The performance of bacterial phytosensing transgenic tobacco under field conditions

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DEDICATION

I dedicate my thesis to my family and friends, who have been supportive throughout my graduate career; especially my parents.

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ABSTRACT

Currently the platforms for wide-area detection of environmental contamination are limited. Therefore, there is interest in developing new platforms, especially for use in crop plants to detect and report the presence of biotic and abiotic stress agents. A biosensor uses a biological organism or substrate to detect the presence of an elicitor (i.e., heavy metal, TNT, or bacteria). The foundational groundwork to create biosensors in transgenic plants exists. The creation of bacterial phytosensing transgenic tobacco containing an orange fluorescent protein (OFP) reporter driven by synthetic pathogen-inducible promoters provides a fluorescent signal when infected with phytopathogens for earlier detection in the field.

This thesis research performed time-course analysis of field grown transgenic phytosensing tobacco plants infected with *Pseudomonas* phytopathogens. Some of the phytosensors responded in predictable ways to a suite of treatments, with more than 2-fold of expression of the OFP reporter driven by two different salicylic acid inducible motifs, SARE and PR1. Specifically, transgenic lines containing synthetic promoters with salicylic acid inducible *cis*-acting regulatory elements showed earlier OFP fluorescence induction by phytopathogen treatments (within 48 hours) than transgenic lines harboring other synthetic promoters; such as the synthetic promoters containing ethylene inducible *cis*-acting regulatory elements (ERE) which induced OFP fluorescence after phytopathogen treatment only at 72 hours post inoculation. Transgenic lines harboring the OFP reporter driven by synthetic promoters containing defense-related *cis*-acting regulatory elements were indicative of plant defenses during phytopathogen interactions. Results reported here indicate the functionality of phytosensors in the field that could play a role in presision agriculture in the future.

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CHAPTER I: INTRODUCTION

Inexpensive, reliable, real-time diagnostic systems for monitoring environmental risks are currently of great interest (Sadanandom and Napier, 2010), and biosensors provide an economic means of detection. Biosensors incorporate a living organism or biological components to detect an analyte; these sensing systems can be used for detection of various chemicals, compounds, and even biological agents (Band *et al.*, 2012; Liu *et al.*, 2013a; Liu *et al.*, 2011; Mazarei *et al.*, 2011). Furthermore, the use of biosensors can be used to understand biological processes; for example, the creation of an auxin monitoring system developed with an inducible biosensing system has greatly advanced the understanding of the phytohormone indole acetic acid (IAA) (Band *et al.*, 2012). In essence, this auxin biosensor used a phytohormone as a signal and produced a change in phenotype (i.e., reduction of fluorescence) that indicated the temporal concentration of IAA (Band *et al.*, 2012). Physiological changes cause transcriptional modifications; the tight regulation of transcriptional modifications allows for the creation of a biosensing system by incorporating a reporter into an inducible system that yields an output relative to the concentration of the analyte of interest. Qualities ideal for a sensor integrate quantitative, realtime, spatial, *in vivo* signals for output and allow temporal monitoring of analyte concentrations (Sadanandom and Napier, 2010). Ideally, sensing systems designed with these qualities in mind, are supplied with an empirical model to determined analyte concentration. Phytopathogen detection lacks early warning systems and could greatly benefit from the creation of a system that allows for early warning of potential phytopathogen outbreaks; furthermore, there is the potential to monitor environmental contaminants (i.e., heavy metals or trinitrotoluene). Many areas using detection methods could benefit from biosensing systems; however, this thesis focuses on biosensing in plant systems; specifically, it will cover the

progress and improvements made towards creating pathogenic biosensors as well as heavy metal biosensors created to understand mechanisms of gene regulation in the presence of an elicitor (i.e., pathogenic agents or heavy metals). Thus, I will cover the potential field applications of such biosensors.

Biological sensors

How effective are current phytopathogen detection methods? The current methods are expensive, and are not always available for field detection. Typically, molecular identification techniques such as PCR or immunological techniques are used to identify the pathogenic agents; these techniques are powerful but do have disadvantages. These techniques are typically post-symptomatic (Liu *et al.*, 2013a) or after substantial losses have already occurred. Even though PCR techniques provide robust detection of low quantities of pathogenic agents, they either have not been developed yet or are still in development for in-field detection. Furthermore, PCR merely detects the presence of DNA of a pathogenic agent but cannot distinguish from viable or inviable cells. Immunological techniques are applicable in the field; however, there are often sensitivity problems with these methods. As an agricultural application, a preventative treatment regimen could be implemented if phytopathogens are detected at an early stage on the farm. It is feasible to use a biological system to detect the presence of plant pathogens by fusing pathogen-inducible *cis*-acting regulatory elements to reporter genes (i.e., those encoding β-glucuronidase, fluorescent proteins, or luciferase). In essence, these pathogen-sensors yield the reporter post-induction through transcriptional changes from pathogen recognition in the plant through pathogen recognition receptors along with secretion of virulence proteins by the pathogen that triggers signal transduction cascades leading to the synthesis of phytohormones (salicylic acid,

jasmonic acid, ethylene, and abscisic acid) involved in plant defenses (Liu *et al.*, 2013b). The synthesis of phytohormones has the capability to induce specific *cis*-acting elements, which can be used to create inducible promoters (Liu *et al.* 2013a; Liu *et al.*, 2011; Mazarei *et al.*, 2008; Rushton *et al.*, 2002).

Cis-acting regulatory elements are short sequences of DNA that yield enhanced transcription in the presence of corresponding transcription factors (Liu *et al.*, 2013b). Multiple *cis*-acting regulatory elements have been determined through experimental methods from promoter analysis studies. They were used in a first generation of phytosensing constructs by fusing tetramers of regulatory elements to a minimal CaMV 35S promoter containing a TATA box designated *-46 35S*, which was then fused to the β-glucuronidase (*Gus)* reporter and nopaline synthase terminator (*NosT*) (Mazarei *et al.*, 2008). Another set of constructs, designated enhanced constructs, were created containing enhancer elements B and A1 from the CaMV 35S promoter (Benfey *et al.*, 1990) flanking tetramers of the regulatory elements. The resulting phytosensing constructs allowed for quantitative and qualitative analysis of synthetic promoters made from distinct regulatory elements through analysis of the *gus* reporter. Mazarei *et al.* (2008) analyzed the induction of these synthetic promoters under various abiotic treatments, which included infiltration with phytohormones (ethylene, methyl jasmonate, and salicylic acid) and with the fungal elicitor chitin. An interesting note on this study is the discovery of the induction of the *gus* reporter after inoculation with the *alfalfa mosaic virus* and *tobacco mosaic virus* (Mazarei *et al.*, 2008). Of particular interest were the *cis*-acting regulatory elements as they displayed the highest level of induction by phytohormone or fungal elicitor treatment (Mazarei *et al.*, 2008): two *cis*-acting regulatory elements were highly induced in the presence of salicylic acid; these *cis*-acting elements were named salicylic acid regulatory element (SARE) (Shah and

Klessig, 1996), pathogenesis related 1 regulatory element (PR1) (Lebel *et al.*), one responsive inducible by ethylene designated ethylene regulatory element (ERE) (Brown *et al.*, 2003; Ohme-Takagi and Shinshi, 1995; Rushton *et al.*, 1996), and a jasmonic acid regulatory element (JAR) was significantly induced by methyl jasmonate (Guerineau *et al.*, 2003; Mazarei *et al.*, 2008). Hence, synthetic promoters containing salicylic acid inducible *cis*-acting regulatory elements (PR1 and SARE) are designated as salicylic acid-responsive promoters, and those containing ethylene or jasmonic regulatory elements (ERE and JAR, respectively) as ethylene/jasmonic-responsive promoters.

Rushton *et al.* (2002) published on a similar system earlier than previously mentioned study. The system was similar to the one employed by Mazarei *et al.* (2008) by using tetramers of regulatory elements fused to the minimal *-46 35S* promoter from *CaMV 35S* promoter upstream of the *gus* reporter fused to the *NosT* terminator (Rushton *et al.*, 2002). However, Rushton *et al.* (2002) evaluated different regulatory elements in their studies; interestingly, they found that some regulatory elements, most importantly box D, remained uninduced post-wounding. Thus, Rushton *et al.*, (2002) found a *cis*-acting regulatory element ideal for bacterial or fungal phytosensing; furthermore, they were able to identify *cis*-acting regulatory elements that could be useful for insect inducible promoters (i.e., synthetic promoters induced post-wounding). Also, Rushton *et al.* (2002) used mutants with modified spacing within the *cis*-acting element to increase the fold change from a 7.2-fold increase to either a 32 or a 75-fold increase; in essence, this abolished basal expression yielding much higher fold induction in the synthetic promoter (Rushton *et al.*, 2002). Nevertheless, Rushton *et al.* (2002) conducted their research within *Arabidopsis* with the *gus* reporter, whose expression cannot be assessed *in vivo*. Therefore, this research was the foundational work for plant synthetic promoters and determined

that bacteria, fungal elicitors or wounding specifically induced these *cis*-acting regulatory elements in this system (Rushton *et al.*, 2002); however, a reporter capable of realtime monitoring was needed to evaluate the applicability of these promoters *in vivo* and under field conditions; therefore, a second generation of the best performing constructs was created with an orange fluorescent protein (OFP) reporter instead of the *gus* reporter.

Studies conducted on transgenic plants yielded further refinement to the existing phytosensing system. A major improvement from previous studies was the implementation of a nondestructive fluorescent reporter. A green fluorescence protein gene driven by a native pathogen-inducible promoter was first evaluated in transgenic tobacco by Kooshki *et al.* (2003); Transgenic lines harboring the pathogen inducible promoter *gn1* fused to a plant optimized green fluorescent protein (*mGFP5-ER*) showed visually induced GFP fluorescence by a salicylic acid phytohormone compared to the mock treatment (Kooshki *et al.*, 2003). Also, these transgenic plants harboring *gn1::mGFP5-ER* displayed induced GFP levels detected through western blot. However, it appeared that transgenic plants containing *gn1::mGFP5-ER* were not able to induce sufficient GFP fluorescence to be measured by spectrofluormetry. Given the relative failure of the native promoter, research was motivated to engineer synthetic promoters for use in phytosensing. Several rounds of improved phytosensing constructs were conducted, which resulted in several synthetic promoters driving a bright orange fluorescent protein (*pporRFP* from *Porites porites* (Alieva *et al.*, 2008; Mann *et al.*, 2012)) allowing *in vivo* monitoring of plant pathogens.

Two previous studies evaluated synthetic pathogen-inducible promoters for treatment with phytohormones and bacterial pathogens (*Pseudomonas syringae* pv. *tomato, P. s.* pv. *tabaci, P. marginalis*) within transgenic tobacco and *Arabidopsis* (Liu *et al.*, 2013a; Liu *et al.*,

2011). These studies used similar systems employed by both Rushton *et al.* (2002) and Mazarei *et al.* (2008), by using tetramers of regulatory elements to drive the expression of the a bright orange fluorescent protein reporter (*pporRFP* (Alieva *et al.*, 2008; Mann *et al.*, 2012)). However, the major improvement here was the use of strongly inducible promoters and the bright orange fluorescent protein (OFP) (Alieva *et al.*, 2008; Mann *et al.*, 2012). Liu *et al.* (2011) elucidated functioning constructs for early induction under a transient expression system; furthermore, Liu *et al.* (2013a) showed that stable transgenic lines had equivalent results of those observed in the transient transformation studies. Nevertheless, there were some discrepancies in induction levels observed between transient and stable transformation; the highest induction level observed in transient expression studies was approximately a 50-fold increase in OFP fluorescence; compared with a 4-fold increase in OFP fluorescence in stably transformed lines. Furthermore, discrepancies between the two studies demonstrated that a transient expression system could not account for the insertional effects during the stable integration of the transgene (Liu *et al.*, 2013a). There are expected significant variations in expression among independent stable transgenic events. Liu *et al.* (2013a) also found that plants responded specifically to the pathogen-host interaction; for example, *Arabidopsis* displayed induced OFP fluorescence in transgenic lines harboring the JAR regulatory elements, but transgenic tobacco harboring the same regulatory elements yielded a lower induction of OFP fluorescence. Furthermore, these synthetic promoters are induced by the endogenous defense mechanisms of the transformed plant; thus, during the creation of phytosensors for field crops would need to be developed in the crop as different plants responses may yield different induction patterns to the same pathogens as observed between *Arabidopsis* and tobacco (Liu *et al.*, 2013a).

Environmental sensors

The detection and identification of toxic concentrations of chemicals in the environment requires specialized equipment; furthermore, the evaluation of mutation-causing agents proves even more difficult (Kovalchuk and Kovalchuk, 2008). So far, various biosensor systems (i.e., mammalian, bacterial, and plant) have been employed to detect environmental pollutants (Kovalchuk and Kovalchuk, 2008). The potential for plants to be used as environmental sensors is appealing for many reasons: plants respond to environmental elicitors (i.e., temperature, herbicides, insecticides, heavy metals and pollutants) and this response could be manipulated to form a biosensor under certain conditions (Volkov and Ranatunga, 2006). These elicitors cause stimulation at the site of contact, and this stimulation causes a bioelectrical impulse that is dispersed throughout the plant (Volkov and Ranatunga, 2006). Thus, plants can be genetically engineered to create an environmental biosensor for the detection of many harmful elicitors by using signal transduction within the plant (Adams *et al.*, 2011; Aksoy *et al.*, 2013; Al-Shayeb *et al.*, 1995). The monitoring of these harmful pollutants is essential to understanding their impact on the environment.

The strategy to create environmental phytosensors reviewed here will focus on the inducible systems such as the inducible systems for phytopathogen detection; therefore, elements employed should be inducible by the elicitor (or pollutant) of interest. Thus, creating a transgenic plant that will yield an altered phenotype (i.e., fluorescing plant) in the presence of the elicitor. The principle of the phytosensor has been demonstrated under general stress conditions (Perera and Jones, 2004), and insect herbivory (Paul *et al.*, 2004), as well as phytopathogens (Liu *et al.*, 2013a; Liu *et al.*, 2011; Mazarei *et al.*, 2008; Rushton *et al.*, 2002)

Recently a study used a protein-based biosensor using fluorescent resonance energy transfer (FRET) (Clegg, 1995) to detect protein conformational changes in the presence of zinc (Adams *et al.*, 2011). The potential to use FRET for future biosensors is promising. FRET requires two fluorescent proteins and the action of changing those fluorescent proteins in proximity for resonance energy transfer to occur (30 - 100 Å) (Clegg, 1995). In essence, an appropriate protein would entail one with a conformational shift in the presence of a heavy metal, pollutant, or pesticide. This study evaluated the effects of zinc on the FRET activity of a fusion protein of two fluorescent proteins and the *PtZNT* protein that shifts between a tense or relax state in the presence or absence of zinc, respectively (Adams *et al.*, 2011). Furthermore, the evaluation of a zinc sensing system demonstrated the ability of transgenic lines of *Arabidopsis* and poplar containing the constructs of a cyan fluorescent protein and a red fluorescent protein flanking the zinc reactive protein *PtZNT* to detect environmental levels of zinc (Adams *et al.*, 2011). Thus, the potential for FRET in biosensing systems could play a role in deciphering protein-interacting molecules or protein-protein interactions; for example, if two proteins form a dimer under exposure of a pollutant the engineering of transgenic plants with each protein labeled with FRET compatible fluorescent proteins would provide a sensing system for that pollutant.

The potential to create heavy metal-inducible synthetic promoters exists; however, the current studies are limited to promoter analyses of native promoter regions inducible by heavy metals (Berna and Bernier, 1999; Lescure *et al.*, 1991; Qi *et al.*, 2007). Interestingly, the stress related gene 2 from bean (*PvSR2*) has been shown to specifically respond to heavy metal stress and not other abiotic stresses (Qi *et al.*, 2007); therefore, the *cis*-acting elements within this promoter may allow for the creation of a synthetic promoter inducible by environmental levels of

heavy metals. Another study showed a similar promoter element to induce the expression of *gus* in the presence of heavy metals; however, the results indicated that other stressors such as heat also induced the expression of the *Gus* reporter (Monciardini *et al.*, 1998). Thus, this system may need further refinement for biosensing of heavy metals. Furthermore, the creation of copper inducible constructs has previously been demonstrated by Mett *et al.* (1993) to allow for controlled expression of transgenes (Mett *et al.*, 1993); thus, if copper concentrations directly correlate to promoter activity it would be feasible to create a copper-sensing system. Hence, one could create heavy metal phytosensing transgenic plants with aptamers of heavy metal-inducible *cis*-acting regulatory elements fused to a fluorescent protein to allow *in vivo* monitoring (i.e., luciferase).

Another approach to create a biosensing transgenic plants employed re-designed periplasmic binding proteins (PBPs) to create a synthetic signal transduction cascade (Antunes *et al.*, 2011). These PBPs can be created as a fusion protein containing a receptor region for elicitor recognition, with a cytoplasmic region fused to a portion of a histidine kinase (Antunes *et al.*, 2011); typically, upon receptor recognition a conformational shift occurs leading to the phosphorylation of the histidine kinase and triggers the synthetic signal transduction cascade (Antunes *et al.*, 2011). Also, it had been shown that receptors could be computationally designed for trinitrotoluene recognition and produced reporter gene expression in the presence of trinitrotoluene (TNT) (Looger *et al.*, 2003). Thus, a synthetic signal transduction cascade created for TNT sensing consisted of *Arabidopsis* plants containing the PBPs with TNT receptors and Trg:PhoR (a histidine kinase) in the cytoplasmic region, upon exogenous TNT contamination leads phosphorylation of PhoB-VP64 and can cause targeted induction of the *PlantPho* promoter (Antunes *et al.*, 2009) to drive *Gus* or a de-greening circuit (Antunes *et al.*, 2006; Antunes *et al.*,

2011). Importantly, the de-greening circuit is detectable from afar, quantitative, and easily observed (Antunes *et al.*, 2011). Therefore, this system could allow detection of landmines in impoverish countries. However, the correlation between *Gus* and TNT concentrations is of particular interest; this correlation explained only 9% ($R^2 = 0.09$) of the variation within this system (Antunes *et al.*, 2011). Thus, the improved system would yield much higher R^2 values.

As for an ideal system, Band *et al.* (2012) developed at first glance a qualitative biosensor for auxin concentrations; while this sensing system was not created for pathogens or environmental factors its further refinement yielded a model and the creation of a quantitative biosensor. In order to achieve an experimentally determined model like in Band *et al.* (2012), one needs to determine all parameters that affect the signal output (i.e.| temperature, UV light, rate-limiting steps, and other stressors). Thus, this study (Band *et al.*, 2012) chose the VENUS reporter, which has been previously characterized with improved protein maturation and chromophore oxidation (Nagai *et al.*, 2002) within *Arabidopsis*; the characteristics of the VENUS reporter contributed to the study's creation of a quantitative model from a qualitative biosensor. Therefore, in future studies for pathogen detection the ideal reporter should be determined; this reporter may or may not be VENUS. However, the ideal reporter would directly correlate to the analyte of interest, which will allow direct quantification of the analyte; inverse correlations between the reporter and elicitor could be used as shown in Band *et al.* (2012). Furthermore, the concentration gradient experiment within the Band *et al.* (2012) study demonstrates the quantitative nature of this biosensor. Future studies, evaluating biotic or heavy metal treatments should use concentration titers to address physiologically relevant concentrations under which that biosensor is quantitative. (Sadanandom and Napier, 2010).

Field trials on phytosensing transgenic plants

The applicability of these systems must be determined under relevant environmental conditions. Transgenic plants as biosensors provide great utility and affordability to sensing technology; however, a laboratory study cannot replicate environmental conditions. In addition, many studies have shown that laboratory characterized traits are not maintained under environmental conditions (Brandle *et al.*, 1995; Conner *et al.*, 1994; de Carvalho *et al.*, 1992; De Wilde *et al.*, 2000; McKersie *et al.*, 1999; Mohamed *et al.*, 2001). Therefore, it is essential that these systems be deployed to field sites as sentinels to evaluate the affect of the environment on transgene inducibility and FRET activity. These systems are based on biological processes that may be adversely affected by conditions such as wind, UV light, heat, cold, drought, and endogenous biological agents or chemicals.

This research is essential in developing plants for detection of plant pathogens. A long term goal of this research would facilitate the development of sentinel plants to detect and forecast pathogen movement from field to field; eventually, this could allow preventative treatments able to treat disease before a phytopathogen outbreak occurs, which would reduce food losses in the field. Field evaluations are essential on these types of systems to determine their applicability in an agricultural system.

Environmental conditions can modify gene expression drastically due to the combination of stressors (Mittler, 2006); therefore, there is a need to evaluate transgenic plants in the field to confirm laboratory results. Before this research, it was unknown if environmental conditions such as UV light, wind, insects, drought, and precipitation would enhance, decrease, or abolish transgene inducibility; furthermore, the best pathogen-inducible synthetic construct had not been determined under field conditions. However, our results demonstrate that some transgenic lines

maintain inducibility under a variety of environmental conditions when challenged with the same phytopathogens as previous laboratory studies (Liu *et al.*, 2013a; Liu *et al.*, 2011). Furthermore, the most specific bacterial phytosensing transgenic lines appear to be those containing 4×PR1 inducible elements.

Research presented here aimed at evaluating pathogenic bacterial phytosensing transgenic tobacco with the following objectives: (i) Pathogenic bacterial phytosensing transgenic tobacco lines will be deployed at East Tennessee Research and Education Center (ETREC) in 2012 and 2013 to verify if laboratory results are maintained under environmental conditions. (ii) Furthermore, we will quantify OFP fluorescence (visually and with spectrofluorometry) over a 72-hour time course study after inoculation with multiple *Pseudomonas* plant pathogens to determine the OFP fluorescence induction from phytobacterial treatments. Ideally, a phytosensor will have a signal commensurate with pathogen load. (iii) Therefore, we will quantify bacterial populations post inoculation of leaf tissue with rifampicin-selected mutants. (iv) Finally, we will perform quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) on a subset of inoculated tissue to confirm *pporRFP* expression localized at the site of infection.

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CHAPTER II: THE PERFORMANCE OF BACTERIAL PHYTOSENSING TRANSGENIC TOBACCO

A version of this chapter was originally submitted by Michael H. Fethe, Wusheng Liu, Jason Burris, Reginald Millwood, Mary Rudis, Mitra Mazarei, Duncan Yeaman, Marion Dubosqueille, and Charles Neal Stewart:

Michael H. Fethe *et al*. "The performance of bacterial phytosensing transgenic tobacco in the field." *Journal of Plant Biotechnology* (Expected 2014).

Michael Harrison Fethe conducted field research for three-years on bacterial phytosensing tobacco. This research was written and revised by Michael Fethe, Wusheng Liu, Mitra Mazarei, and Charles Neal Stewart. Michael Fethe, Jason Burris and Reginald Millwood conducted field design. Mary Rudis transformed transgenic tobacco plant lines. Michael Fethe, Duncan Yeaman, and Marion Dubosqueille collected data and images for publication. Charles Neal Stewart conceived and approved the idea.

Abstract

Phytosensors are useful for rapid-on-the-plant detection of contaminants and agents that cause plant stress. Previously, we produced a series of plant pathogen-inducible synthetic promoters fused to an orange fluorescent protein (OFP) reporter gene and transformed them into tobacco and *Arabidopsis thaliana* plants; in these transgenic lines an OFP signal is expressed commensurate with the presence of plant pathogens. We report here the results of two-years of field experiments using a subset of these bacterial phytosensing tobacco plants. Time-course analysis of field grown phytosensors showed that a subset of plants responded predictably to treatments with *Pseudomonas* phytopathogens. There was a 2-fold induction in the OFP

fluorescence driven by two distinct salicylic acid-responsive synthetic promoters, 4×PR1 and 4×SARE. Most notably, transgenic plants containing 4×PR1 displayed the earliest and highest OFP induction at 48 and 72 hours post inoculation (hpi) upon inoculation with two phytopathogens *Pseudomonas syringae* pv. *tomato* and *P. s.* pv. *tabaci*, respectively. These results demonstrate transgenic tobacco harboring a synthetic inducible promoter-driven OFP could be used to facilitate monitoring and early-warning reporting of phytopathogen infections in agricultural fields.

Introduction

Each year plant pathogens are responsible for approximately ten percent loss in global food production (Strange, 2005). Currently, plant pathogens are typically detected post-symptomatically in the field. Therefore, new solutions for early detection of pathogens are needed to address the post-symptomatic pathogen losses by facilitating preventive treatments and monitoring pathogen movement across agricultural ecosystems (Liu *et al.*, 2013a; Lucas, 2010; Skottrup *et al.*, 2008). The engineering of transgenic plants to create phytopathogen phytosensors may help address this need.

Cis-acting regulatory elements employed in previous studies showed significant inducibility after application of phytohormones and phytopathogens (Mazarei *et al.*, 2008; Rushton *et al.*, 2002). Mazarei *et al.* (2008) defined the four most highly inducible regulatory elements, i.e., a salicylic acid-inducible element (SARE; Shah and Klessig, 1996), a pathogenesis related (PR1) element (Lebel *et al.*, 1998), an ethylene-responsive element (ERE; Brown *et al.*, 2003; Ohme-Takagi and Shinshi, 1995; Rushton *et al.*, 1996), and a jasmonic acid-responsive (JAR) element (Guerineau *et al.*, 2003) that were identified as candidates for pathogen-inducible

synthetic promoters. The tetramers (i.e., 4 head-to-tail copies; Fig. 1 and Table 1) of these *cis*-acting elements were used to create synthetic pathogen-inducible promoters 4×SARE, 4×PR1, 4×ERE and 4×JAR, which were placed upstream of the minimal CaMV 35S promoter (i.e., -46 35S) to drive an orange fluorescent protein gene [OFP; i.e., *pporRFP* from *Porites porites* (Alieva *et al.*, 2008; Mann *et al.*, 2012)] (Liu *et al.*, 2011; 2013a). Transformation of tobacco and *Arabidopsis* with these synthetic promoters allowed evaluation of these synthetic constructs *in planta* for bacterial pathogen phytosensing under controlled growth chamber conditions (Liu *et al.*, 2011; 2013a). These synthetic constructs in stable and transient phytosensors displayed significantly induced expression of the reporter gene post treatment with phytohormones and bacterial pathogens such as *Pseudomonas syringae* pv. *tomato* (*Pto*), *P. s.* pv. *tabaci* (*Pst*), and *P. marginalis* (*Pm*) (Liu *et al.*, 2011; 2013a). *Pto* is a rod-shaped gram-negative bacterium with polar flagellum that causes a hypersensitive response (HR) in tobacco during an incompatible plant-pathogen interaction (Wei *et al.*, 2007). *Pst* causes the common wildfire symptom in tobacco during a compatible interaction (Wei *et al.*, 2007). Soil-borne *Pm* causes "soft-rot" disease.

To date, phytosensors have been well characterized in the laboratory and growth chamber conditions wherein environmental conditions have been tightly controlled. There are instances in which transgenic plants have exhibited vastly different transgene expression between greenhouse and field conditions; i.e., attenuated transgene expression in the field (Brandle *et al.*, 1995; Conner *et al.*, 1994; de Carvalho *et al.*, 1992; De Wilde *et al.*, 2000). Thus, it is important to field-test transgenic plants to assess the robustness and predictability of transgene expression. We were interested in determining the degree and specificity of inducibility of synthetic promoters over two field seasons in an environmentally- and agronomically-relevant site for

tobacco cultivation. A suite of transgenic tobacco phytosensors was treated with the 3 phytobacterial pathogens (i.e., *Pto, Pst,* and *Pm*) to assess the utility of these phytosensors in the field. To our knowledge, this was the first field assessment of phytosensors for plant pathogens or any other target contaminant.

Results

We conducted a two-year field trial of transgenic plants expressing an OFP reporter (i.e., *pporRFP*) driven by one of the four different inducible promoters (i.e., 4×SARE, 4×PR1, 4×ERE, and 4×JAR), with or without the B and A1 enhancer domains of the CaMV 35S promoter, for bacterial pathogen phytosensing at East Tennessee Research and Education Center (ETREC), Knoxville, TN, USA in 2012 and 2013 (Table 2). We selected a subset of transgenic tobacco lines produced earlier that contained inducible *cis*-regulatory elements driving the *pporRFP* reporter gene and showed high inducibility upon treatments with different phytopathogens under controlled growth chamber conditions (Liu *et al.*, 2011; 2013a). We included two T_2 homozygous transgenic tobacco lines containing one of the four regulatory elements (with and without the B and A1 domains) in our field studies in 2012. In addition, one T_2 homozygous transgenic line containing *-46 35S::pporRFP*, *B_A::pporRFP* or *35S::pporRFP* was included in our 2012 field experiments. In 2013, we added one additional set of T_2 homozygous lines containing *4×PR1::pporRFP* and *4×ERE::pporRFP*, and did not include any transgenic lines containing enhancer elements, *B_A::pporRFP,* or *4×JAR::pporRFP* since they showed little to no observable inducibility in the 2012 field experiments (Table 2). Four-to-six week-old plants were transplanted from greenhouse to ETREC in June of 2012 and 2013, and grown in the field for seven weeks in 2012 and six

weeks in 2013 prior to inoculation with phytopathogens. We applied phytopathogens *Pto* and *Pst* in the field in 2012 and 2013, and included *Pm* as an additional treatment in 2013 (Table 2). Our observations yielded no visual (non-fluorescence) phenotypic difference in any transgenic lines. We observed infrequent insect herbivory in the field in 2012 and 2013 (data not shown). There was also above-average precipitation during both growing seasons (USDA, 2012 - 2013) (Table 2). Below average temperature was observed in 2012 (USDA, 2012 - 2013), and no difference from normal temperature was observed in 2013 (Table 2). We did not observe symptoms of any naturally-occurring plant pathogens; i.e., the experimental treatments of plant pathogens accounted for all of the plants' responses in the field during our two-year study.

Inducibility of salicylic acid-responsive promoters

Phytopathogen treatments with *Pto, Pst,* and *Pm* of control lines containing either *-46 35S::pporRFP*, *B_A::pporRFP* (data not shown), or *35S::pporRFP* displayed OFP fluorescence and reporter transcript levels comparable to mock treatment during our time-course analysis (Figs. 2; 3; S1). Salicylic acid-responsive promoters 4×SARE and 4×PR1 have previously been shown to have the highest inducibility by HR-inducing *Pto*, followed by *Pst*, which causes disease development in tobacco (Liu *et al.*, 2011; 2013a). In our 2012 field trial, *Pto* treatment significantly induced OFP fluorescence at 48 and 72 hpi in transgenic line S1 (S designates lines containing 4×SARE) harboring 4×SARE motifs (Figs. 2; S2). At 48 hpi with *Pto* treatment of the S1 line, the OFP fluorescence was 2.0-fold higher (Tukey mean separation, $P < 0.01$; Fig. 2) and *pporRFP* mRNA was 1.5-fold (unpaired Student's T-test, $P < 0.05$) higher than in mock treated samples (Tukey mean separation, $P < 0.01$; Fig. 2). Another line S2 containing 4×SARE only

showed significant induction in OFP fluorescence of 1.8-fold over the mock treated samples at 72 hpi (Tukey mean separation, $P < 0.01$) while the *pporRFP* mRNA level was apparently slightly increased at 48 hpi (Fig. 2). In 2013, the *Pto* treatment of the S1 line resulted in induced OFP fluorescence to 1.4-fold at 48 hpi (Tukey mean separation, $P < 0.01$) and 1.5-fold at 72 hpi (Tukey mean separation, $P < 0.001$) over the mock treated samples (Fig. 3). *Pto* treatment of the S2 line showed a late induction with a 2.0-fold increase in OFP fluorescence compared to mock treated samples at 72 hpi (Tukey mean separation, $P < 0.001$; Fig. 3). In 2012, we applied the *Pst* treatment on plants previously inoculated with *Pto;* this allowed evaluation of the OFP fluorescence inducibility after plants had been infected with a HR-inducing phytopathogen. Our results indicated that the *Pst* treatment did not cause detectable OFP fluorescence induction in transgenic lines S1 and S2 in 2012 (Fig. S1) after inoculating plants with *Pto*. Following *Pst* treatment in 2013, transgenic line S1 exhibited 1.3-fold of OFP fluorescence above mock treated samples at 48 hpi (Tukey mean separation, $P < 0.05$), followed by a slight 1.2-fold induction in OFP fluorescence above the mock treated samples at 72 hpi (Tukey mean separation, $P < 0.001$; Fig. 3). OFP fluorescence in the S2 line after *Pst* treatment remained unchanged in 2013 (Fig. 3).

Most notable was the *Pto*-induced OFP fluorescence in transgenic lines harboring 4×PR1 regulatory elements, in which the OFP fluorescence in 2012 showed significant induction of approximately 1.5-fold at 48 hpi (Tukey mean separation, $P < 0.01$) and 2.0-fold at 72 hpi (Tukey mean separation, $P < 0.001$) compared to the mock treated samples, even though the OFP fluorescence induction remained undetected at 24 hpi (Fig. 2). Transcript analysis of OFP expression in transgenic lines P1 and P2 (P designates lines containing 4×PR1) containing 4×PR1 showed that *Pto* inoculation significantly induced *pporRFP* mRNA by 3.7-fold in the P1 line and 2.4-fold in the P2 line at 24 hpi compared to the mock treated samples (unpaired

Students *t*-test, $P < 0.05$), and *pporRFP* transcription was reduced yet remained slightly elevated compared to mock samples at 48 hpi in P1 and P2 lines (Fig. 2). Interestingly, the line P1 displayed a significant induction of *pporRFP* transcript of 2-fold higher than the mock treated samples at 72 hours post *Pto* inoculation (unpaired Student's *t*-test, $P < 0.05$; Fig. 2). Line P3 was added to our experimental design in 2013. In 2013, relative *pporRFP* expression was elevated in two of three transgenic lines containing 4×PR1 by phytopathogen treatments *Pto* and *Pst* by 24 hpi (Fig. S3). *Pto* inoculation significantly induced OFP fluorescence in transgenic line P1 to approximately 1.4-fold at 48 hpi (Tukey mean separation, $P < 0.05$), and showed a significant 2 to 3-fold increase in the OFP fluorescence in transgenic lines P1, P2, and P3, which was observed at 72 hpi with *Pto* over the mock treated samples (Tukey mean separation, $P < 0.001$). No difference in the OFP fluorescence between *Pto* and mock treated samples was detected at 24 hpi in all the three lines (Fig. 3). The *Pst* treatment following *Pto* inoculation in 2012 on transgenic lines harboring 4×PR1 yielded OFP fluorescence levels comparable to mock treatment in 2012; however, we observed significant induction in transgenic lines harboring 4×PR1 post-*Pst* inoculation in 2013. All lines harboring 4×PR1 yielded significantly induced OFP fluorescence by 72 hpi (Tukey mean separation, $P < 0.001$, Fig. 3; S4). Furthermore, transgenic lines P1 and P3 displayed OFP induction of approximately 1.5-fold over mock treated samples as early as 48 hpi with *Pst* (Tukey mean separation, $P < 0.001$, Fig. 3). Thus, transgenic lines containing 4×PR1 displayed the earliest, highest, and most consistent induction in our 2-year studies.

Treatment of transgenic phytosensing tobacco with *Pm* in 2013 yielded no significant induction in the OFP fluorescence in transgenic lines containing either salicylic acid-responsive promoter (i.e., 4×SARE or 4×PR1; Fig. 3).

Inducibility of ethylene/jasmonate-responsive promoters

Previous studies showed that transgenic lines harboring ethylene/jasmonate-responsive promoters exhibited induced OFP fluorescence following treatments with necrotizing *Pto,* and also with soft-rot causing bacteria *Pm* (Liu *et al.*, 2011; 2013a). In our 2012 field trial, the OFP fluorescence remained unchanged in transgenic lines harboring 4×ERE at 24 or 48 hpi after all phytopathogen treatments, but line E1 (E designates lines containing 4×ERE) exhibited significant induction in the OFP fluorescence of 1.3-fold increase over mock treatments at 72 hpi with *Pto* treatments (Tukey mean separation, $P < 0.05$; Fig. 2). Transcript analysis also showed a 1.9-fold induction in the *pporRFP* mRNA level in the transgenic line E1 at 48 hpi with *Pto* treatments. In 2013, an additional line containing 4×ERE was added to our experimental design and designated E3. *Pto* treatment in 2013 induced all three transgenic lines containing 4×ERE motifs at 72 hpi with induction from 1.5 to 2.0-fold above the mock treated samples (Tukey mean separation, $P < 0.001$) (Fig. 3). In 2012, the *Pst* treatment applied following *Pto* treatment in 2012 yielded OFP fluorescence comparable to mock treatments. In 2013, a similar pattern was observed with *Pst* treatment of transgenic line E1– the OFP fluorescence induction was 2.0-fold of mock fluorescence at 72 hpi. The remaining transgenic lines containing 4×ERE (i.e., E2 and E3) treated with *Pst* showed no statistical difference from mock treated samples. No lines containing 4×ERE displayed induction at 24 or 48 hpi with any phytopathogen treatment (Figs. 2; 3). At 72 hpi with *Pst*, the induction in the OFP fluorescence in line E1 was 1.9-fold above the mock treatment. All transgenic lines harboring 4×ERE exhibited a significantly induced OFP fluorescence from 1.5 to 2.0-fold over mock treatments at 72 hpi after *Pto* treatment (Tukey mean separation, $P < 0.001$) (Fig. 3). All ethylene-responsive promoters remained comparable to

mock treated samples post-infiltration of *Pm* throughout our time course analysis in 2013 (Fig. 3).

Our analysis was unable to detect significant induction in the OFP fluorescence in transgenic lines containing 4×JAR with any phytopathogen treatment at any time points in 2012 (Fig. S2), with the exception of the line J1 (J designates lines containing 4×JAR) harboring 4×JAR which displayed significantly elevated *pporRFP* mRNA level of 1.6-fold at 48 hpi with *Pto* infection over the mock treated samples (unpaired Student's *t*-test, $P < 0.05$) (Fig. 2). Therefore, transgenic lines containing 4×JAR were excluded from our 2013 field studies.

Correlation analysis of the OFP fluorescence and transgene transcription following Pto treatment

Most transgenic lines harboring the synthetic promoters displayed significant induction in OFP fluorescence after *Pto* treatment (Figs. 2; 3). Therefore, we calculated Pearson's Rho (R) between the OFP transcript and the OFP fluorescence to determine the correlation between transcriptional changes and protein fluorescence. Our analysis detected a significant correlation between *pporRFP* mRNA from samples collected 24 hours prior to OFP fluorescent measurements ($P < 0.001$, $R = 0.4428$, $R^2 = 0.1968$) and samples collected at the same time points of OFP fluorescent ($P < 0.001$, $R = 0.28$, $R^2 = 0.078$) of OFP measurements. Our results suggest that a direct relation exists between the *pporRFP* transcript levels and the OFP fluorescence measurements.

Bacterial growth and disease development

We sampled *Pseudomonas* populations in *Pto-, Pst-,* and *Pm*-inoculated leaves using *Pseudomonad* selective King's B media supplemented with rifampicin. Bacterial counts were taken from subsamples within each bacterial treatment (N = 12). Results of bacterial concentration significantly correlated with the OFP fluorescence signal in transgenic lines P1, P3, and E1. However, the significant correlations between bacterial populations and OFP fluorescence signal in P1 ($R = 0.17$, $R^2 = 0.030$) and P3 lines ($R = 0.17$, $R^2 = 0.029$) and line E1 ($R = 0.14$, $R^2 = 0.0196$) ($P < 0.05$) were weak. Bacterial populations obtained from infiltrated leaf tissue indicated that symptoms were indeed caused by bacterial infiltration treatments.

We noticed that *Pto* caused necrosis by 24 hpi with cellular collapse that developed between 24 to 48 hpi. Growth of *Pto* was observed within 24 hpi. *Pto* levels showed a steady decrease from 24 hpi to 72 hpi from 3.37×10^9 CFU g^{-1} to 2.33×10^9 CFU g^{-1} throughout the time-course analysis (Fig. 4). Bacterial enumeration of *Pto* showed a steady decrease in this population after 24 hpi, however, the levels of *Pto* remained high in the field, above 10^9 CFU g^{-1} (Fig. 4).

Bacterial symptoms progressed on the field-grown plants at the same rate as in our growth chamber experiments (Liu *et al.*, 2013a) after application with *Pst*. The inoculation of *Pst* caused wildfire disease characterized by chlorosis at 24 hpi, followed by necrosis and hyponasty of the infected area at 72 hpi (Liu *et al.*, 2013a). *Pst*-inoculated tissue displayed bacterial growth within 24 hpi. The *Pst* population at 24 hpi with 2.6×10^9 CFU g^{-1} proliferated minimally to 2.74×10^9 CFU g^{-1} at 48 hpi and decreased by 72 hpi to 2.35×10^9 CFU g^{-1} (Fig. 4). Bacterial counts of *Pst* were consistently above 10^9 CFU g^{-1} in the field-grown inoculated plants.

Pm showed minimal growth on plants, the absence of necrosis or chlorosis, and was asymptomatic in our treated samples (Fig. S5). *Pm* enumeration showed a steady decrease in bacterial counts after 24 hpi: with 8.47×10^5 CFU/g at 24 hpi and ending at 72 hpi with 5.99×10^4 CFU/g (Fig. 4). Thus, *Pm* inoculum levels showed no proliferation and remained below 10^6 CFU g^{-1} in our time-course study.

Discussion

Our study evaluated the field performance of transgenic tobacco plants for bacterial pathogen phytosensing over the course of two years of field experiments to determine if synthetic promoters maintain inducibility under field conditions. Since synthetic inducible promoters have seldom been used under field conditions, it was unclear how relevant environmental factors (i.e., UV stress, insect herbivory, wind stress, drought, or high precipitation) would affect the inducibility of the OFP fluorescence in transgenic lines. The field-grown transgenic tobacco lines containing either 4×SARE, 4×PR1, or 4×ERE maintained similar fluorescence induction patterns and levels as observed in our previous studies (Liu *et al.*, 2011; 2013a) when subjected to phytopathogen attack by hemi-biotroph *Pto* and biotroph *Pst.* In particular, transgenic lines evaluated here harboring 4×PR1 may prove useful for future studies on bacterial pathogen phytosensing because of detectable inducibility and transcriptional analysis across multiple years and transgenic lines that indicated consistent and specific inducibility by phytobacterial pathogens under field conditions. To our knowledge, this report represents the first field experiments to evaluate the performance of transgenic plants designed for pathogen phytosensing.

The hypersensitive response in tobacco during an incompatible plant-pathogen interaction with *Pto* develops within 24 - 48 hpi (Wei *et al.*, 2007). We observed that the induction in OFP fluorescence was consistent with the known pathogenic mechanisms of bacterial treatments. For example, transgenic lines harboring salicylic acid-inducible constructs displayed higher induction than lines containing 4×ERE after the HR-inducing *Pto* treatments; thus, supporting previous research that indicates salicylic acid's role in the HR-response (Liu *et al.*, 2013c). Also noteworthy, lines P1 harboring 4×PR1 and S2 harboring 4×SARE were induced within 48 hpi with *Pto*; these lines displayed a HR-induced OFP fluorescence within 48 hpi (Figs. 2; 3). The biotic treatment with *Pst* caused a gradual increase in OFP fluorescence as previously observed (Liu *et al.*, 2011; 2013a), even though induction of OFP fluorescence was lower than that observed in the *Pto* treatments; however, this noteworthy response was observed significantly earlier than that of the *Pto* treatments in lines P3 and S1 in the field. Furthermore, the absence of induction from *Pm* treatments was consistent with no observable disease symptoms. Therefore, the OFP fluorescence measurements indicate the level of the plants' defenses involved in a pathogen attack under field conditions when the OFP reporter is controlled by pathogen-defense related regulatory elements.

The ethylene-responsive promoter 4×ERE showed a late OFP induction at 72 hpi with *Pto;* which further demonstrates that necrotic tissue development increases ethylene synthesis (Bari and Jones, 2009). Nevertheless, in 2012 *Pst* treatment did not induce transgenic lines harboring synthetic pathogen-inducible promoters (Figs. S1; S2) yet induced lines harboring 4×SARE, 4×PR1, and 4×ERE in 2013 (Fig. 3). Variation between years could be the result of environmental effects on transgene expression or age of plants at the time of inoculation (Table 2). Liu *et al.* (2013a) discussed the effect of plant age on transgene inducibility in this system

and the increased age of plants treated with *Pst* in 2012 likely reduced OFP fluorescence inducibility in field-grown plants.

Field trials on multiple lines containing the same synthetic constructs allowed for observation of line-to-line variations (Figs. 2; 3). For example, two lines containing 4×SARE displayed different OFP fluorescence induction patterns after the same biotic treatment (Fig. 3): line S1 harboring 4×SARE showed induction at 48 and 72 hpi with *Pst* whereas line S2 harboring the same synthetic construct showed no induction post-treatment with *Pst.* Transgenic lines containing 4×PR1 displayed the earliest and highest induction, but discrepancies between lines still existed (Fig. 3). Moreover, only line E1 harboring 4×ERE displayed OFP fluorescence induced by *Pst* treatment at 72 hpi. Insertional or epigenetic effects may possibly explain line-to-line variations observed within each construct (Butaye *et al.*, 2005).

Field evaluations yielded different induction patterns from previous growth chamber studies on phytosensing transgenic lines containing the same constructs (Liu *et al.*, 2011; 2013a), but OFP induction in different constructs remained time-specific to phytopathogen treatments. In Liu *et al.* (2013a), OFP induction in T_1 hemizygous lines was reported within 24 hours after *Pto* treatment, but in field-tested T_2 homozygous lines it took at least 48 hpi for transgenic lines harboring the salicylic acid-responsive constructs to display the fluorescent signal induction. Moreover, OFP fluorescence remained significantly induced in growth chamber-tested transgenic lines harboring 4×SARE after 24 hpi with *Pto*, and OFP fluorescence decreased in transgenic lines containing 4×PR1 after 24 hpi with *Pto* (Liu *et al.*, 2013a); field evaluations yielded significantly induced OFP fluorescence only at or after 48 hours post phytobacterial treatments. Thus, field-tested T_2 homozygous transgenic lines harboring salicylic acid-responsive promoters (4×SARE and 4×PR1) displayed a later significant induction in OFP fluorescence

than growth-chamber studies. Also, Liu *et al.* (2013a) showed that *Pm* treatment induced OFP fluorescence in transgenic plants containing 4×ERE, but the field-tested transgenic lines harboring the same construct displayed no induction when inoculated with *Pm*. Discrepancies between field and laboratory studies have been extensively documented (McKersie *et al.*, 1999; Mohamed *et al.*, 2001), and our field studies demonstrate the need for evaluation of transgenic plants under combinations of different stresses before their application in the field (Mittler, 2006).

The differences in results between the prior laboratory experiments and field experiments could have numerous causes. The synthetic promoter containing different *cis*-regulatory elements could respond differently to transcriptional and translational modifications during phytohormone or phytopathogen treatments (Liu *et al.*, 2011; 2013a; Mazarei *et al.*, 2008). Of these synthetic constructs, SARE contains multiple activation motifs that are inducible by salicylic acid (Shah and Klessig, 1996). Furthermore, the PR1 regulatory element contains sequences, which negatively and positively regulate transcription (Pape *et al.*, 2010). It has been noted that regulatory elements with multiple *cis*-acting elements are better suited for specific pathogen induction (Rushton *et al.*, 2002), and these promoters maintain pathogen-specific inducibility in the field. However, the ERE regulatory elements contain an *ERE* (AGCCGCC) sequence similar to the drought responsive sequence *DRE* (GGCCGAC and TACCGAC) (Stockinger *et al.*, 1997) and abiotic stresses may have induced this *cis*-acting element from the involvement of the ET/JA pathway in abiotic stress responses (Fraire-Velázquez *et al.*, 2011). Thus, the slight induction observed in the laboratory by *Pm* treatment was minimized by environmental factors affecting activity of ERE regulatory elements. These observations agree

with previous research (Rushton *et al.*, 2002) indicating synthetic promoters containing multiple *cis*-acting elements maintain specific inducibility.

Moreover, other environmental conditions may have altered the inducibility of synthetic pathogen-inducible promoters in transgenic lines. For instance, precipitation was approximately 13 and 28 centimeters above average in 2012 and 2013, respectively, during the growing period of this study (Table 2). Field temperatures fluctuate by the hour, in contrast Liu *et al.* (2013a) in which experiments were maintained at constant temperatures (25°C). Thus, the results presented here indicate that these promoters maintained specific inducibility to phytopathogens under a variety of conditions including high precipitation, insect herbivory, UV stress, and other environmental stressors.

This study illustrates some of the inherent weaknesses present in the current phytosensor system, along with some possible suggested improvements. Previous studies (Rushton *et al.*, 2002) showed that spacing and the number of regulatory element could dramatically affect the inducibility of synthetic constructs, which would also be interesting to test under field conditions with the goal of improving detectable inducible signal. Furthermore, the regulatory elements used in our studies may be improved upon by coupling these regulatory elements with others such as the box D element described in Rushton *et al.* (2002). It is important that regulatory elements in synthetic constructs are as specific as possible to eliminate false positives; experimental testing and threshold determination of an OFP fluorescence signal indicative of phytopathogen infections can help avoid false positives. Thus, these field results should be considered as early research phytosensing systems for environmental detection.

Of particular interest are the potential applications of bacterial phytosensing transgenic lines evaluated here; for example, these lines may be useful as sentinel plants for phytobacterial

detection in agricultural fields for tobacco or other crops that could be damaged by *P. syringae.* For example, soybean, pea, oat, bean, pepper, potato, and tomato, among other crops are susceptible to the pathovars of *P. syringae* we tested here. Thus, it might be possible to plant phytosensing tobacco plants as gridded sentinels against pathogens or transform the crop of interest with appropriate phytosensing constructs. In addition, if might be useful to monitor incidence of other plant pathogens. Specific lines may be better suited for inducing OFP fluorescence by compatible or HR-inducing pathogens in the field; for instance, line S2 may be useful for detecting HR-inducing pathogens while line P3 may display earlier induction during compatible phytobacterial pathogen interactions. The development of insect- or fungal-specific phytosensors is possible through a similar system using insect or fungal-inducible promoters. In particular, previous research indicates the ERE regulatory element is inducible by the fungal elicitor chitin (Mazarei *et al.,* 2008). Therefore, while transgenic lines harboring 4×ERE displayed late induction of OFP fluorescence after phytobacterial pathogen treatments here, they may prove useful for fungal pathogens.

Clearly, phytosensors have the potential to be used as early-detection tools as part of precision agriculture systems in commercial crop production. Integrating plant biotechnology and synthetic biology tools with detectors and global positioning system services to for better pest management (Lucas, 2010). Plant synthetic biology tools are proliferating at a seemingly rapid pace (Liu *et al*., 2013b), that includes synthetic promoters (Venter, 2007; Venter and Botha, 2010) but also genome editing tools; these tools could be used to make advanced phytosensors. The problem of phytosensor signal detection also has several potential solutions. One potential detection strategy would be to periodically take samples of leaves for fluorescence measurements using a GFP-meter (Millwood *et al.,* 2003; Opti-Sciences, Hudson, NH USA). A

second possibility would be to use laser-induced fluorescence imaging (Stewart *et al.*, 2005) that might mounted on unmanned aircraft. A third example for possible monitoring of induced plant fluorescence is the Rover Fluorocam (Photon Systems Instruments, Drasov, Czech Republic). It is a fluorescent imaging system capable of large scale field monitoring, which can produce fluorescent images comparable to those presented here (Fig. S4). Taken together these technologies could allow precise applications of agrochemicals in time and space (Skottrup *et al.*, 2008) to decrease pesticide footprints and increase yields.

Experimental procedures

Plasmid construction, plant materials and transformation

All stable transgenic tobacco plants used in these experiments were described previously (Liu *et al.* 2013a). Specifically, constructs containing each of the four distinct *cis*-acting regulatory elements of salicylic acid-responsive element (SARE), pathogenesis-related (PR1), ethylene-responsive element (ERE), and jasmonic acid-responsive element (JAR), with and without B and A1 domains of the CaMV 35S promoter, driving an OFP reporter [i.e., *pporRFP* from *Porites porites;* (Alieva *et al.*, 2008)] were used in this study. Transgenic tobacco plants harboring empty vectors (*-46 35S::pporRFP, B_A::pporRFP,* and *35S::pporRFP)* were also used for this study. After transformation into *Nicotiana tabacum* (cv. Xanthi) plants, homozygous lines containing each of the above-mentioned constructs were obtained by screening T_1 seeds of hemizygous lines yielding approximately 1:3 segregation of gentamycin resistance on Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with gentamycin (Sigma Aldrich, St. Louis, Mo., USA) at 200-µg ml^{-1}. Homozygous T_2 transgenic tobacco lines were

germinated and grown in float trays (London Tobacco Market, London, Ky., USA) for at least 1 month. In 2012, plants were transplanted at the field site and allowed to establish for 7 weeks then treatment plots were infiltrated with *Pto* and control plots with 10 mM $MgCl_2$; following *Pto* treatments leaves were removed for sample and data collection then the plants in treatment plots were infiltrated with *Pst* and control plots with 10 mM $MgCl_2$ (Fig. S6). In 2013, plants were established in the field for 6 weeks after transplanting and treated with phytopathogen treatments on corresponding plots (Fig. S7).

Bacterial growth

Bacterial cultures used for biotic treatment were grown under the same conditions as in Liu *et al.* (2011). Cultures of *P. s.* pv. *tabaci (Pst), P. s.* pv. *tomato (Pto),* and *P. marginalis (Pm)* were grown in tryptic soy broth (TSB), with constant shaking (250 RPM), at 28° C overnight. Rif^R mutants for 2013 were selected for in TSB with rifampicin at a concentration of 50-mg L^{-1}.

A 0.1-g sample of leaf tissue obtained from each treatment was homogenized in sterile distilled water. Serial dilutions were plated on King's B (KB) agar medium supplemented with rifampicin at a concentration of 50-mg L^{-1}. Each biological sample was replicated 3 times and four experimental replicates were used to calculate colony-forming units (CFUs) per treatment.

Biotic treatment

Infiltration solutions were prepared as described in Liu *et al.* (2011). Leaves of the same age and size were selected for infiltration (5 – 7-cm). *Pst, Pto,* and *Pm* cells were collected through centrifugation of cultures at 5,000 RPM for 10 minutes. Cells were washed two times in 10-mM $MgCl_2$. Final solutions of *Pto* and *Pm* were diluted to 2×10^8-CFU ml^{-1}, while *Pst* solutions were diluted to 2×10^7-CFU ml^{-1} (Table 2). Leaves were inoculated through infiltration with a

needleless syringe using approximately 200-µL of corresponding solution on each side of the midrib. Ten millimolar $MgCl_2$ was used for mock treatments.

Experimental design

Field site locations at East Tennessee Research and Education Center (ETREC) in Knoxville, Tenn., USA were used in 2012, and 2013 for our study (Figs. S6; S7). In 2012, our field site was broadcast fertilized with N (200-kg ha^{-1}), P (25-kg ha^{-1}), and K (100-kg ha^{-1}), which was done according to recommendations for agronomic tobacco cultivation. In 2013, urea was applied at a rate of 200-kg ha^{-1}. We used a randomized complete block design with a strip-split plot arrangement. In 2012, two biotic treatments (*Pst, Pto*) (Fig. S6) and in 2013, three biotic treatments (*Pst, Pto,* and *Pm*) (Fig. S7) were applied to blocks. In 2012, subplots contained two independent transgenic lines harboring *4×SARE::pporRFP, 4×PR1::pporRFP, 4×ERE::pporRFP, 4×JAR::pporRFP,* enhanced version of lines, and one transgenic line harboring empty vectors *-46 35S::pporRFP, B_A::pporRFP, 35S::pporRFP* (Table 2; Fig. S6). In 2013, subplots contained three transgenic lines harboring *4×PR1::pporRFP* and *4×ERE::pporRFP,* two lines harboring *4×SARE::pporRFP,* and one line harboring empty vector *-46 35S::pporRFP* and *35S::pporRFP* (Fig. S7). Replicates and treatment plots were separated with 3.0-m buffer zones. Weeds were controlled with mechanical disruption and application of pre-emergent herbicides [Prowl (BASF, Fremont, Calif., USA) and Command (DuPont, Wilmington, DE, USA)].

Statistical analysis

All statistical analyses were performed using R (R Developement Core Team, 2005). Mixed model package 'lme4' (Bates *et al.*, 2008) was used to determine significant differences ($P <$

0.05) in OFP fluorescence. The constructs that exhibited statistically significant induction in OFP fluorescence was analyzed post-hoc with mean comparisons with the 'multcomp' package (Hothorn *et al.*, 2007). Correlations were conducted using the 'cor.test' function. For relative quantitative reverse-transcriptase polymerase chain reaction (relative qRT-PCR) calculations, Expression Suite (Life Technologies, Carlsbad, CA, USA) was used.

Fluorometric spectroscopy

A Fluorolog®-3 system (Jobin Yvon and Glen Spectra, Edison, NJ, USA) was used to quantify *pporRFP* in infiltrated sections of leaves through fluorometric spectroscopy. Samples were excited with green light (λ_{ex} = 530-nm) and emission was measured from λ_{em} = 560 − 605-nm to yield a spectral scan. Spectral scans were standardized as described in Millwood *et al.* (2003) to mock treated *-46 35S::pporRFP* at 24 hpi. After normalization, the *pporRFP* peak (λ = 591-nm) was used to quantify fluorescence in an arbitrary unit, counts per second (CPS).

Fluorescent imaging

Fluorescent images were captured on a closed Fluorocam system (Photon Systems Instruments, Drasov, Czech Republic). Samples were excited with green light (λ_{ex}= 535 – 540-nm) while images were captured through a single-band pass filter lens 593/46-nm (Semrock, Rochester, NY, USA). Images were captured with an exposure time of 10-ms, sensitivity at 14%, and light intensity at 13%.

RNA extraction

Infiltrated leaf tissue was collected in the field at 24, 48, and 72 hpi, kept on ice and transported back to the laboratory. Leaf tissue was flash frozen in liquid N_2 and stored in a -80° C freezer for

RNA extraction after fluorescent image and spectrofluorometer data collection. Approximately 100-mg of frozen infiltrated plant tissue was ground in a 15-ml polyethylene tube (Corning, Edison, NJ, USA) on liquid N_2. One milliliter of Tri-reagent (Molecular Research Center, Cincinnati, OH, USA) was then added to the ground tissue of each sample. RNA extraction proceeded following manufacturer's instructions. After resuspension of RNA in RNase free water, analysis of RNA integrity was observed through ethidium bromide gel electrophoresis on a 1% agarose gel. RNA was quantified with spectroscopy on a Synergy HT Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA) using a Take3 Micro-Volume plate (BioTek).

cDNA synthesis

RNA was treated with DNase I (Ambion, Austin, TX, USA) prior to cDNA synthesis following manufacturer's instructions. Ten-microliters of DNase I treated RNA extract was reverse transcribed with a high capacity cDNA synthesis kit (Applied Biosystems, Foster City, CA, USA) following manufacturer's instructions. cDNA was synthesized under the following conditions: 10 minutes at 25° C, 120 minutes at 37° C, 5 minutes at 85° C. Samples were kept at 4° C overnight then stored at -20° C. cDNA quality was observed spectroscopically with the OD 260/230 ratio > 1.8, and the OD 260/280 ratios ≈1.8 ± 0.02.

Quantitative real-time polymerase chain reaction

Relative quantification PCR was performed using Power SYBR Green chemistry (Applied Biosystems) on a 7900 HT Fast Real-time PCR system (Applied Biosystems). Standard curves were used to calculate efficiency with appropriate primers (Table S1). PCR was performed in MicroAmp Optical 384-well reaction plates (Life Technologies). Reactions contained appropriate primer concentrations (Table S1), 0.75-µl of cDNA reaction, 2.5-µl of 2× Power

SYBR Green (Life Technologies), 1.75-µl water. A hot-start began the cycle at 95° C for 10 minutes. Forty cycles of 15s at 95° C and 120s at 60° C were run while fluorescent (520-nm) measurements were taken post-elongation phase. Melting curves were obtained by heating samples to 95° C, cooling to 60° C, followed by a 1°C sec^{-1} increase to 95° C under fluorescent measurement. Primer design was done with Primer Express software (Life Technologies); and reference gene primers were obtained from Schmidt and Delaney (2012) (Table S1). Data analysis was performed using Expression Suite software (Life Technologies) using the ΔΔCt method (Schmittgen and Livak, 2008).

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Appendix:

Tables:

Table 1. Cis-acting regulatory elements (RE) used in construction of phytosensing cassettes [subtracted from (Liu et al., 2013a)]. The core sequences in each RE are in bold.

RE	**Sequence (5' > 3')**	**Promoter Origin**	**Species Origin**	**Source**
SARE	**TTCGACCTCC**AAAGAG GACCCAGAAT	*PR2-d*	*N. tabacum*	(Shah and Klessig, 1996)
PR1	**ACGTCA**TAGATGT**GGC GGC**ATATATTCTTCA**G GACTTTTC**	*PR1*	*A. thaliana*	(Lebel *et al.*, 1998)
ERE	**C**AGCCGCC**AAAGAGGA CCCAGAAT**	chitinase	*N. tabacum*	(Brown *et al.*, 2003; Ohme-Takagi and Shinshi, 1995; Rushton *et al.*, 1996)
JAR	CAACGA**CACGCCAAAT** TCTAATTTAGCACAGTC TC**ACGTG**	*VSP1*	*A. thaliana*	(Guerineau *et al.*, 2003)

Table 2. Treatments, constructs, plant age, and environmental conditions for the 2-year field experiments.

Treatments per year	2012	2013
P. s. pv. *tomato*	+	+
P. s. pv. *tabaci*	+	+
P. marginalis	-	+
Number of independent lines used each year		
-46 35S	1	1
35S	1	1
B_A	1	0
4×SARE	2	2
B_4×SARE_A	2	0
4×PR1	2	3
B_4×PR1_A	2	0
4×ERE	2	3
B_4×ERE_A	2	0
4×JAR	2	0
B_4×JAR_A	2	0
Total	19	10
Plant age and climate each year		
Plant age at time of treatment (wks after transplanting)	7 - 15	6 - 9
Precipitation (deviation from normal in cm)	+13	+28
Temperature (deviation from normal in °C)	-1.1	0

Table 3. Comparison of the characteristics of compatible and incompatible interactions between tobacco and Pseudomonas [adapted from (Liu et al., 2013a)].

Pathogen	***Nicotiana tabacum***
P.s. pv. *tomato*	Non-Host; Hypersensitive response (HR) within 24 hpi, necrosis at OD_{600}=0.3
P.s. pv. *tabaci*	Host; Normal-sensitive "Wildfire" symptom within 48-72 hpi at OD_{600}=0.03
P. marginalis	Non-host; Very mild or no symptom at OD_{600}=0.3

Figures:

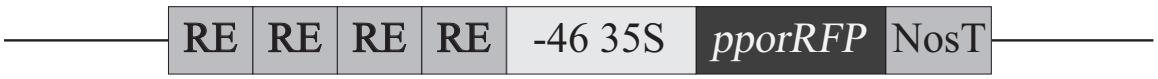


Figure 1. Synthetic promoter design [adapted from (Liu *et al.*, 2011)].

Tetramer of regulatory element sequence was placed upstream of the minimal CaMV 35S (i.e., -46 35S) promoter and orange fluorescent protein reporter (*pporRFP*) from *Porites porites* (Alieva *et al.*, 2008).

Figure 2. Time course analysis of OFP fluorescence and mRNA expression levels in stable transgenic tobacco following *Pseudomonas syringae* pv. *tomato* (*Pto*) treatment in 2012 field trial.

Left: qRT-PCR was performed using RNA extracted from leaf tissue inoculated with *Pto* and 10 mM $MgCl_2$ at 24, 48, and 72 hpi. Expression of the *pporRFP* reporter was normalized to the relative transcript abundance of the tobacco *L25* gene and *Tac9* gene, and relative quantification of *pporRFP* was performed using the ΔΔCt method (Schmittgen and Livak, 2008).

Right: Fluorescent measurements of the OFP reporter were conducted on leaf tissue infiltrated with *Pto* (OD_{600} = 0.3) and 10 mM $MgCl_2$ at 24, 48, and 72 hpi. Quantifications of the *pporRFP* OFP were made on a Fluorolog®-3 system (Jobin Yvon and Glen Spectra, Edison, NJ, USA) by exciting leaf tissue at 530 nm and scanning the emission from 560 to 605 nm. Data was normalized to the mock-treated plants harboring -46 35S. Counts per second were quantified at 591 nm (Liu *et al.*, 2011). Error bars represent standard error from two measurements obtained per biological replicate with two biological replicates per experiment; experiments were replicated 3 times (N = 6, n=12). Asterisks indicate statistical significant (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Lines indicated by first alphabetic character in *cis*-acting regulatory elements in promoter followed by line number in-text (i.e., line 1 containing 4×SARE is S1). Line harboring -46 35S. Lines harboring 4×SARE: line 1, S1; line 2, S2. Lines harboring 4×PR1: line 1, P1; line 2, P2. Lines harboring 4×ERE: line 1, E1; line 2, E2. Lines harboring 4×JAR: line 1, J1; line 2, J2.

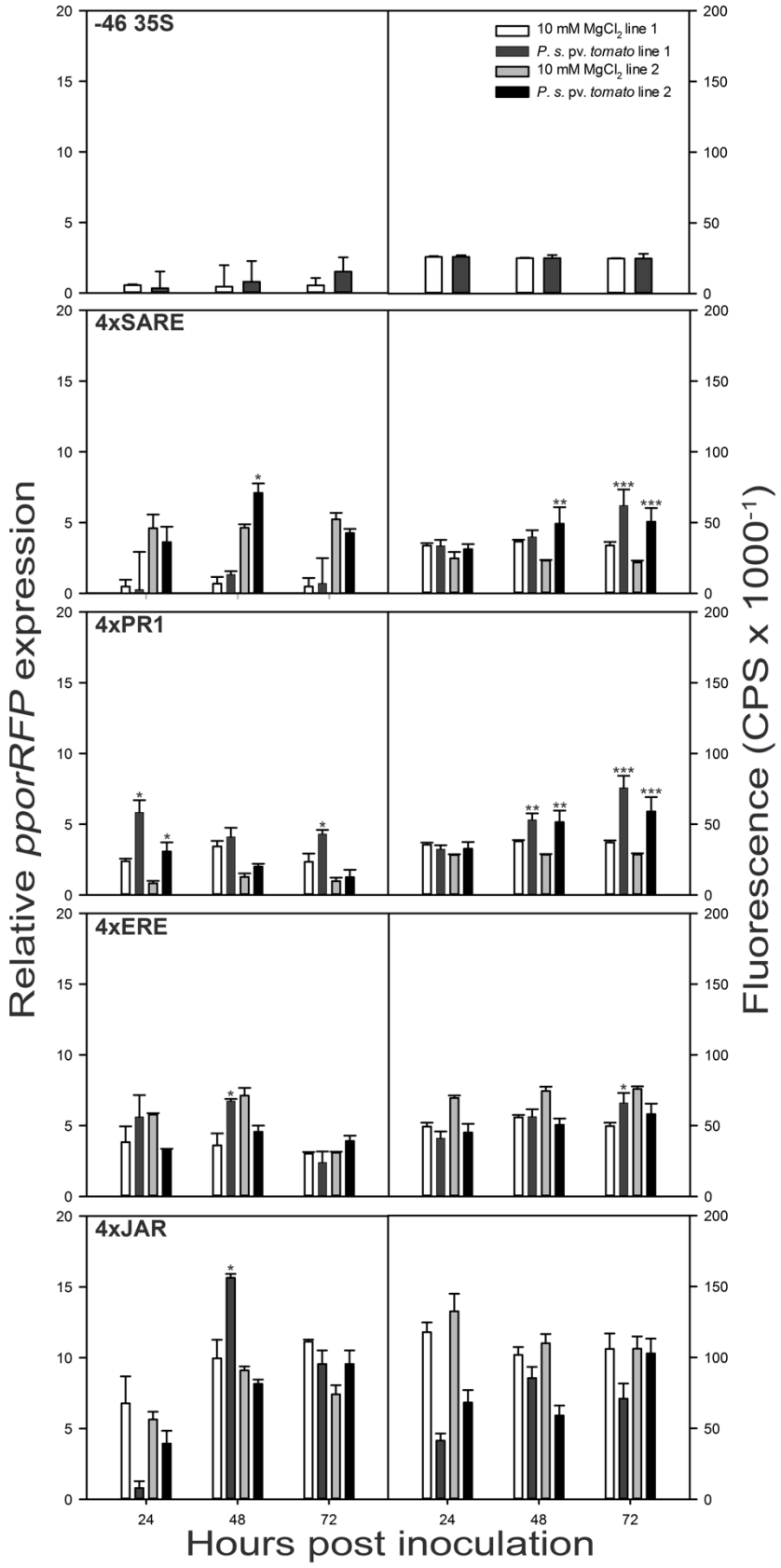

-46 35S
4xSARE
4xPR1
4xERE
4xJAR
10 mM MgCl2 line 1
P. s. pv. tomato line 1
10 mM MgCl2 line 2
P. s. pv. tomato line 2
Relative pporRFP expression
Fluorescence (CPS x 1000-1)
Hours post inoculation
24
48
72

Figure 3. Time course analysis of OFP fluorescence in stable transgenic tobacco following treatments with *P. s.* pv. *tomato* (*Pto*), *P. s.* pv. *tabaci* (*Pst*), and *P. marginalis* (*Pm*) in 2013 field trial.

Fluorescent measurements of the orange fluorescent protein (OFP) reporter were conducted on leaf tissue infiltrated with *Pto* (OD_{600} = 0.3), *Pst* (OD_{600} = 0.03), *Pm* (OD_{600} = 0.3), and 10 mM $MgCl_2$ at 24, 48, and 72 hpi. OFP fluorescence was determined using a Fluorolog®-3 system (Jobin Yvon and Glen Spectra, Edison, NJ, USA) by exciting leaf tissue at 530 nm and scanning the emission from 560 to 605-nm. Data was normalized to mock treated plants harboring -46 35S. Counts per second (CPS) were quantified at 591 nm (Liu *et al.*, 2011). Error bars indicate standard error from two measurements per biological replicate with three biological replicates per experiment; experiments were replicated 4 times (N = 12, n = 24). Significant induction was observed in all constructs. Asterisks indicate statistical significance (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). Each designated line name is indicated above each graph. OFP fluorescent measurement post-bacterial treatments of transgenic lines containing: line harboring -46 35S; line harboring 35S; lines S1 and S2 containing 4×SARE; lines P1, P2, and P3 containing 4×PR1; lines E1, E2, and E3 containing 4×ERE.

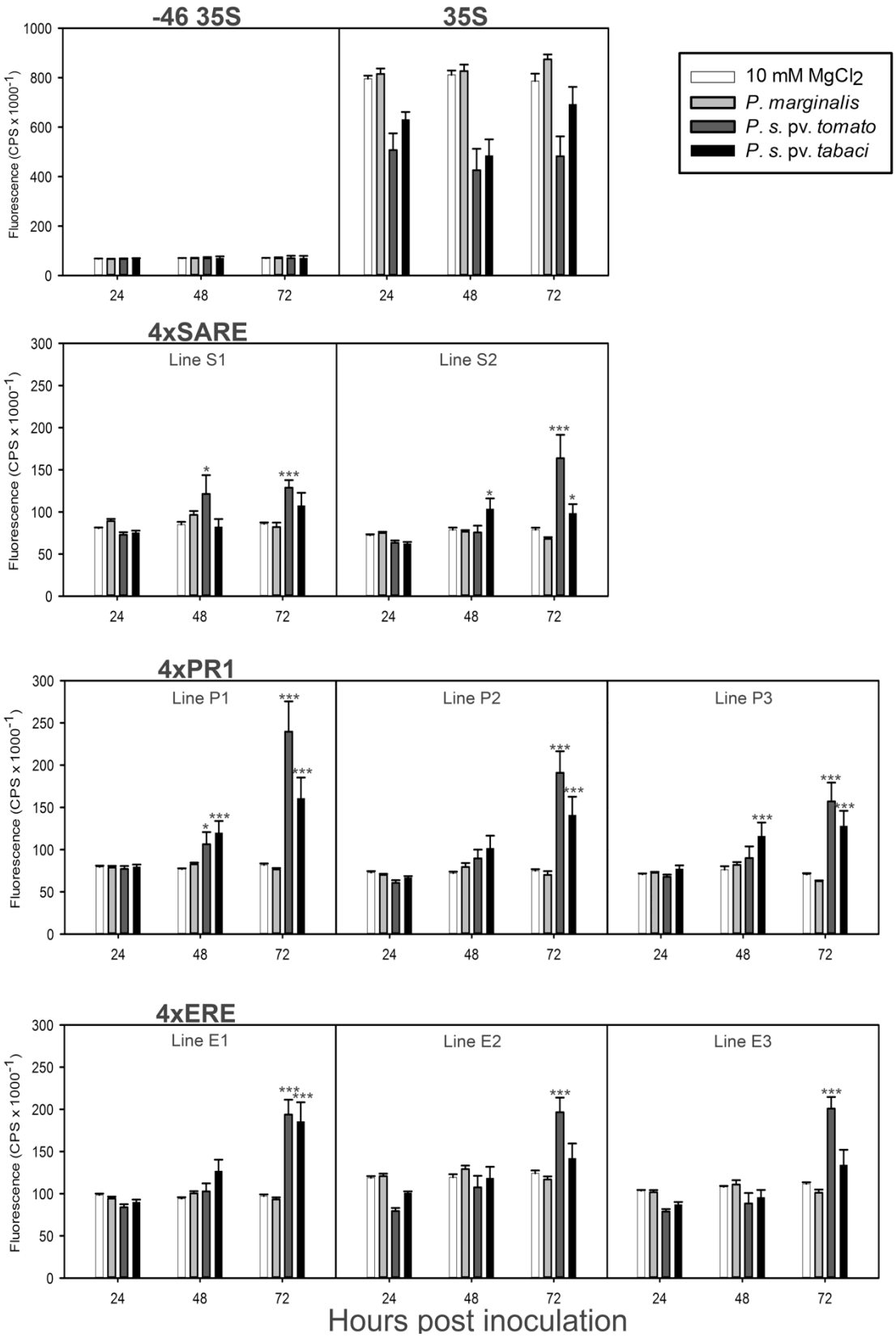
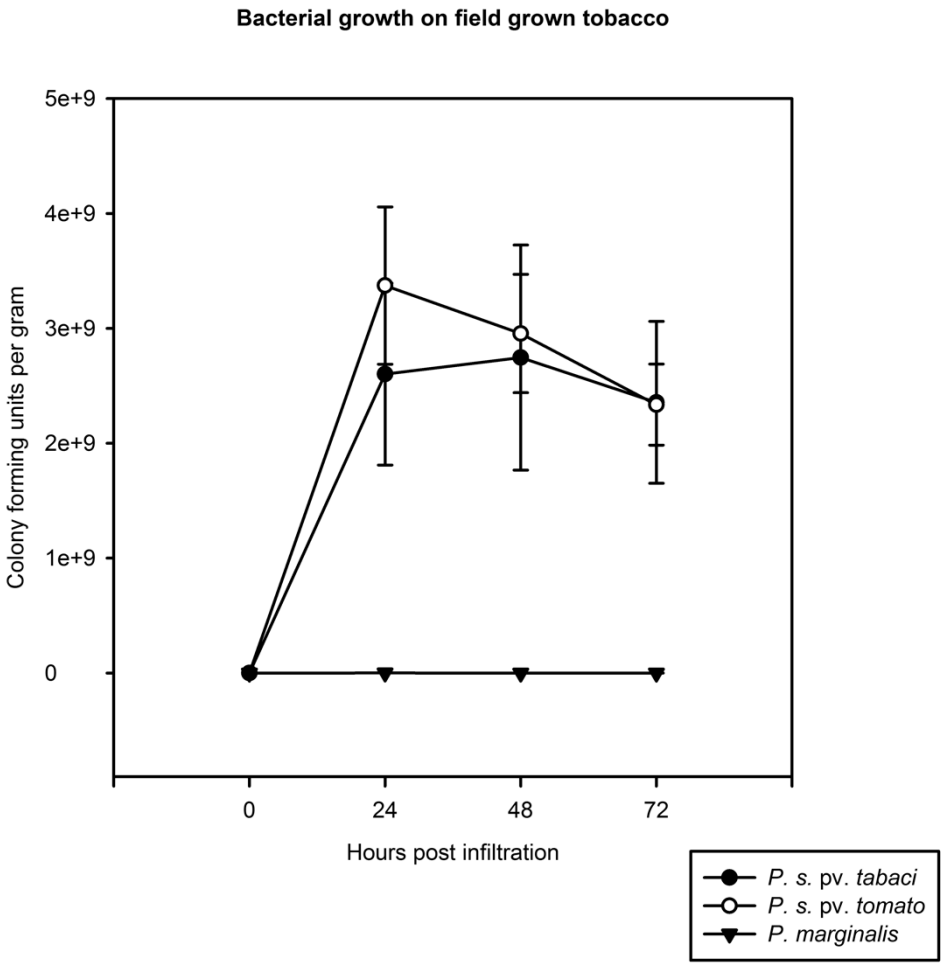

-46 35S
35S
10 mM MgCl2
P. marginalis
P. s. pv. tomato
P. s. pv. tabaci
Fluorescence (CPS x 1000-1)
4xSARE
Line S1
Line S2
4xPR1
Line P1
Line P2
Line P3
4xERE
Line E1
Line E2
Line E3
Hours post inoculation

Figure 4. Time course analysis of bacterial growth of three phytopathogens under field conditions in 2013.

Leaves were infiltrated with pathogens suspended in 10 mM $MgCl_2$ at corresponding OD_{600} (*P. s.* pv. *tomato* = 0.3, *P. s*. pv. *tabaci* = 0.03, *P. marginalis* = 0.3). Each point represents the mean of 3 biological replicates per experiment; experiments were replicated 4 times per treatment and time point (N=12). Bacterial counts were obtained from 0.1 g of infiltrated leaf tissue homogenized in 10 ml of 10 mM $MgCl_2$. Serial dilutions were plated onto King's B medium supplemented with 50 mg L^{-1} rifampicin and bacterial populations were quantified after incubation period (20 - 24 hr for *P. marginalis,* and 42 - 48 hr for *P. s.* pv. *tomato* and *P. s.* pv. *tabaci*). Error bars represent standard errors.


Bacterial growth on field grown tobacco
5e+9
4e+9
3e+9
2e+9
1e+9
0
Colony forming units per gram
0
24
48
72
Hours post infiltration
P. s. pv. tabaci
P. s. pv. tomato
P. marginalis

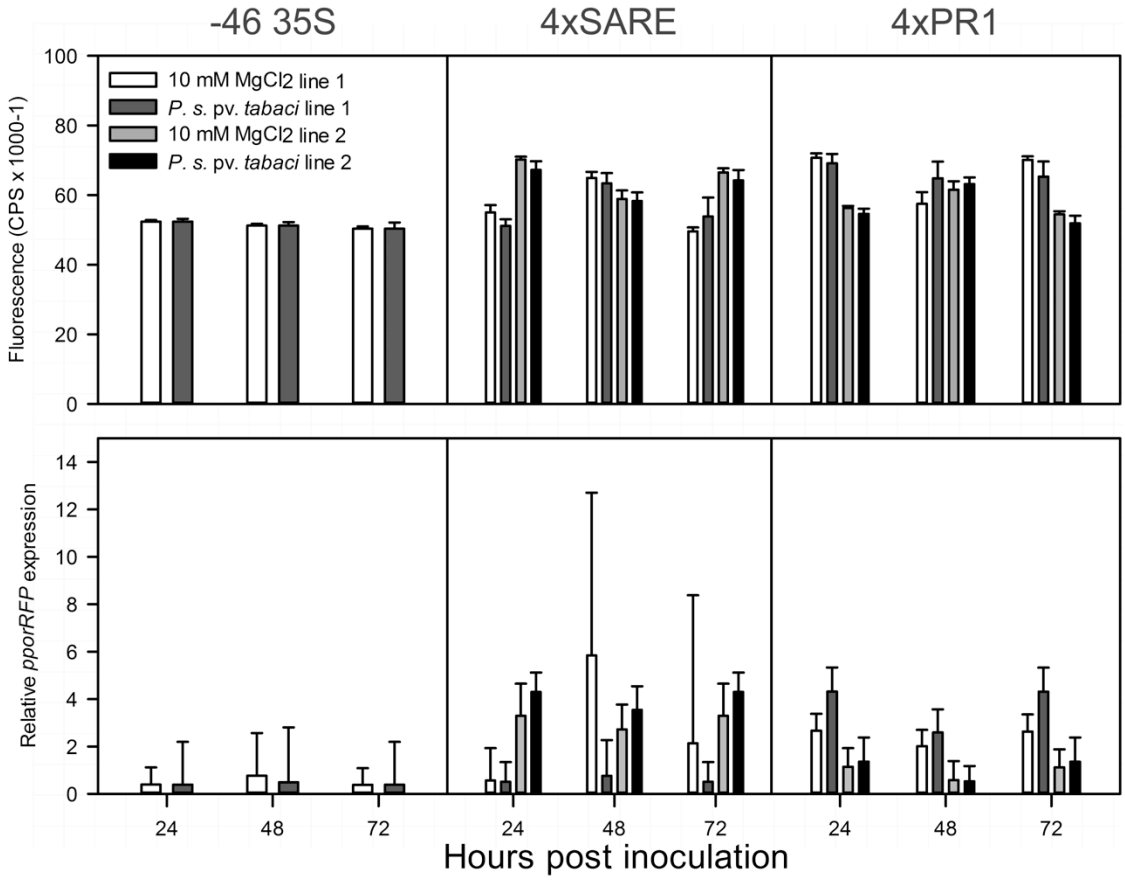
Supporting information:

Table S1. qRT-PCR Primers. Primer sets used for relative quantification of *pporRFP* transcription.

Gene	Accession No.	Function	Primer sequence	Reference
pporRFP	DQ206380	Reporter	5'-CATGGCCTTGAAGTTGGAGAAC-3' 5'-GTTTGTGGTCAACGAAGTGATACG-3'	
L25	L18908	Ribosomal protein	5'-CCCCTCACCACAGAGTCTGC-3' 5'-AAGGGTGTTGTTGTCCTCAATCTT-3'	(Schmidt *et al.*, 2012)
Tac9	X69885	Actin	5'-GAGACGTCAAAGACCAGCTCTTC-3' 5'-GGACCTCAGGACAACGGAAA-3'	(Schmidt *et al.*, 2012)

Figure S1. OFP reporter (i.e., pporRFP) fluorescence and reporter transcript response to Pseudomonas syringae pv. tabaci (Pst) treatment in transgenic lines containing -46 35S, 4×SARE, and 4×PR1 in 2012.

Time course analysis over 72 hours of OFP fluorescence in independent transgenic tobacco lines treated with *Pst* or mock treatment (10 mM $MgCl_2$). Top row: Orange fluorescent protein reporter (OFP) was quantified with a Fluorolog®-3 system (Jobin Yvon and Glen Spectra, Edison, NJ, USA) in leaf tissue infiltrated with *P. s.* pv. *tabaci* (OD_{600} = 0.03) and 10 mM $MgCl_2$ at 24, 48, and 72 hpi, by exciting leaf tissue at 530-nm and scanning emission from 560 – 605-nm. Two measurements were obtained per biological replicate. Error bars represent standard error from two biological replicates per experiment; experiments were replicated 3 times (N=6, n = 12). Data was normalized to mock treated line containing empty vector -46 35S. Counts per second were quantified at 591-nm (Liu *et al.*, 2011). Bottom row: Relative *pporRFP* expression quantified with qRT-PCR normalized to two reference genes (*L25* and *Tac9*) mRNA levels. Lines designated in-text as S stands for 4×SARE, P for 4×PR1; line numbers represent numerical character of in-text designated lines (i.e., line S1 represents line 1 containing 4×SARE). No lines or constructs displayed significant induction throughout our time course analysis. Negative control -46 35S. Lines containing 4×SARE: line 1, S1; line 2, S2. Lines containing 4×PR1: line 1, P1; line 2, P2.


-46 35S
4xSARE
4xPR1
10 mM MgCl2 line 1
P. s. pv. tabaci line 1
10 mM MgCl2 line 2
P. s. pv. tabaci line 2
Fluorescence (CPS x 1000-1)
Relative pporRFP expression
24
48
72
Hours post inoculation

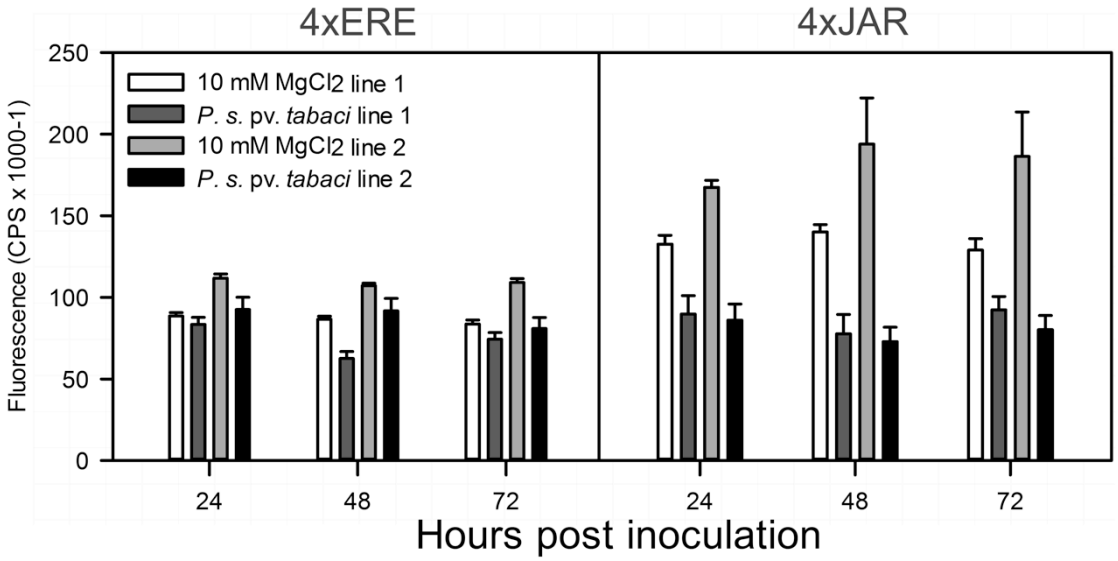


Figure S2. Orange fluorescent protein (OFP) reporter response to *Pseudomonas syringae* pv. *tabaci* (*Pst*) treatment in transgenic tobacco lines containing 4×ERE and 4×JAR in 2012.

OFP fluorescence was quantified with a Fluorolog®-3 system (Jobin Yvon and Glen Spectra, Edison, NJ, USA) in *Pst* and mock-infiltrated leaf tissue (*Pst*: OD_{600} = 0.03; mock treatment: 10 mM $MgCl_2$) at 24, 48, and 72 hours post inoculation, by exciting leaf tissue at 530-nm and scanning emission from 560 − 605-nm. Two measurements were obtained per biological replicate. Error bars represent standard errors from two biological replicates per experiment; experiments were replicated 3 times (N = 6, n = 12). Data was normalized to mock treated line containing empty vector -46 35S. Counts per second were quantified at 591-nm (Liu *et al.,* 2011). Lines designated in-text as E stands for 4×ERE, J for 4×JAR; line numbers represent numerical character of in-text designated lines (i.e., line E1 represents line 1 containing 4×ERE). Lines containing 4×ERE: line 1, E1; line 2, E2. Lines containing 4×JAR: line 1, J1; line 2, J2.

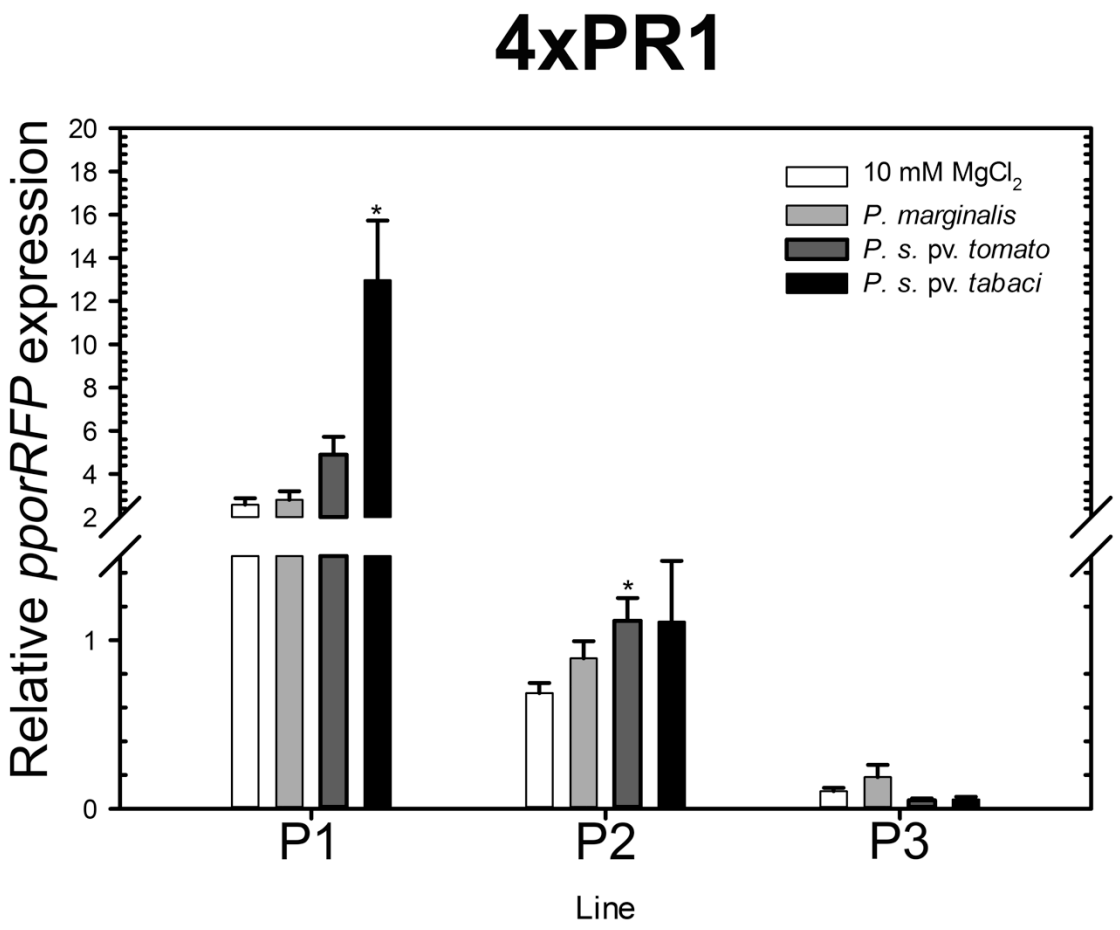


Figure S3. Relative *pporRFP* expression in three transgenic lines harboring 4×PR1 24 hours post treatment with *Pseudomonas marginalis* (*Pm*), *P. syringae* pv. *tomato* (*Pto*), and *P. s.* pv. *tabaci* (*Pst*) in 2013.

Relative *pporRFP* expression quantified with qRT-PCR normalized to two reference genes (*L25* and *Tac9*) mRNA levels with the ΔΔCt method (Schmittgen and Livak, 2008). Error bars represent standard error from three biological replicates. Asterisks indicate statistical significance (* $P < 0.05$).

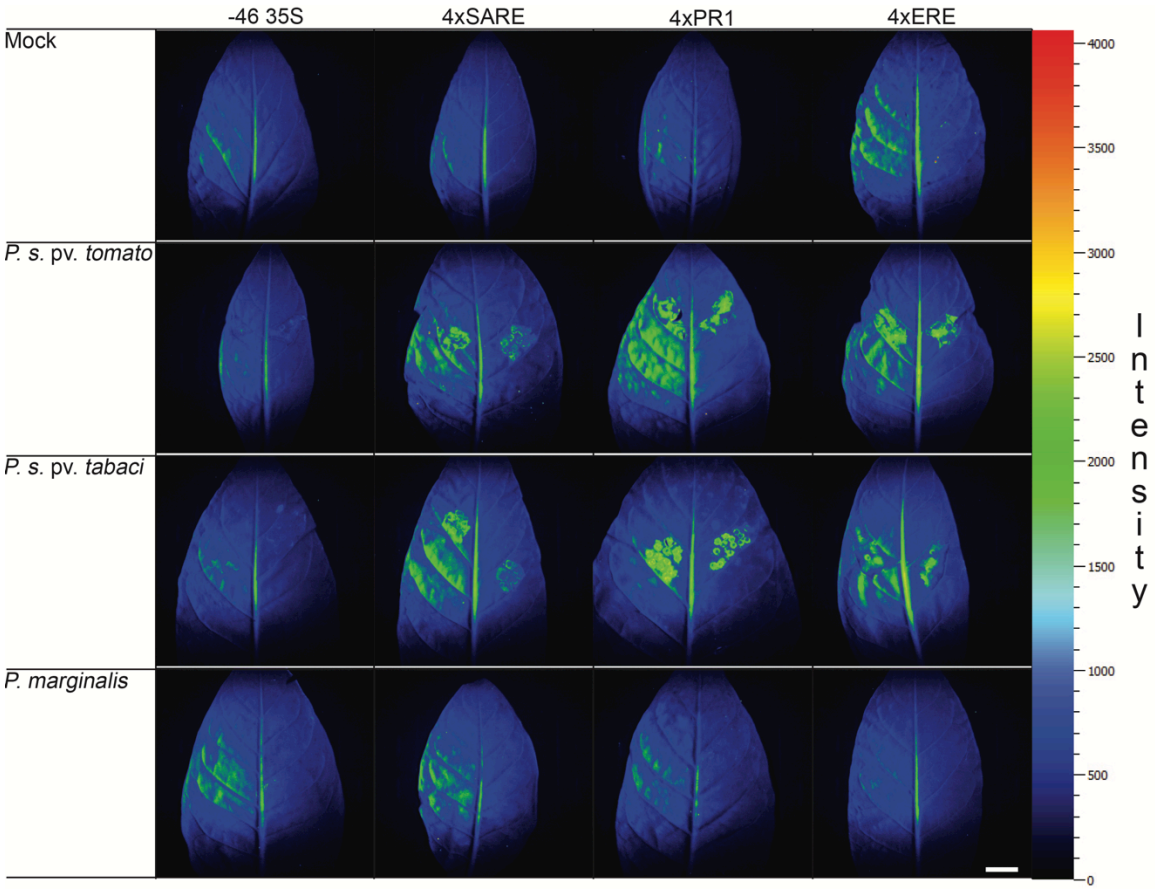


Figure S4. . Images of the orange fluorescent protein reporter fluorescing in treated leaf tissue from 2013 field study.

Images (representative) of treated leaf tissue were captured at 72 hours post inoculation with a closed Fluorocam (Photon Systems Instruments, Drasov, Czech Republic) under green light at 13% intensity (535 – 540-nm) through a 593/46 single band pass filter (Semrock, Rochester, NY, USA) exposure time was 10-ms, and sensitivity was set to 14%. Scale bar represents 2-cm. Stable transgenic tobacco lines containing synthetic promoter constructs are labeled at the top of the figure; phytobacterial treatment label (left); fluorescence heatmap intensity in arbitrary units (right).

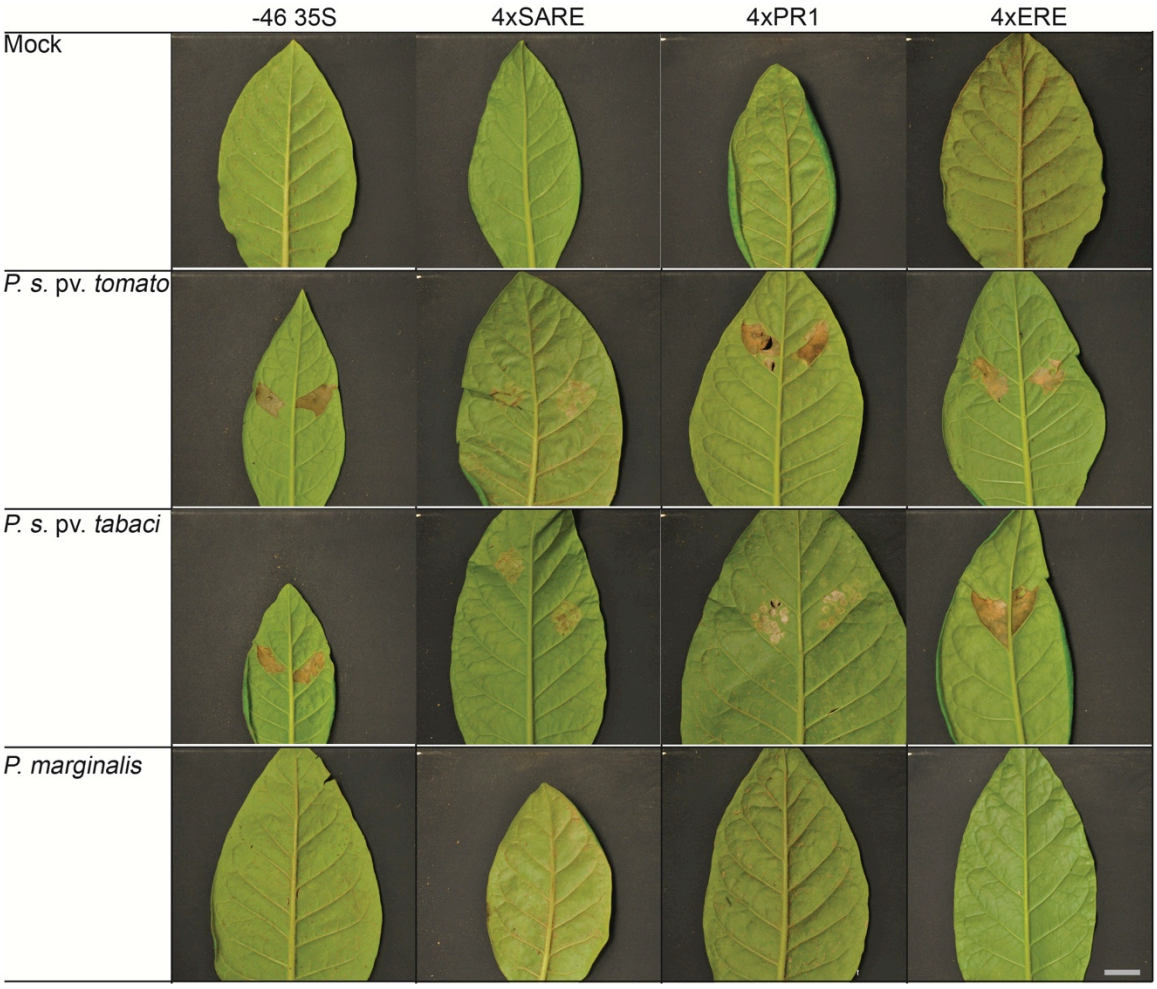


Figure S5. Corresponding visual images of Figure S3.

Captured on Nikon D90 through an 18.0 – 105.0-mm lens. Scale bars represent 2-cm.

Figure S6. Field design used in 2012 of a completely randomized block design at the Plant Sciences East Tennessee Research and Education Center in Knoxville, TN, USA. Bacterial treatments applied to treatment plots alongside mock treated plots.

Constructs indicated by pattern (4×SARE = ▒, 4×PR1 = ■, 4×ERE = ▨, 4×JAR = □) and plant lines by numeric character (1 & 2 indicate lines without enhancer; 3 & 4 indicate lines with enhancers; 5, 6, and 7 are controls -46 35S, 35S, B_A respectively). Experiments were replicated three times; horizontal lines separate each replicate. Plots were 13 × 40-m. Subplots were 0.5 × 0.5-m and contained 3 biological replicates of corresponding line and construct. A 3-meter buffer zone separated mock from treatment plots and replicates. N = 36 per line.

Treatment plots

3	1	2	4
5	4	3	7
4	7	4	1
1	5	7	3
6	2	5	6
2	6	6	5
7	3	1	2
6	6	7	3
2	7	1	1
3	1	6	4
7	3	4	7
5	2	2	2
1	5	5	5
4	4	3	6

4	2	7	1
7	4	2	5
1	1	3	7
3	5	5	2
5	3	6	4
6	7	1	6
2	6	4	3
3	4	7	6
2	1	6	1
5	3	2	2
4	2	4	7
1	6	1	4
6	7	5	5
7	5	3	3

4	3	6	2
7	4	4	1
3	7	1	7
6	6	3	6
5	1	2	4
1	5	5	3
3	2	7	5
2	1	5	1
6	4	6	3
1	2	4	7
7	3	3	6
5	6	7	5
4	7	1	4
3	5	2	2

3 m

Mock plots

7	2	3	2
6	3	2	6
3	1	4	3
1	7	5	1
5	4	7	7
2	5	6	5
4	6	1	4
5	2	5	7
1	1	3	1
3	3	4	2
2	4	1	4
4	6	2	3
6	5	6	6
7	7	7	3

5	4	5	1
3	5	2	5
7	3	4	6
4	2	7	3
1	7	1	7
2	1	6	4
6	6	3	2
4	3	2	3
6	2	6	7
2	6	7	2
3	3	4	6
1	1	1	5
5	4	3	1
7	7	5	4

4	3	4	4
1	5	2	1
3	7	1	3
2	6	6	2
5	4	7	5
7	1	5	7
6	2	3	6
5	4	3	5
7	2	5	7
4	1	7	4
6	6	6	6
3	7	4	3
1	5	1	1
2	3	2	2

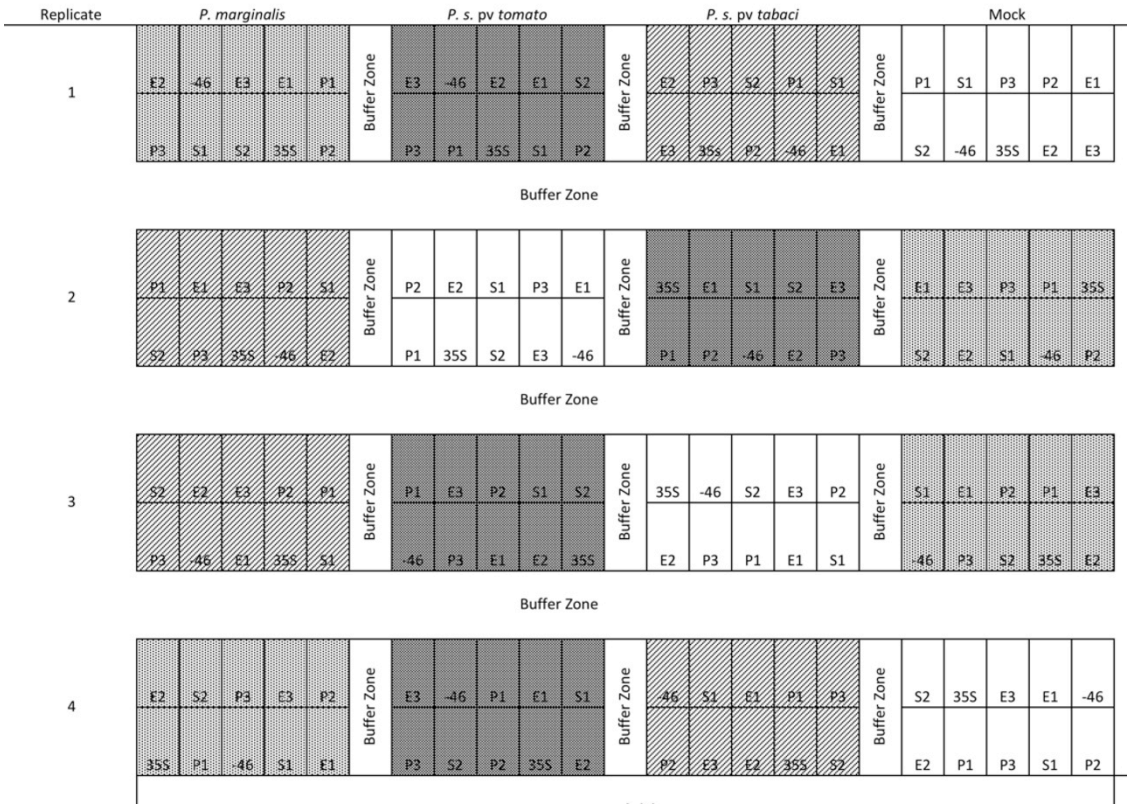


Figure S7. Field layout used in 2013 of a completely randomized block design at the Plant Sciences East Tennessee Research and Education Center in Knoxville, TN.

Treatments labeled by pattern (top), and lines containing constructs labeled by letter (-46 = -46 35S, 35S = 35S, S= 4×SARE, P = 4×PR1, E = 4×ERE). Lines of each construct labeled by numeric character. Controls -46 35S and 35S contain only one line so no line number is indicated here. A 3-m buffer zone separated each treatment and replicate. Treatment plots were 10 m by 3 m. Subplots of individual lines within treatment plots were 2 × 1.6-m. Each subplot contained 3 biological replicates of corresponding line. Experimental design contained 4 replicates. N = 48 per individual line.

CHAPTER III: CONCLUSION

To determine if transgenic lines harboring synthetic pathogen-inducible promoters would maintain their OFP fluorescence inducibility after phytopathogen treatments, I conducted a two-year field study with these transgenic lines. The study was conducted with a rigorous experimental design of a completely randomized block design and a treatment design of a split-split plot. The plots were split on phytopathogen treatment and also on transgenic lines containing different synthetic inducible promoters. Then, a time-course study of this system was conducted to monitor the induction of OFP fluorescence over a 72-hour period. Therefore, this design was able to evaluate the effect of transgenic construct or line, time, and most importantly phytopathogens on the OFP fluorescence induction; in addition, this study determined the best transgenic lines for bacterial phytosensing within the 72-hour time course study and if the OFP reporter induction was specific to a phytopathogen treatment. It was found that the transgenic lines containing 4×PR1 were the most consistent in inducing OFP reporter fluorescence after *Pto* and *Pst* treatments.

Differences from previous growth-chamber studies and field experiments were shown (Liu et al., 2013; Liu et al., 2011). For example, transgenic lines harboring 4×JAR did not display OFP fluorescence induction after any phytopathogen treatment or at any time point in the field (Figure S2). Furthermore, growth-chamber studies yielded significant OFP fluorescence induction in transgenic lines harboring enhancer elements. The field research, however, showed that most transgenic lines harboring enhancer elements actually yielded no induction under field conditions (data not shown). This discovery led to the removal of these constructs from future studies. Furthermore, the early induction that was observed by Liu *et al*. (2013) in transgenic lines containing salicylic acid-responsive promoters was not observed after *Pto* treatments under

field conditions. These same transgenic lines in the field showed the earliest induction at 48 hpi and increased by 72 hpi instead of the laboratory-characterized induction at 24 hpi and either maintained or reduced OFP fluorescence after 24 hpi (Liu et al., 2013).

Therefore, it is clear that the phytosensing system employed here needs further refinements when producing the second-generation of pathogen-sensing plants. Previous research showed that combinations of different *cis*-acting regulatory elements could yield a more specific response to phytopathogens (Rushton et al., 2002). The present system does yield significant induction, however, a larger fold-change may be needed in an inducible system for clear 'digital' detection. A solution to this could employ an auto-feedback loop incorporating repressor to minimize runaway expression (Czarnecka et al., 2012); the resulting system may have lower background expression and yield higher fold-change. Another issue with the current phytosensing plants is the reporter (i.e., *pporRFP*), which could also be improved. While previous studies indicated *pporRFP* as one of the brightest reporters in plants (Mann et al., 2012), there have been no studies to characterize the rate-limiting step for fluorescence production in plants, which is chromophore oxidation or protein maturation. Thus, for future studies two options exist to better characterize phytosensor's OFP fluorescence inducibility: use a well characterized reporter such as mRFP1 (Campbell et al., 2002) or VENUS (Nagai et al., 2002), or conduct studies to characterize the protein maturation, chromophore oxidation, and degradation of OFP. Finally, with rate-limiting steps determined a model would need to be constructed to determine how fluorescence or reporter levels relate to pathogenic bacteria levels in the field as previously done with auxin levels (Band et al., 2012).

Although this study's scope was to determine the OFP fluorescence induction caused by bacterial pathogens it could be of interest to conduct similar studies on fungal and insect

pathogens in the field. A potential application of an insect phytosensor would allow monitoring of the herbivorous insect population levels and facilitate precise applications of insecticides in the field. Thus, insect phytosensors could reduce costs and negative environmental effects of detrimental chemical applications (Skottrup et al., 2008).

The concept of biosensing is feasible with any elicitor that induces gene expression or a quantitative phenotypic change; thus most hazardous chemicals cause induction of certain genes or changes in phenotype and would allow the creation of biosensors for many hazardous materials (biological or chemical). The potential impact of biosensors could allow more precise and targeted applications of pesticides, herbicides, or facilitate remediation of polluted areas. Therefore, the potential of biosensors has yet to be fully realized. There is great work to be done in the field of biosensors. While most biosensors incorporate enzymatic substrates to detect concentrations of elicitors the incorporation of biological systems as biosensors is a cheaper alternative. The annual market for biosensors is expected to exceed $100 billion USD (Ford, 2013); while this includes medical biosensors (i.e.,blood glucose monitoring system) it demonstrates that the market for affordable detection is growing at a rapid rate.

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VITA

Michael Harrison Fethe was born in Knoxville, TN to Chuck and Mary Fethe. He showed interest in math and science from a very young age: subjects he exceled at. He attended Farragut High School from 2003 to 2007. After high school, he attended the University of Tennessee and obtained a Bachelors degree in Microbiology. During his undergraduate career, he obtained a job within the Stewart lab. This position eventually led to a graduate position on field research with transgenic tobacco. This thesis culminates his work of three years at the Stewart lab.