lab used *in vitro* methods to measure the MTBE binding affinity of the MTBE receptor and found the receptor has a relatively low binding affinity, K_d= 6 µM (unpublished data), compared to that of the TNT receptor (K_d= 2 nM).

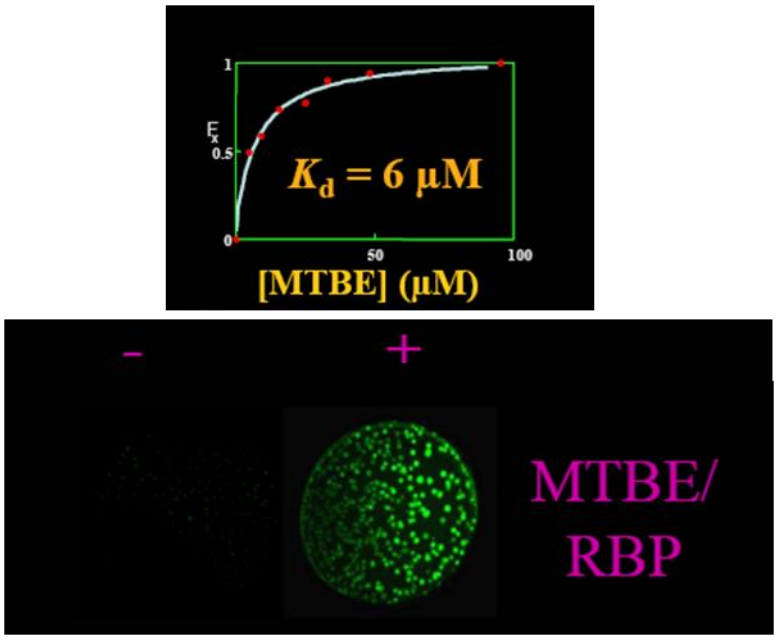

Figure 4.2 Data collected and provided by Dr. Hellinga's lab showing the MTBE receptor is functional in bacteria. The top image depicts the binding affinity for the MTBE receptor. The bottom image shows GFP fluorescence increases in the presence of MTBE (unpublished, Hellinga).

2. Can the MTBE receptor be used in plants?

To determine the feasibility of using plants to detect the environmental pollutant MTBE, the computationally designed MTBE receptor was expressed in plants with the complete synthetic signaling system described in Chapter One. Recall the complete synthetic signal system contains the synthetic fusion HK Fls-Trg-PhoR, the response regulator PhoB-VP64, and the *PlantPho* promoter controlling transcription of response genes. The Pex secretory sequence was used to target the MTBE receptor to the plant apoplast, as was done with the previously described computationally designed TNT receptor (Chapter One).

Two reporter systems were used to examine the plants ability to respond to MTBE. The previously described β-glucuronidase (GUS) reporter gene was one system and the second is a de-greening circuit (Antunes et al., 2006) that was used as the read-out in the TNT detector plants (Antunes et al., 2011). In this case, the de-greening circuit is controlled by the *PlantPho* promoter and consists of *chlorophyllase*, *red chlorophyllide catabolite reductase* (*RCCR*), and an RNAi to *protochlorophyllide oxidoreductase (POR C)* (Antunes et al., 2006). Chlorophyllase and RCCR are key proteins that act in the degradation of chlorophyll (Benedetti and Arruda, 2002). Chlorophyllase removes the hydrophobic tail and RCCR cleaves the porphyrin ring of chlorophyll (Pruzinska et al., 2005). POR C is an enzyme that catalyzes the conversion of protochlorophyllide a to chlorophyllide a during chlorophyll synthesis (Masuda et al., 2003). Upon transcriptional activation of the *PlantPho* promoter, these components both initiate chlorophyll break-down and disrupt synthesis of new chlorophyll, causing the detector plants to lose their green color (Antunes et al., 2011).

I placed the MTBE detection system with each, separate reporter system in both Arabidosis and tobacco. The previously described excised leaf assay was used to screen initial transformants with progeny of these plants tested under a variety of assay conditions. I developed a hydroponic growing and assay protocol to test the plants ability to take up MTBE in the roots and transport MTBE to the leaves where the readout of the detection system could be visualized. Responses to MTBE were measured by quantifying GUS activity, and in plants with the de-greening circuit, the responses were measured visually with a digital camera and quantitatively using a Fluorcam (Photon Systems Instruments, Brno, Czech Republic). The Fluorcam measures chlorophyll fluorescence which is used to calculate the efficiency of photosystem II (F_v/F_m). The F_v/F_m parameter is a useful measurement because activation of the de-greening circuit should correspond to reduced photosynthetic efficiency in Photosystem II. With these data collection methods I determined the feasibility of using plants to detect MTBE.

Transgene silencing can be caused by a variety of factors including suboptimal codon usage from heterologously expressed genes (Matzke et al., 1996). All of the synthetic signaling components contain the original bacterial nucleotide sequence, which make-up codons that are most commonly used in bacteria. Therefore, the DNA sequence of each signaling component was codon optimized for *Arabidopsis thaliana* to circumvent transgene silencing in the detector plants. Upon synthesis of codon optimized signaling components, including the MTBE receptor, all components were cloned and tested in the same fashion as the original synthetic signaling components.

The goal of this work was to determine if the MTBE receptor can be used in plants with our synthetic signaling system to detect MTBE.

II. Materials and Methods

A. Plasmid construction

1. To test the MTBE receptor with the complete synthetic signaling system for transcriptional activation of the GUS reporter gene, plants were transformed with two plasmids harboring the synthetic signaling components. A previously described plasmid (Chapter Two) constructed by Dr. Mauricio Antunes, contains the response regulator, *PhoB-VP64*, and the *GUS* reporter gene under transcriptional control of the *PlantPho* promoter, in the pCambia 2300 backbone, referred to as the 'RR/GUS plasmid' (Figure 2.4) (Antunes et al., 2011). I constructed a plasmid containing *ssMTBE* and the synthetic fusion HK, *Fls-Trg-PhoR,* referred to as the 'MTBE/HK plasmid'. The Pex secretory sequence (ss) was fused to the MTBE receptor, by Dr. Kevin Morey, using overlapping extension PCR and I cloned the resulting *ssMTBE* gene downstream of the *CaMV35S* promoter in the pCB302-3 plant transformation vector (Xiang et al., 1999) and verified the DNA sequence from a commercial service provider (Macrogen USA). I then inserted the *PNOS-Fls-Trg-PhoR-TNOS-TB* fragment, digested from a previously constructed plasmid (Figure 2.3), into the pCB302-3 plasmid with *ssMTBE* (Figure 4.3). Codon optimized signaling components were cloned in the same manner as described above. The bacterial nucleotide sequence of the MTBE receptor was codon optimized for *Arabidopsis thaliana* and synthesized by GeneArt with my specific design components.

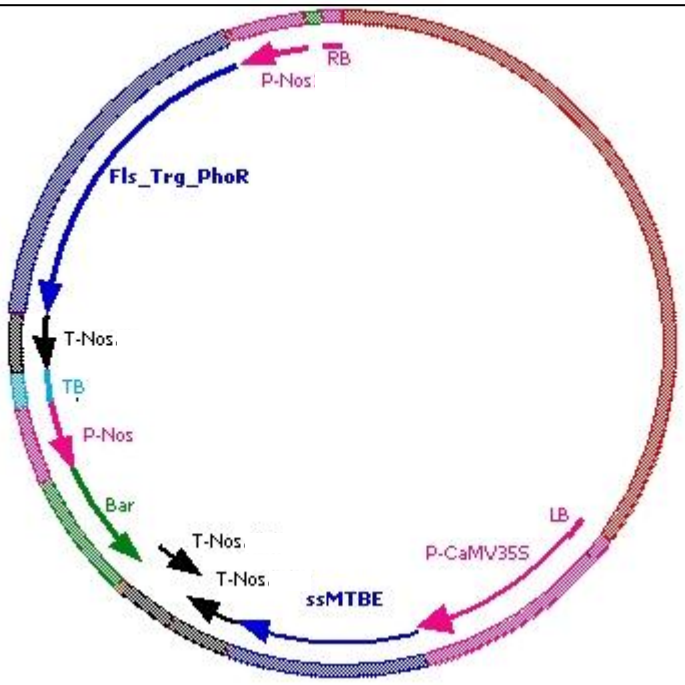

Figure 4.3 Map of MTBE/HK Plasmid. Plant transformation vector, pCB302-3, that encodes the synthetic HK *Fls-Trg-PhoR* and computationally designed *MTBE receptor*. *Fls-Trg-PhoR*: Fls signal peptide fusion to synthetic HK, Trg-PhoR; *P-Nos*: *Nopaline Synthetase promoter*; TB: *Transcription block*; *Bar*: selectable marker, conveys resistance to the herbicide glufosinate ammonium; *T-Nos*: *Nopaline Synthetase* terminator; *ssMTBE*: *Pex secretory sequence* fused to computationally designed *MTBE receptor*; *P*-CaMV35S: Cauliflower Mosaic Virus *35S promoter*; LB: left border; RB: right border.

2. To test the MTBE receptor with the complete synthetic signaling system for transcriptional activation of the de-greening circuit, plants were transformed with two plasmids. One plasmid harbors the synthetic signaling components with the MTBE receptor and the other plasmid harbors the de-greening circuit transcriptionally controlled by the *PlantPho promoter*. I inserted constitutively expressed *PhoB-VP64* into the above described MTBE/HK plasmid using blunt-end non-directional cloning to produce the “signaling plasmid” in the pCB302-3 plant transformation vector (Figure 4.4), which contains the *Bar* gene conveying resistance to the herbicide glufosinate ammonium also known as Basta.

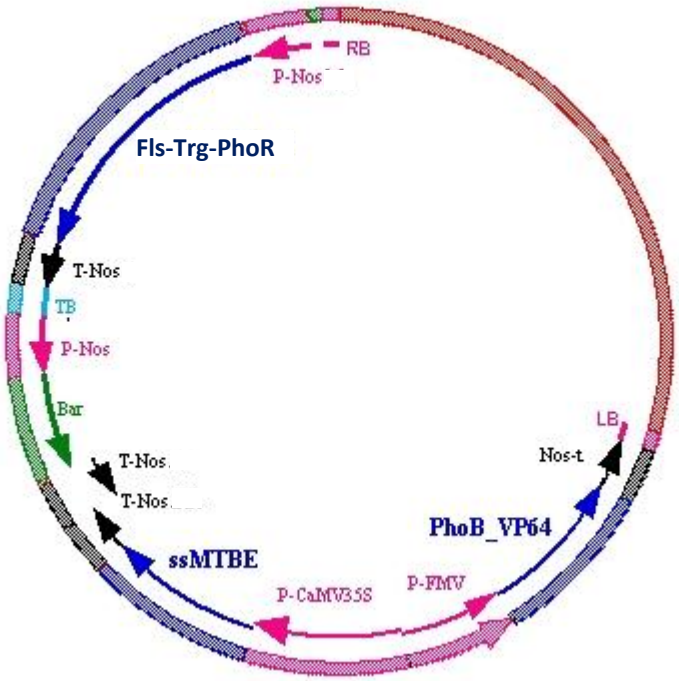

Figure 4.4 Signaling Plasmid Map. Plant transformation vector, pCB302-3, that encodes the synthetic signaling components *Fls-Trg-PhoR,* the computationally designed *MTBE receptor*, and *PhoB-VP64*. *Fls-Trg-PhoR*: Fls signal peptide fusion to synthetic HK, Trg-PhoR; *P-Nos*: *Nopaline Synthetase* promoter; TB: *Transcription block*; *Bar*: selectable marker, coveys resistance to the herbicide glufocinate ammonium; *T-Nos*: *Nopaline Synthetase* terminator; *ssMTBE*: *Pex secretory sequence* fused to computationally designed *MTBE receptor*; *P-CaMV35S*: *Cauliflower Mosaic Virus 35S promoter*; *P-FMV*: *Figwort Mosaic Virus promoter*; *PhoB-VP64*: response regulator *PhoB* fused to the eukaryotic transcriptional activator *VP64*; *LB*: Left border; *RB*: Right border.

The second plasmid used was made by Dr. Mauricio Antunes and contains the de-greening circuit components, *chlorophyllase*, *RCCR*, and an RNAi to *POR C,* all under transcriptional control of the *PlantPho promoter*, in the pCambia 2300 plant transformation vector, referred to as the “readout plasmid” (Figure 4.5). The pCambia 2300 plant transformation vector contains the *NptII* gene, which conveys resistance to the antibiotic kanamycin sulfate.

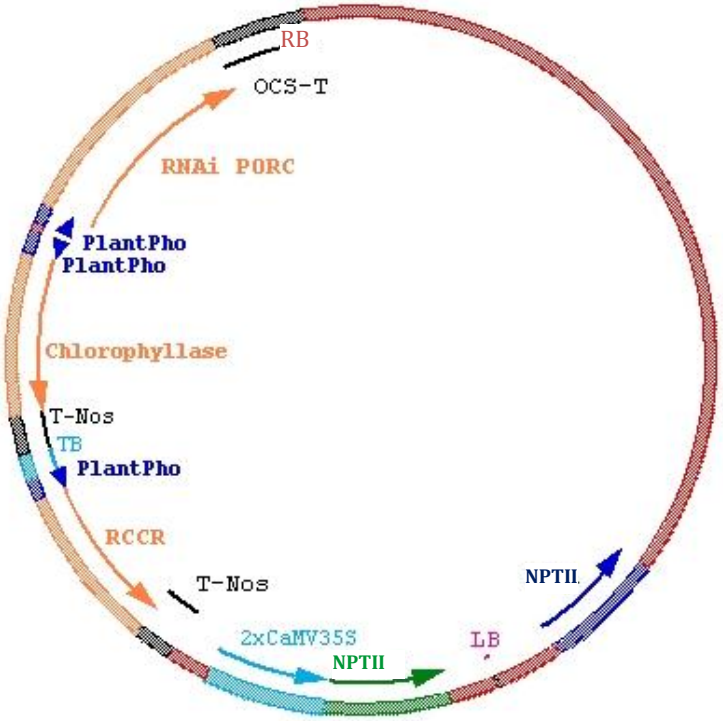

Figure 4.5 Readout Plasmid Map. Plant transformation vector pCambia 2300, that encodes the de-greening circuit components *Chlorophyllase*, *RCCR*, and an RNAi to *PorC,* all controlled by the *PlantPho* promoter. *T-OCS*: octopine synthase terminator; *PlantPho*: PlantPho promoter; *T-Nos*: Nopaline synthetase terminator; *TB*: Transcription block; *RCCR*: red chlorophyllide catabolite reductase; *2xCaMV35S*: enhanced Cauliflower Mosaic Virus 35S promoter; *NPTII*: selectable marker, conveys resistance to the antibiotic kanamycin sulfate; *LB*: Left border; *RB:* right border.

B. Plant Material and Transformation

1. Arabidopsis

Arabidopsis thaliana, ecotype Columbia (*Col-0*) was used for these experiments. Plants were transformed with *Agrobacterium tumefaciens* GV3011 following the standard floral dip procedure (Clough and Bent, 1998). *Agrobacterium* was transformed by electroporation with the plasmids described above.

2. Tobacco

Nicotiana tabacum (Petit Havana SR-1) were transformed using *Agrobacterium tumefaciens* GV3011 mediated gene transfer. Wounded leaves were inoculated with

Agrobacterium harboring the above described plasmids, followed by tissue culture of transformed cells as described by Dandekar *et al* (Dandekar and Fisk, 2005). Upon root development transformed plants were transferred to soil and grown for seed.

C. Growth Conditions

1. Growth on Agar Plates

Transgenic seeds were sterilized and cold treated to synchronize germination overnight at 4°C, and were grown at 23-25°C under 16 hours light (70-100 $\mu E.m^{-2}.s^{-1}$ fluorescent light)/ 8 hours dark cycle, in either a Percival AR75L growth chamber or light shelf. Seeds were germinated on Murashige and Skoog (MS) media supplemented with 50mg/l kanamycin sulfate, (Sigma-Aldrich, St. Louis, MO) for selection of plants containing the pCAMBIA 2300 T-DNA, and 5mg/l Glufosinate ammonium (Crescent Chemical Islandia, NY) for selection of the pCB302-3 T-DNA.

2. Growth in Hydroponic System

Some plants were grown and assayed in a hydroponic system. These plants were germinated and selected on MS agar plates as described above then transferred to the hydroponic system. Several variations of the components that make up the hydroponic system were experimented with until conditions were optimized for this situation. Parameters that were of importance are minimizing the stress caused by transfer to the hydroponic system and uniform growth among individual plants. All plants in this system were grown in standard Hoagland's solution, contained in plastic containers that were spray painted black to reduce growth of algae. Individual plants were transferred from MS plates into a longitudinal slit in small foam stoppers, and then

placed into a 1.5 mL microcentrifuge tube with the bottom removed. The tubes were supported by the lid of the plastic container with holes cut to fit the tubes. An additional hole was made in the lid to accommodate a hose attached to an aquarium air pump for aeration of the Hoagland's solution (Figure 4.6).

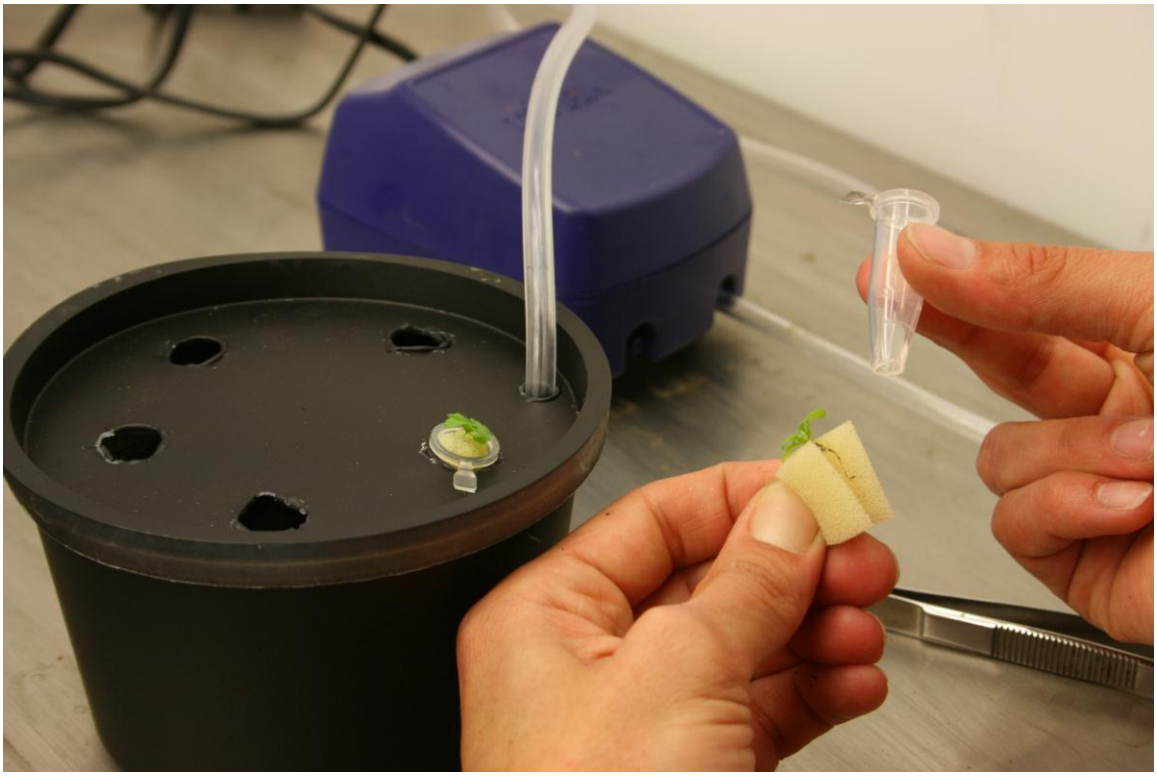

Figure 4.6 Photograph of the hydroponic growing system.

Hoagland's solution was changed weekly or prior to induction with MTBE. Upon completion of an assay plants were transferred to soil and grown for seed.

D. Assay Conditions

The excised leaf assay uses leaves one and two of two-week old Arabidopsis plants or leaves from three-week old tobacco plants. One leaf from each plant was incubated for 14-16 hours, in water with Tween 20 (control) and the other leaf in a 50 µM MTBE (ChemService, West Chester, PA) in water, with Tween20, to facilitate leaf

contact with the ligand. Subsequently, the leaves were prepared for the appropriate assay, either the previously described GUS assay (Chapter Two) or the de-greening assay, described below.

The root-to-shoot assay relies on the plants ability to take the experimental ligand up through the roots and moved into the leaves to activate the response. For MTBE induction, these experiments were done using the hydroponic system. The low vaporization temperature of MTBE (55.2°C) makes successful addition of MTBE to warm liquid agar unlikely. Therefore, MTBE was added to the room temperature Hoagland's solution for induction in the root-to-shoot assays. Negative controls for these experiments included transgenic plants, of the same line being induced, that were grown in Hoagland's solution without MTBE added, and wild type plants that were exposed to MTBE. Plant roots were exposed to MTBE for a minimum of 24 hours prior to leaf excision for GUS assays.

Upon completion of either the excised leaf assay or root-to-shoot assay, plants were selected for analysis in the next generation based on the assay results. Individual plants were placed into one of four categories based on the amount of GUS activity in the leaf incubated with the experimental ligand, relative to that of the corresponding control leaf incubated in water.

E. Analysis of De-greening

Analysis of de-greening was done using both the excised leaf assay and the root-to-shoot assay. For the excised leaf assay, leaves were removed from the plant and data was collected (described below). Next, one leaf was incubated in water plus

Tween20 and the other in 50 μM MTBE solution plus Tween20, in 24 well plates and placed on a light shelf. After 14-16 hours of incubation the leaves were removed from the solution and data was collected, then again every 24 hours for up to five days. After data collection, the leaves were returned to freshly prepared solutions and placed back on the light shelf.

Data were collected using two different types of methods. Plants were imaged with a digital camera and the efficiency of photosystem II was measured with a Fluorcam (Photon Systems Instruments, Brno, Czech Republic). Induction of the de-greening gene circuit causes chlorophyll degradation in plastid photosystems (Antunes et al., 2006) therefore, we quantified changes in F_v/F_m, the maximum efficiency of photosystem II. Specifically, F_v/F_m values were calculated using the Quenching Analysis protocol provided with the manufacturer's software (Photon Systems Instruments, Brno, Czech Republic). The calculated F_v/F_m data are presented spatially, where individual pixels of the image display the intensity of F_v/F_m values according to the color table shown in each figure, and data for some plants are presented graphically as well.

III. Results

A. Analysis of transcriptional response to MTBE in transgenic Arabidopsis plants containing the complete synthetic signaling system and MTBE receptor

1. Measurement of response through quantification of GUS activity.

Arabidopsis plants containing the complete synthetic signaling system with the computationally MTBE receptor and the GUS reporter gene under transcriptional control of the *PlantPho* promoter were tested for response to MTBE. Primary transformants

(T_0) were tested using the excised leaf assay followed by quantification of GUS activity. Individual plants were placed into one of four categories based on the amount of GUS activity in the leaf incubated with the experimental ligand, relative to that of the corresponding control leaf incubated in water. Individual plants were considered to have significant GUS activity if the amount of 4-MU (product of GUS enzymatic activity) was greater than or equal to 10 nmoles 4-MU^{-1} mg $protein^{-1}$ hour. This value was determined, from the experimental data in Chapter One, to be the threshold for background GUS activity. Leaves exposed to the experimental ligand having GUS activity 1.4 fold higher or more above that of control leaves are considered to show induction. Plants exposed to the experimental ligand having GUS activity 0.6 or less of that of the control leaves are considered to show repression. Plants with both experimental and control samples having GUS activity above 10 nmoles 4-MU^{-1} mg $protein^{-1}$ hour but, not fitting into the repression or induction group, are considered to have some GUS activity that is relatively equivalent between the control and induced samples. Plants with both experimental and control samples having values below 10 nmoles 4-MU^{-1} mg $protein^{-1}$ hour, are considered to have no significant GUS activity.

Of the 124 T_0 Arabidopsis plants assayed, for transcriptional response to MTBE exposure, four plants show higher GUS activity in leaves exposed to exogenous MTBE than that of leaves incubated in water and three plants show some GUS activity that is relatively equivalent between both leaves. The other 117 T_0 plants show little to no GUS activity (Figure 4.7 $t(123)=0.23$, $p=0.421$). Six T_0 individuals were excluded from data analysis due to errors during sample preparation.

Each of the four plants that showed induction in response to MTBE exposure were transferred to soil and grown for seed. All of these plants were stunted and produced very little seed. The T_1 seed was germinated on MS agar with selection and each line showed reduced germination and survival rate. Surviving plants appeared stunted and were transferred to MS agar without selection to try and save the plants due to limited seed supply. However, the plants remained stunted and none survived to produce seed. This was repeated and a few selected plants were assayed, but the plants were too small to accurately quantify GUS activity from crude extract. Plants of these lines were also germinated on MS agar without selection and again produced stunted seedlings with reduced survival rate, similar to germination on media with selective agents. This is an unexpected result that has not been observed in other plants that contain any of the synthetic signaling components. However, due to the limited amount of seed and reduced survival rate, no further data could be collected about these plants.

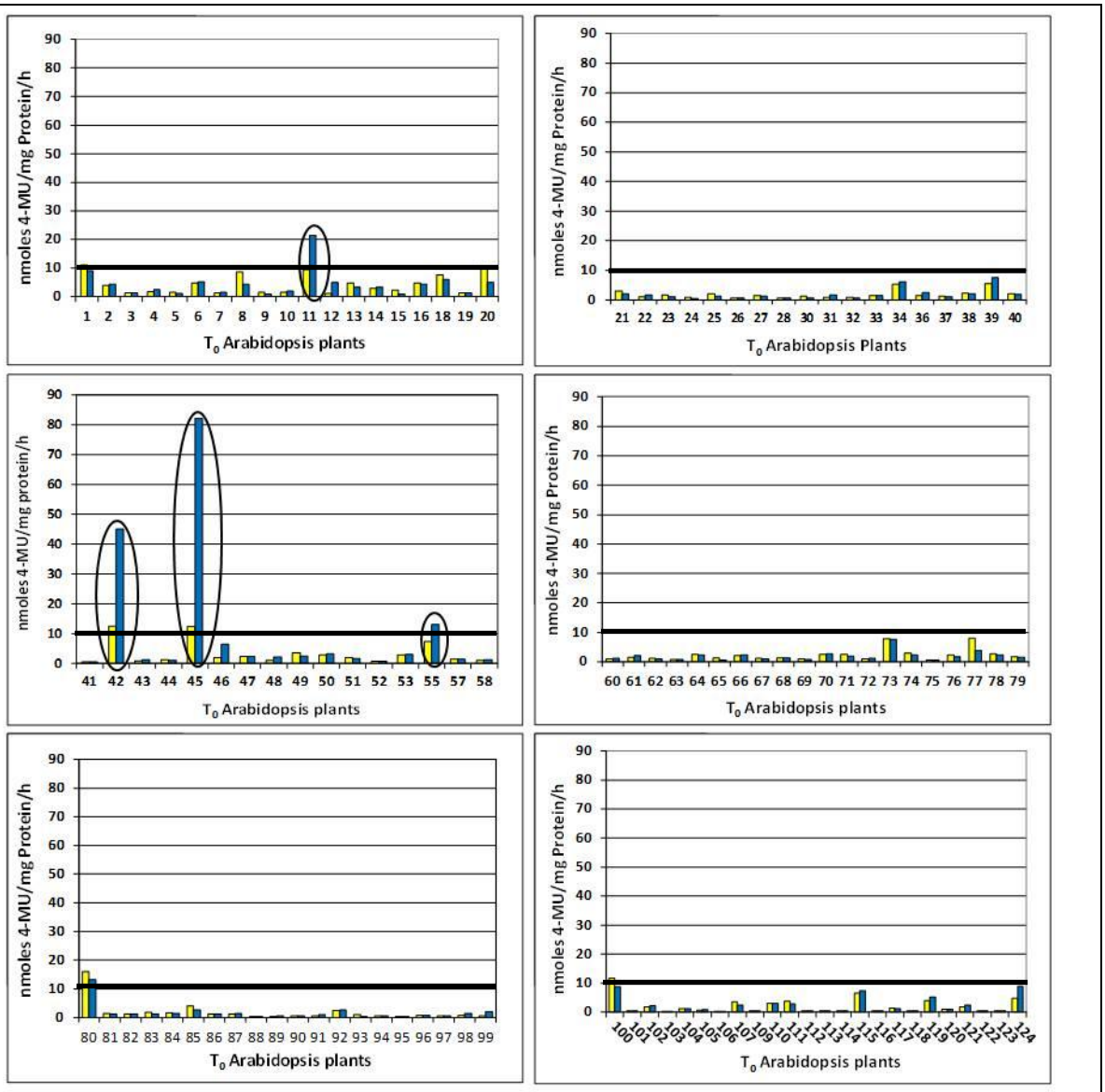

Figure 4.7 Response of T_0 Arabidopsis Plants to Exogenous MTBE. Plants tested using the excised leaf assay. Charts show quantification of GUS activity in leaves of Arabidopsis plants containing the computationally designed MTBE receptor, synthetic HK Fls-Trg-PhoR, PhoB-VP64 and the GUS reporter gene under transcriptional control of the *PlantPho* promoter. Yellow bars represent GUS activity of leaves incubated in water and blue bars represent that of the corresponding leaf incubated in 50μM MTBE. Individuals selected for analysis in the next generation are circled.

2. Measurement of de-greening response to MTBE

Arabidopsis plants that contain the complete synthetic signaling system, MTBE receptor and de-greening gene circuit were analyzed for loss of chlorophyll in response to exogenous exposure to MTBE. Twenty-six T_0 Arabidopsis plants were tested using

the excised leaf assay. Nearly every leaf tested from the 26 independent plants showed some amount of de-greening (Figure 4.8).

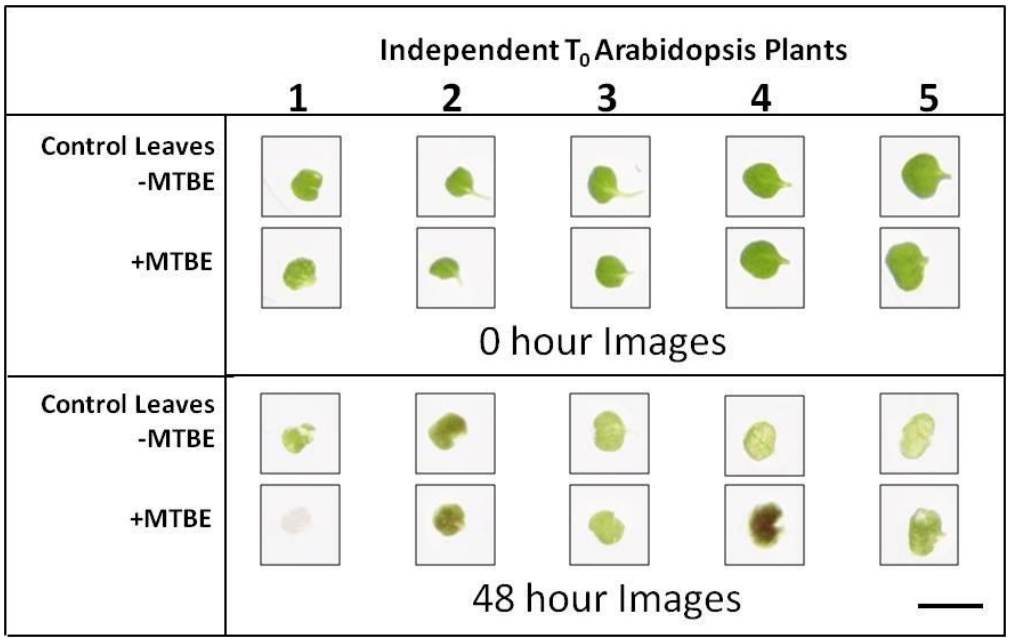

Figure 4.8 De-greening response in T_0 Arabidopsis Plants to MTBE. Representative experiment testing the de-greening response in T_0 Arabidopsis that contain the complete synthetic signaling system with the MTBE receptor and the de-greening gene circuit transcriptionally controlled by the *PlantPho* promoter in response to 50μM MTBE exposure. Plants were tested using the excised leaf assay. T_0 individuals one and four were selected for analysis in the next generation. Scale bar=1 cm

Some leaves had anthocyanin accumulation as well, making it difficult to visualize de-greening with the naked eye. Plants with leaves incubated in MTBE solution that appeared to de-green more than the corresponding leaf incubated in water, were transferred to soil and grown for seed. No Fluorcam data were collected during this set of experiments due to limited equipment availability. Two plants among the 26 T_0 plants tested were selected for further analysis (Figure 4.8).

The T_1 progeny of the two selected T_0 lines were then re-tested using the excised leaf assay. Twenty individuals from each line were analyzed for response to exogenous MTBE exposure. Similar to the T_0 results, most leaves show some de-greening. Of the

40 T_1 individuals assayed, one leaf turned completely white, however this leaf was exposed to only water (Figure 4.9) suggesting an artifact. None of the other leaves of that line show a similar magnitude of de-greening. FluorCam data were collected for these experiments and revealed a response similar to that seen in the digital camera images (Figure 4.9).

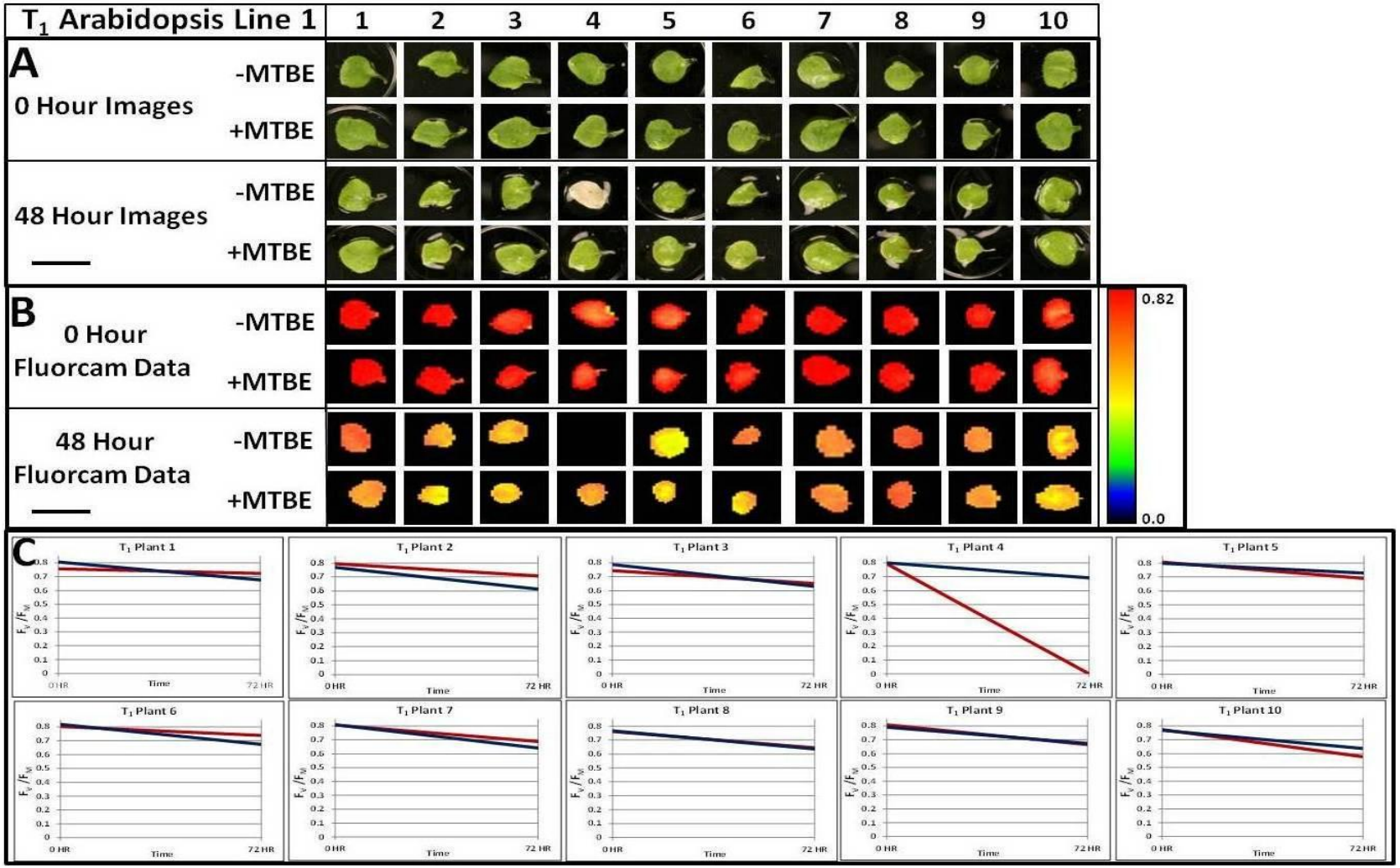

Figure 4.9 T_1 Arabidopsis De-greening Response to Exogenous MTBE. Visual images and Fluorcam data in leaves of T_1 Arabidopsis Line one individuals using the excised leaf assay. Control leaves were incubated in water and the corresponding leaf was incubated in 50μM MTBE. Data is shown for the zero and 48 hour time points. (A) Digital camera images of Arabidopsis leaves. Scale bar=1 cm. (B) Spatial representation of F_V/F_M values collected with the Fluorcam. Scale bar=1 cm. (C) Graphical representation of F_V/F_M values at zero and 48 hours. Red line represents F_V/F_M values of control leaves incubated in water and blue line represents that of the corresponding leaf incubated in MTBE.

B. Analysis of transcriptional response to MTBE in transgenic tobacco plants containing the complete synthetic signaling system and MTBE receptor.

1. Measurement of response through quantification of GUS activity.

Tobacco plants containing the complete synthetic signaling system with the computationally MTBE receptor and the GUS reporter gene under transcriptional control of the *PlantPho* promoter were tested for response to MTBE. Fifty primary transformants (T_0) were tested using the excised leaf assay followed by quantification of GUS activity (Figure 4.10). Of the fifty T_0 tobacco plants that were tested one of those individuals showed induction with GUS activity above background levels. All other individuals tested have little to no GUS activity. Despite showing low GUS activity, three independent transgenic tobacco plants, in addition to the one plant that showed induction, were transferred to soil and grown for seed. These three individuals appeared to show induction but, have GUS activity values below what is considered to be background (Figure 4.10 $t(49)=0.27$, $p=0.383$). Due to the lack of plants that responded to MTBE, a more relaxed criterion was applied to ensure that no responding plants were overlooked.

The T_1 progeny of each of the four selected lines were tested with both the excised leaf assay and root-to-shoot assay. Using the excised leaf assay, five individuals from Line 3 ($t(4)=0.06$, $p=0.463$) were tested and none showed induction in response to exogenous MTBE exposure (Figure 4.11). Ten individuals each from Line 5 ($t(9)=0.15$, $p=0.423$) and Line 16 ($t(9)=0.21$, $p=0.423$) were tested, and one plant from Line 5 shows induction. Fifteen individuals from Line 30 ($t(14)=0.79$, $p=0.218$) were tested and two plants show induction in response to exogenous MTBE exposure.

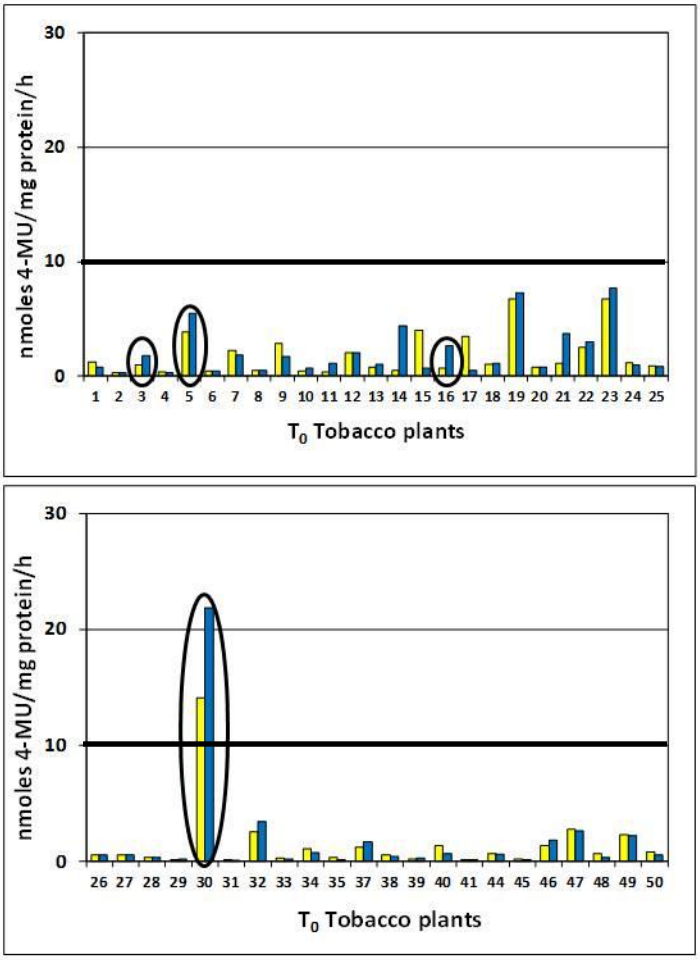

Figure 4.10 Response of T_0 Tobacco Plants to Exogenous MTBE. Plants tested using the excised leaf assay. Charts show quantification of GUS activity in leaves of Tobacco plants containing the computationally designed MTBE receptor, synthetic HK Fls-Trg-PhoR, PhoB-VP64 and the GUS reporter gene under transcriptional control of the *PlantPho* promoter. Yellow bars represent GUS activity of leaves incubated in water and blue bars represent that of the corresponding leaf incubated in 50μM MTBE. Individuals selected for analysis in the next generation are circled.

Also, all individuals from Line 30 show GUS activity that exceeds background levels, with most plants having relatively equivalent values between leaves incubated in water and leaves exposed to MTBE. One plant from Line 30 shows repression (Figure 4.11).

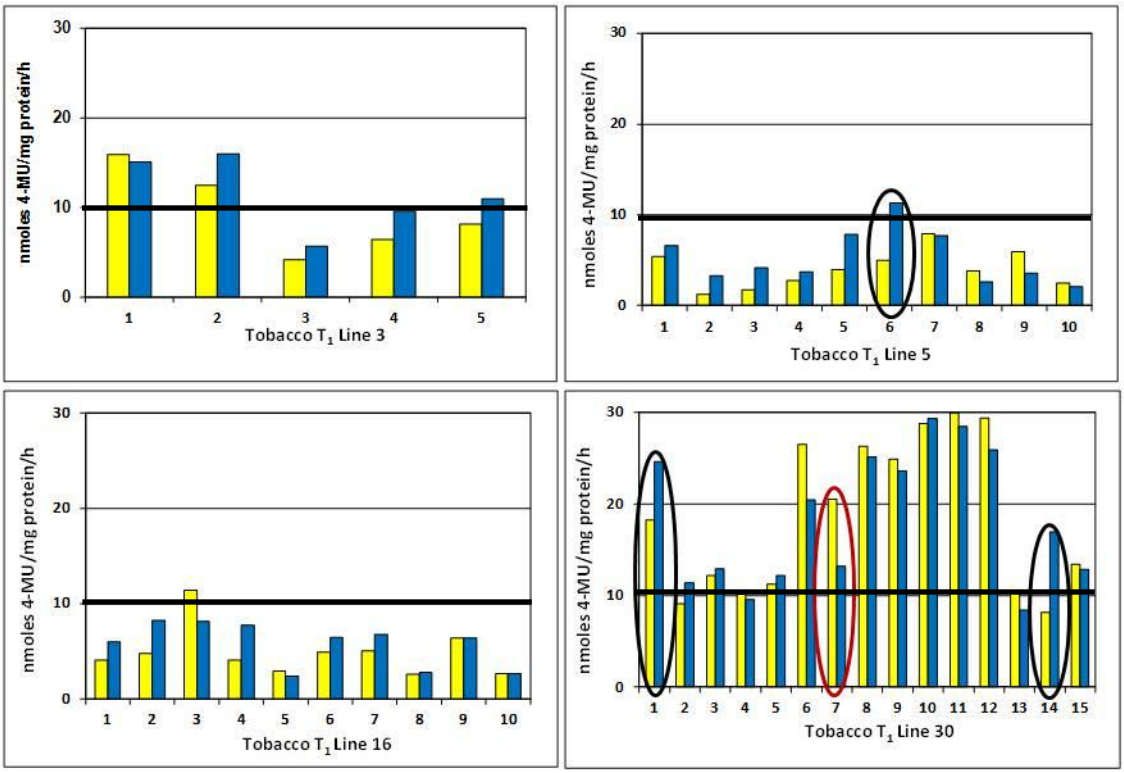

Figure 4.11 Response of T_1 Tobacco Plants to Exogenous MTBE. Plants tested using the excised leaf assay. Charts show quantification of GUS activity in leaves of Tobacco plants containing the computationally designed MTBE receptor, synthetic HK Fls-Trg-PhoR, PhoB-VP64 and the GUS reporter gene under transcriptional control of the *PlantPho* promoter. Yellow bars represent GUS activity of leaves incubated in water and blue bars represent that of the corresponding leaf incubated in 50μM MTBE. Individual plants showing induction are circled in black and the plant showing repression is circled in red.

The same four selected T_1 tobacco lines tested above, using the excised leaf assay, were also tested using the root-to-shoot assay. To increase the likely-hood that the plants are taking enough MTBE into their leaves to initiate signaling and response, the concentration of MTBE in the hydroponic solution plants were grown in was increased to 500 μM. Among the 60 individuals assayed, only three plants had GUS activity above background however, these plants were not exposed to MTBE (negative controls). Six plants from Line 5 that were induced with MTBE, show GUS activity that is higher than that of negative control plants, however this level does not exceed

background levels (Figure 4.12 3:t(10)=0.41, 0.349; 5:t(30)=0.6, p=0.277; 16:t(8)=0.27, p=0.386; 30:t(8)=0.36, p=0.350).

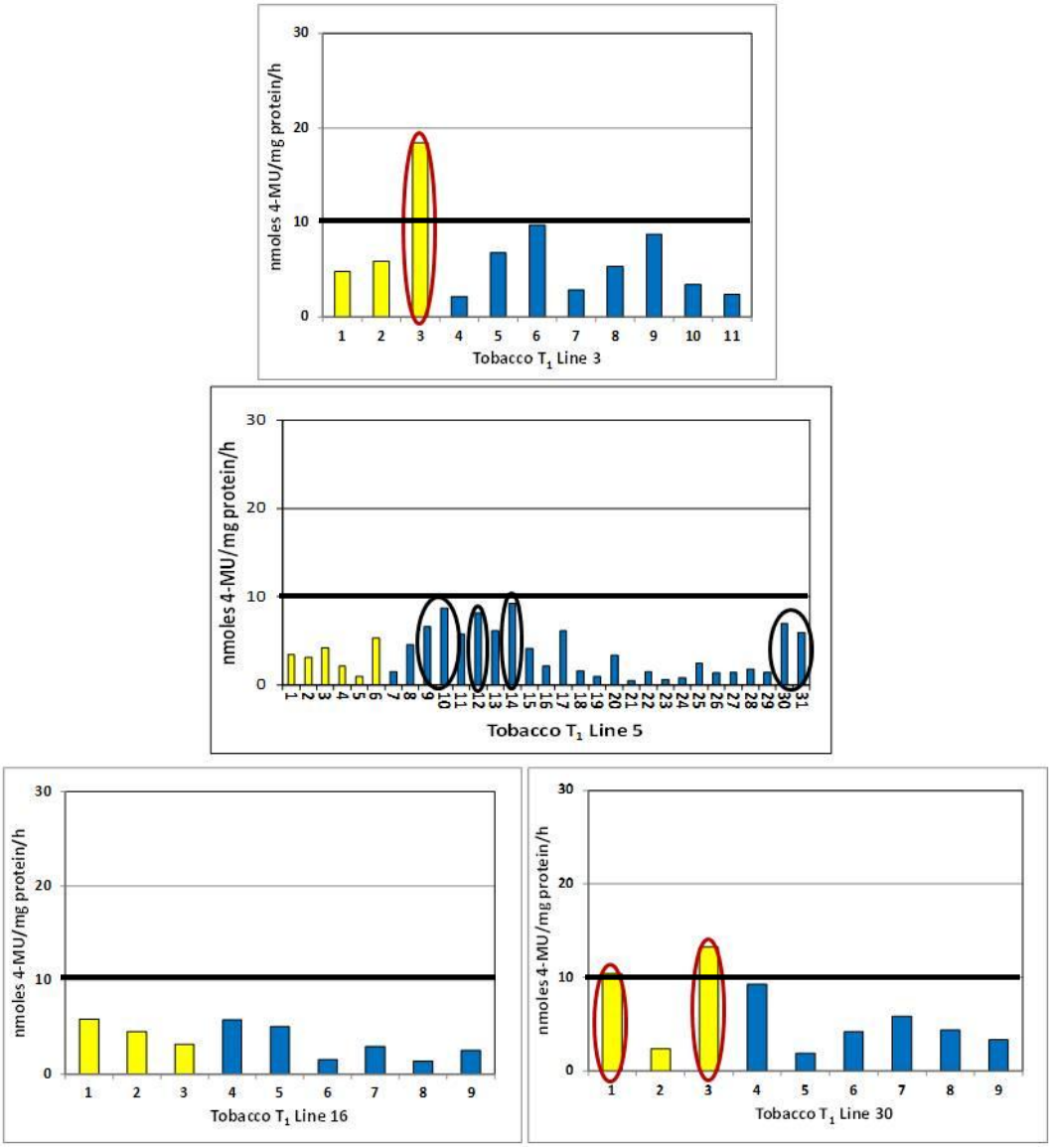

Figure 4.12 Response of T_1 Tobacco Leaves to MTBE Exposure Using the Root-to-shoot Assay. Charts show quantification of GUS activity in leaves of Tobacco plants containing the computationally designed MTBE receptor, synthetic HK Fls-Trg-PhoR, PhoB-VP64 and the GUS reporter gene under transcriptional control of the *PlantPho* promoter. Yellow bars represent GUS activity of transgenic control plants that were grown in Hoagland's solution without MTBE and blue bars represent that of plants grown in Hoagland's solution plus 500μM MTBE. Individuals circled in black show induction but GUS activity does not exceed background levels. Individuals circled in red show GUS activity above background but were not exposed to MTBE.

2. Measurement of de-greening response to MTBE

Tobacco plants that contain the complete synthetic signaling system and de-greening gene circuit were analyzed for loss of chlorophyll in response to exogenous exposure to MTBE. Ninety-six tobacco plants were tested when they had established roots and 4-6 leaves, using the excised leaf assay. As seen in Arabidopsis, nearly all leaves showed some de-greening likely due to being submerged in water for extended periods of time. Resulting images from the digital camera and data collected using the FluorCam of a representative experiment are shown in Figure 4.13. Nine T_0 tobacco plants that appeared to show more de-greening in leaves exposed to MTBE than leaves exposed to water were selected for analysis in the next generation, transferred to soil and grown for seed.

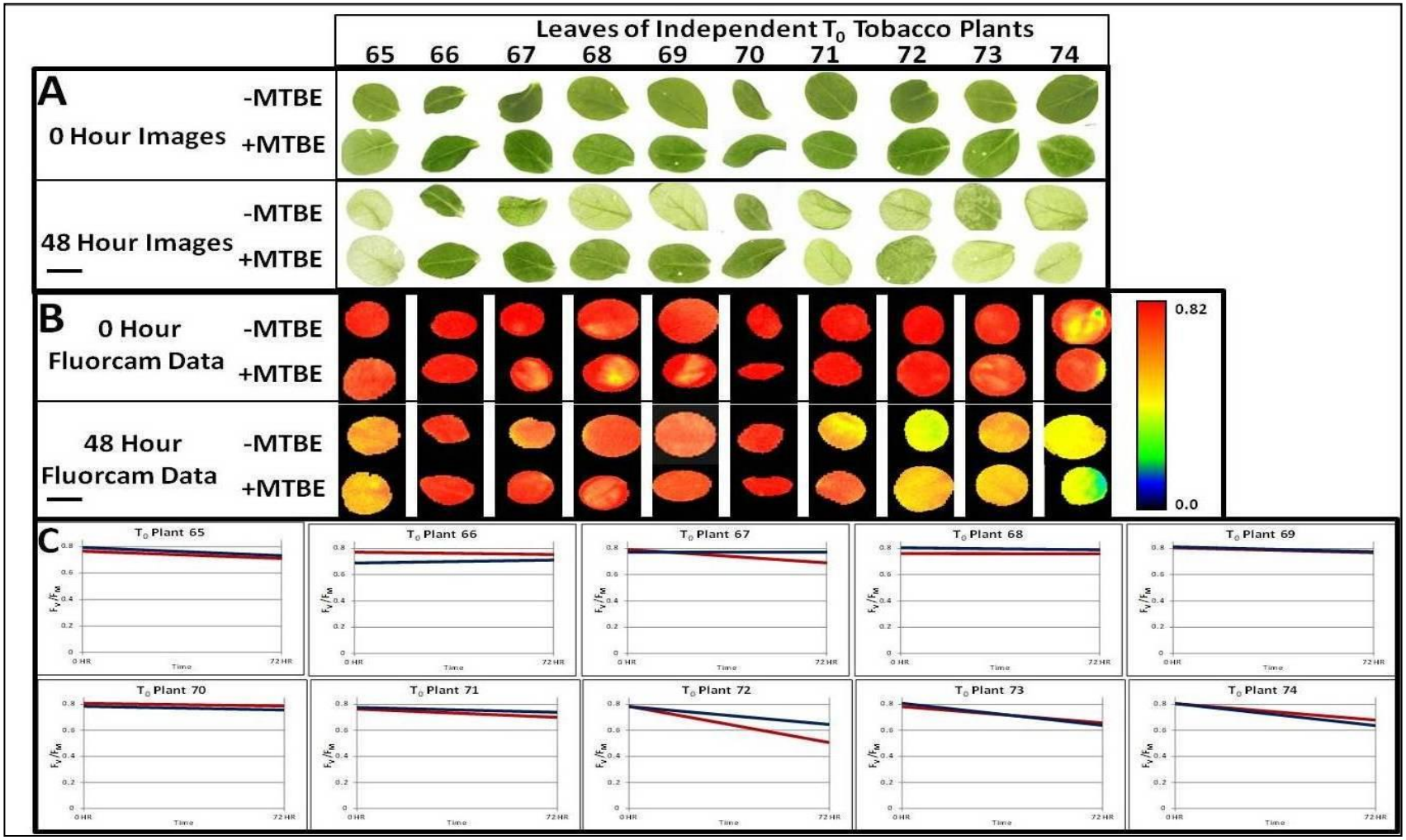

Figure 4.13 T_0 Tobacco De-greening Response to Exogenous MTBE. Visual images and Fluorcam data in leaves of T_0 Tobacco plants using the excised leaf assay. Control leaves were incubated in water and the corresponding leaf was incubated in 50µM MTBE. Data is shown for the zero and 48 hour time points. (A) Digital camera images of Arabidopsis leaves. Scale bar=1 cm. (B) Spatial representation of F_V/F_M values collected with the Fluorcam. Scale bar=1 cm. (C) Graphical representation of F_V/F_M values at zero and 48 hours. Red line represents F_V/F_M values of control leaves incubated in water and blue line represents that of the corresponding leaf incubated in MTBE.

Most of the T_1 tobacco lines were tested with the root-to-shoot assay and consequently were grown in the hydroponic system. Wild-type SR1 tobacco was included in each experiment to ensure that the high concentration of MTBE (500μM) or growth in the hydroponic system does not have adverse effects on the health of the plants. Nine T_1 lines were tested, totaling 136 plants assayed for de-greening response to MTBE exposure in the roots. Very slight de-greening was observed in some plants that were exposed to MTBE and in some transgenic lines that were not exposed to MTBE. Resulting images for a representative line assayed are shown in Figure 4.14.

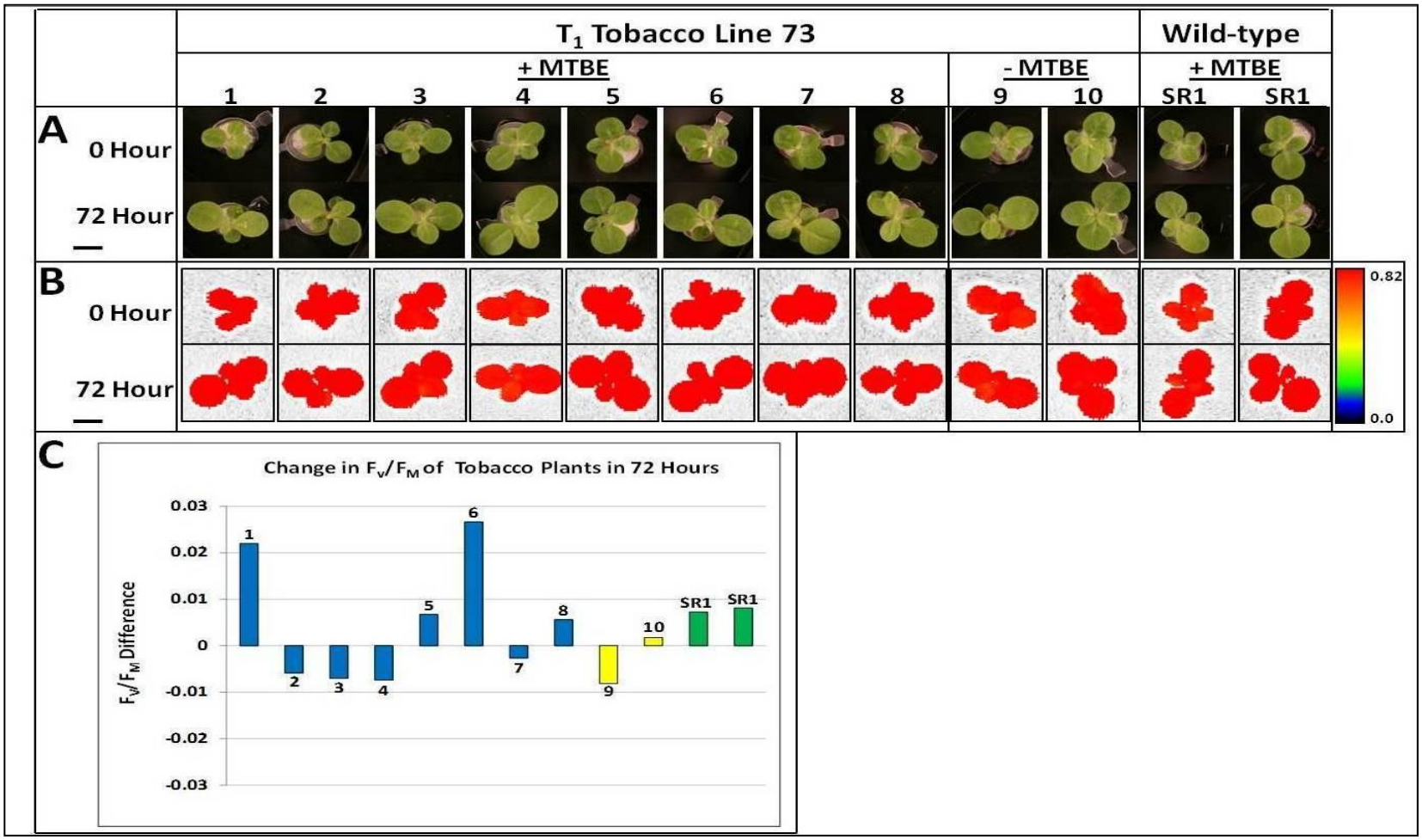

Figure 4.14 T_1 Tobacco De-greening Response of Leaves to MTBE Exposure in Roots. Visual images and Fluorcam data of T_1 Tobacco Line 73 plants were collected using the root-to-shoot assay. Plants 1-8 were grown in solution that contained 500µM MTBE. Plants 9 and 10 were grown in solution that did not contain MTBE and serve as negative controls. Wild-type (SR1) plants were grown in solution that contained 500µM MTBE. Data is shown for the zero and 72 hour time points. (A) Digital camera images of tobacco plants. Scale bar=1 cm. (B) Spatial representation of F_V/F_M values collected with the Fluorcam. Scale bar=1 cm. (C) Graphical representation of changes in F_V/F_M values over 72 hours. Blue bars represent changes in F_V/F_M values of plants induced with MTBE. Yellow bars represent changes in F_V/F_M values of plants of the same transgenic line that were not induced with MTBE. Green bars represent changes in F_V/F_M values of wild-type tobacco that were exposed to MTBE.

C. Codon Optimized Signaling Components

Tobacco plants containing the codon optimized complete synthetic signaling system with the computationally MTBE receptor and the GUS reporter gene under transcriptional control of the *PlantPho* promoter were tested for response to MTBE. The excised leaf assay was used to test 141 selected T_0 plants for an increase in GUS activity in response the exogenous MTBE exposure. Of the 141 plants tested only six individuals had GUS activity above what we consider to be background signaling (Figure 4.15). Of those 6 plants, 2 show induction in response to MTBE and the other four show repression. All other leaves had GUS activity below ten nmoles 4-MU^{-1} mg $protein^{-1}$ hour (Figure 4.15 $t(140)=0.81$, $p=0.212$).

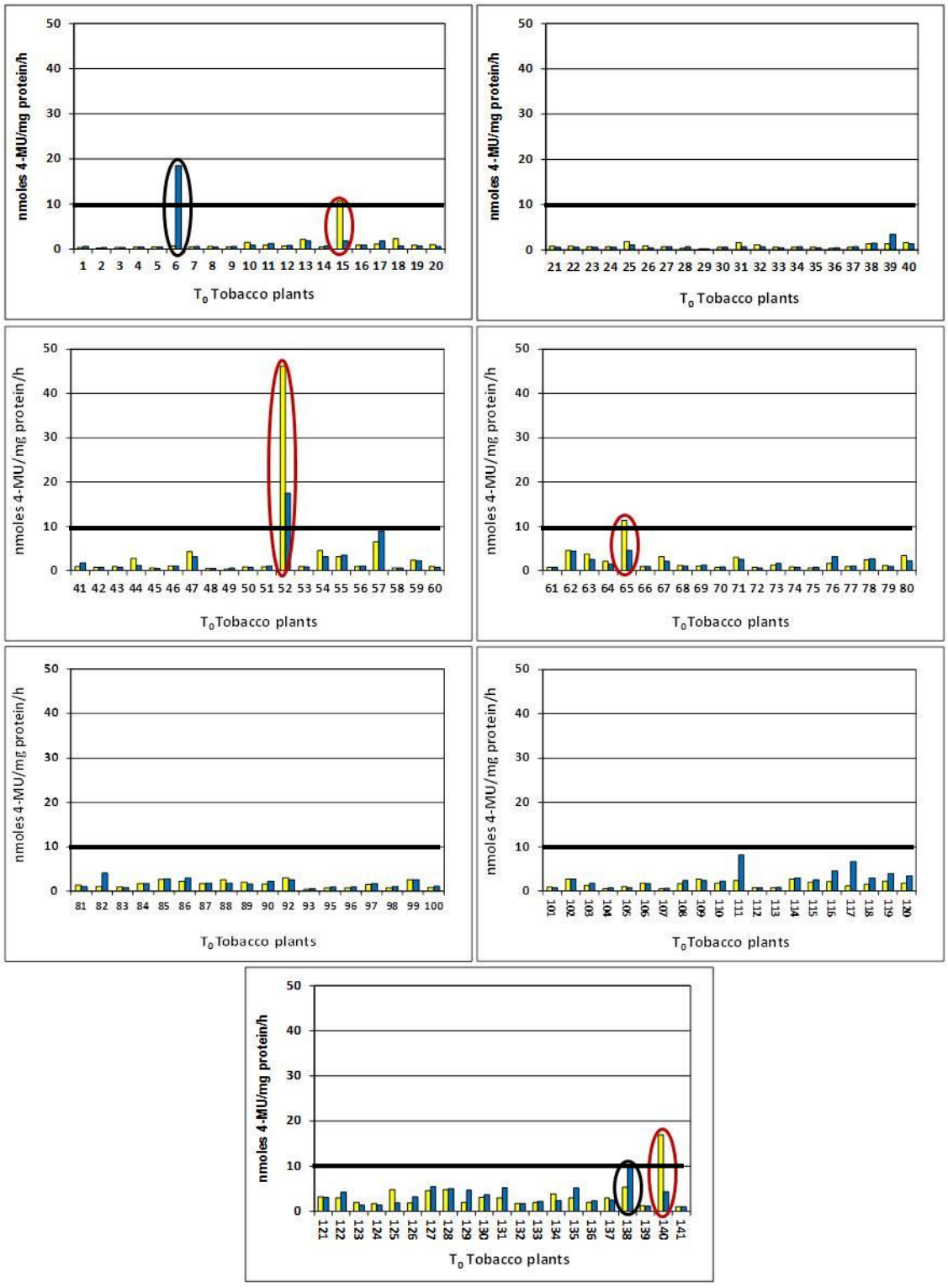

Figure 4.15 Response of T_0 Tobacco Plants to MTBE. Plants containing the codon optimized *MTBE receptor*, synthetic HK *Fls-Trg-PhoR*, *PhoB-VP64* and the GUS reporter gene under transcriptional control of the *PlantPho* promoter were tested using the excised leaf assay. Yellow bars represent GUS activity of leaves incubated in water and blue bars represent that of the corresponding leaf incubated in 50μM MTBE. Plants showing induction are circled in black and those showing repression are circled in red.

IV. Conclusions

The goal of this work was to determine the feasibility of producing MTBE detector plants by testing the computationally designed MTBE receptor with our complete synthetic signaling system *in planta*. Individual transgenic Arabidopsis and tobacco plants were tested using the excised leaf assay to examine the response of MTBE exposure in the leaves, and the root-to-shoot assay to examine the response in leaves to MTBE exposure in the roots. To analyze the response of plants to MTBE exposure, two distinct transcriptional reporters were quantified; the activity of the GUS reporter gene product or changes in chlorophyll fluorescence caused by activation of the de-greening gene circuit. A few individual plants showed an increase in GUS activity (Figures 4.7, 4.10, 4.11 and 4.15, individuals circled in black) or de-greening (Figures 4.8 and 4.9) in response to MTBE. However, just as many plants showed a decrease in GUS activity relative to negative controls (Figure 4.11, 4.12 and 4.15, individuals circled in red) or de-greening in the absence of MTBE (Figures 4.8, 4.9 and 4.13). Most individuals tested showed no response to MTBE exposure (Figures 4.7-4.15).

As seen in results of several experiments using the excised leaf assay, leaves that have not been exposed to MTBE showed high levels of GUS activity or de-greening after being submerged in water for extended periods of time (Figure 4.8, 4.9, 4.11, 4.13, 4.15). This is especially evident in the experiments measuring de-greeing in response to MTBE (Figures 4.8, 4.9 and 4.13). Analysis of the de-greening results using the excised leaf assay suggests that the assay itself may be causing de-greening. Most Arabidopsis and tobacco leaves, including those that were not exposed to MTBE, show some de-greening after 24 hours. Perhaps removal of the leaf from the plant and

submergence in liquid for extended periods of time can cause de-greening. However, no de-greening was observed when testing for GUS activity in transgenic plants that do not contain the de-greening circuit using the excised leaf assay. These results suggest that the excised leaf assay produces artifacts and may not be appropriate for testing transcriptional responses to MTBE in plants.

Plants tested using the root-to-shoot assay consistently show no response to MTBE. The root-to-shoot assay requires the MTBE ligand be taken up in the roots and transported to the leaves to be perceived by the apoplastically localized MTBE receptor. This assay does not produce the artifacts described above in the excised leaf assay. Yet individual plants tested, do not show an increase in GUS activity exceeding background levels (Figure 4.12) or de-greening (Figure 4.14) in response to MTBE in the hydoponic growing solution. These data raise the question of why the plants do not respond to MTBE. Among the many possibilities, MTBE may not be present in high enough concentrations in the apoplast to be perceived, or the computationally designed MTBE receptor is not functioning as reported or does not function properly *in planta*.

Taken together the above described results show that plants containing the computationally designed MTBE receptor with the complete synthetic signaling system do not respond to MTBE in a reproducible and reliable manner. These data raise questions about whether the assays used were suitable for testing the system or if the MTBE receptor functions as reported. Conclusions about the feasibility of producing MTBE detector plants cannot be drawn from these data and further analysis of the MTBE receptor is needed.

V. Discussion

A. Does the MTBE receptor work as originally reported?

The lack of response to MTBE in the detector plants leads to questions about the functionality of the receptor. The bacterial data indicating the MTBE receptor does in fact bind MTBE and initiate downstream signaling was never published by Dr. Hellinga. Despite having the bacterial cells, HH3000, used in the Dr. Hellinga's experiments in our possession, other members of the lab have not been able to produce functional signaling with these cells. Also, Dr. Hellinga's testing system in bacteria does not use components from our synthetic signaling system. Furthermore, questions about the stability and binding ability of other computationally designed receptors produced by the Hellinga lab have been raised (Hayden, 2009; Schreier et al., 2009). This will be discussed further in Chapter Five. It may be worth while to test the MTBE receptor in bacteria with our synthetic signaling components to show that the receptor is functional with our system.

There are many properties of MTBE and aspects of our plant testing system that make drawing conclusions about the response to MTBE challenging. The results of my experiments do not indicate that the MTBE receptor is functional in plants; however, it is difficult to determine the reason(s) for the lack of response to MTBE in the plants tested. One possibility is that the ligand is not contacting the apoplast-localized receptor. Other research using hydroponic growing systems has shown that MTBE is taken up by roots and moves through the transpiration path with water (Zhang et al., 2001; Yu and Gu, 2006). However, MTBE is very unstable and could be degraded before being taken up by the roots. Because I did not measure MTBE in the plants, it is not known if sufficient

concentrations of MTBE were present in the apoplast to cause induction of a measurable response. Another possibility is that the MTBE receptor simply does not bind MTBE as reported and the bacterial results provided by Dr. Hellinga's laboratory could be an artifact.

As previously shown, the synthetic signal transduction system can be activated by exogenous cytokinin application (Figure 2.2) (Antunes et al., 2009), therefore I hypothesize that components of the cytokinin signaling pathway can interact with PhoB-VP64 (Chapter Three). Potential cross-talk between endogenous plant signaling pathway(s) and PhoB-VP64 complicates interpretation of the above described results making it challenging to determine the cause of transcriptional responses observed in plants.

B. Bacterial Testing System

Using plants as a testing system presents many unknown variables that could affect the function of our synthetic signaling components and make drawing conclusions from this data challenging. Testing the MTBE receptor in bacteria using our synthetic signaling components will determine whether or not the MTBE receptor is functional in our system. Furthermore, with a bacterial system, assay methods can be tested for suitability by using wild-type RPB as a positive control for the function of synthetic signaling components. Also, bacteria do not contain the plant hormone signaling pathway that can activate PhoB (Antunes et al., 2009). Therefore cross-talk should not interfere with data interpretation when testing in the appropriate bacterial strain. Finally, ensuring contact between the ligand and the receptor should not be difficult in bacteria because *in vivo* transport of the ligand is not required, as it was in the root-to-shoot

assay in plants. Considering the MTBE receptor was not shown to be functional in plants, and the uncertainties surrounding the computationally designed receptors, we should revisit the question of whether or not the MTBE receptor is functional in bacteria.

Chapter Five

Characterization of Computationally Designed Periplasmic Binding Proteins in Bacteria

I. Introduction

A. Computationally Designed PBPs

1. Design

Each design for a periplasmic binding protein (PBP) with altered recognition described in the previous chapters was generated using an eight-part computational design algorithm (Looger et al., 2003) produced by Dr. Homme Hellinga's laboratory at Duke University. Dr. Hellinga and his team have used this method to design protein-ligand binding specificities in several PBPs, including maltose binding protein (MBP), glucose binding protein (GBP), ribose binding protein (RBP), arabinose binding protein (ABP), glutamine binding protein (QBP), and histidine binding protein (HBP). PBPs have been designed to bind various non-cognate ligands including 2,4,6-trinitrotoluene (TNT), L-lactate, serotonin (Looger et al., 2003), zinc (Marvin and Hellinga, 2001) and methyl tert-butyl ether (MTBE) (unpublished). The computationally designed PBPs, used by the Medford laboratory, tailored to bind TNT and MTBE were produced within the RBP scaffold.

The eight-part computational design algorithm produced by the Hellinga laboratory uses high-resolution three-dimensional structures to identify amino acid alterations predicted to form a complementary surface between the PBP and the new

ligand (Looger et al., 2003). First, the amino acid side chains within the binding pocket of the PBP that directly contact the original cognate ligand are trimmed back and changed to alanine. This generates a docking region for the new ligand. A cubic grid is placed within the docking region and an ensemble representing all rotational degrees of freedom within the grid is generated. Next the new ligand is docked within the cubic grid testing different poses to select those that do not form unfavorable steric interactions. Each ligand docked ensemble is ranked according to the interaction energy between the new ligand and receptor. For each of the top 10,000 ensembles, all possible side-chain conformations are calculated, taking into account global minimum energy of the ligand/receptor interaction, van der Waals forces, hydrogen bonding, and hydrophobic effects. The protein backbone is kept fixed during this process and the surface of the newly designed binding pocket is complementary to the new ligand. Finally the resulting receptor designs are ranked according to the previous calculations then a small number of the top-ranked designs are experimentally tested (Looger et al., 2003).

2. Experimental Testing of Computationally designed PBPs

Completed designs were tested in Dr. Hellinga's laboratory using both *in vitro* and *in vivo* methods. The *in vitro* assays use purified computationally designed PBP that was modified with a thiol-reactive styryl fluorophore introduced into the "hinge-bending" region of the PBP. Fluorescence emission intensity of the dye responds to the closure of the PBP binding. Ligand-binding affinities (K_d) for each computationally designed PBP were determined by measuring the fluorescence of the dye in response to the new ligand (Looger et al., 2003).

The *in vivo* assay used by the Hellinga laboratory employs a synthetic signal transduction pathway that links extra-cellular ligand detection to transcriptional activation in bacteria. Wild-type PBPs mediate the activity of two component signal transduction pathways controlling chemotaxis. To test the computational designs that use RBP as a scaffold, a synthetic histidine kinase (HK), Trg-EnvZ, was used. The synthetic HK is composed of the extracellular chemotactic receptor Trg fused to the cytoplasmic portion of the osmoregulator EnvZ (Baumgartner et al., 1994). The synthetic HK is phosphorylated by the appropriate ligand-RBP scaffold complex and transmits a phosphate signal to the bacterial response regulator OmpR. Upon phosphorylation, OmpR binds the *OmpC* promoter that regulates the expression of the β-galactosidase reporter gene (Looger et al., 2003). This synthetic signaling system was used to test the computationally designed TNT.R3 receptor *in vivo* along with several other computational designs (Looger et al., 2003).

Due to high background produced by the wild-type *OmpC* promoter, *Lac operator* elements were added to the *OmpC* promoter allowing the Lac repressor to repress expression of the reporter gene (in this case GFP). The Lac repressor was placed under control of the *OmpF* promoter that is activated by low concentrations of phosphorylated OmpR and repressed by high concentrations of phosphorylated OmpR. In the absence of ligand mediated signal low concentrations of phosphorylated OmpR cause production of the Lac repressor that represses expression of GFP and when the ligand is present, high concentrations of phosphorylated OmpR decrease expression of the Lac repressor removing repression of the *OmpC* promoter controlling GFP. Also, the *OmpC* promoter is responsive to high concentrations of phosphorylated OmpR activating transcription of

GFP (Figure 5.1) (unpublished, Smith *et. al*). Use of this synthetic signaling system sets a threshold and suppresses biological noise. Hellinga's laboratory was able to show ligand dependent GFP expression for the MTBE receptor (Figure 3.2, unpublished) using this synthetic signaling system in bacteria.

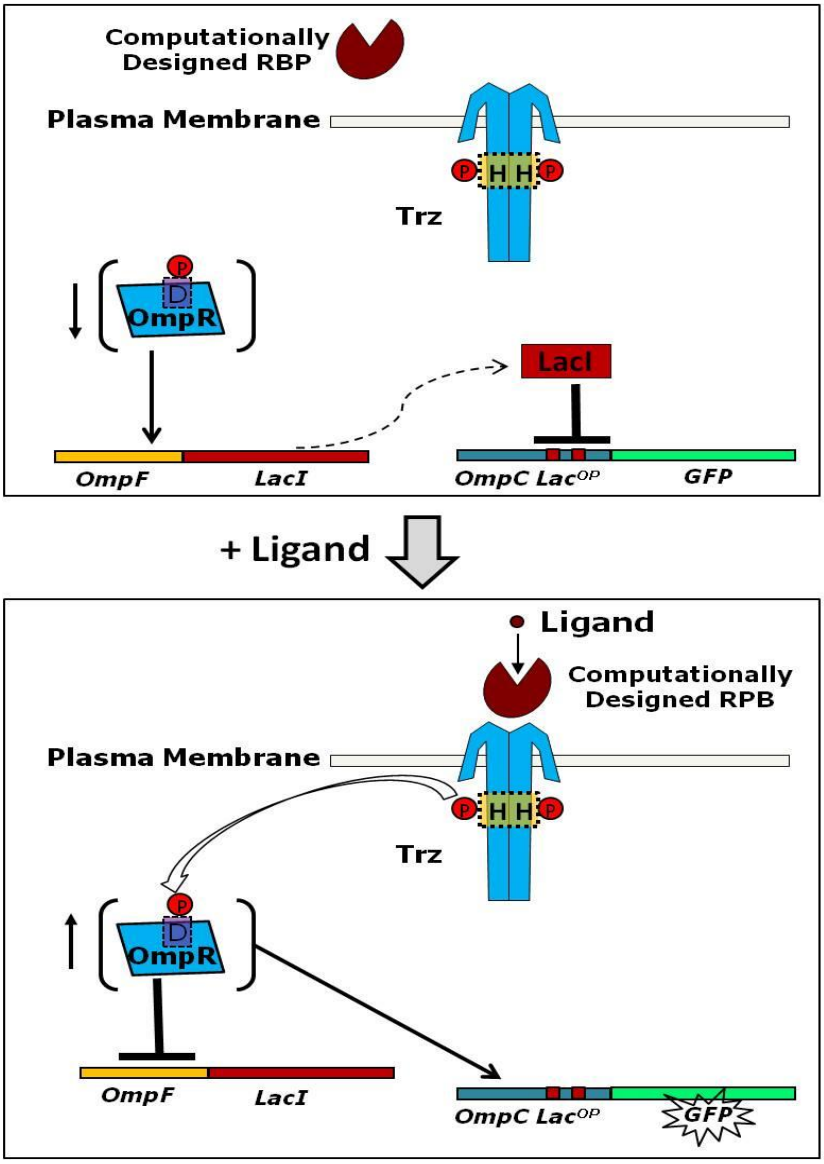

Figure 5.1 Schematic of synthetic signaling system used to test the computationally designed MTBE receptor. To test the computationally designed MTBE receptor *in vivo* the Hellinga laboratory used a RBP responsive synthetic HK fusion, Trg-EnvZ (Trz). Upon ligand-RBP complex binding, Trz is autophosphorylated initiating a phospho-relay to the RR OmpR. Low concentrations of phosphorylated OmpR induce the *OmpF* promoter while high concentrations of phosphorylated OmpR repress the *OmpF* promoter that controls transcription of the Lac repressor. Repression of the *OmpC Lac*^{OP} promoter is relieved allowing high concentrations of phosphorylated OmpR to activate transcription of the *OmpC* promoter stimulating GFP production (unpublished, Smith *et.al.*).

B. Other analyses of Computationally Designed PBPs

In vitro analyses of the structure of some computationally designed PBPs has been performed by the Hocker laboratory (Schreier et al., 2009). These analyses did not include the TNT.R3 receptor or the MTBE receptor used in our laboratory. However, other computational designs of Dr. Hellinga's were analyzed by the Hocker laboratory. They reported that four of the five designed receptors tested could not be crystallized due to aggregation or instability, yet all of the wild-type scaffold PBPs have been crystallized (Phillips et al., 1976; Vyas et al., 1988; Bjorkman and Mowbray, 1998). Hence other methods were used to determine the characteristics of five of the computationally designed receptors. They used far-UV circular dichroism to measure the content of secondary structure, isothermal titration calorimetry to determine binding constants, and NMR spectroscopy to measure the structural changes in computationally designed and wild-type PBPs caused by saturation with the appropriate ligand (Schreier et al., 2009). The Hocker laboratory suggested that direct measurement of binding would better characterize the computationally designed PBPs compared to the indirect *in vitro* measurements of binding collected by the Hellinga laboratory using a fluorophore. Both the Hellinga and Hocker laboratories reported that each of the designed receptors tested were found to be less stable than the corresponding wild-type scaffold PBP (Looger et al., 2003; Schreier et al., 2009). Also, no binding was detected for the designed receptors, whereas structural changes in the wild-type PBPs due to ligand binding were observed in the presence of the appropriate sugar using the same methods (Schreier et al., 2009).

The above described analyses on computationally designed PBPs were performed *in vitro*. This requires the generation of large amounts of purified protein for experiments. The Hocker laboratory and the Hellinga laboratory used the same protein expression and purification methods for the *in vitro* studies of the computationally designed receptors. In both laboratories, *in vitro* experiments used receptors in which the N-terminal signal sequence that targets the protein to the periplasm has been deleted (de Lorimier et al., 2002; Looger et al., 2003; Schreier et al., 2009). Consequently the proteins were expressed in the cytoplasm prior to purification. This could affect the stability and binding ability of the computationally designed receptors. The Hellinga laboratory was able to show binding of the respective ligands using the fluorophore (Looger et al., 2003) while Hocker's experiments did not show binding of the respective ligands by the computationally designed receptors (Schreier et al., 2009). Analyses using the fluorophore might be susceptible to artifacts as the ligand or solvent might affect the fluorophore. In general, *in vitro* analyses may not be representative of the cellular environment or provide accurate measurements of the computationally designed receptors.

C. Advantages of testing in bacteria

The above described *in vitro* analyses did not include the computationally designed PBPs that our lab has used in the TNT detector plants or the MTBE detector plants. However, these experiments do present questions about the binding ability and methods used to analyze the computational designs produced by Dr. Hellinga. Dr. Hellinga's laboratory used a synthetic signaling system based on EnvZ-OmpR phospho-relay (Figure 5.1) to test the computationally designed PBPs *in vivo*. This system relies

on the same RBP-Trg interaction as our synthetic signaling system. However, the fusion HK and downstream RR differ from that of our synthetic signaling system (Figure 5.2). Considering the above described results from the Hocker and Hellinga laboratories and my work with the MTBE receptor in plants, I decided to test the computationally designed MTBE receptor and TNT receptor in a bacterial testing system using our synthetic signaling system (Figure 5.2).

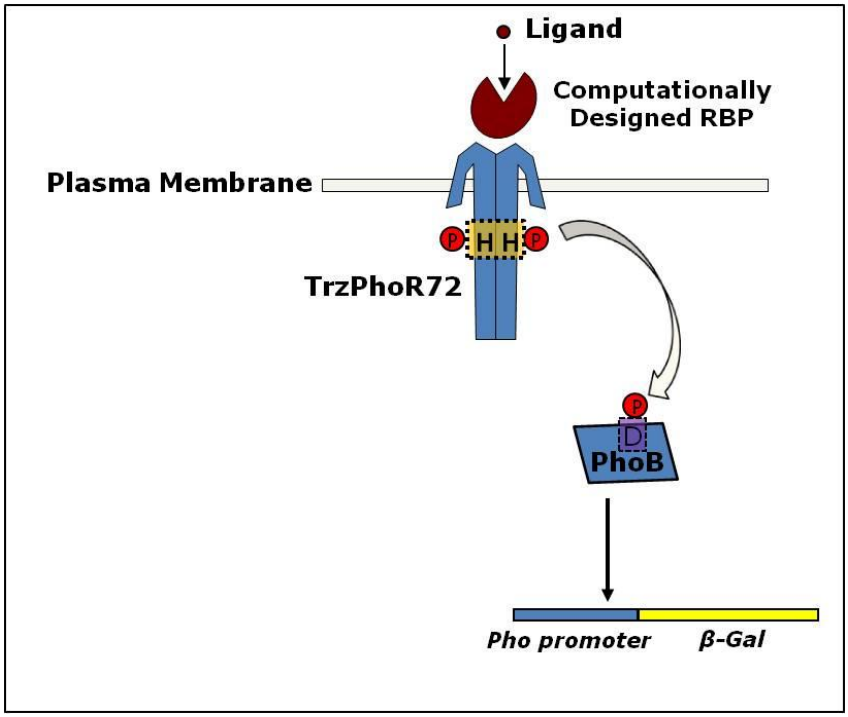

Figure 5.2 Schematic of Bacterial testing system for Computationally Designed Receptors. Bacterial cell containing synthetic signal transduction components, the synthetic fusion HK Trz-PhoR72 (described below), bacterial RR, PhoB, and a computationally designed RBP. The proposed mechanism for signal transduction follows: the computationally designed receptor binds the ligand, the ligand-receptor complex binds and activates the fusion HK Trz-PhoR72 that is autophosphorylated, then the phosphate (P) is transferred from the histidine (H) on Trz-PhoR72 to the aspartate (D) on PhoB. Phosphorylaqted PhoB binds the *Pho promoter* sequence and activates transcription of the *β-galactosidase (β-gal)* reporter gene.

Testing the MTBE and TNT receptors in bacteria with our synthetic signaling components could eliminate questions about the *in vivo* function of the computationally designed receptors used in the detector plants. As previously discussed, testing the

receptors in plants introduces variables such as positional effects of transgene insertion and cross-talk with endogenous plant signaling pathways that may complicate data interpretation. By testing the computationally designed receptors in bacteria we can avoid some of these variables which could produce more uniformity in both response to the ligand and background signaling. Another advantage to testing the computationally designed receptors in bacteria is that we can use wild-type RBP, the scaffold PBP of the computationally designed MTBE and TNT receptors, as a positive control for the function of the synthetic signaling system. The goal of the work described in this chapter was to determine if the computationally designed MTBE and TNT receptors function as previously reported with our synthetic signaling system.

II. Materials and Methods

A. BW cell line

For assays the plasmids described below were expressed in the BW23423 *E.coli* cell line (Haldimann et al., 1996). Deletions in this cell line help to reduce the interaction of components of our synthetic signal transduction system with other bacterial signaling components. It is important to reduce the number of possible signaling components that might activate PhoB in the absence of the ligand being tested to reduce complexity in data interpretation. The BW23423 cell line has a deletion in the PhoBR operon, Δ*PhoBR580*, so that expression of wild-type PhoB and PhoR will not interfere with experiments. There is a deletion in the CreBCD operon , Δ*CreBCD153*, as CreC has been shown to interact with PhoB (Kim et al., 1996; Lee et al., 2007). Also, high levels of acetyl phosphate have been shown to activate PhoB in the absence of PhoR and

CreC (Kim et al., 1996), and the BW23423 cell line contains deletions in the phosphotransacetylase (pta) and acetate kinase (ackA) genes to prevent accumulation of high levels of acetyl phosphate (Haldimann et al., 1996; Metcalf et al., 1996). There is also a deletion in the Pho regulon, *phn(EcoB)*, to ensure that phosphate starvation response genes are not activated during experiments. Finally, a transcriptional fusion inserted into the lac locus has the reporter gene *β-galacosidase* controlled by a pho-responsive promoter (Haldimann et al., 1996) to allow for measurement of PhoB-regulated changes in transcription.

B. Improved Synthetic Fusion HK

I examined the *in vivo* function of the computationally designed MTBE and TNT receptors using a bacterial testing system originally developed by Dr. Kevin Morey. This system was first developed in order to test different versions of the synthetic signal transduction components. Using this system Dr. Morey was able to identify a fusion point between the Trg and PhoR portions of the synthetic HK that results in improved function. The original fusion HK (described in Chapters One and Two), designed by Dr. Jeff Smith of the Hellinga laboratory, was shown to have high un-induced levels of reporter gene activity while showing a small increase in activity in response to ligand exposure in bacteria (Antunes et al., 2011). The new fusion point discovered by Dr. Morey produced a synthetic fusion HK that has lower un-induced levels of reporter gene activity, relative to the original fusion, and produces a larger increase in activity in response to ligand exposure (unpublished, Dr. Kevin Morey). The new and improved synthetic fusion HK will be referred to as Trz-PhoR72.

C. Plasmid Construction

All synthetic signaling components were expressed on a single plasmid in the pACYC177 backbone that carries the *β-lactamase* gene providing resistance to the antibiotic carbenicillin. The original plasmid was obtained from Dr. Hellinga's laboratory, and was adapted for use in these experiments. The original plasmid expresses the fusion HK, Trg-EnvZ (Baumgartner et al., 1994) and the bacterial RR, OmpR, in an operon controlled by the *LacI promoter*, and wild-type RBP also under control of the *LacI promoter*. To express our synthetic signaling components, Dr. Kevin Morey replaced the HK/RR operon from the original plasmid with our synthetic fusion HK, Trz-PhoR72 and RR PhoB operon downstream of the *LacI promoter* (Figure 5.3), hereafter referred to as the "RBP plasmid".

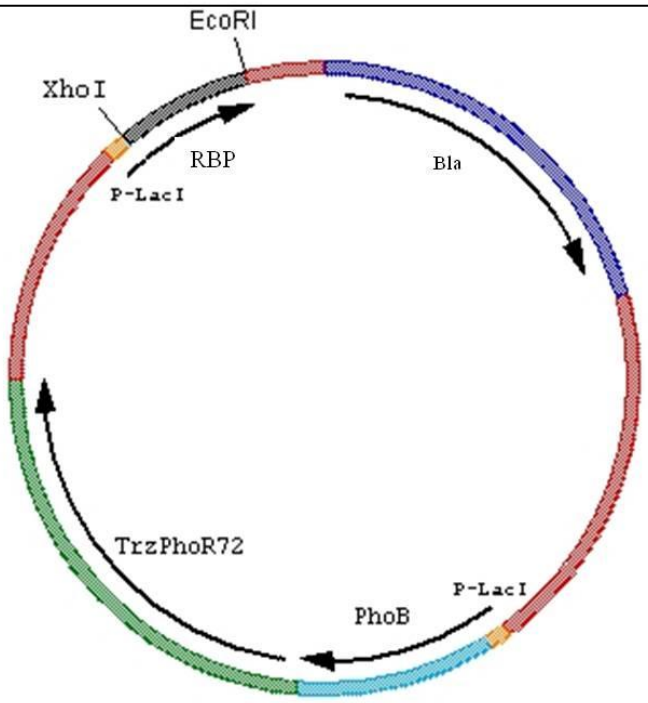

Figure 5.3 Map of RBP Plasmid Bacterial expression vector pACYC, which contains wild-type *ribose binding protein,* synthetic fusion HK *Trz-PhoR72*, and bacterial RR, *PhoB*. RBP: ribose binding protein; P-LacI: *LacI promoter*; Bla: selectable marker, conveys resistance to the antibiotic carbenicillin.

To test the *in vivo* function of the computationally designed MTBE receptor and TNT receptor, the sequence coding for wild-type RBP was replaced with the sequence coding for each of the computationally designed receptors individually. The MTBE receptor and TNT receptor were PCR amplified with appropriate restriction sites and cloned into the above described plasmid. Three plasmids were produced for testing the computationally designed receptors, all three contain our synthetic signaling components, *Trz-PhoR72* and *PhoB*. The plasmid containing wild-type RBP was used as a positive control to develop and optimize the quantitative β-galactosidase assay protocol (described below). The plasmids containing each of the computationally designed receptors, MTBE receptors, referred to as the "MTBE plasmid", and TNT receptor, referred to as the "TNT plasmid", were tested individually for response to the appropriate ligand.

D. Bacterial Transformation and Growth Conditions

All bacteria were transformed by electroporation with the above described plasmids. Transformants were selected and grown in M9 minimal media supplemented with casamino acids (M9CA) (J. Sambrook, 1989), maltose as a carbon source, and carbenicillin for selection of the pACYC177 plasmid on agar plates. For assays, M9CA broth with carbenicillin was inoculated with a freshly transformed (requires 48 hours to grow on agar plate at 30°) single colony and was grown overnight at 30°C in an incubator with shaking at 220 rpm. To measure β-galactosidase activity in response to exposure to the appropriate ligand, 20 mL of M9CA broth with carbenicillin and varying concentrations of the appropriate ligand was inoculated with 1-5 µL of the un-induced

overnight culture. To decrease growth rate variability among cultures, each of the test cultures was inoculated from the same overnight culture with a uniform volume for each assay. Cells containing the plasmid expressing the synthetic signaling components with wild-type RBP were exposed to ribose concentrations ranging from 10μM to 13mM. Cells containing the MTBE plasmid were exposed to MTBE concentrations ranging from 100nM to 500μM. Cells containing the TNT plasmid were exposed to TNT concentrations ranging from 1pM to 10μM. Because the MTBE and TNT ligands are sensitive to photo-degradation, the cultures were protected from light by covering the incubator with a black cloth.

E. β-galactosidase Assay

I measured β-galactosidase activity in BW23423 cells harboring one of the three above described plasmids in response to the appropriate ligand. β-galactosidase activity was measured using the substrate 4-methylumbelliferyl-β-D-galactopyranoside (MUG) because it has been shown to be a more sensitive substrate than *O*-nitrophenyl-β-D-galactopyranoside (ONPG) (Armenta et al., 1985; Honeyman et al., 2002). I adapted a protocol from Honeyman *et.al.* that measures β-galactosidase activity using the MUG substrate (Honeyman et al., 2002).

Several variations of the assay protocol were analyzed (see Results section below) with the following being the final protocol. After growth, cultures were centrifuged in 100mL tubes in a Beckman J2-21M Induction Drive centrifuge at 10000 x g for 10 minutes at 4°C. Liquid was removed, and the pelleted cells were washed by re-suspending with 4mL TES buffer (10mM Tris, 1mM EDTA, 150mM NaCl). Next the cells were centrifuged again, buffer removed and re-suspended in 1.5mL TES buffer.

The suspension was transferred to 2mL microcentrifuge tubes with 1.5mg of glass beads and cells were mechanically lysed using a MiniBeadBeater-8 (BioSpec Products) set to homogenize for 1 minute increments of shaking then cooling, totaling 3 minutes of shaking. Lysate was cleared in a microcentrifuge for 10 minutes at 10000 x g at 4°C. Five µL of lysate was aliquoted in duplicate into a 96-well plate, with 40µL of AB buffer (60mM K_2HPO_4, 40mM KH_2PO_4, 100mM NaCl, pH 8.0), and 10µL of freshly prepared MUG solution (0.4mg mL^{-1} in DMSO). After mixing each sample by pipetting up and down, the plate was wrapped in plastic to prevent evaporation and incubated at 37°C for 45 minutes. Following incubation, the β-galactosidase reaction was slowed by diluting each sample with 200µL of AB buffer so that 4-methylumbelliferyl (MU) fluorescence could be accurately measured. The samples were covered to protect them from light while an MU standard curve was prepared. Eight standards were prepared ranging from 0µg mL^{-1} to 4.0µg mL^{-1} MU in AB buffer. Methylumbelliferyl fluorescence was measured using a Synergy HT microplate reader (BioTek Instruments, Inc), kindly provided by the Kanatous laboratory, using a 355nm excitation filter and 460nm emission filter.

Each sample was normalized based on total protein in the lysate described above. Bradford reagent (Bio-Rad Laboratories) was used to quantify total protein and was measured with a Beckman Coulter DU 5.3 Life Science spectrophotometer, also kindly provided by the Kanatous Laboratory, or Thermo Scientific Nano Drop 2000c spectrophotometer at 595nm wavelength. Total protein was standardized according to a standard curve with bovine serum albumin (BSA) concentrations ranging from 0mg

mL^{-1} to 0.9mg mL^{-1} prepared in TES buffer. β-galactosidase activity is expressed in μg 4-MU mg $protein^{-1}$.

III. Results

A. Developing the Assay using Ribose Binding Protein

1. RBP was used to develop β-galactosidase assay protocol.

The RBP plasmid, containing wild-type RBP and our synthetic signaling components, *Trz-PhoR72* and *PhoB*, was used for the development of the β-galactosidase assay protocol and as a positive control for the function of the synthetic signaling components. This plasmid was transformed into the BW23423 cell line described above and used first in preliminary experiments to develop assay protocol.

The typical quantitative β-galactosidase assay uses culture optical density (OD) to normalize enzyme activity values (J. Sambrook, 1989). That works well for the ONPG assay because the culture is lysed directly and cells are not washed before lysis. However, in this case cells are washed before lysis and inevitably variable amounts of cells are lost during the washing process. Therefore, I wanted to use a normalization parameter that is a direct measurement of the lysate used to measure β-galactosidase activity. Similar to the protocol described by Honeyman *et. al.,* I decided to quantify total protein in the lysate as a normalization method. Honeyman *et.al*. uses the BCA (Pierce) protein assay to quantify total protein in the lysate (Honeyman et al., 2002). We tried using this reagent and found, that once added to our samples the BCA solution was rather viscous and the resulting color was not uniform throughout the sample. In the past our lab has used the Bradford reagent (Bio-Rad Laboratories) to quantify total

protein in samples of ground plant tissue, and did not see the aggregation problems seen with the BCA reagent. After successfully measuring total protein in our bacterial samples with the Bradford reagent, I decided to use this method for normalization of β-galactosidase activity in these experiments.

Next we needed to determine a cell lysis method that allows for both measurement of β-galactosidase activity and protein quantification with the Bradford reagent. The lysis method used for the typical β-galactosidase assay with ONPG substrate, involves lysis with SDS/chloroform and toluene (J. Sambrook, 1989). However, when quantifying protein these chemicals should not be used due low percent recovery of protein and low reliability (De Mey et al., 2008). De Mey *et.al.* found that when using the Bradford reagent for protein quantification commercial lysing agents, BugBuster (Novagen) and EasyLyse (Epicentre Biotechnologies), as well as mechanical lysis such as sonication, are the most reliable lysis methods with highest percent recovery of protein. However, Dudak *et.al.* tested various chemical lysis reagents and showed that commercial lysing agents have a negative effect on β-galactosidase activity (Dudak et al., 2009). Furthermore, I tested the commercial lysis agent BugBuster (Novagen) using the RBP plasmid in the BW23423 cell line. While I was able to successfully quantify protein in cultures, I could not detect β-galactosidase activity. Therefore, I concluded that mechanical lysis is the most appropriate method for this assay. Preliminary experiments used sonication as a lysis method. However, this method proved to be very time consuming when used for experiments with multiple cultures and biological replicates. Therefore, our laboratory purchased a

MiniBeadBeater-8 (BioSpec Products) for mechanical lysis of cultures and this instrument was used for all subsequent experiments.

2. Effects of growth phase of cultures on the response to ribose.

Preliminary experiments were done to determine the effects of the growth phase at which the cultures are harvested, as indicated by optical density or absorbance at wavelength 600 nm (OD_{600}), on the response to ribose. Optical density of a culture correlates with cell density of a culture which can indirectly indicate the growth phase of the cells (Monod, 1949; Scullard and Meynell, 1966). To determine the effect of growth phase, hereafter referred to as OD_{600}, on response to ribose, four pairs of cultures were prepared, with one sample grown without ribose and the other grown with 13mM ribose. Each pair was grown to a different approximate OD_{600} of 0.2, 0.4 (standard deviation = 0.005), 0.6 (standard deviation = 0.052), and 1.0 (standard deviation = 0.036), then the β-galactosidase activity of each culture was measured. The highest fold increase in β-galactosidase activity in response to ribose is observed in the cultures grown to an OD_{600} of approximately 0.4, corresponding to an early log growth phase (Appendix C). The cultures grown to OD_{600} of approximately 0.2 did not have measurable amounts of protein and 4-MU. The cultures grown to OD_{600} of approximately 0.6 and higher, show an increase in β-galactosidase activity in response to ribose but the fold increase is not as high as that of cultures grown to OD_{600} of 0.4 (Figure 5.4 OD0.4: $t(4)=20.5$, $p=0.0001$; OD0.6: $t(4)=24.4$, $p=0.0001$; OD1.0: $t(4)=8.62$, $p=0.001$). This experiment was performed two other additional times with the results showing similar trends; cultures grown to an OD_{600} of approximatly 0.4 show the highest fold induction in response to the ribose ligand.

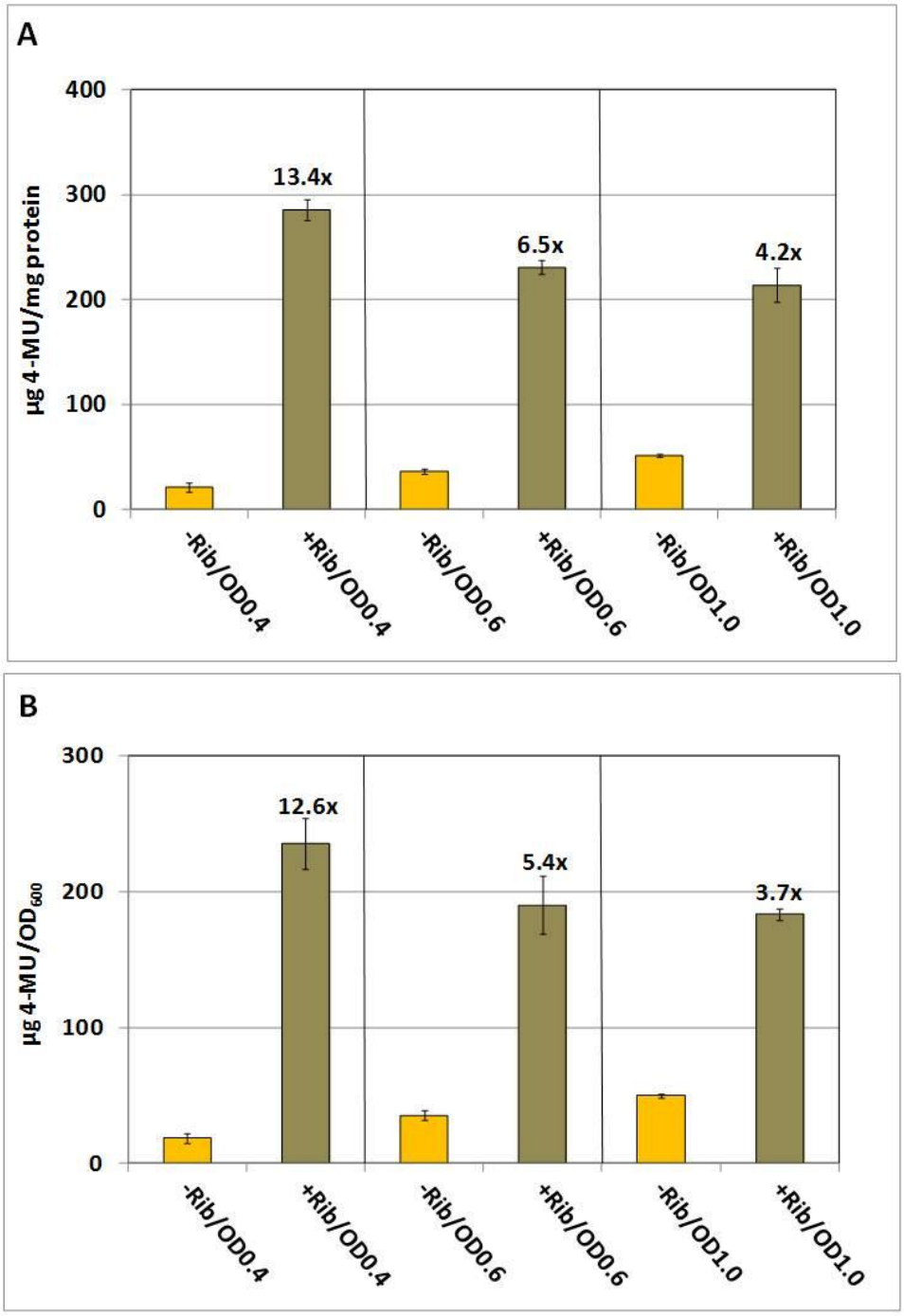

Figure 5.4 Trial to determine optimal growth phase of cultures. β-galactosidase activity in BW23423 cells harboring the RBP plasmid grown in pairs of cultures to an approximate OD_{600} of 0.4, 0.6, or 1.0. One culture of each pair was grown without ribose (yellow bars) and the other was grown with 13mM ribose (tan bars). The numbers at the top of the tan bars represent the fold increase in β-galactosidase activity in response to ribose. Error bars represent (+/-) 1 standard deviation among three biological replicates for each category. Panal A shows β-galactosidase values normalized using total protein in the lysate. Panal B shows β-galactosidase values normalized using OD_{600}.

As shown above, variation in OD_{600} among cultures affects the magnitude of response to the ligand (in this case ribose) and cultures should be grown uniformly to ensure accurate comparison of response among multiple cultures. To aid in reducing variation I tested different inoculation methods with the goal of achieving uniform growth among cultures. I tested inoculation with a single freshly transformed colony grown on M9CA agar plates. I used various tools including an inoculation loop, toothpick, and wooden dowel for transfer of a colony to each liquid culture. None of these methods produced uniform growth among cultures. I found the method that produces the most uniform growth, is to inoculate a single liquid culture without the ligand, with a single freshly transformed colony and grow it overnight at 30°C, then use a defined amount of this liquid culture to inoculate the experimental cultures. This method produces relatively uniform growth among the different cultures and was chosen for the assay protocol.

3. Ribose dependent concentration curve

To determine whether or not our synthetic signaling system produces increasing amounts of β-galactosidase in response to increasing concentrations of ribose, BW23423 cells harboring the RBP plasmid were used to inoculate M9CA broth with concentrations of ribose ranging from zero to 13mM. This experiment was repeated seven times and shows that β-galactosidase activity increases in response to increasing concentrations of the ribose ligand. I was able to show this response in cultures grown to, high OD_{600} values ranging from 0.75 to 1.0 (Figure 5.5) and low OD_{600} values ranging from 0.4 to 0.6 (Figure 5.6).

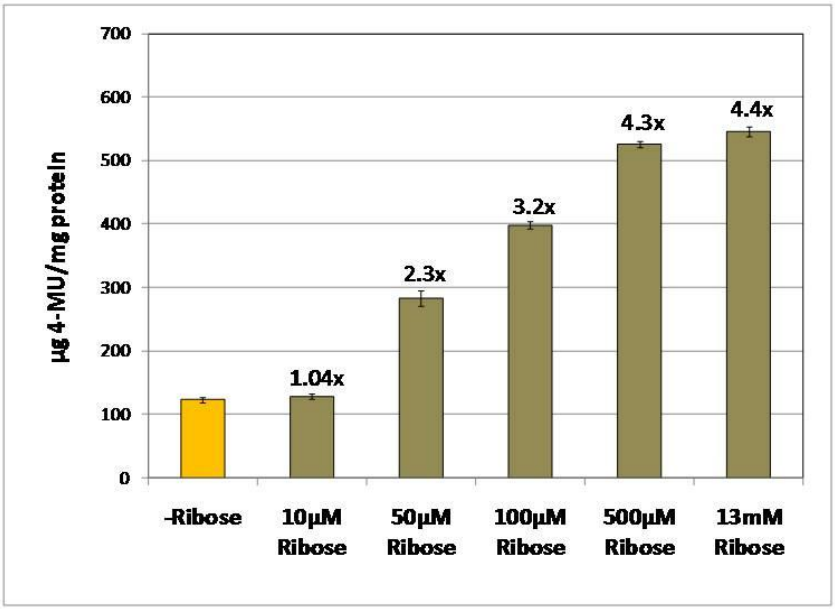

Figure 5.5 Ribose dependent concentration curve β-galactosidase activity in cultures of BW23423 cells harboring the RBP plasmid grown to high OD_{600} values. The yellow bar represents the control culture grown without ribose and the tan bars represent cultures grown with increasing concentrations of ribose. The numbers above the tan bars indicate the fold increase in β-galactosidase activity relative to the control for each concentration of ribose. Error bars represent (+/-) 1 standard deviation among two biological replicates for each concentration.

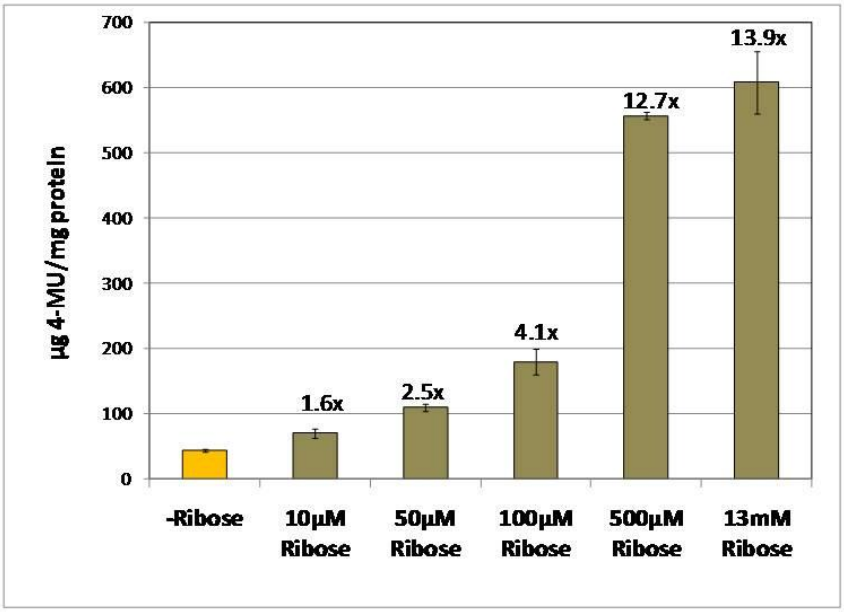

Figure 5.6 Ribose dependent concentration curve. β-galactosidase activity in cultures of BW23423 cells harboring the RBP plasmid uniformly grown to low OD_{600} values. The yellow bar represents the control culture grown without ribose and the tan bars represent cultures grown with increasing concentrations of ribose. The numbers above the tan bars indicate the fold increase in β-galactosidase activity relative to the control for each concentration of ribose. Error bars represent (+/-) 1 standard deviation among three biological replicates for each concentration.

Statistical analysis of data shown in Figure 5.6 is as follows: 10μM – t(4)=3.11, p=0.036; 50μM – t(4)=8.51, p=0.0010; 100μM – t(4)=5.84, p=0.0043; 500μM – t(4)=69.8, p=0.0001; 13mM – t(4)=10.3, p=0.0005. As previously mentioned all of these cultures are in the early log growth phase of the BW23423 cell line according to the OD_{600} values (Appendix C). Similar to previous experiments the response to ribose is larger in cultures with lower OD_{600} values. For example, the fold increase at the 13mM ribose concentration in the cultures grown to higher OD_{600} is 4.4, while the fold increase is 13.9 in the cultures grown to lower OD_{600} with the same concentration of ligand.

These data confirm that our synthetic signaling system components, Trz-PhoR72 and PhoB, are functional in bacteria. Furthermore, the system shows predictability in that increasing concentrations of ribose cause an increase in β-galactosidase activity. This system and assay protocol should be suitable to test the computationally designed MTBE and TNT receptors for ligand dependent initiation of our synthetic signaling system in bacteria.

B. Computationally Designed MTBE Receptor

To test the computationally designed MTBE receptor with our synthetic signaling system for ligand dependent response, the MTBE plasmid was transformed into the BW23423 cell line. Cultures were grown in the same manner as the control RBP cultures described above. Broth containing increasing concentrations of MTBE, ranging from zero to 500μM, was inoculated with a defined volume of a liquid culture of BW23423 cells containing the MTBE plasmid. Then β-galactosidase activity was quantified to determine the response to the MTBE ligand. These results do not show a significant

increase in β-galactosidase activity at any concentration of MTBE (Figure 5.7, 100nM – t(6)=0.146, p=0.89; 10μM – t(6)=0.468, p=0.66; 100μM – t(6)=0.035, p=0.97; 500μM – t(6)=0.462, p=0.66). This experiment was repeated five times with each experiment showing similar results. Each experiment included cultures harboring the RBP plasmid, with and without ribose, as a positive control for the assay. Each positive control showed an increase in β-galactosidase activity in response to ribose. However, cultures of cells containing the MTBE plasmid grown under the same conditions do not show a ligand dependent response. Possible reasons for this are examined in the discussion.

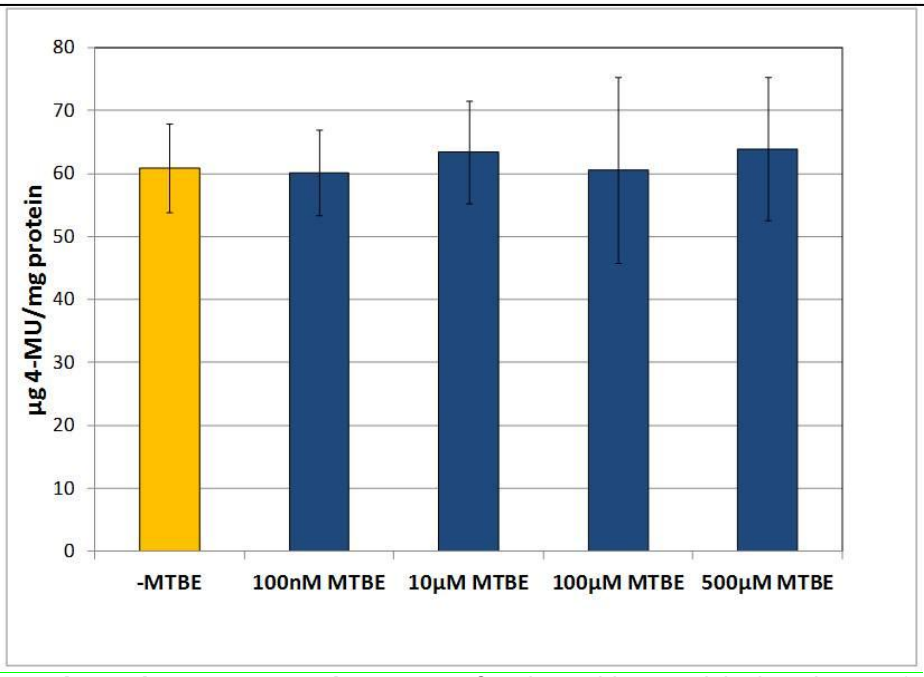

Figure 5.7 MTBE dependent concentration curve. β-galactosidase activity in cultures of BW23423 cells harboring the MTBE plasmid grown to low OD_{600} values. The yellow bar represents the control culture grown without MTBE and the blue bars represent cultures grown with increasing concentrations of MTBE. Error bars represent (+/-) 1 standard deviation among four biological replicates for each concentration.

C. Computationally Designed TNT Receptor

1. Preliminary Experiments

Preliminary experiments were done to test the TNT receptor with the synthetic signaling system for ligand dependent increase in transcriptional activtation. The TNT plasmid was transformed into the BW23423 cell line and grown with increasing concentrations of TNT, ranging from 1pM to 10μM. In these experiments OD_{600} of the cultures was not measured, and the results show an increase in β-galactosidase activity where the error bars do not overlap, at the 100 pM TNT concentration (Figure 5.8). Because OD_{600} values were not collected for these cultures I question the validity of the results. The results could be due to differences in OD_{600} values between cultures, as I have shown that cultures with higher OD_{600} values also have higher β-galactosidase activity (Figure 5.4; compare Figures 5.10 and 5.11).

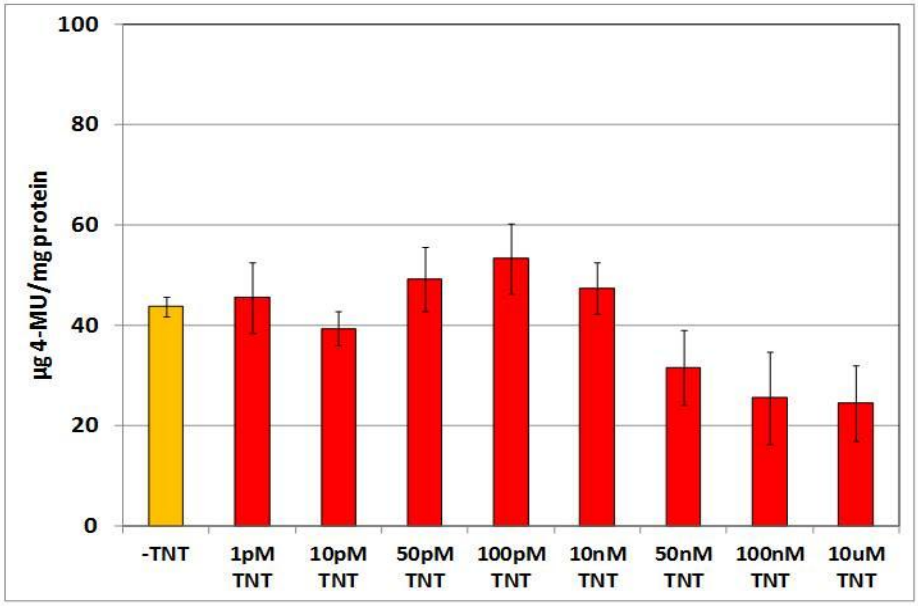

Figure 5.8 Preliminary TNT dependent concentration curve. β-galactosidase activity in cultures of BW23423 cells harboring the TNT plasmid. The yellow bar represents the control culture grown without TNT and the red bars represent cultures grown with increasing concentrations of TNT. Error bars represent +/-1 standard deviation among five biological replicates for each concentration

Statistical analyses of the data presented in Figure 5.8 suggest that there is no significant increase in GUS activity of cultures grown with the TNT ligand: 1pM – t(8)=0.492, p=0.64; 10pM – t(8)=0.51, p=0.63; 50pM – t(8)=0.64 p=0.55; 100pM – t(8)=1.09, p=0.31; 10nM – t(8)=0.65, p=0.54.

2. Effects of growth phase of cultures on response to TNT ligand.

To test whether the growth phase affects the magnitude of the response to TNT, as shown previously with the RBP plasmid, four pairs of cultures were prepared. Each pair contains one culture grown in the absence of TNT and the other grown with 100pM TNT. Each pair of cultures was grown to a different approximate OD_{600} of 0.2, 0.4 (0.42, 0.46), 0.6 (0.64, 0.63) and 1.0 (1.0, 0.96). Again the cultures grown to OD_{600} of approximately 0.2 did not have measurable amounts of protein and 4-MU. Cultures grown to the approximate OD_{600} of 0.4 show the highest increase in β-galactosidase activity in response to TNT (Figure 5.9). This trend is similar to the results seen with the RBP plasmid in response to ribose. Cultures grown to the approximate OD_{600} of 0.6 show a 1.3 fold increase in response to TNT. Unlike results seen with the RBP plasmid in response to ribose, cultures grown to an approximate OD of 1.0 did not show an increase in β-galactosidase activity in response to TNT (Figure 5.9). Similar to results described above using the RBP plasmid (Figure 5.4), the largest increase in β-galactosidase activity observed in our synthetic signaling system due to ligand exposure occurs when the cells are grown to an approximate OD_{600} of 0.4.

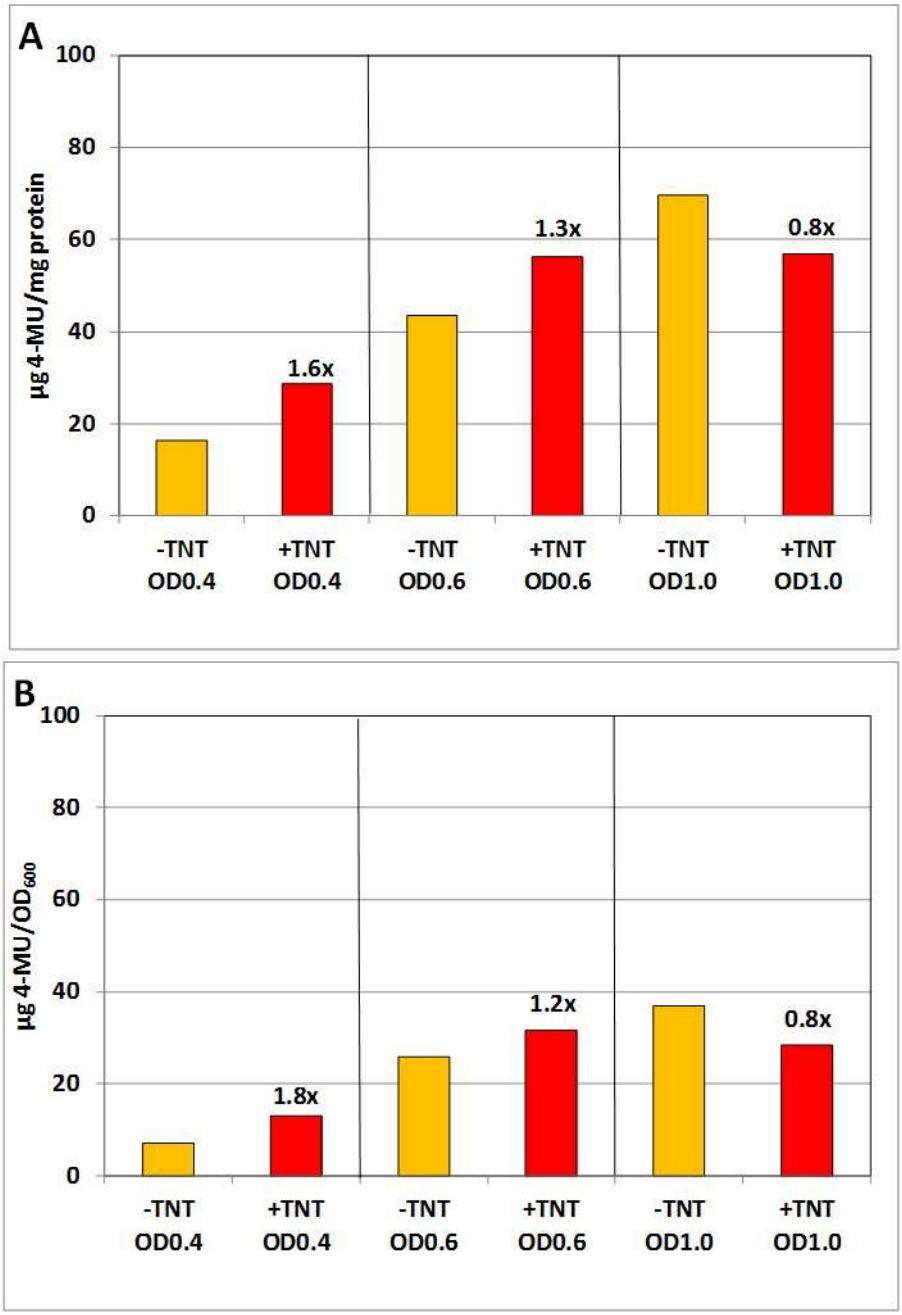

Figure 5.9 Trial to determine effect of growth phase on β-galactosidase activity. β-galactosidase activity in BW23423 cells harboring the TNT plasmid grown in pairs of cultures to an approximate OD_{600} of 0.2, 0.4, 0.6, or 1.0. One culture of each pair was grown without TNT (yellow bars) and the other was grown with 100pM TNT (red bars). The numbers above the red bars represent the fold change in β-galactosidase activity relative to that of the control culture. Panel A shows β-galactosidase values normalized using total protein in the lysate. Panel B shows β-galactosidase values normalized using OD_{600}.

2. Concentration Curve Experiments

To test the computationally designed TNT receptor with our synthetic signaling system for ligand dependent response, the TNT plasmid was transformed into the BW23423 cell line and grown with increasing concentrations of TNT, ranging from 1pM to 10μM. Subsequently, β-galactosidase activity was quantified for each culture to determine the response to TNT. Cultures grown to low ODs ranging from 0.34 to 0.45, do not show a significant increase in β-galactosidase activity in response to increasing concentrations of TNT (Figure 5.10).

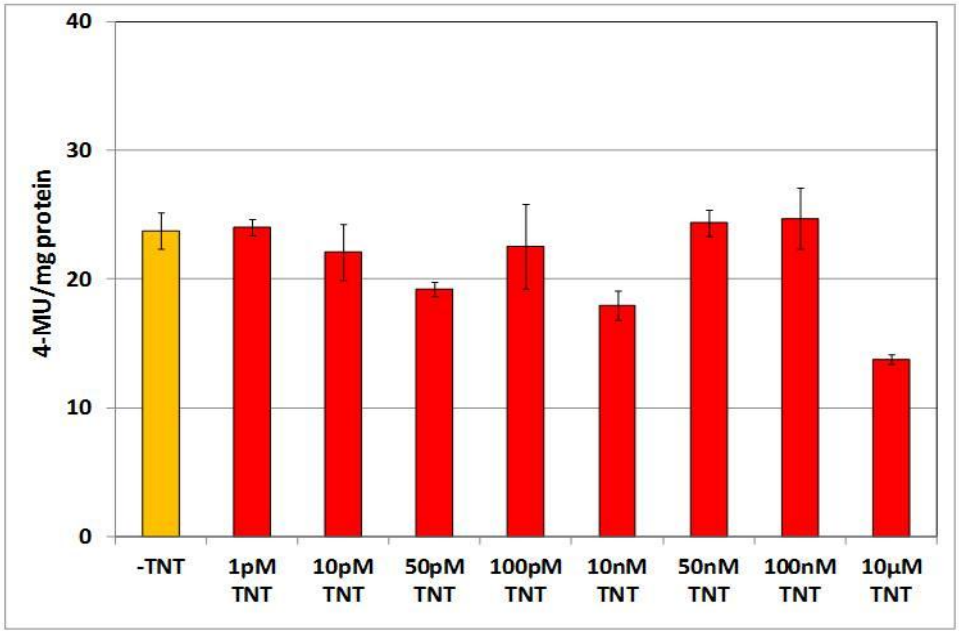

Figure 5.10 TNT dependent concentration curve. β-galactosidase activity in cultures of BW23423 cells harboring the TNT plasmid uniformly grown to low OD_{600} values. The yellow bar represents the control culture grown without TNT and the red bars represent cultures grown with increasing concentrations of TNT.

A small increase in β-galactosidase activity is observed at the 1pM, 50nM and 100nM TNT concentrations, however these increases do not exceed the error bars for these measurements.

The TNT concentration curve experiment was repeated measuring β-galactosidase activity of cultures grown to high ODs ranging from 1.1 to 1.4. Again, these data do not show a significant increase in β-galactosidase activity in response to increasing concentrations of TNT (Figure 5.11).

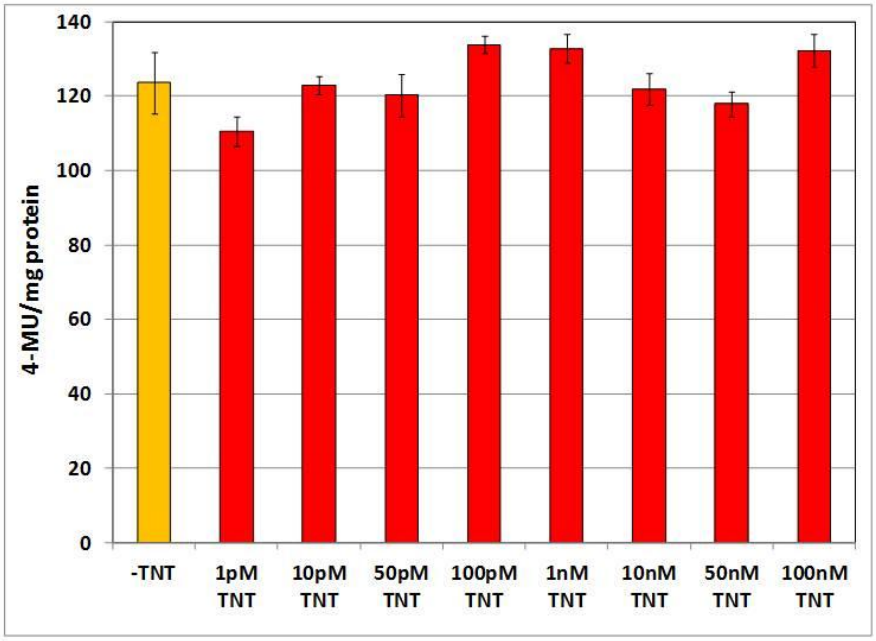

Figure 5.11 TNT dependent concentration curve. β-galactosidase activity in cultures of BW23423 cells harboring the TNT plasmid grown to high OD_{600} values. The yellow bar represents the control culture grown without TNT and the red bars represent cultures grown with increasing concentrations of TNT.

These results show an increase in β-galactosidase activity at the 100pM, 1nM and 100nM TNT concentrations however, these increases do not exceed the error bars for these measurements.

Combined statistical analyses of the data presented in Figues 5.10 and 5.11 show that there is no significant increase in GUS activity of cultures grown with the TNT ligand: 1pM – $t(6)=0.17$, $p=0.87$; 10pM – $t(6)=0.029$, $p=0.97$; 50pM – $t(6)=0.095$, $p=0.93$; 100pM – $t(6)=0.104$, $p=0.92$; 10nM – $t(6)=0.09$, $p=0.93$; 50nM – $t(6)=0.064$, $p=0.95$; 100nM – $t(6)=0.113$, $p=0.91$. These results suggest that the TNT receptor does not show a significant response to TNT with the synthetic signaling system.

Furthermore, the system does not show a predictable response in that increasing concentrations of the TNT ligand do not cause increases in β-galactosidase activity (Figures 5.10 and 5.11), as seen with the wild-type RBP in response to the ribose ligand (Figures 5.5 and 5.6).

3. Effect of General Periplasmic Chaperone on response to TNT ligand

In consideration of the possibility that protein stability may be an issue with the computationally designed receptors (Looger et al., 2003; Schreier et al., 2009), we hypothesized that the aid of a chaperone may increase stability of the computationally designed receptors, resulting in an increase in signaling initiated by the TNT receptor. Dr. Kevin Morey suggested the use of the skp periplasmic chaperone. Unlike most periplasmic chaperones, skp has a broad substrate range and acts as a general periplasmic molecular chaperone (Missiakas et al., 1996; Baneyx, 1999). Overexpression of skp has been shown to improve folding (Bothmann and Pluckthun, 1998) and increase solubility (Hayhurst and Harris, 1999) of aggregation-prone single chain secreted antibodies. The skp periplasmic chaperone is required for proper folding of several outer membrane proteins in gram-negative bacteria (Schafer et al., 1999). Therefore, in an effort to improve the stability of the TNT receptor, I expressed the coding sequence for skp in the pBR322 plasmid and co-transformed the plasmid with the TNT plasmid into the BW23423 cell line. As above, cultures were grown with and without 100pM TNT, then β-galactosidase activity was quantified. Neither cultures with or without the skp chaperone show a significant increase in β-galactosidase activity in response to TNT (Figure 5.12 –chaperone: $t(6)=0.39$, $p=0.71$; +chaperone: $t(6)=0.67$, $p=0.53$).

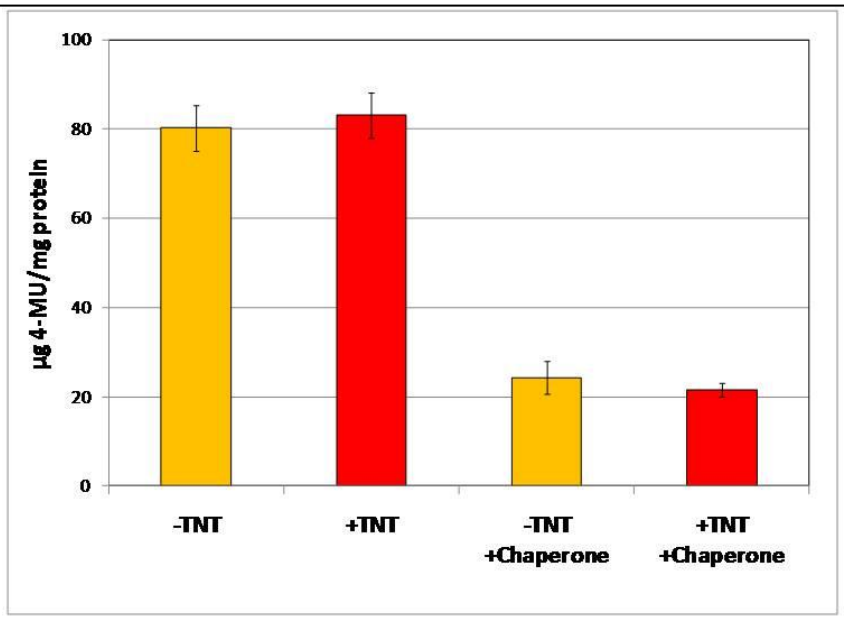

Figure 5.12 Test with general molecular chaperone. β-galactosidase activity in BW23423 cells harboring the TNT plasmid and the plasmid expressing the skp chaperone. The yellow bars represent control cultures grown without TNT and the red bars represent cultures grown with 100pM TNT. Error bars represent (+/-) 1 standard deviation among four biological replicates for each culture.

The cells harboring both the chaperone plasmid and the TNT plasmid grow much more slowly than those harboring only the TNT plasmid. Consequently β-galactosidase activity of the cultures were measured at different OD_{600} values. Cultures of cells harboring only the TNT plasmid had OD_{600} values ranging from 0.80 to 0.92, and those harboring both the TNT plasmid and chaperone plasmid had OD_{600} values ranging from 0.44 to 0.52. These experiments included cultures harboring the RBP plasmid as a positive control. This experiment was repeated and again results show no significant increase in β-galactosidase activity. Over-expression of the skp chaperone does not improve the response to TNT of the computationally designed TNT receptor with our synthetic signaling system.

4. Multiple Biological Replicates

I have measured a small increase in β-galactosidase activity in cultures that contain 100pM TNT in the above results (Figure 5.9 and 5.11). The largest increase in β-galactosidase activity observed was at the 100pM TNT concentration of cultures grown to an approximate OD_{600} of 0.4 (see Figure 5.9). To replicate this response, experiments were set up with four to six biological replicates. One experiment testing multiple biological replicates at the 100pM and 10nM TNT concentrations shows a 1.3 fold increase in β-galactosidase activity in the culture with 100pM TNT. However, the increase is not statistically significant ($t(10)=2.72$, $p=0.22$). No increase is observed in the culture with 10nM TNT ($t(10)=-1.48$, $p=0.17$) (Figure 5.13).

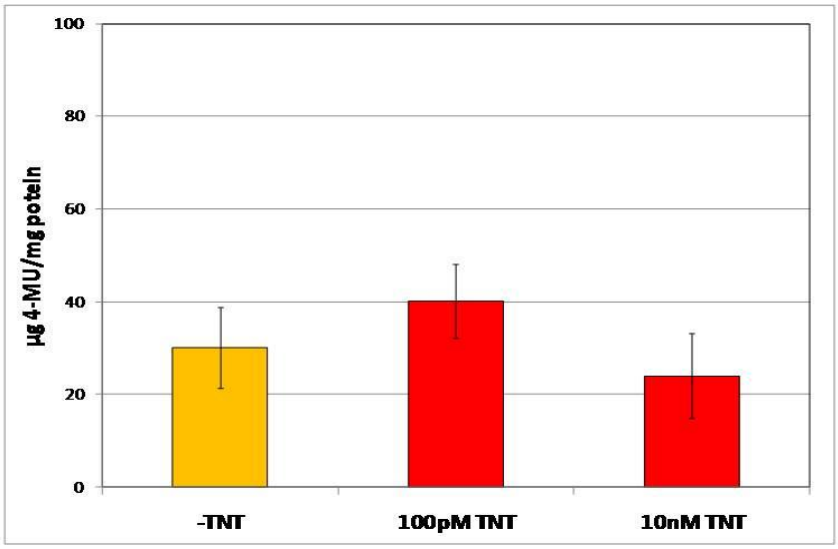

Figure 5.13 Test with multiple biological replicates. β-galactosidase activity in BW23423 cells harboring the TNT plasmid grown to low OD_{600} values. The yellow bar represents control cultures grown without TNT and the red bars represent cultures grown with TNT. Error bars represent (+/-) 1 standard deviation among six biological replicates for each concentration.

This experiment was repeated three more times testing only the cultures grown without TNT and those grown with 100pM TNT. None of these experiments show a

significant increase in β-galactosidase activity in response to TNT. Figure 5.14 shows the results for one of these experiments with no significant increase in β-galactosidase activity ($t(6)=0.253$, $p=0.81$). Testing of multiple biological replicates does not show a clear increase in β-galactosidase activity in response to TNT.

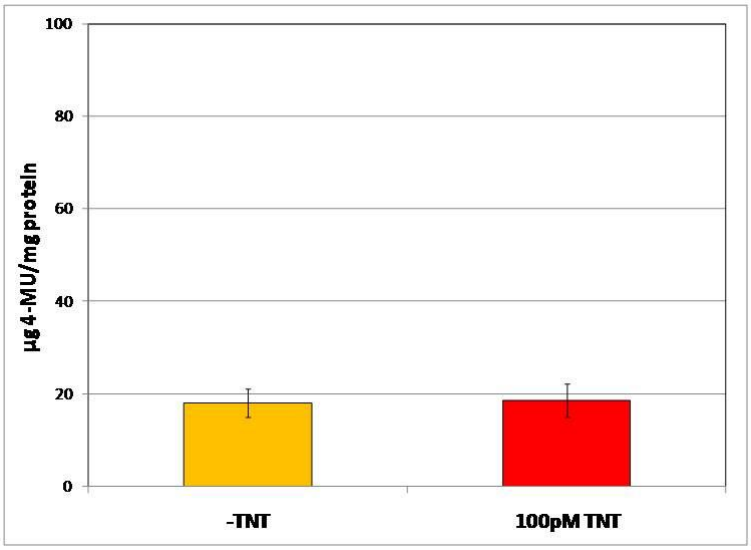

Figure 5.14 Test with multiple biological replicates. β-galactosidase activity in BW23423 cells harboring the TNT plasmid grown to low OD_{600} values. The yellow bar represents control cultures grown without TNT and the red bars represent cultures grown with TNT. Error bars represent (+/-) 1 standard deviation among four biological replicates for each concentration.

IV. Conclusions

A. Ribose binding protein with the synthetic signaling system

The use of the RBP plasmid was important in the identification of optimal assay conditions for testing the computationally designed receptors with the synthetic signaling system. Using the BW23423 cell line harboring the RBP plasmid, I examined different cell lysis methods and protein quantification methods to determine which methods would work for testing our particular system. We also determined the inoculation method that produces the most uniform growth among cultures, and the

optimal OD_{600} at which to harvest the cultures in order to see the largest increase in β-galactosidase activity in response to the ligand. I found that the optimal OD_{600} at which to harvest the cells is approximately 0.4 (Figure 5.4). However, as shown in Figures 5.4 and 5.5, an increase in β-galactosidase activity in response to the ribose ligand is observed at all ODs that were measured. The use of wild-type RBP enabled the development of an assay protocol suitable for testing the computationally designed RBPs.

The testing of BW23423 cells harboring the RPB plasmid with increasing concentrations of ribose in the growth media shows our synthetic signal transduction components, *Trz-PhoR72* and *PhoB* are functional in bacteria. As shown in Figures 5.5 and 5.6, increasing β-galactosidase activity corresponds to increasing concentrations of ribose, showing that our synthetic signaling system works in a predictable manner.

B. Function of the MTBE receptor in our synthetic signaling system.

The BW23423 cell line harboring the MTBE plasmid was tested for an increase in β-galactosidase activity in response to MTBE concentrations ranging from 100nM to 500μM. This experiment was performed five times, and none of the cultures show a significant increase in β-galactosidase activity in response to MTBE. Experiments that tested cultures grown to OD_{600} of approximately 0.4 did not show an increase in β-galactosidase activity in response to any of the tested concentrations of MTBE (Figure 5.7). Hence, the function of the computationally designed MTBE receptor is not verifiable with our synthetic signaling system in bacteria.

C. Function of the TNT receptor in our synthetic signaling system.

Seven experiments were performed to test BW23423 cells harboring the TNT plasmid for a response to increasing TNT concentrations ranging from 1pM to 10μM. Some preliminary experiments show an increase in β-galactosidase activity compared to that of cultures grown without TNT (Figure 5.8). However, OD_{600} of these cultures was not measured and increases in β-galactosidase activity could have been caused by differences in OD_{600}. Experiments in which cultures were uniformly grown and OD_{600} was measured did not show a significant increase in β-galactosidase activity in cultures containing TNT relative to those without TNT (Figures 5.10 and 5.11).

An experiment was done to test the BW23423 cells harboring the TNT plasmid at a range of OD_{600} values to determine at which OD the cultures show the largest increase in β-galactosidase activity. Similar to the results shown using the RBP plasmid, the largest increase in β-galactosidase activity relative to cultures without TNT, is seen at OD_{600} of approximately 0.4 (Figure 5.9). This experiment shows the largest increase in β-galactosidase activity, 1.6 fold, in response to TNT seen with the bacterial testing system. However, there were no biological replicates in this experiment and this response could not be replicated in following experiments.

Experiments were done with multiple (4-6) biological replicates of cultures without TNT and cultures with 100pM TNT. One of these experiments shows an increase in β-galactosidase activity in response to TNT yet the increase does not exceed the error bars for these measurements (Figure 5.13). This experiment was repeated three more times and none of the cultures show a significant increase in β-galactosidase activity in response to TNT.

The computationally designed TNT receptor initially reported to function well (Looger et al., 2003), then questioned in later studies (Schreier et al., 2009), was tested with our synthetic signaling system in bacteria. I found that the TNT receptor does not function in a consistent or predictable manner with our synthetic signaling system.

V. Discussion

A. Evaluation of Approach

Despite the ability to detect a response to ribose in cells harboring the RBP plasmid, a response to MTBE in cells harboring the MTBE plasmid was not detected and a response to TNT was not consistently detected in cells harboring the TNT plasmid. There are several factors that might contribute to the lack of consistent response observed using the computationally designed receptors in our bacterial testing system. These factors include, but are not limited to, a lack of contact between the ligand and receptor, the ligand is degraded, the receptor is unstable or not secreted properly, the receptor-ligand complex does not activate the fusion HK Trz-PhoR72, or our bacterial testing system in not sensitive enough to detect responses. However with the data collected here, we do not have enough information to determine why we do not consistently see a response to the ligand of interest. Other experiments would need to be done examining localization of the computationally designed receptors in bacteria, stability of the computationally designed receptors in the periplasm, and the interaction between the ligand-receptor complex and the fusion HK Trz-PhoR72.

Among the possibilities for the lack of response seen using the computationally designed receptors in our bacterial testing system is ligand degradation. Both MTBE

and TNT are subject to degradation by oxygen and could be degraded in the cultures oxygenated during incubation by shaking at 220 rpm. However, the TNT receptor was shown to bind TNT degradation products 2,4 dinitrotoluene, 2,6 dinitrotoluene, and trinitrobenzene with decreased binding affinities at 8.4μM, 15μM, and 1μM respectively (Looger et al., 2003). Perhaps the TNT in the cultures is degraded over time and the small increase in β-galactosidase activity observed in the results of some experiments is due to the TNT receptor binding TNT degradation products and initiating signaling. This is only one possibility among many, but ligand degradation could explain the results seen using the computationally designed receptors.

As described in other chapters, we use our synthetic signaling system in plants and bacteria serve as a testing platform for components of the system. In order to continue the development and optimization of our synthetic signal transduction system in plants, we need a reliable receptor to initiate signal transduction. Because the receptor component is the first step in the signal transduction cascade, it is difficult to determine the functionality of downstream components if the receptor is not reliable. We have shown that wild-type RBP serves as a positive control for the assay and function of the synthetic signaling system in bacteria. Perhaps RBP could be used in plants with our synthetic signaling system to show a response to ribose, enabling us to collect data about downstream synthetic signaling components *in-planta.*

B. RBP *in-planta*

A reliable means to activate our synthetic signaling system *in-planta* would allow for testing different versions of the synthetic signaling components and provide

quantitative data used for modeling the system. Previous work in the Medford lab has shown that bacterial RBP can be expressed in plants and can be secreted outside of the plant cell using the Pex secretory sequence (Antunes et al., 2011). Also, ribose is highly water soluble, non-toxic and stable in the air and light making it an easy ligand to work with. However, little is known about the possibility of transport and metabolism of free ribose in plant cells. Therefore, to determine if RBP can be used to activate our synthetic signaling system in plants, the receptor component of our synthetic signal transduction pathway has been replaced with RBP. For optimal expression in plant cells, the coding sequence for wild-type RBP was codon optimized for *Arabidopsis thaliana* and synthesized by GeneArt. To support apoplastic localization the codon optimized RBP (AtRBP) was synthesized with the Pex secretory sequence attached to the 5' end of the gene (AtssRBP). Upon arrival I cloned *AtssRBP* into the pCB302-3 plant transformation vector under control of the cauliflower mosaic virus 35S (CaMV35) constitutive promoter. After addition of other synthetic signal transduction components (see below) this plasmid was transformed into Arabidopsis for testing of ribose detector plants.

2. Optimal fusion HK Trz-PhoR72

For initial testing of ribose detector plants, we want to use the best synthetic signaling components available to us. The fusion HK Trz-PhoR72, designed by Dr. Kevin Morey, shows a more substantial increase in β-galactosidase activity than that of the original fusion HK Trg-PhoR in response to ribose in the bacterial testing system (unpublished data). Because Trz-PhoR72 functions better than the original fusion HK in bacteria it was chosen for use in our ribose detector plants. I cloned the version of *Fls*-

Trz-PhoR72 that was codon optimized for Arabidopsis into the above described plasmid with *AtssRBP*.

3. Less stable reporter gene

As previously discussed, the GUS reporter gene is very stable in plant cells and can persist for up to three days (Jefferson et al., 1987), leading to the concern that a small amount of leaky gene expression could cause a build-up of the reporter gene in plant cells. To avoid this possibility, the less stable luciferase reporter gene (Thompson et al., 1991; de Ruijter et al., 2003) was used in our ribose detector plants. The plant, firefly luciferase gene was kindly provided by the Reddy laboratory. Upon receipt, I the inserted luciferase reporter gene downstream of the *PlantPho promoter* in the previously constructed pCambia 2300 plant transformation vector that contains the codon optimized version of *PhoB-VP64*.

The two above described plasmids were co-transformed into Arabidopsis plants using the same methods described in Chapter Two, producing ribose detector plants (Figure 5.15). These plants are being tested for an increase in luciferase activity in response to ribose in the growth media using the luciferase imaging system, by other members of the Medford laboratory.

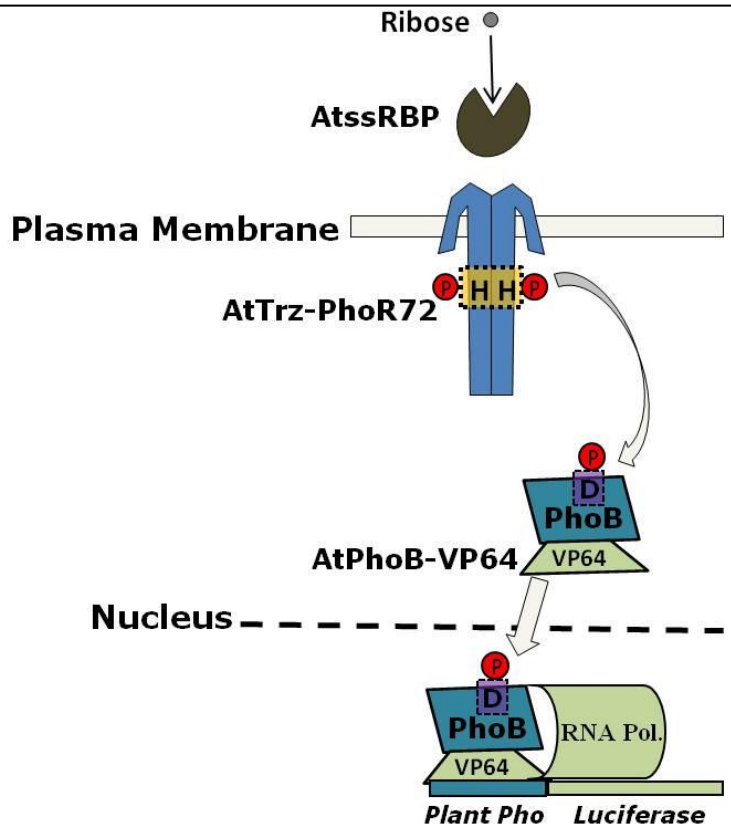

Figure 5.15 Diagram of Ribose Detector Plant System Plant cell with synthetic signal transduction components AtssRBP, fusion HK AtFls-Trz-PhoR72, and response regulator AtPhoB-VP64. The proposed mechanism of signal transduction diagramed follows; upon binding, the ribose-RBP complex activates the fusion HK Trz-PhoR72 which is autophosphorylated, then the high energy phosphate (P) is transferred from the histidine (H) on Trz-PhoR72 to the aspartate (D) on PhoB. Activated PhoB-VP64 moves into the nucleus, binds the *PlantPho promoter* and activates transcription of the luciferase reporter gene.

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APPENDIX 1

Table A1.1 Segregation analysis of T_0 transgenic Arabidopsis plants. $P \geq 0.05$

PhoB mutant	Observed		Expected			Accept/Reject
T_0 Individual	Resistant	Susceptible	3:1 segregation		χ^2 value	$\chi^2 \leq 3.84$
D53A-1	48	2	37.5	12.5	11.760	Reject
D53A-2	99	1	75.0	25.0	30.720	Reject
D53A-3	93	7	75.0	25.0	17.280	Reject
D53A-4	97	3	75.0	25.0	25.813	Reject
D53A-5	92	6	73.5	24.5	18.626	Reject
D53A-6	64	35	74.3	24.8	5.660	Reject
D53A-7	76	27	77.3	25.8	0.081	Accept
D53A-10	29	19	36.0	12.0	5.444	Reject
D53A-11	55	37	69.0	23.0	11.362	Reject
D53A-12	60	30	67.5	22.5	3.333	Accept
D53A-13	43	4	35.3	11.8	6.816	Reject
D53A-17	89	6	71.3	23.8	17.688	Reject
D53A-18	50	0	37.5	12.5	16.667	Reject
D53A-19	49	1	37.5	12.5	14.107	Reject
D53A-20	27	17	33.0	11.0	4.364	Reject
D53A-21	46	3	36.8	12.3	9.313	Reject
D53A-22	45	5	37.5	12.5	6.000	Reject
D53A-26	41	9	37.5	12.5	1.307	Accept
D53A-27	29	14	32.3	10.8	1.310	Accept
D53A-29	35	14	36.8	12.3	0.333	Accept
D53A-30	42	8	37.5	12.5	2.160	Accept
D53A-31	35	11	34.5	11.5	0.029	Accept
D53A-32	38	11	36.8	12.3	0.170	Accept
D53A-33	45	8	39.8	13.3	2.774	Accept
D53A-34	38	11	36.8	12.3	0.170	Accept
D53A-35	45	5	37.5	12.5	6.000	Reject
D53A-36	37	13	37.5	12.5	0.027	Accept
D53A-37	39	8	35.3	11.8	1.596	Accept
D53A-38	37	12	36.8	12.3	0.007	Accept
D53A-41	33	16	36.8	12.3	1.531	Accept
D53A-42	42	17	44.3	14.8	0.458	Accept
D53A-44	35	13	36.0	12.0	0.111	Accept
D53A-45	41	8	36.8	12.3	1.966	Accept
D53A-46	38	11	36.8	12.3	0.170	Accept
D53A-47	32	15	35.3	11.8	1.199	Accept
D53A-48	39	11	37.5	12.5	0.240	Accept

Table A1.2 Segregation analysis of T_0 transgenic Arabidopsis plants. $P \geq 0.05$

PhoB mutant	Observed		Expected			Accept/Reject
T_0 Individual	Resistant	Susceptible	3:1 segregation		χ^2 value	$\chi^2 \leq 3.84$
D100A-1	34	15	36.8	12.3	0.823	Accept
D100A-2	39	10	36.8	12.3	0.551	Accept
D100A-3	49	1	37.5	12.5	14.107	Reject
D100A-4	37	13	37.5	12.5	0.027	Accept
D100A-5	35	11	34.5	11.5	0.029	Accept
D100A-6	40	10	37.5	12.5	0.667	Accept
D100A-7	35	11	34.5	11.5	0.029	Accept
D100A-8	38	12	37.5	12.5	0.027	Accept
D100A-9	43	7	37.5	12.5	3.227	Accept
D100A-10	35	15	37.5	12.5	0.667	Accept
D100A-11	41	5	34.5	11.5	4.899	Reject
D100A-12	46	4	37.5	12.5	7.707	Reject
D100A-14	39	9	36.0	12.0	1.000	Accept
D100A-15	36	12	36.0	12.0	0.000	Accept
D100E-1	44	5	36.8	12.3	5.721	Reject
D100E-2	46	3	36.8	12.3	9.313	Reject
D100E-3	43	7	37.5	12.5	3.227	Accept
D100E-4	38	10	36.0	12.0	0.444	Accept
D100E-5	38	12	37.5	12.5	0.027	Accept
D100E-6	37	12	36.8	12.3	0.007	Accept
D100E-7	35	12	35.3	11.8	0.007	Accept
D100E-8	23	26	36.8	12.3	20.578	Reject
D100E-9	39	11	37.5	12.5	0.240	Accept
D100E-10	38	12	37.5	12.5	0.027	Accept
D100E-11	47	2	36.8	12.3	11.435	Reject
D100E-12	35	13	36.0	12.0	0.111	Accept
D100E-13	42	8	37.5	12.5	2.160	Accept
D100E-14	37	11	36.0	12.0	0.111	Accept
D100E-15	37	11	36.0	12.0	0.111	Accept

Table A1.3 Segregation analysis of T_0 transgenic Arabidopsis plants. $P \geq 0.05$

PhoB mutant	Observed		Expected			Accept/Reject
T_0 Individual	Resistant	Susceptible	3:1 segregation		χ^2 value	$\chi^2 \leq 3.84$
H144A-3	31	15	34.5	11.5	1.420	Accept
H144A-5	48	2	37.5	12.5	11.760	Reject
H144A-7	35	13	36.0	12.0	0.111	Accept
H144A-8	33	15	36.0	12.0	1.000	Accept
H144A-10	34	16	37.5	12.5	1.307	Accept
H144A-13	34	16	37.5	12.5	1.307	Accept
H144A-15	43	8	38.3	12.8	2.359	Accept
H144A-16	36	14	37.5	12.5	0.240	Accept
H144A-17	38	10	36.0	12.0	0.444	Accept
H144A-21	39	10	36.8	12.3	0.551	Accept
H144A-22	38	17	41.3	13.8	1.024	Accept
H144A-23	39	13	39.0	13.0	0.000	Accept
H144A-24	36	14	37.5	12.5	0.240	Accept
H144A-25	32	16	36.0	12.0	1.778	Accept

Table A1.4 Segregation analysis of T_0 transgenic Arabidopsis plants. $P \geq 0.05$

PhoB mutant	Observed		Expected				χ^2 value		Accept/Reject
T_0 Individual	Res	Sus	9:7		3:1		9:7	3:1	$\chi^2 \leq 7.82$
D53A-1	72	27	55.7	43.3	74.3	24.8	10.92208	0.272727	Accept
D53A-2	79	22	56.8	44.2	75.8	25.3	19.80591	0.557756	Accept
D53A-3	44	55	55.7	43.3	74.3	24.8	5.606702	49.2963	Accept
D53A-4	56	41	54.6	42.4	72.8	24.3	0.086565	15.42612	Accept
D53A-5	29	71	56.3	43.8	75.0	25.0	30.17397	112.8533	Reject
D53A-7	32	62	52.9	41.1	70.5	23.5	18.83755	84.09929	Reject
D53A-8	30	69	55.7	43.3	74.3	24.8	27.08369	105.4848	Reject
D53A-11	80	20	56.3	43.8	75.0	25.0	22.92063	1.333333	Accept
D53A-12	76	20	54.0	42.0	72.0	24.0	20.48677	0.888889	Accept
D53A-13	41	40	45.6	35.4	60.8	20.3	1.044288	25.68313	Accept
D53A-14	77	23	56.3	43.8	75.0	25.0	17.49587	0.213333	Accept
D53A-16	75	26	56.8	44.2	75.8	25.3	13.30835	0.029703	Accept
D53A-17	52	48	56.3	43.8	75.0	25.0	0.733968	28.21333	Accept
D53A-19	96	3	55.7	43.3	74.3	24.8	66.70274	25.48485	Reject
D53A-20	52	42	52.9	41.1	70.5	23.5	0.033097	19.41844	Accept

Table A1.5 Segregation analysis of T_0 transgenic Arabidopsis plants. $P \geq 0.05$

PhoB mutant	Observed		Expected				χ^2 value		Accept/Reject
T_0 Individual	Res	Sus	9:7		3:1		9:7	3:1	$\chi^2 \leq 7.82$
D100A-1	95	3	55.1	42.9	73.5	24.5	65.929	25.156	Reject
D100A-2	71	30	56.8	44.2	75.8	25.3	8.098	1.191	Accept
D100A-3	84	15	55.7	43.3	74.3	24.8	32.902	5.121	Accept
D100A-4	73	28	56.8	44.2	75.8	25.3	10.542	0.399	Accept
D100A-7	50	50	56.3	43.8	75.0	25.0	1.587	33.333	Accept
D100A-8	51	12	35.4	27.6	47.3	15.8	15.621	1.190	Accept
D100A-10	88	11	55.7	43.3	74.3	24.8	42.855	10.185	Reject
D100A-13	74	25	55.7	43.3	74.3	24.8	13.764	0.003	Accept
D100A-15	145	53	111.4	86.6	148.5	49.5	23.204	0.330	Accept
D100E-1	81	16	54.6	42.4	72.8	24.3	29.280	3.742	Accept
D100E-3	65	25	50.6	39.4	67.5	22.5	9.330	0.370	Accept
D100E-4	52	29	45.6	35.4	60.8	20.3	2.079	5.041	Accept
H144A-1	64	23	48.9	38.1	65.3	21.8	10.597	0.096	Accept
H144A-4	68	32	56.3	43.8	75.0	25.0	5.610	2.613	Accept
H144A-6	79	26	59.1	45.9	78.8	26.3	15.383	0.003	Accept
H144A-8	13	90	57.9	45.1	77.3	25.8	79.667	213.751	Reject
H144A-9	72	27	55.7	43.3	74.3	24.8	10.922	0.273	Accept
H144A-10	40	47	48.9	38.1	65.3	21.8	3.731	39.084	Accept
H144A-11	68	30	55.1	42.9	73.5	24.5	6.873	1.646	Accept
H144A-12	76	24	56.3	43.8	75.0	25.0	15.850	0.053	Accept
H144A-13	78	19	54.6	42.4	72.8	24.3	23.012	1.515	Accept
H144A-14	72	24	54.0	42.0	72.0	24.0	13.714	0.000	Accept
H144A-15	72	29	56.8	44.2	75.8	25.3	9.280	0.743	Accept
H144A-16	74	21	53.4	41.6	71.3	23.8	18.085	0.425	Accept
H144A-17	51	21	40.5	31.5	54.0	18.0	6.222	0.667	Accept

APPENDIX 2

```
Optimized      7   ATGGACCTGGTTCAAAAACAAAAATCGCTGCAAGACTACACGAAATCCCTGTTCCTGGAA
Original       7   ATGGATTTGGTTCAGAAGCAGAAGAGTTTGCAAGATTACACCAAATCACTCTTCTTAGAA

Optimized     67   GGCATCCTGGACTCCCAATTCCTGCAACTGCAGCAACTGCAGGATGAAAGCAACCCGGAC
Original      67   GGGATTTTGGACAGCCAGTTCTTGCAGCTGCAACAACTACAAGATGAAAGCAATCCAGAT

Optimized    127   TTTGTGTCTCAGGTGGTTACCCTGTTTTTCCAAGATTCCGACCGTATTCTGAATGATCTG
Original     127   TTTGTTTCACAAGTTGTCACACTCTTCTTCCAAGACTCTGATAGGATTCTCAATGATCTC

Optimized    187   TCACTGTCGCTGGATCAGCAAGTCGTGGACTTCAAAAAAGTGGATCCGCATGTTCACCAG
Original     187   TCACTTTCCCTAGATCAACAAGTTGTAGACTTTAAAAAAGTTGATCCCCATGTTCATCAA

Optimized    247   CTGAAAGGCAGCTCTAGTTCCATCGGTGCGCAGCGTGTTAAAAACGCCTGCGTTGTCTTT
Original     247   CTCAAAGGTAGCAGCTCCAGTATAGGAGCACAGAGAGTTAAGAATGCTTGTGTTGTCTTC

Optimized    307   CGCAGCTTCTGTGAACAGCAAAATGTCGAAGCGTGCCATCGTTGTCTGCAGCAAGTCAAA
Original     307   CGCAGCTTCTGCGAGCAGCAAAATGTCGAAGCATGTCATAGATGTTTGCAACAAGTGAAG

Optimized    367   CAGGAATATTACCTGGTGAAAAACCGCCTGGAAACGCTGTTTAAACTGGAACAACAAATC
Original     367   CAAGAGTATTATCTTGTGAAAAACAGATTAGAGACTCTGTTCAAGCTGGAGCAACAGATT

Optimized    427   GTGGCAAGTGGTGGTATGATCCCGGCTGTGGAACTGGGTTTCTAA
Original     427   GTAGCTTCTGGTGGAATGATCCCGGCCGTCGAACTCGGATTTTGA
```

Figure A2.1 Sequences of AHP 1 codon optimized for expression in *E.coli* (GenScript). Nucleotides in red were changed during the optimization process.

```
Optimized      7   ATGGACGCCCTGATCGCACAACTGCAACGCCAATTCCGTGACTATACGATTTCCCTGTAC
Original       7   ATGGACGCTCTCATTGCTCAGCTTCAGAGACAATTTCGTGATTACACCATTTCTCTCTAC

Optimized     67   CAACAAGGCTTCCTGGATGACCAATTCACCGAACTGAAAAAACTGCAGGATGACGGCTCC
Original      67   CAACAGGGGTTTTTGGATGATCAATTTACTGAGTTGAAAAAGCTACAAGATGATGGAAGT

Optimized    127   CCGGATTTTGTGTCAGAAGTTCTGTCGCTGTTTTTCGAAGACTGCGTTAAACTGATTAGC
Original     127   CCTGATTTTGTGTCTGAAGTGCTTTCACTTTTCTTTGAAGATTGTGTGAAGCTTATCAGT

Optimized    187   AACATGGCGCGTGCCCTGGATACCACGGGTACCGTGGACTTTAGCCAGGTCGGTGCATCT
Original     187   AACATGGCTAGAGCTTTGGACACGACAGGAACTGTAGATTTTAGTCAGGTAGGTGCTAGT

Optimized    247   GTGCATCAACTGAAAGGCAGCTCTAGTTCCGTTGGTGCTAAACGTGTCAAAACGCTGTGT
Original     247   GTGCATCAATTGAAGGGTAGTAGCTCAAGTGTTGGTGCCAAGAGGGTCAAAACTTTGTGT

Optimized    307   GTGAGTTTCAAAGAATGCTGTGAAGCGAAAAATTATGAAGGTTGCGTTCGCTGTCTGCAG
Original     307   GTTAGCTTCAAGGAATGTTGTGAAGCTAAGAACTACGAAGGGTGTGTGAGATGTTTGCAG

Optimized    367   CAAGTCGATATCGAATACAAAGCACTGAAAACGAAACTGCAAGATATGTTCAATCTGGAA
Original     367   CAAGTGGATATTGAGTACAAGGCGTTAAAGACAAAGCTTCAAGATATGTTCAATCTTGAG

Optimized    427   AAACAAATCATTCAAGCAGGTGGTATCGTGCCGCAGGTGGACATCAACTAA
Original     427   AAACAGATCATTCAAGCTGGTGGTATAGTTCCTCAAGTGGATATTAACTAA
```

Figure A2.2 Sequences of AHP 2 codon optimized for expression in *E.coli* (GenScript). Nucleotides in red were changed during the optimization process.

```
Optimized      7   ATGGATGCTCTGATTGCTCAGCTGCAACGCCAGTTCCGTGATTATACGATTTCTCTGTAC
Original       7   ATGGACGCTCTCATTGCTCAGCTTCAGAGACAATTTCGTGATTACACCATTTCTCTCTAC

Optimized     67   CAACAAGGCTTTCTGGACGACCAGTTTACCGAACTGAAAAAACTGCAGGATGACGGCTCC
Original      67   CAACAGGGGTTTTTGGATGATCAATTTACTGAGTTGAAAAAGCTACAAGATGATGGAAGT

Optimized    127   CCGGATTTTGTGTCAGAAGTTCTGTCGCTGTTTTTCGAAGACTGCGTTAAACTGATTAGC
Original     127   CCTGATTTTGTGTCTGAAGTGCTTTCACTTTTCTTTGAAGATTGTGTGAAGCTTATCAGT

Optimized    187   AACATGGCGCGTGCCCTGGATACCACGGGTACCGTGGACTTTAGCCAGGTCGGTGCATCT
Original     187   AACATGGCTAGAGCTTTGGACACGACAGGAACTGTAGATTTTAGTCAGGTAGGTGCTAGT

Optimized    247   GTGCATCAACTGAAAGGCAGCTCTAGTTCCGTTGGTGCTAAACGTGTCAAAACGCTGTGT
Original     247   GTGCATCAATTGAAGGGTAGTAGCTCAAGTGTTGGTGCCAAGAGGGTCAAAACTTTGTGT

Optimized    307   GTGAGTTTCAAAGAATGCTGTGAAGCGAAAAATTATGAAGGTTGCGTTCGCTGTCTGCAG
Original     307   GTTAGCTTCAAGGAATGTTGTGAAGCTAAGAACTACGAAGGGTGTGTGAGATGTTTGCAG

Optimized    367   CAAGTCGATATCGAATACAAAGCACTGAAAACGAAACTGCAAGATATGTTCAACCTGGAA
Original     367   CAAGTGGATATTGAGTACAAGGCGTTAAAGACAAAGCTTCAAGATATGTTCAATCTTGAG

Optimized    427   AAACAGATTATTCAAGCCGGTGGTATCGTGCCGCAAGTGGACATCAACTAA
Original     427   AAACAGATCATTCAAGCTGGTGGTATAGTTCCTCAAGTGGATATTAACTAA
```

Figure A2.3 Sequences of AHP 3 codon optimized for expression in *E.coli* (GenScript). Nucleotides in red were changed during the optimization process.

```
Optimized      7   ATGGACGCCCTGATCGCACAACTGCAACGCCAATTTCGTGATTATACCATTTCGCTGTAC
Original       7   ATGGACGCTCTCATTGCTCAGCTTCAGAGACAATTTCGTGATTACACCATTTCTCTCTAC

Optimized     67   CAACAAGGCTTTCTGGATGACCAATTTACCGAACTGAAAAAACTGCAGGATGACGGCTCC
Original      67   CAACAGGGGTTTTTGGATGATCAATTTACTGAGTTGAAAAAGCTACAAGATGATGGAAGT

Optimized    127   CCGGATTTTGTGTCAGAAGTTCTGTCGCTGTTTTTCGAAGACTGCGTTAAACTGATTAGC
Original     127   CCTGATTTTGTGTCTGAAGTGCTTTCACTTTTCTTTGAAGATTGTGTGAAGCTTATCAGT

Optimized    187   AACATGGCGCGTGCCCTGGATACCACGGGTACCGTGGACTTTAGCCAGGTCGGTGCATCT
Original     187   AACATGGCTAGAGCTTTGGACACGACAGGAACTGTAGATTTTAGTCAGGTAGGTGCTAGT

Optimized    247   GTGCATCAACTGAAAGGCAGCTCTAGTTCCGTTGGTGCTAAACGTGTCAAAACGCTGTGT
Original     247   GTGCATCAATTGAAGGGTAGTAGCTCAAGTGTTGGTGCCAAGAGGGTCAAAACTTTGTGT

Optimized    307   GTGAGTTTCAAAGAATGCTGTGAAGCGAAAAATTATGAAGGTTGCGTTCGCTGTCTGCAG
Original     307   GTTAGCTTCAAGGAATGTTGTGAAGCTAAGAACTACGAAGGGTGTGTGAGATGTTTGCAG

Optimized    367   CAAGTCGATATCGAATACAAAGCACTGAAAACGAAACTGCAAGATATGTTCAATCTGGAA
Original     367   CAAGTGGATATTGAGTACAAGGCGTTAAAGACAAAGCTTCAAGATATGTTCAATCTTGAG

Optimized    427   AAACAAATCATTCAGGCAGGTGGTATCGTCCCGCAGGTTGACATTAACTAA
Original     427   AAACAGATCATTCAAGCTGGTGGTATAGTTCCTCAAGTGGATATTAACTAA
```

Figure A2.4 Sequences of AHP 5 codon optimized for expression in *E.coli* (GenScript). Nucleotides in red were changed during the optimization process.

ATATGAACTGGGCACTCAACAATCATCAAGAAGAAGAAGAAGAGCCACGAAGAATTGAAATTTCTGATTCCGAGT
CACTAGAAAACTTGAAAAGCAGCGATTTTTATCAACTGGGTGGTGGTGGTGCTCTGAATTCGTCAGAAAAGCCGA
GAAAGATCGATTTTTGGCGTTCGGGGTTGATGGGTTTTGCGAAGATGCAGCAGCAGCAACAGCTTCAGCATTCAG
TGGCGGTGAAGATGAACAATAATAATAATAACGATCTAATGGGTAATAAAAAAGGGTCAACTTTCATACAAGAAC
ATCGAGCATTGTTACCAAAAGCTTTGATTCTGTGGATCATCATTGTTGGGTTTATAAGCAGTGGGATTTATCAGTG
GATGGATGATGCTAATAAGATTAGAAGGGAAGAGGTTTTGGTCAGCATGTGTGATCAAAGAGCTAGAATGTTGC
AGGATCAATTTAGTGTTAGTGTTAATCATGTTCATGCTTTGGCTATTCTCGTCTCCACTTTTCATTACCACAAGAACC
CTTCTGCAATTGATCAGGAGACATTTGCGGAGTACACGGCAAGAACAGCATTTGAGAGACCGTTGCTAAGTGGAG
TGGCTTATGCTGAAAAAGTTGTGAATTTTGAGAGGGAGATGTTTGAGCGGCAGCACAATTGGGTTATAAAGACAA
TGGATAGAGGAGAGCCTTCACCGGTTAGGGATGAGTATGCTCCTGTTATATTCTCTCAAGATAGTGTCTCTTACCTT
GAGTCACTCGATATGATGTCAGGCGAGGAGGATCGTGAGAATATTTTGCGAGCTAGAGAAACCGGAAAAGCTGT
CTTGACTAGCCCTTTTAGGTTGTTGGAAACTCACCATCTCGGAGTTGTGTTGACATTCCCTGTCTACAAGTCTTCTCT
TCCTGAAAATCCGACTGTCGAAGAGCGTATTGCAGCCACTGCAGGGTACCTTGGTGGTGCGTTTGATGTGGAGTC
TCTAGTCGAGAATTTACTTGGTCAGCTTGCTGGTAACCAAGCAATAGTTGTGCATGTGTATGATATCACCAATGCAT
CAGATCCACTTGTCATGTATGGTAATCAAGATGAAGAAGCCGACAGATCTCTCTCTCATGAGAGCAAGCTCGATTT
TGGAGACCCCTTCAGGAAACATAAGATGATATGCAGGTACCACCAAAAGGCACCAATACCGTTGAATGTGCTCAC
AACTGTGCCATTGTTCTTTGCGATTGGTTTCTTGGTGGGTTATATACTGTATGGTGCAGCTATGCACATAGTAAAAG
TCGAAGATGATTTCCATGAAATGCAAGAGCTTAAAGTGCGAGCAGAAGCTGCTGATGTCGCTAAATCGCAGTTTCT
TGCTACCGTGTCTCACGAGATCAGGACACCAATGAATGGCATTCTCGGAATGCTTGCTATGCTCCTAGATACAGAA
CTAAGCTCGACACAGAGAGATTACGCTCAAACCGCTCAAGTATGTGGTAAAGCTTTGATTGCATTGATAAATGAGG
TTCTTGATCGCGCCAAGATTGAAGCTGGAAAGCTGGAGTTGGAATCAGTACCATTTGATATCCGTTCAATATTGGA
TGATGTCCTTTCTCTATTCTCTGAGGAGTCAAGGAACAAAGGCATTGAGCTCGCGGTTTTCGTTTCAGACAAAGTA
CCAGAGATAGTCAAAGGAGATTCAGGGAGATTTAGACAGATAATCATAAACCTTGTTGGAAATTCGGTTAAATTC
ACAGAGAAAGGACATATCTTTGTTAAAGTCCATCTTGCGGAACAATCAAAAGATGAATCTGAACCGAAAAATGCA
TTGAATGGTGGAGTGTCTGAAGAAATGATCGTTGTTCCAAACAGTCAAGTTACAACACATTGAGCGGTTACGAA
GCTGCTGATGGTCGGAATAGCTGGGATTCATTCAAGCATTTGGTCTCTGAGGAGCAGTCATTATCGGAGTTTGATA
TTTCTAGCAATGTTAGGCTTATGGTTTCAATCGAAGACACGGGTATTGGAATCCCTTTAGTTGGACAAGGCCGTGT
GTTTATGCCGTTTATGCAAGCAGATAGCTCGACTTCAAGAAACTATGGAGGTACTGGTATTGGTTTGAGTATAAGC
AAGTGTCTTGTTGAACTTATGCGTGGTCAGATAAATTTCATAAGCCGGCCTCATATTGGAAGCACGTTCTGGTTCAC
GGCTGTTTTAGAGAAATGCGATAAATGCAGTGCGATTAACCATATGAAGAAACCTAATGTGGAACACTTGCCTTCT
ACTTTTAAAGGAATGAAAGCTATAGTTGTTGATGCTAAGCCTGTTAGAGCTGCTGTGACTAGATACCATATGAAAA
GACTCGGAATCAATGTTGATGTCGTGACAAGTCTCAAAACCGCTGTTGTTGCAGCTGCTGCGTTTGAAAGAAACG
GTTCTCCTCTCCCAACAAAACCGCAACTTGATATGATCTTAGTAGAGAAAGATTCATGGATTTCAACTGAAGATAAT
GACTCAGAGATTCGTTTATTGAATTCAAGAACCAACGGAAACGTTCATCACAAGTCTCCGAAACTAGCTCTATTCGC
AACAAACATCACAAATTCGGAGTTCGACAGAGCTAAATCCGCAGGATTTGCAGATACGGTAATAATGAAACCGTT
AAGAGCAAGCATGATTGGGGCGTGTCTGCAACAAGTTCTCGAGCTGAGAAAAACAAGACAACAACATCCAGAAG
GATCATCACCCGCAACTCTCAAGAGCTTGCTTACAGGGAAGAAGATTCTTGTGGTTGATGATAATATAGTTAACAG
GAGAGTAGCTGCAGGAGCTCTCAAGAAATTTGGAGCAGAAGTGGTTTGTGCAGAGAGTGGTCAAGTTGCTTTGG
GTTTGCTTCAGATTCCACACACTTTCGATGCTTGCTTCATGGATATTCAAATGCCACAGATGGACGGATTTGAAGCA
ACTCGTCAGATAAGAATGATGGAGAAGGAAGCTAAAGAGAAGACAAATCTCGAATGGCATTTACCGATTCTAGCG
ATGACTGCGGATGTGATACACGCGACCTACGAGGAATGTCTGAAAAGTGGGATGGATGGTTACGTCTCCAAACCT
TTTGAAGAAGAGAATCTCTATAAATCCGTTGCCAAATCATTCAAACCTAATCCTATCTCACCTTCGTCGTAATCCAAT
CTTCCGGCGAGTTTTTTTTCTCTCTCCGCAGCCGGAAGAGTGGACCGATTCTGCTGATTGATATGCATTTTGGTTTC
TGTACATACAGTAGGTTCACAATCTAGAGATTTTGAAGGTTTTTTTTTCTTTCACCGAAGTAATGTAGCTTGCCATG
ACTAGTGTATGTTGTTAAACGACAACGTCTAAGACGACGGTTCAGTGTTGATCTTAGCGTAAGTATTAATCCCACG

GGATCGTTTGTACTGTATCAGATTTGGTTAGTCGTTTAAACATTGTAATGTTCTAATAATAACTTTTCCATATATAAC
ATCTTCTTATAACTTGAGACGAGACCATTTTGATT

Figure A2.5 Sequencing results (Macrogen, USA) of attempt to constitutively express *AHK4* in pBR322 vector. Nucleotides highlighted in yellow are inverted and in the wrong position in the gene. Nucleotides highlighted in grey represent deletions. Nucleotides highlighted in green are point mutations. Nucleotides highlighted in blue are duplicated later in the sequence.

Original Sample:

ggataaccagaagcaataaaaaatcaaatcggatttcactatataatctcactttatctaagatgaatccgatggaagcatc
ctgttttctctcaatttttttatctaaaacccagcgttcgatgcttctttgagcgaacgatcaaaaataagtgccttcccatcaaaa
aaatattctcaacataaaaaactttgtgtaatacttgtaacgctacatggagattaactcaatctagctagagaggctttacac
tttatgcttccggctcgtataatgtgtggaattgtgagcggataacaatttcacacaggaaacagctatgaccatgattacgg
attcactggaactctagataacgaggcgcaaaaaatgaaaaagacagctatcgcgattgcagtggcactggctggtttcg
ctaccgtagcgcaggccggaattccaagcttgATGAACTGGGCACTCAACAATCATCAAGAAGAAGAAGAAGAGCC
ACGAAGAATTGAAATTTCTGATTCCGAGTCACTAGAAAACTTGAAAAGCAGCGATTTTTATCAACTGGGTGGTGGT
GGTGCTCTGAATTCGTCAGAAAAGCCGAGAAAGATCGATTTTTGGCGTTCGGGGTTGATGGGTTTTGCGAAGATG
CAGCAGCAGCAACAGCTTCAGCATTCAGTGGCGGTGAAGATGAACAATAATAATAATAACGATCTAATGGGTAAT
AAAAAAGGGTCAACTTTCATACAAGAACATCGAGCATTGTTACCAAAAGCTTTGATTCTGTGGATCATCATTGTTG
GGTTTATAAGCAGTGGGATTTATCAGTGGATGGATGATGCTAATAAGATTAGAAGGGAAGAGGTTTTGGTCAGCA
TGTGTGATCAAAGAGCTAGAATGTTGCAGGATCAATTTAGTGTTAGTGTTAATCATGTTCATGCTTTGGCTATTCTC
GTCTCCACTTTTCATTACCACAAGAACCCTTCTGCAATTGATCAGGAGACATTTGCGGAGTACACGGCAAGAACAG
CATTTGAGAGACCGTTGCTAAGTGGAGTGGCTTATGCTGAAAAAGTTGTGAATTTTGAGAGGGAGATGTTTGAGC
GGCAGCACAATTGGGTTATAAAGACAATGGATAGAGGAGAGCCTTCACCGGTTAGGGATGAGTATGCTCCTGTTA
TATTCTCTCAAGATAGTGTCTCTTACCTTGAGTCACTCGATATGATGTCAGGCGAGGAGGATCGTGAGAATATTTT
GCGAGCTAGAGAAACCGGAAAAGCTGTCTTGACTAGCCCTTTTAGGTTGTTGGAAACTCACCATCTCGGAGTTGTG
TTGACATTCCCTGTCTACAAGTCTTCTCTTCCTGAAAATCCGACTGTCGAAGAGCGTATTGCAGCCACTGCAGGGTA
CCTTGGTGGTGCGTTTGATGTGGAGTCTCTAGTCGAGAATTTACTTGGTCAGCTTGCTGGTAACCAAGCAATAGTT
GTGCATGTGTATGATATCACCAATGCATCAGATCCACTTGTCATGTATGGTAATCAAGATGAAGAAGCCGACAGAT
CTCTCTCTCATGAGAGCAAGCTCGATTTTGGAGACCCCTTCAGGAAACATAAGATGATATGCAGGTACCACCAAAA
GGCACCAATACCGTTGAATGTGCTCACAACTGTGCCATTGTTCTTTGCGATTGGTTTCTTGGTGGGTTATATACTGG
TATGGGTGCAGCTATGCACATAGTAAAAGTCGAAGATGATTTCCATGAAATGCAAGAGCTTAAAGTTCGAGCAGA
AGCTGCTGATGTCGCTAAATCGCAGTTTCTTGCTACCGTGTCTCACGAGATCAGGACACCAATGAATGGCATTCTC
GGAATGCTTGCTATGCTCCTAGATACAGAACTAAGCTCGACACAGAGAGATTACGCTCAAACCGCTCAAGTATGTG
GTAAAGCTTTGATTGCATTGATAAATGAGGTTCTTGATCGCGCCAAGATTGAAGCTGGAAAGCTGGAGTTGGAAT
CAGTACCATTTGATATCCGTTCAATATTGGATGATGTCCTTTCTCTATTCTCTGAGGAGTCAAGGAACAAAAGCATT
GAGCTCGCGGTTTTCGTTTCAGACAAAGTACCAGAGATAGTCAAAGGAGATTCAGGGAGATTTAGACAGATAATC
ATAAACCTTGTTGGAAATTCGGTTAAATTCACAGAGAAAGGACATATCTTTGTTAAAGTCCATCTTGCGGAACAAT
CAAAAGATGAATCTGAACCGAAAAATGCATTGAATGGTGGAGTGTCTGAAGAAATGATCGTTGTTTCCAAACAGT
CAAGTTACAACACATTGAGCGGTTACGAAGCTGCTGATGGTCGGAATAGCTGGGATTCATTCAAGCATTTGGTCTC
TGAGGAGCAGTCATTATCGGAGTTTGATATTTCTAGCAATGTTAGGCTTATGGTTTCAATCGAAGACACGGGTATT
GGAATCCCTTTAGTTGCGCAAGGCCGTGTGTTTATGCCGTTTATGCAAGCAGATAGCTCGACTTCAAGAAACTATG

GAGGTACTGGTATTGGTTTGAGTATAAGCAAGTGTCTTGTTGAACTTATGCGTGGTCAGATAAATTTCATAAGCCG
GCCTCATATTGGAAGCACGTTCTGGTTCACGGCTGTTTTAGAGAAATGCGATAAATGCAGTGCGATTAACCATATG
AAGAAACCTAATGTGGAACACTTGCCTTCTACTTTTAAAGGAATGAAAGCTATAGTTGTTGATGCTAAGCCTGTTA
GAGCTGCTGTGACTAGATACCATATGAAAAGACTCGGAATCAATGTTGATGTCGTGACAAGTCTCAAAACCGCTGT
TGTTGCAGCTGCTGCGTTTGAAAGAAACGGTTCTCCTCTCCCAACAAAACCGCAACTTGATATGATCTTAGTAGAG
AAAGATTCATGGATTTCAACTGAAGATAATGACTCAGAGATTCGTTTATTGAATTCAAGAACCAACGGAAACGTTC
ATCACAAGTCTCCGAAACTAGCTCTATTCGCAACAAACATCACAAATTCGGAGTTCGACAGAGCTAAATCCGCAGG
ATTTGCAGATACGGTAATAATGAAACCGTTAAGAGCAAGCATGATTGGGGCGTGTCTGCAACAAGTTCTCGAGCT
GAGAAAAACAAGACAACAACATCCAGAAGGATCATCACCCGCAACTCTCAAGAGCTTGCTTACAGGGAAGAAGAT
TCTTGTGGTTGATGATAATATAGTTAACAGGAGAGTAGCTGCAGGAGCTCTCAAGAAATTTGGAGCAGAAGTGGT
TTGTGCAGAGAGTGGTCAAGTTGCTTTGGGTTTGCTTCAGATTCCACACACTTTCGATGCTTGCTTCATGGATATTC
AAATGCCACAGATGGACGGATTTGAAGCAACTCGTCAGATAAGAATGATGGAGAAGGAAACTAAAGAGAAGACA
AATCTCGAATGGCATTTACCGATTCTAGCGATGACTGCGGATGTGATACACGCGACCTACGAGGAATGTCTGAAA
AGTGGGATGGATGGTTACGTCTCCAAACCTTTTGAAGAAGAGAATCTCTATAAATCCGTTGCCAAATCATTCAAAC
CTAATCCTATCTCACCTTCGTCGTAATCCAATCTTCCGGCGAGTTTTTTTTCTCTCTCCGCAGCCGGAAGAGTGGACC
GATTCTGCTGATTGATATGCATTTTGGTTTCTGTACATACAGTAGGTTCACAATCTAGAGATTTTGAAGGTTTTTTTT
TCTTTCACCGAAGTAATGTAGCTTGCCATGACTAGTGTATGTTGTTAAACGACAACGTCTAAGACGACGGTTCAGT
GTTGATCTTAGCGTAAGTATTAATCCCACGGGATCGTTTGTACTGTATCAGATTTGGTTAGTCGTTTAAACATTGTA
ATGTTCTAATAATAACTTTTCCATATATAACATCTTCTTATAACTTGAGACGAGACCATTTTGATT

Experimental Sample:

ggataaccagaagcaataaaaaatcaaatcggatttcactatataatctcactttatctaagatgaatccgatggaagcatc
ctgttttctctcaatttttttatctaaaacccagcgttcgatgcttctttgagcgaacgatcaaaaataagtgccttcccatcaaaa
aaatattctcaacataaaaaactttgtgtaatacttgtaacgctacatggagattaactcaatctagctagaggctttacac
tttatgcttccggctcgtataatgtgtggaattgtgagcggataacaatttcacacaggaaacagctatgaccatgattacgg
attcactggaactctagataacgaggcgcaaaaaatgaaaaagacagctatcgcgattgcagtggcactggctggtttcg
ctaccgtagcgcaggccggaattccaagcttgATGAACTGGGCACTCAACAATCATCAAGAAGAAGAAGAAGAGCC
ACGAAGAATTGAAATTTCTGATTCCGAGTCACTAGAAAACTTGAAAAGCAGCGATTTTTATCAACTGGGTGGTGGT
GGTGCTCTGAATTCGTCAGAAAAGCCGAGAAAGATCGATTTTTGGCGTTCGGGGTTGATGGGTTTTGCGAAGATG
CAGCAGCAGCAACAGCTTCAGCATTCAGTGGCGGTGAAGATGAACAATAATAATAATAACGATCTAATGGGTAAT
AAAAAAGGGTCAACTTTCATACAAGAACATCGAGCATTGTTACCAAAAGCTTTGATTCTGTGGATCATCATTGTTG
GGTTTATAAGCAGTGGGATTTATCAGTGGATGGATGATGCTAATAAGATTAGAAGGGAAGAGGTTTTGGTCAGCA
TGTGTGATCAAAGAGCTAGAATGTTGCAGGATCAATTTAGTGTTAGTGTTAATCATGTTCATGCTTTGGCTATTCTC
GTCTCCACTTTTCATTACCACAAGAACCCTTCTGCAATTGATCAGGAGACATTTGCGGAGTACACGGCAAGAACAG
CATTTGAGAGACCGTTGCTAAGTGGAGTGGCTTATGCTGAAAAAGTTGTGAATTTTGAGAGGGAGATGTTTGAGC
GGCAGCACAATTGGGTTATAAAGACAATGGATAGAGGAGAGCCTTCACCGGTTAGGGATGAGTATGCTCCTGTTA
TATTCTCTCAAGATAGTGTCTCTTACCTTGAGTCACTCGATATGATGTCAGGCGAGGAGGATCGTGAGAATATTTT
GCGAGCTAGAGAAACCGGAAAAGCTGTCTTGACTAGCCCTTTTAGGTTGTTGGAAACTCACCATCTCGGAGTTGTG
TTGACATTCCCTGTCTACAAGTCTTCTCTTCCTGAAAATCCGACTGTCGAAGAGCGTATTGCAGCCACTGCAGGGTA
CCTTGGTGGTGCGTTTGATGTGGAGTCTCTAGTCGAGAATTTACTTGGTCAGCTTGCTGGTAACCAAGCAATAGTT
GTGCATGTGTATGATATCACCAATGCATCAGATCCACTTGTCATGTATGGTAATCAAGATGAAGAAGCCGACAGAT
CTCTCTCTCATGAGAGCAAGCTCGATTTTGGAGACCCCTTCAGGAAACATAAGATGATATGCAGGTACCACCAAAA
GGCACCAATACCGTTGAATGTGCTCACAACTGTGCCATTGTTCTTTGCGATTGGTTTCTTGGTGGGTTATATACTGG

TATGGGTGCAGCTATGCACATAGTAAAAGTCGAAGATGATTTCCATGAAATGCAAGAGCTTAAAGTTCGAGCAGA
AGCTGCTGATGTCGCTAAATCGCAGTTTCTTGCTACCGTGTCTCACGAGATCAGGACACCAATGAATGGCATTCTC
GGAATGCTTGCTATGCTCCTAGATACAGAACTAAGCTCGACACAGAGAGATTACGCTCAAACCGCTCAAGTATGTG
GTAAAGCTTTGATTGCATTGATAAATGAGGTTCTTGATCGCGCCAAGATTGAAGCTGGAAAGCTGGAGTTGGAAT
CAGTACCATTTGATATCCGTTCAATATTGGATGATGTCCTTTCTCTATTCTCTGAGGAGTCAAGGAACAAAAGCATT
GAGCTCGCGGTTTTCGTTTCAGACAAAGTACCAGAGATAGTCAAAGGAGATTCAGGGAGATTTAGACAGATAATC
ATAAACCTTGTTGGAAATTCGGTTAAATTCACAGAGAAAGGACATATCTTTGTTAAAGTCCATCTTGCGGAACAAT
CAAAAGATGAATCTGAACCGAAAAATGCATTGAATGGTGGAGTGTCTGAAGAAATGATCGTTGTTTCCAAACAGT
CAAGTTACAACACATTGAGCGGTTACGAAGCTGCTGATGGTCGGAATAGCTGGGATTCATTCAAGCATTTGGTCTC
TGAGGAGCAGTCATTATCGGAGTTTGATATTTCTAGCAATGTTAGGCTTATGGTTTCAATCGAAGACACGGGTATT
GGAATCCCTTTAGTTGCGCAAGGCCGTGTGTTTATGCCGTTTATGCAAGCAGATAGCTCGACTTCAAGAAACTATG
GAGGTACTGGTATTGGTTTGAGTATAAGCAAGTGTCTTGTTGAACTTATGCGTGGTCAGATAAATTTCATAAGCCG
GCCTCATATTGGAAGCACGTTCTGGTTCACGGCTGTTTTAGAGAAATGCGATAAATGCAGTGCGATTAACCATATG
AAGAAACCTAATGTGGAACACTTGCCTTCTACTTTTAAAGGAATGAAAGCTATAGTTGTTGATGCTAAGCCTGTTA
GAGCTGCTGTGACTAGATACCATATGAAAAGACTCGGAATCAATGTTGATGTCGTGACAAGTCTCAAAACCGCTGT
TGTTGCAGCTGCTGCGTTTGAAAGAAACGGTTCTCCTCTCCCAACAAAACCGCAACTTGATATGATCTTAGTAGAG
AAAGATTCATGGATTTCAACTGAAGATAATGACTCAGAGATTCGTTTATTGAATTCAAGAACCAACGGAAACGTTC
ATCACAAGTCTCCGAAACTAGCTCTATTCGCAACAAACATCACAAATTCGGAGTTCGACAGAGCTAAATCCGCAGG
ATTTGCAGATACGGTAATAATGAAACCGTTAAGAGCAAGCATGATTGGGGCGTGTCTGCAACAAGTTCTCGAGCT
GAGAAAAACAAGACAACAACATCCAGAAGGATCATCACCCGCAACTCTCAAGAGCTTGCTTACAGGGAAGAAGAT
TCTTGTGGTTGATGATAATATAGTTAACAGGAGAGTAGCTGCAGGAGCTCTCAAGAAATTTGGAGCAGAAGTGGT
TTGTGCAGAGAGTGGTCAAGTTGCTTTGGGTTTGCTTCAGATTCCACACACTTTCGATGCTTGCTTCATGGATATTC
AAATGCCACAGATGGACGGATTTGAAGCAACTCGTCAGATAAGAATGATGGAGAAGGAAACTAAAGAGAAGACA
AATCTCGAATGGCATTTACCGATTCTAGCGATGACTGCGGATGTGATACACGCGACCTACGAGGAATGTCTGAAA
AGTGGGATGGATGGTTACGTCTCCAAACCTTTTGAAGAAGAGAATCTCTATAAATCCGTTGCCAAATCATTCAAAC
CTAATCCTATCTCACCTTCGTCGTAATCCAATCTTCCGGCGAGTTTTTTTTCTCTCTCCGCAGCCGGAAGAGTGGACC
GATTCTGCTGATTGATATGCATTTTGGTTTCTGTACATACAGTAGGTTCACAATCTAGAGATTTTGAAGGTTTTTTTT
TCTTTCACCGAAGTAATGTAGCTTGCCATGACTAGTGTATGTTGTTAAACGACAACGTCTAAGACGACGGTTCAGT
GTTGATCTTAGCGTAAGTATTAATCCCACGGGATCGTTTGTACTGTATCAGATTTGGTTAGTCGTTTAAACATTGTA
ATGTTCTAATAATAACTTTTCCATATATAACATCTTCTTATAACTTGAGACGAGACCATTTTGATT

Figure A2.6 Sequencing results (Macrogen, USA) of the pIN-III-OmpA3 vector with AHK4. Regions of the vector sequenced included the *lpp-LacOP* regulatory elements, *Omp-A3* signal peptide and *AHK4.* The AHK4 sequence is shown in capital letters. Nucleotides highlighted in grey represent deletions.

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGCTGGTTTCGCTACCGTAGCGCAGGCC

Figure A2.7 OmpA3 signal peptide sequence: (www.ncbi.nlm.nih.gov/nuccore/3929644)

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Optimized    13   TCCCTGATGAACTGGGCACTGAACAACCACCAAGAAGAAGAAGAAGAACCGCGTCGTATT
Original     13   AGCTTGATGAACTGGGCACTCAACAATCATCAAGAAGAAGAAGAAGAGCCACGAAGAATT

Optimized    73   GAAATCTCGGACTCGGAAAGCCTGGAAAACCTGAAAAGCTCTGATTTTTATCAGCTGGGC
Original     73   GAAATTTCTGATTCCGAGTCACTAGAAAACTTGAAAAGCAGCGATTTTTATCAACTGGGT

Optimized   133   GGTGGCGGTGCGCTGAATAGTTCCGAAAAACCGCGCAAAATTGATTTTTGGCGTTCCGGC
Original    133   GGTGGTGGTGCTCTGAATTCGTCAGAAAAGCCGAGAAAGATCGATTTTTGGCGTTCGGGG

Optimized   193   CTGATGGGTTTCGCCAAAATGCAGCAACAGCAACAGCTGCAACATTCAGTGGCAGTTAAA
Original    193   TTGATGGGTTTTGCGAAGATGCAGCAGCAGCAACAGCTTCAGCATTCAGTGGCGGTGAAG

Optimized   253   ATGAACAACAACAACAACAACGATCTGATGGGCAACAAAAAAGGTAGTACCTTCATTCAG
Original    253   ATGAACAATAATAATAATAACGATCTAATGGGTAATAAAAAAGGGTCAACTTTCATACAA

Optimized   313   GAACACCGCGCCCTGCTGCCGAAAGCACTGATCCTGTGGATTATCATTGTGGGCTTTATT
Original    313   GAACATCGAGCATTGTTACCAAAAGCTTTGATTCTGTGGATCATCATTGTTGGGTTTATA

Optimized   373   TCATCGGGTATCTATCAATGGATGGATGACGCCAATAAAATTCGTCGCGAAGAAGTCCTG
Original    373   AGCAGTGGGATTTATCAGTGGATGGATGATGCTAATAAGATTAGAAGGGAAGAGGTTTTG

Optimized   433   GTGTCTATGTGCGATCAGCGTGCACGCATGCTGCAAGACCAGTTTAGCGTTTCTGTCAAC
Original    433   GTCAGCATGTGTGATCAAAGAGCTAGAATGTTGCAGGATCAATTTAGTGTTAGTGTTAAT

Optimized   493   CATGTTCACGCGCTGGCCATTCTGGTCTCGACCTTCCATTATCACAAAAATCCGAGCGCG
Original    493   CATGTTCATGCTTTGGCTATTCTCGTCTCCACTTTTCATTACCACAAGAACCCTTCTGCA

Optimized   553   ATCGATCAGGAAACGTTTGCCGAATACACCGCACGTACGGCATTCGAACGTCCGCTGCTG
Original    553   ATTGATCAGGAGACATTTGCGGAGTACACGGCAAGAACAGCATTTGAGAGACCGTTGCTA

Optimized   613   AGCGGTGTTGCATACGCTGAAAAAGTGGTTAACTTTGAACGTGAAATGTTCGAACGCCAG
Original    613   AGTGGAGTGGCTTATGCTGAAAAAGTTGTGAATTTTGAGAGGGAGATGTTTGAGCGGCAG
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Optimized   673   CATAATTGGGTGATTAAAACCATGGATCGCGGTGAACCGTCCCCGGTGCGTGACGAATAT
Original    673   CACAATTGGGTTATAAAGACAATGGATAGAGGAGAGCCTTCACCGGTTAGGGATGAGTAT

Optimized   733   GCGCCGGTTATCTTTTCACAGGATAGTGTCTCCTACCTGGAATCTCTGGATATGATGAGT
Original    733   GCTCCTGTTATATTCTCTCAAGATAGTGTCTCTTACCTTGAGTCACTCGATATGATGTCA

Optimized   793   GGCGAAGAAGACCGCGAAAACATTCTGCGTGCCCGCGAAACCGGTAAAGCAGTGCTGACG
Original    793   GGCGAGGAGGATCGTGAGAATATTTTGCGAGCTAGAGAAACCGGAAAAGCTGTCTTGACT

Optimized   853   AGCCCGTTTCGTCTGCTGGAAACCCATCACCTGGGCGTCGTGCTGACGTTCCCGGTCTAT
Original    853   AGCCCTTTTAGGTTGTTGGAAACTCACCATCTCGGAGTTGTGTTGACATTCCCTGTCTAC

Optimized   913   AAAAGCTCTCTGCCGGAAAATCCGACCGTGGAAGAACGTATCGCAGCAACGGCTGGTTAC
Original    913   AAGTCTTCTCTTCCTGAAAATCCGACTGTCGAAGAGCGTATTGCAGCCACTGCAGGGTAC

Optimized   973   CTGGGCGGTGCGTTTGATGTGGAAAGCCTGGTTGAAAACCTGCTGGGCCAACTGGCCGGT
Original    973   CTTGGTGGTGCGTTTGATGTGGAGTCTCTAGTCGAGAATTTACTTGGTCAGCTTGCTGGT

Optimized   1033  AATCAGGCAATCGTTGTCCATGTTTACGATATTACCAACGCGAGTGACCCGCTGGTCATG
Original    1033  AACCAAGCAATAGTTGTGCATGTGTATGATATCACCAATGCATCAGATCCACTTGTCATG

Optimized   1093  TACGGCAATCAGGATGAAGAAGCCGACCGTTCACTGTCGCACGAATCCAAACTGGATTTT
Original    1093  TATGGTAATCAAGATGAAGAAGCCGACAGATCTCTCTCTCATGAGAGCAAGCTCGATTTT

Optimized   1153  GGTGACCCGTTCCGCAAACATAAAATGATTTGCCGTTATCACCAGAAAGCACCGATCCCG
Original    1153  GGAGACCCCTTCAGGAAACATAAGATGATATGCAGGTACCACCAAAAGGCACCAATACCA

Optimized   1213  CTGAACGTCCTGACCACGGTGCCGCTGTTTTTCGCGATTGGCTTTCTGGTTGGTTATATC
Original    1213  TTGAATGTGCTCACAACTGTGCCATTGTTCTTTGCGATTGGTTTCTTGGTGGGTTATATA

Optimized   1273  CTGTACGGCGCAGCTATGCATATTGTGAAAGTTGAAGATGACTTCCACGAAATGCAAGAA
Original    1273  CTGTATGGTGCAGCTATGCACATAGTAAAAGTCGAAGATGATTTCCATGAAATGCAAGAG

Optimized   1333  CTGAAAGTGCGCGCCGAAGCGGCCGATGTTGCAAAATCGCAGTTTCTGGCTACCGTGAGC
Original    1333  CTTAAAGTGCGAGCAGAAGCTGCTGATGTCGCTAAATCGCAGTTTCTTGCTACCGTGTCT

Optimized   1393  CATGAAATTCGTACGCCGATGAACGGCATCCTGGGTATGCTGGCGATGCTGCTGGATACC
Original    1393  CACGAGATCAGGACACCAATGAATGGCATTCTCGGAATGCTTGCTATGCTCCTAGATACA
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Optimized   1453   GAACTGAGTTCCACGCAGCGCGACTATGCACAAACCGCTCAGGTCTGTGGCAAAGCGCTG
Original    1453   GAACTAAGCTCGACACAGAGAGATTACGCTCAAACCGCTCAAGTATGTGGTAAAGCTTTG

Optimized   1513   ATTGCCCTGATCAATGAAGTGCTGGATCGTGCTAAAATTGAAGCGGGTAAACTGGAACTG
Original    1513   ATTGCATTGATAAATGAGGTTCTTGATCGCGCCAAGATTGAAGCTGGAAAGCTGGAGTTG

Optimized   1573   GAATCTGTGCCGTTTGACATTCGCAGTATCCTGGATGACGTTCTGTCACTGTTCTCGGAA
Original    1573   GAATCAGTACCATTTGATATCCGTTCAATATTGGATGATGTCCTTTCTCTATTCTCTGAG

Optimized   1633   GAAAGCCGTAACAAAGGTATTGAACTGGCCGTCTTTGTGTCTGATAAAGTTCCGGAAATC
Original    1633   GAGTCAAGGAACAAAGGCATTGAGCTCGCGGTTTTCGTTTCAGACAAAGTACCAGAGATA

Optimized   1693   GTCAAAGGCGACAGTGGTCGTTTCCGCCAGATCATTATCAACCTGGTGGGCAATAGCGTT
Original    1693   GTCAAAGGAGATTCAGGGAGATTTAGACAGATAATCATAAACCTTGTTGGAAATTCGGTT

Optimized   1753   AAATTCACCGAAAAAGGTCATATCTTCGTTAAAGTCCACCTGGCGGAACAATCGAAAGAT
Original    1753   AAATTCACAGAGAAAGGACATATCTTTGTTAAAGTCCATCTTGCGGAACAATCAAAAGAT

Optimized   1813   GAAAGCGAACCGAAAAACGCCCTGAATGGCGGTGTTTCCGAAGAAATGATCGTGGTTTCA
Original    1813   GAATCTGAACCGAAAAATGCATTGAATGGTGGAGTGTCTGAAGAAATGATCGTTGTTTCC

Optimized   1873   AAACAGTCATCGTATAACACCCTGTCGGGCTACGAAGCAGCTGATGGTCGCAATTCTTGG
Original    1873   AAACAGTCAAGTTACAACACATTGAGCGGTTACGAAGCTGCTGATGGTCGGAATAGCTGG

Optimized   1933   GACAGTTTTAAACATCTGGTGTCTGAAGAACAGAGCCTGAGCGAATTTGATATTAGCTCT
Original    1933   GATTCATTCAAGCATTTGGTCTCTGAGGAGCAGTCATTATCGGAGTTTGATATTTCTAGC

Optimized   1993   AATGTCCGTCTGATGGTGTCTATCGAAGACACCGGCATTGGTATCCCGCTGGTCGCGCAA
Original    1993   AATGTTAGGCTTATGGTTTCAATCGAAGACACGGGTATTGGAATCCCTTTAGTTGCACAA

Optimized   2053   GGTCGTGTGTTTATGCCGTTCATGCAGGCCGATAGTTCCACCAGCCGTAACTATGGCGGT
Original    2053   GGCCGTGTGTTTATGCCGTTTATGCAAGCAGATAGCTCGACTTCAAGAAACTATGGAGGT

Optimized   2113   ACGGGCATTGGTCTGAGTATCTCCAAATGTCTGGTTGAACTGATGCGCGGCCAGATTAAT
Original    2113   ACTGGTATTGGTTTGAGTATAAGCAAGTGTCTTGTTGAACTTATGCGTGGTCAGATAAAT

Optimized   2173   TTCATCTCCCGTCCGCATATTGGTTCAACCTTTTGGTTCACGGCAGTTCTGGAAAAATGC
Original    2173   TTCATAAGCCGGCCTCATATTGGAAGCACGTTCTGGTTCACGGCTGTTTTAGAGAAATGC
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Optimized  2293   TTTAAGGGTATGAAAGCTATTGTCGTGGATGCAAAACCGGTTCGTGCAGCAGTCACGCGC
Original   2293   TTTAAAGGAATGAAAGCTATAGTTGTTGATGCTAAGCCTGTTAGAGCTGCTGTGACTAGA

Optimized  2353   TACCACATGAAACGTCTGGGCATCAACGTGGATGTTGTCACCTCTCTGAAAACGGCGGTG
Original   2353   TACCATATGAAAAGACTCGGAATCAATGTTGATGTCGTGACAAGTCTCAAAACCGCTGTT

Optimized  2413   GTTGCAGCTGCGGCCTTTGAACGTAATGGTAGTCCGCTGCCGACCAAACCGCAGCTGGAT
Original   2413   GTTGCAGCTGCTGCGTTTGAAAGAAACGGTTCTCCTCTCCCAACAAAACCGCAACTTGAT

Optimized  2473   ATGATTCTGGTTGAAAAAGACTCCTGGATCTCAACCGAAGATAACGACTCGGAAATTCGC
Original   2473   ATGATCTTAGTAGAGAAAGATTCATGGATTTCAACTGAAGATAATGACTCAGAGATTCGT

Optimized  2533   CTGCTGAATAGCCGTACGAACGGCAATGTGCATCACAAAAGCCCGAAACTGGCACTGTTT
Original   2533   TTATTGAATTCAAGAACCAACGGAAACGTTCATCACAAGTCTCCGAAACTAGCTCTATTC

Optimized  2593   GCTACCAACATCACCAACAGCGAATTTGATCGCGCGAAAAGTGCCGGCTTCGCAGACACC
Original   2593   GCAACAAACATCACAAATTCGGAGTTCGACAGAGCTAAATCCGCAGGATTTGCAGATACG

Optimized  2653   GTTATTATGAAACCGCTGCGTGCCAGCATGATCGGTGCATGCCTGCAACAGGTGCTGGAA
Original   2653   GTAATAATGAAACCGTTAAGAGCAAGCATGATTGGGGCGTGTCTGCAACAAGTTCTCGAG

Optimized  2713   CTGCGCAAAACGCGCCAACAGCATCCGGAAGGCTCATCGCCGGCGACCCTGAAATCTCTG
Original   2713   CTGAGAAAAAACAAGACAACAACATCCAGAAGGATCATCACCCGCAACTCTCAAGAGCTTG

Optimized  2773   CTGACGGGCAAGAAAATTCTGGTCGTGGATGACAACATCGTGAATCGTCGCGTTGCAGCT
Original   2773   CTTACAGGGAAGAAGATTCTTGTGGTTGATGATAATATAGTTAACAGGAGAGTAGCTGCA

Optimized  2833   GGCGCCCTGAAAAAATTCGGTGCTGAAGTTGTCTGCGCGGAAAGCGGCCAAGTGGCACTG
Original   2833   GGAGCTCTCAAGAAATTTGGAGCAGAAGTGGTTTGTGCAGAGAGTGGTCAAGTTGCTTTG

Optimized  2893   GGTCTGCTGCAGATTCCGCACACCTTTGATGCGTGTTTCATGGACATCCAAATGCCGCAG
Original   2893   GGTTTGCTTCAGATTCCACACACTTTCGATGCTTGCTTCATGGATATTCAAATGCCACAG

Optimized  2953   ATGGATGGTTTTGAAGCAACCCGTCAGATTCGTATGATGGAAAAAGAAGCTAAAGAAAAA
Original   2953   ATGGACGGATTTGAAGCAACTCGTCAGATAAGAATGATGGAGAAGGAAGCTAAAGAGAAG

Optimized  3013   ACGAATCTGGAATGGCATCTGCCGATTCTGGCTATGACCGCGGATGTTATCCACGCGACG
Original   3013   ACGAATCTCGAATGGCATTTACCGATTCTAGCGATGACTGCGGATGTGATACACGCGACC
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Optimized   3073   TATGAAGAATGTCTGAAATCCGGCATGGACGGTTACGTGTCAAAACCGTTTGAAGAAGAA
Original    3073   TACGAGGAATGTCTGAAAAGTGGGATGGATGGTTACGTCTCCAAACCTTTTGAAGAAGAG

Optimized   3133   AACCTGTATAAATCAGTCGCAAAATCATTCAAACCGAACCCGATCTCTCCGTCG
Original    3133   AATCTCTATAAATCCGTTGCCAAATCATTCAAACCTAATCCTATCTCACCTTCG
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Figure A2.8 Sequence of the AHK4 coding region codon optimized for *E.coli* (GenScript). Nucleotides in red were changed in the optimization process.

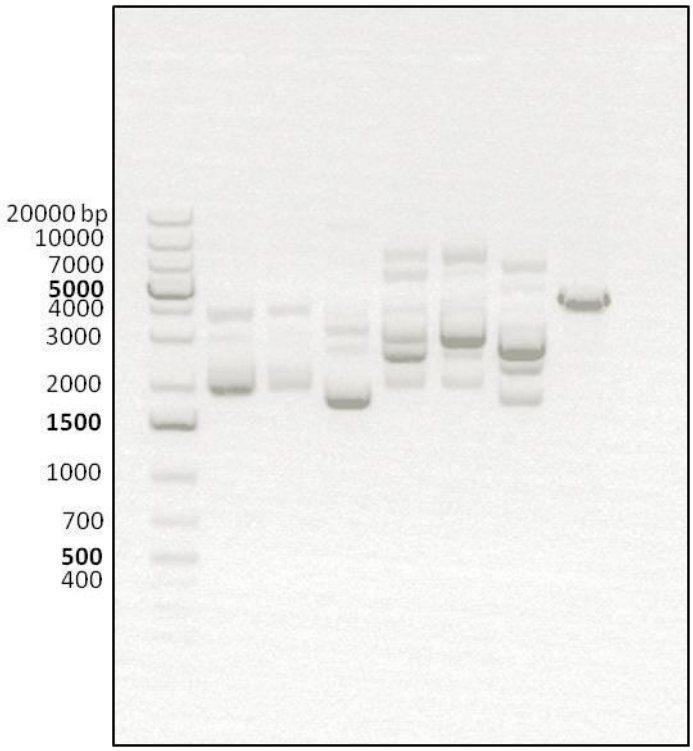

Figure A2.9 Image of agarose gel showing results for digest screen of six putative clones constitutively expressing *EcAHK4* in pBR322. The digest should have resulted in two bands at 4.1 kb and 3.4 kb. As shown below, restriction digests resulted in multiple bands of incorrect size. The lane furthest to the left shows the reference ladder (Fermentas, Gene Ruler PLUS 1kb DNA ladder). The lane furthest to the right shows the digested empty pBR322 vector as an additional reference.

CCTCACGTCGACCAAATAAAACGAAAGGCCCAGTCGAAAGACTGGGCCTTTCGTTT
TATCTGTTGTTTGTCGGTGAACGCTCTCCTGAGTAGGACAAATCCGCCGGGAGCG
GATTTGAACGTTGCGAAGCAACGGCCCGGAGGGTGGCGGGCAGGACGCCCGCCA
TAAACTGCCAGGCATCAAATTAAGCAGAAGGCCATCCTGACGGATGGCCTTTTTGC
GTTTCTACAAACTCTTCCTGTCGTCATATCTACAAGCCATGTACTCTTTCCCGACTG
GAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCA
CCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGG
ATAACAATTTCACACAGGGCAGCCATGATCTAGATAACGAGGG

Figure A2.10 rrnB transcriptional terminator and Lac promoter operator (*rrnBT1T2-Lac^{PO}*) sequence (Anthony et al., 2004). Fragment was synthesized by Integrated DNA Technologies for inducible regulation of *EcAHK4* in the bacterial expression vector.

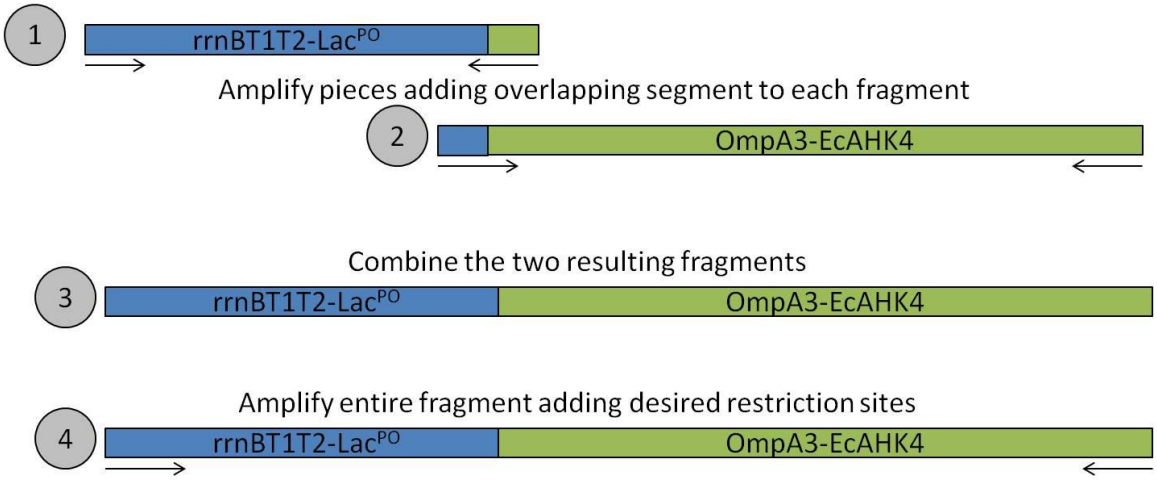

Figure A2.11 Diagram of overlapping PCR

cctcacgtcgaccaaataaaacgaaaggcccagtcgaaagactgggcctttcgttttatctgttgtttgtcggtgaacgctct
cctgagtaggacaaatccgccgggagcggatttgaacgttgcgaagcaacggcccggagggtggcgggcaggacgc
ccgccataaactgccaggcatcaaattaagcagaaggccatcctgacggatggccttttgcgtttctacaaactcttcctgt
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cattaggcaccccaggctttacactttatgcttccggctcgtatgttgtgtggaattgtgagcggataacaatttcacacaggg
cagccatgatctagataacgagggTCCCTGATGAACTGGGCACTGAACAACCACCAAGAAGA
AGAAGAAGAACCGCGTCGTATTGAAATCTCGGACTCGGAAAGCCTGGAAAACCTG
AAAAGCTCTGATTTTTATCAGCTGGGCGGTGGCGGTGCGCTGAATAGTTCCGAAAA
ACCGCGCAAAATTGATTTTTGGCGTTCCGGCCTGATGGGTTTCGCCAAAATGCAGC
AACAGCAACAGCTGCAACATTCAGTGGCAGTTAAAATGAACAACAACAACAACAAC
GATCTGATGGGCAACAAAAAAGGTAGTACCTTCATTCAGGAACACCGCGCCCTGC
TGCCGAAAGCACTGATCCTGTGGATTATCATTGTGGGCTTTATTTCATCGGGTATCT
ATCAATGGATGGATGACGCCAATAAAATTCGTCGCGAAGAAGTCCTGGTGTCTATG
TGCGATCAGCGTGCACGCATGCTGCAAGACCAGTTTAGCGTTTCTGTCAACCATGT
TCACGCGCTGGCCATTCTGGTCTCGACCTTCCATTATCACAAAAATCCGAGCGCGA
TCGATCAGGAAACGTTTGCCGAATACACCGCACGTACGGCATTCGAACGTCCGCT
GCTGAGCGGTGTTGCATACGCTGAAAAAGTGGTTAACTTTGAACGTGAAATGTTCG
AACGCCAGCATAATTGGGTGATTAAAACCATGGATCGCGGTGAACCGTCCCCGGT
GCGTGACGAATATGCGCCGGTTATCTTTTCACAGGATAGTGTCTCCTACCTGGAAT
CTCTGGATATGATGAGTGGCGAAGAAGACCGCGAAAACATTCTGCGTGCCCGCGA
AACCGGTAAAGCAGTGCTGACGAGCCCGTTTCGTCTGCTGGAAACCCATCACCTG
GGCGTCGTGCTGACGTTCCCGGTCTATAAAAGCTCTCTGCCGGAAAATCCGACCG
TGGAAGAACGTATCGCAGCAACGGCTGGTTACCTGGGCGGTGCGTTTGATGTGGA
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TGAAGAAGCCGACCGTTCACTGTCGCACGAATCCAAACTGGATTTTGGTGACCCG
TTCCGCAAACATAAAATGATTTGCCGTTATCACCAGAAAGCACCGATCCCGCTGAA
CGTCCTGACCACGGTGCCGCTGTTTTTCGCGATTGGCTTTCTGGTTGGTTATATCC
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CCGTGAGCCATGAAATTCGTACGCCGATGAACGGCATCCTGGGTATGCTGGCGAT
GCTGCTGGATACCGAACTGAGTTCCACGCAGCGCGACTATGCACAAACCGCTCAG
GTCTGTGGCAAAGCGCTGATTGCCCTGATCAATGAAGTGCTGGATCGTGCTAAAAT
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CGTCTTTGTGTCTGATAAAGTTCCGGAAATCGTCAAAGGCGACAGTGGTCGTTTCC
GCCAGATCATTATCAACCTGGTGGGCAATAGCGTTAAATTCACCGAAAAAGGTCAT
ATCTTCGTTAAAGTCCACCTGGCGGAACAATCGAAAGATGAAAGCGAACCGAAAAA
CGCCCTGAATGGCGGTGTTTCCGAAGAAATGATCGTGGTTTCAAAACAGTCATCGT
ATAACACCCTGTCGGGCTACGAAGCAGCTGATGGTCGCAATTCTTGGGACAGTTTT
AAACATCTGGTGTCTGAAGAACAGAGCCTGAGCGAATTTGATATTAGCTCTAATGT
CCGTCTGATGGTGTCTATCGAAGACACCGGCATTGGTATCCCGCTGGTCGCGCAA
GGTCGTGTGTTTATGCCGTTCATGCAGGCCGATAGTTCCACCAGCCGTAACTATGG
CGGTACGGGCATTGGTCTGAGTATCTCCAAATGTCTGGTTGAACTGATGCGCGGC
CAGATTAATTTCATCTCCCGTCCGCATATTGGTTCAACCTTTTGGTTCACGGCAGTT
CTGGAAAAATGCGATAAATGTTCGGCTATCAACCATATGAAAAAACCGAATGTGGA

ACACCTGCCGAGCACCTTTAAGGGTATGAAAGCTATTGTCGTGGATGCAAAACCG
GTTCGTGCAGCAGTCACGCGCTACCACATGAAACGTCTGGGCATCAACGTGGATG
TTGTCACCTCTCTGAAAACGGCGGTGGTTGCAGCTGCGGCCTTTGAACGTAATGG
TAGTCCGCTGCCGACCAAACCGCAGCTGGATATGATTCTGGTTGAAAAAGACTCCT
GGATCTCAACCGAAGATAACGACTCGGAAATTCGCCTGCTGAATAGCCGTACGAA
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ACAGCGAATTTGATCGCGCGAAAAGTGCCGGCTTCGCAGACACCGTTATTATGAAA
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TGACGGGCAAGAAAATTCTGGTCGTGGATGACAACATCGTGAATCGTCGCGTTGC
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GTGGCACTGGGTCTGCTGCAGATTCCGCACACCTTTGATGCGTGTTTCATGGACAT
CCAAATGCCGCAGATGGATGGTTTTGAAGCAACCCGTCAGATTCGTATGATGGAAA
AAGAAGCTAAAGAAAAAACGAATCTGGAATGGCATCTGCCGATTCTGGCTATGACC
GCGGATGTTATCCACGCGACGTATGAAGAATGTCTGAAATCCGGCATGGACGGTT
ACGTGTCAAAACCGTTTGAAGAAGAAAACCTGTATAAATCAGTCGCAAAATCATTCA
AACCGAACCCGATCTCTCCGTCG

Figure A2.12 Sequencing results (Macrogen, USA) for one of the four putative clones of *rrnBT1T2-Lac^{PO}-OmpA3-EcAHK4* in the bacterial expression vector. Uppercase letters represent *EcAHK4*. Nucleotides highlighted in grey represent deletions. Nucleotides highlighted in green are point mutations.

AHP1 XhoI forward: 5'-*GACTCGAGATGGATTTGGTTC*-3' Tm=60.5°C
AHP1 EcoRI reverse: 5'-*CATCCATTTATTAGTTAATATCCACTTG*-3' Tm=63.2°C
AHP2 XhoI forward: 5'-*CTCGAGATGGACGCTCTCATT*-3' Tm=60.9°C
AHP2 EcoRI reverse: 5'-*GAATTCAAAAATGCCCCGCTTACGCAGGGCATC CATTTATTAGTTAATATCCACTTG*-3' Tm=66.9°C
AHP3 XhoI forward: 5'-*TTCAGGGTGGTGAATCTCGAGATGGACACAC TCATT*-3' Tm=65.2°C
AHP3 EcoRI reverse: 5'-*CTGCGAATTCCTTGCTGTTCTGTTGTCGATAGG*-3' Tm=64.0°C
AHP5 XhoI forward: 5'-*GACTCGAGATGAACACCATCGTCGTTGCT*-3'
AHP5 EcoRI reverse: 5'-*GAATTCAAAAATGCCCCGCTTACGCAGGGCATC CATTTACTAATTTATATCCACTTGA*-3'

BamHI LacI EcAHK4 forward: 5'-*GGATCCGACACCATCGAATGGCGCAAAA CCTTTCGCGGTATGGCATGATAGCGCCCGGAAGAGAGTCAATTCAGGGTGGTGAA TTCTAGATAACGAGGGCAAAAAATGAAAAAGACAGC*-3' Tm=72.2°C
EcAHK4 GlmS SalI reverse: 5'-*GTCGACAAAAATGCCCCGCTTACGCAGGGC ATCCATTTACACAATGTGCGCCATTTTTCACTTCACAGGTACTA*-3' Tm=71.6°C

SalI rrnB forward: 5'-*CCTCACGTCGACCAAATAAAACGAAAGGCCCAG*-3' Tm=64.8°C
Sigpep LacPO reverse: 5'-*CTCGTTATCTAGACGATGGCTGCCCTG*-3' Tm=62.9°C
LacPO Sigpep forward: 5'-*ACAGGGCAGCCATCGTCTAGATAACG*-3' Tm=61.9°C
BAMHI GlmS EcAHK4 reverse: 5'-*AGAGGATCCAAAAATGCCCCGCTTAC GCAGGGCATCCATTTACACAATGTGCGCCATTTTTCACTTC*-3' Tm=71.6

Figure A2.13 Primer sequences. All oligos synthesized by Integrated DNA Technologies.

APPENDIX 3

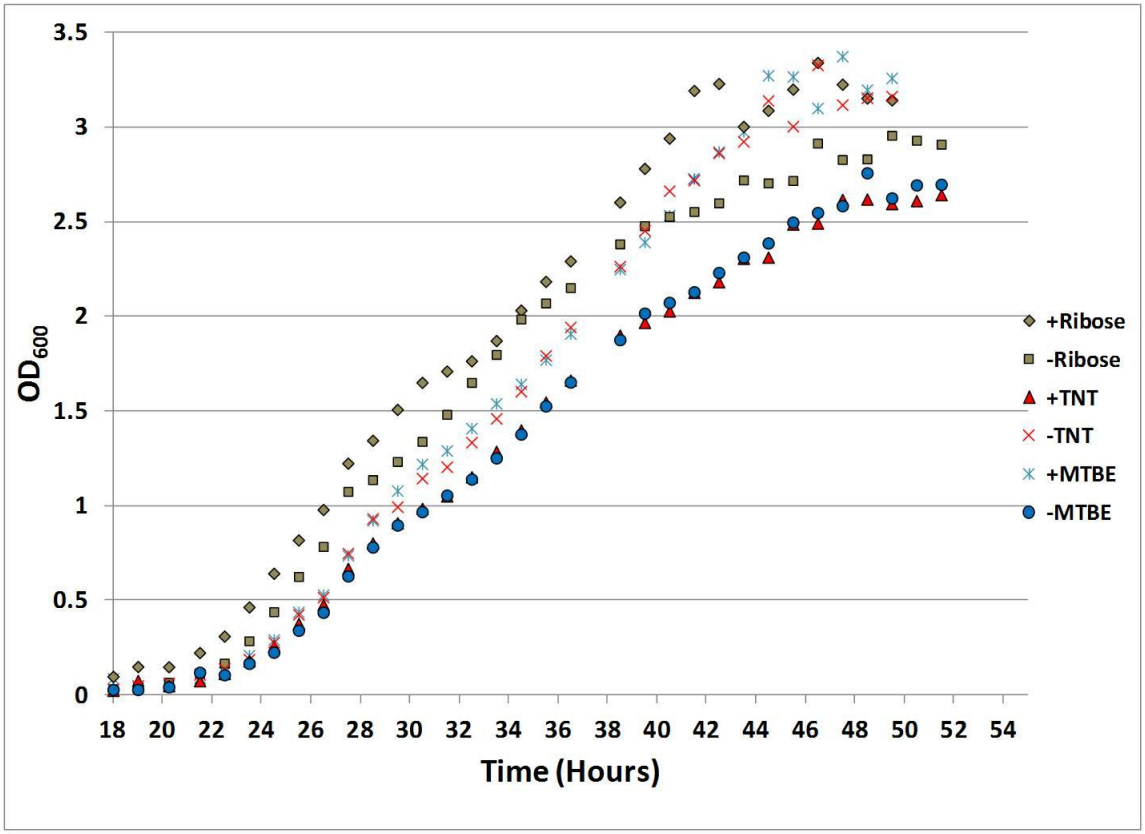

Figure A3.1 Indirect Growth Curves for BW23423 Cell line. Cells harboring the RBP plasmid, MTBE plasmid and TNT plasmid were cultured as described in Chapter Five, with and without the respective ligand until reaching stationary phase.