

The performance of pathogenic bacterial phyto-sensing transgenic tobacco in the field

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Summary

Phyto-sensors 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 2 years of field experiments using a subset of these bacterial phyto-sensing tobacco plants. Time-course analysis of field-grown phyto-sensors showed that a subset of plants responded predictably to treatments with *Pseudomonas* phytopathogens. There was a twofold 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 h postinoculation (h p.i.) upon inoculation with two phytopathogens *Pseudomonas syringae* pv. *tomato* and *P. syringae* pv. *tabaci*, respectively. These results demonstrate transgenic tobacco harbouring a synthetic inducible promoter-driven OFP could be used to facilitate monitoring and early-warning reporting of phytopathogen infections in agricultural fields.

Keywords: transgenic plants, phyto-sensor, synthetic promoters, phytopathogen sensing, phyto-bacterial pathogens, field tested.

Introduction

Estimates indicate plant pests and diseases account for 20%–40% reduction of annual global agricultural production (Ronald and Adamchak, 2008). Currently, plant pathogens are typically detected postsymptomatically in the field. Therefore, new solutions for early detection of pathogens are needed to address the postsymptomatic 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 phyto-sensors may help address this need.

Cis-acting regulatory elements employed in previous studies showed significant inducibility after application of phyto-hormones and phytopathogens (Mazarei *et al.*, 2008; Rushton *et al.*, 2002). Mazarei *et al.* (2008) defined the four most highly inducible regulatory elements, that is, 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. four head-to-tail copies; Figure 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 phyto-sensing under controlled growth chamber conditions (Liu *et al.*, 2011, 2013a). These synthetic constructs in stable and transient phyto-sensors displayed significantly induced expression of the reporter gene post-treatment with phyto-hormones and bacterial pathogens such as *Pseudomonas syringae* pv. *tomato* (*Pto*), *P. syringae* 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). Soilborne *Pm* causes ‘soft-rot’ disease.

To date, phyto-sensors 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, that is, attenuated transgene expression in the field (Brandle *et al.*, 1995; de Carvalho *et al.*, 1992; Conner *et al.*, 1994; 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 phyto-sensors was treated with the 3 phyto-bacterial pathogens (i.e. *Pto*, *Pst* and *Pm*) to assess the utility of these phyto-sensors in the field. To our knowledge, this was the first field assessment of phyto-sensors for plant pathogens or any other target contaminant.



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).

Results

We conducted a 2-year field trial of transgenic plants expressing an OFF 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, Tenn, 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 T₂ 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 T₂ 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 T₂ 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* as 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 7 weeks in 2012 and 6 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 (nonfluorescence) 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; that is, the experimental treatments of plant pathogens accounted for all of the plants' responses in the field during our 2-year study.

Table 1 *Cis*-acting regulatory elements (RE) used in construction of phytosensing cassettes [subtracted from Liu *et al.* (2013a)]

| RE | Sequence (5' > 3') | Promoter origin | Species origin | Source |
|------|--|-----------------|-----------------------------|--|
| SARE | TTCGACTCCAAAGAGGACCCAGAAT | <i>PR2-d</i> | <i>Nicotiana tabacum</i> | Shah and Klessig (1996) |
| PR1 | ACGTCATAGATGTGGCGGCATATATTCTTCAGGACTTTTC | <i>PR1</i> | <i>Arabidopsis thaliana</i> | Lebel <i>et al.</i> (1998) |
| ERE | CAGCCGCCAAAGAGGACCCAGAAT | Chitinase | <i>N. tabacum</i> | Brown <i>et al.</i> (2003); Ohme-Takagi and Shinshi (1995); Rushton <i>et al.</i> (1996) |
| JAR | CAACGAC ACGCCAAAT TCTAATTTAGCACAGTCT ACGTG | <i>VSP1</i> | <i>A. thaliana</i> | Guerineau <i>et al.</i> (2003) |

ERE, ethylene-responsive element; JAR, jasmonic acid-responsive element; SARE, salicylic acid-inducible element.

The core sequences in each RE are in bold.

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

| Treatments per year | 2012 | 2013 |
|--|------|------|
| <i>Pseudomonas syringae</i> pv. <i>tomato</i> | + | + |
| <i>P. syringae</i> pv. <i>tabaci</i> | + | + |
| <i>P. marginalis</i> | – | + |
| 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 (weeks after transplanting) | 7–15 | 6–9 |
| Precipitation (deviation from normal in cm) | +13 | +28 |
| Temperature (deviation from normal in °C) | -1.1 | 0 |

ERE, ethylene-responsive element; JAR, jasmonic acid-responsive element; SARE, salicylic acid-inducible element.

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 OFF fluorescence and reporter transcript levels comparable to mock treatment during our time-course analysis (Figures 2 and 3, Figures S1 and S2). 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 OFF fluorescence at 48 and 72 h p.i. in transgenic line S1 (S designates lines containing 4 × SARE) harbouring 4 × SARE motifs (Figure 2). At 48 h p.i. with *Pto* treatment of the S2 line, the OFF fluorescence was twofold higher (Tukey mean separation, *P* < 0.01; Figure 2) and *pporRFP* mRNA was 1.5-fold higher than in mock-treated samples (unpaired Student's *t*-test, *P* < 0.05, Figure S2). Another line S1

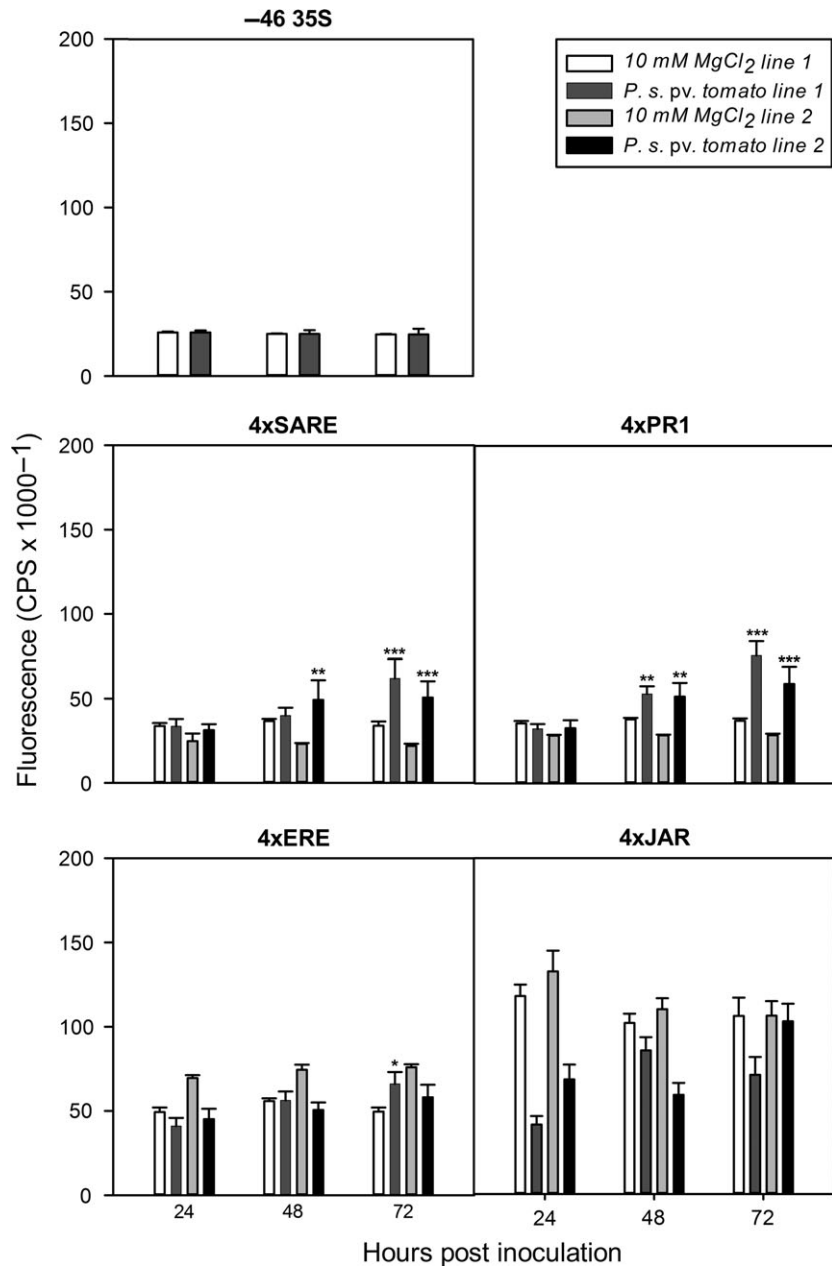


Figure 2 Time-course analysis of orange fluorescent protein (OFP) fluorescence levels in stable transgenic tobacco following *Pseudomonas syringae* pv. *tomato* (*Pto*) treatment in 2012 field trial. Fluorescent measurements of the OFP reporter were conducted on leaf tissue infiltrated with *Pto* ($OD_{600} = 0.3$) and 10 mM $MgCl_2$ at 24, 48 and 72 h p.i. Quantifications of the *pporRFP* OFP were made on a Fluorolog[®]-3 system (Jobin Yvon and Glen Spectra) by exciting leaf tissue at 530 nm and scanning the emission from 560 to 605 nm. Data were normalized to the mock-treated plants harbouring -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 three times ($N = 6$, $n = 12$). Asterisks indicate statistical significance (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). 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). Control line harbouring -46 35S. Lines harbouring 4 × SARE: line 1, S1; line 2, S2. Lines harbouring 4 × PR1: line 1, P1; line 2, P2. Lines harbouring 4 × ERE: line 1, E1; line 2, E2. Lines harbouring 4 × JAR: line 1, J1; line 2, J2.

containing 4 × SARE only showed significant induction in OFP fluorescence of 1.8-fold over the mock-treated samples at 72 h p.i. (Tukey mean separation, $P < 0.01$), while the *pporRFP* mRNA level was apparently slightly increased at 48 h p.i. (Figure S2). In 2013, the *Pto* treatment of the S1 line resulted in induced OFP fluorescence to 1.4-fold at 48 h p.i. (Tukey mean separation, $P < 0.01$) and 1.5-fold at 72 h p.i. (Tukey mean separation, $P < 0.001$) over the mock-treated samples (Figure 3). *Pto* treatment of the S2 line showed a late induction with a twofold increase in OFP fluorescence compared with mock-treated samples at 72 h p.i. (Tukey mean separation, $P < 0.001$; Figure 3). However, the *Pst* treatment did not cause detectable OFP fluorescence induction in transgenic lines S1 and S2 in 2012 (Figure S1). Following *Pst* treatment in 2013, transgenic line S2 exhibited 1.3-fold of OFP fluorescence above mock-treated samples at 48 h p.i. (Tukey mean separation, $P < 0.05$), followed by a slight 1.2-fold induction in OFP fluorescence above the

mock-treated samples at 72 h p.i. (Tukey mean separation, $P < 0.001$; Figure 3). OFP fluorescence in the S1 line after *Pst* treatment remained unchanged in 2013 (Figure 3).

Most notable was the *Pto*-induced OFP fluorescence in transgenic lines harbouring 4 × PR1 regulatory elements, in which the OFP fluorescence in 2012 showed significant induction of approximately 1.5-fold at 48 h p.i. (Tukey mean separation, $P < 0.01$) and twofold at 72 h p.i. (Tukey mean separation, $P < 0.001$) compared with the mock-treated samples, even though the OFP fluorescence induction remained undetected at 24 h p.i. (Figure 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 h p.i. compared with the mock-treated samples (unpaired Student's *t*-test, $P < 0.05$), and *pporRFP* transcription was reduced yet remained slightly increased compared with mock

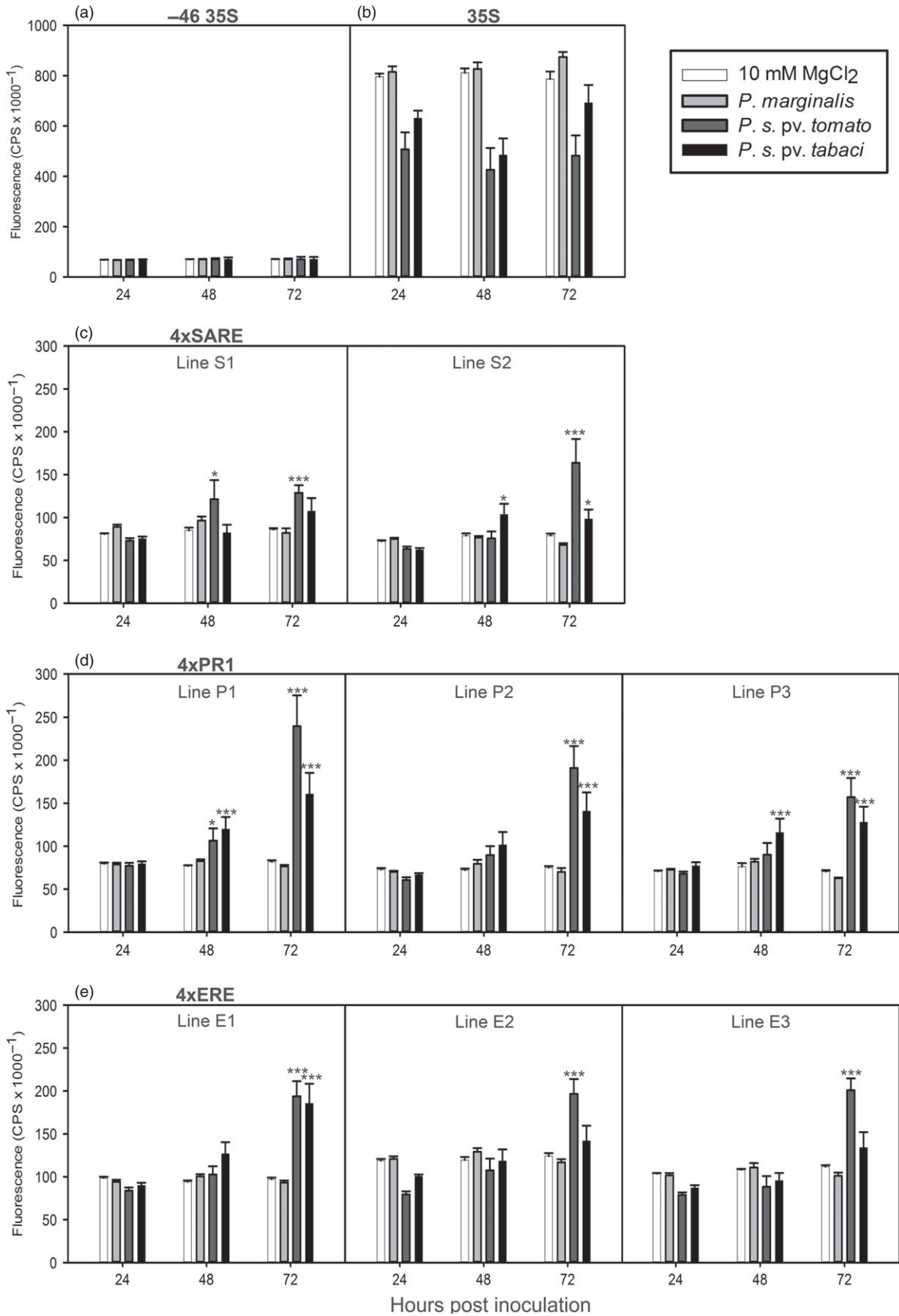


Figure 3 Time-course analysis of orange fluorescent protein (OFP) fluorescence in stable transgenic tobacco following treatments with *Pseudomonas syringae* pv. *tomato* (*Pto*), *P. syringae* pv. *tabaci* (*Pst*) and *P. marginalis* (*Pm*) in 2013 field trial. Fluorescent measurements of the OFP reporter were conducted on leaf tissue infiltrated with *Pto* (OD₆₀₀ = 0.3), *Pst* (OD₆₀₀ = 0.03), *Pm* (OD₆₀₀ = 0.3) and 10 mM MgCl₂ at 24, 48 and 72 h p.i. OFP fluorescence was determined using a Fluorolog[®]-3 system (Jobin Yvon and Glen Spectra) by exciting leaf tissue at 530 nm and scanning the emission from 560 to 605 nm. Data were normalized to mock-treated plants harbouring -46 35S. Counts per second 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 four 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 postbacterial treatments of transgenic lines containing: control line harbouring -46 35S; positive control line harbouring 35S; lines S1 and S2 containing 4 × SARE; lines P1, P2 and P3 containing 4 × PR1; lines E1, E2 and E3 containing 4 × ERE.

samples at 48 h p.i. in P1 and P2 lines (Figure S2). Interestingly, the line P1 displayed a significant induction of *pporRFP* transcript of twofold higher than the mock-treated samples at 72 h post-*Pto* inoculation (unpaired Student's *t*-test, $P < 0.05$; Figure S2). Line P3 was added to our experimental design in 2013. In 2013, relative *pporRFP* expression was increased in two of three transgenic lines containing 4 × PR1 by phytopathogen treatments *Pto* and *Pst* at 24 h p.i. (Figure S4). *Pto* inoculation significantly induced OFP fluorescence in transgenic line P1 to approximately 1.4-fold at 48 h p.i. (Tukey mean separation, $P < 0.05$) and showed a significant twofold to threefold increase in the OFP fluorescence in transgenic lines P1, P2 and P3, which was observed at 72 h p.i. 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 h p.i. in all the three lines (Figure 3). While *Pst* treatment on transgenic lines harbouring 4 × PR1 yielded OFP fluorescence levels comparable to mock treatment in 2012, we observed significant induction in transgenic lines harbouring 4 × PR1 post-*Pst* inoculation in 2013. All lines harbouring 4 × PR1 yielded significantly induced OFP fluorescence at 72 h p.i. (Tukey mean separation, $P < 0.001$, Figure 3; Figure S5). Furthermore, transgenic lines P1 and P3 displayed OFP induction of approximately 1.5-fold over mock-treated samples as early as 48 h p.i. with *Pst* (Tukey mean separation, $P < 0.001$, Figure 3). Thus, transgenic lines containing 4 × PR1 displayed the earliest, highest and most consistent induction in our 2-year studies.

Treatment of transgenic phyto-sensing 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; Figure 3).

Inducibility of ethylene-/jasmonate-responsive promoters

Previous studies showed that transgenic lines harbouring 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 harbouring 4 × ERE at 24 or 48 h p.i. 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 h p.i. with *Pto* treatments (Tukey mean separation, $P < 0.05$; Figure 2). Transcript analysis also showed a 1.9-fold induction in the *pporRFP* mRNA level in the transgenic line E1 at 48 h p.i. with *Pto* treatments (unpaired Student's *t*-test, $P < 0.05$, Figure S2). 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 h p.i. with induction from 1.5-fold to 2.0-fold above the mock-treated samples (Tukey mean separation, $P < 0.001$) (Figure 3). A similar pattern was observed with *Pst* treatment of transgenic line E1—the OFP fluorescence induction was twofold of mock fluorescence at 72 h p.i. 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 h p.i. with any phytopathogen treatment (Figures 2 and 3). At 72 h p.i. with *Pst*, the induction in the OFP fluorescence in line E1 was 1.9-fold above the mock treatment. All transgenic lines harbouring 4 × ERE exhibited a significantly induced OFP fluorescence from 1.5-fold to 2.0-fold over mock treatments at 72 h p.i. after *Pto* treatment (Tukey mean separation, $P < 0.001$) (Figure 3). All ethylene-responsive promoters remained comparable to mock-treated samples postinfiltration of *Pm* throughout our time-course analysis in 2013 (Figure 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 (Figure S3), with the exception of the line J1 (J designates lines containing 4 × JAR) harbouring 4 × JAR which displayed significantly increased *pporRFP* mRNA level of 1.6-fold at 48 h p.i. with *Pto* infection over the mock-treated samples (unpaired Student's *t*-test, $P < 0.05$) (Figure S2). 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 harbouring the synthetic promoters displayed significant induction in OFP fluorescence after *Pto* treatment (Figures 2 and 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 h 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 h p.i. with cellular collapse that developed between 24 and 48 h p.i. Growth of *Pto* was observed within 24 h p.i. *Pto* levels showed a steady decrease from 24 to 72 h p.i. from 3.37×10^9 to 2.33×10^9 colony-forming units (CFU)/g throughout the time-course analysis (Figure 4). Bacterial enumeration of *Pto* showed a steady decrease in this population after 24 h p.i.; however, the levels of *Pto* remained high in the field, above 10^9 CFU/g (Figure 4).

Bacterial symptoms progressed on the field-grown plants at the same rate as in our growth chamber experiments (Table 3) after application with *Pst* (Liu et al., 2013a). The inoculation of *Pst* caused wildfire disease characterized by chlorosis at 24 h p.i., followed by necrosis and hyponasty of the infected area at 72 h p.i. (Liu et al., 2013a). *Pst*-inoculated tissue displayed bacterial growth within 24 h p.i. The *Pst* population at 24 h p.i. with 2.6×10^9 CFU/g proliferated minimally to 2.74×10^9 CFU/g at 48 h p.i. and decreased by 72 h p.i. to 2.35×10^9 CFU/g

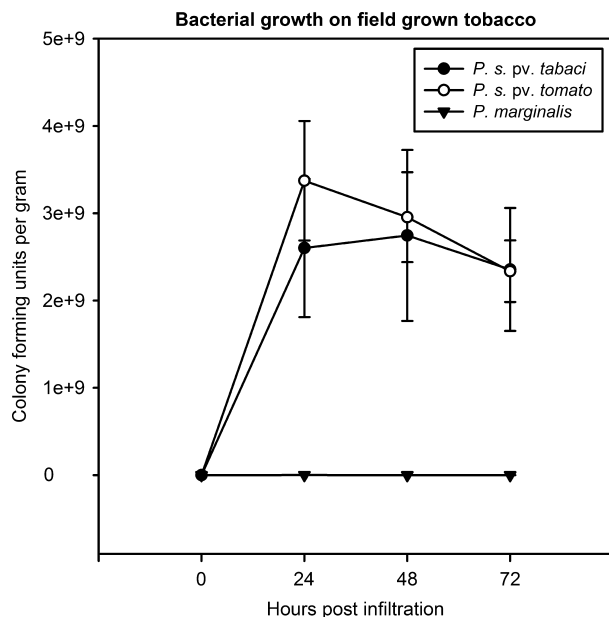


Figure 4 Time-course analysis of bacterial growth of three phytopathogens under field conditions in 2013. Leaves were infiltrated with pathogens suspended in 10 mM MgCl₂ at corresponding OD₆₀₀ (*Pseudomonas syringae* pv. *tomato* = 0.3, *P. syringae* pv. *tabaci* = 0.03 and *P. marginalis* = 0.3). Each point represents the mean of three biological replicates per experiment; experiments were replicated four 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 MgCl₂. Serial dilutions were plated onto King's B medium supplemented with 50 mg/L rifampicin, and bacterial populations were quantified after incubation period (20–24 h for *P. marginalis* and 42–48 h for *P. syringae* pv. *tomato* and *P. syringae* pv. *tabaci*). Error bars represent standard errors.

(Figure 4). Bacterial counts of *Pst* were consistently above 10^9 CFU/g 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 (Figure S6). *Pm* enumeration showed a steady decrease in bacterial counts after 24 h p.i., with 8.47×10^5 CFU/g at 24 h p.i. and ending at 72 h p.i. with 5.99×10^4 CFU/g (Figure 4). Thus, *Pm* inoculum levels showed no proliferation and remained below 10^6 CFU/g in our time-course study.

Discussion

Our study evaluated the field performance of transgenic tobacco plants for bacterial pathogen phytosensing over the course of 2 years of field experiments to determine whether synthetic promoters maintain inducibility under field conditions. As 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 harbouring 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 phyto-bacterial 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 HR in tobacco during an incompatible plant–pathogen interaction with *Pto* develops within 24–48 h p.i. (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 harbouring 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 (Liu et al., 2013c). Also noteworthy, lines P1 harbouring 4 × PR1 and S2 harbouring 4 × SARE were induced within 48 h p.i. with *Pto*; these lines displayed a HR-induced OFP fluorescence

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

| Pathogen | <i>Nicotiana tabacum</i> |
|---|--|
| <i>Pseudomonas syringae</i> pv. <i>tomato</i> | Nonhost; Hypersensitive response (HR) within 24 h p.i., necrosis at OD ₆₀₀ = 0.3 |
| <i>P. syringae</i> pv. <i>tabaci</i> | Host; Normal sensitive 'Wildfire' symptom within 48–72 h p.i. at OD ₆₀₀ = 0.03 |
| <i>P. marginalis</i> | Nonhost; Very mild or no symptom at OD ₆₀₀ = 0.3 |

within 48 h p.i. (Figures 2 and 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' defences involved in a pathogen attack under field conditions when the OFP reporter is controlled by pathogen defence-related regulatory elements.

The ethylene-responsive promoter 4 × ERE showed a late OFP induction at 72 h p.i. 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 harbouring synthetic pathogen-inducible promoters (Figures S1 and S3) yet induced lines harbouring 4 × SARE, 4 × PR1 and 4 × ERE in 2013 (Figure 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 (Figures 2 and 3). For example, two lines containing 4 × SARE displayed different OFP fluorescence induction patterns after the same biotic treatment (Figure 3): line S1 harbouring 4 × SARE showed induction at 48 and 72 h p.i. with *Pst*, whereas line S2 harbouring 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 (Figures 2 and 3). Moreover, only line E1 harbouring 4 × ERE displayed OFP fluorescence induced by *Pst* treatment at 72 h p.i. Insertional or epigenetic effects may possibly explain line-to-line variations observed within each construct (Butaye *et al.*, 2005; Matzke and Matzke, 1998).

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.*'s study (2013a), OFP induction in T₁ hemizygous lines was reported within 24 h after *Pto* treatment, but in field-tested T₂ homozygous lines, it took at least 48 h p.i. for transgenic lines harbouring the salicylic acid-responsive constructs to display the fluorescent signal induction. Moreover, OFP fluorescence remained significantly induced in growth chamber-tested transgenic lines harbouring 4 × SARE after 24 h p.i. with *Pto*, and OFP fluorescence decreased in transgenic lines containing 4 × PR1 after 24 h p.i. with *Pto* (Liu *et al.*, 2013a); field evaluations yielded significantly induced OFP fluorescence only at or after 48 h postphytobacterial treatments. Thus, field-tested T₂ homozygous transgenic lines harbouring 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 harbouring 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 centimetres 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 on 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 phyto-bacterial 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, it 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 phyto-bacterial 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 harbouring 4 × ERE displayed late induction of OFP fluorescence after phyto-bacterial 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 (Adams *et al.*, 2011). 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) and 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). A second possibility would be to use laser-induced fluorescence imaging (Stewart *et al.*, 2005) that might mount 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 (Figure S5). 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) element, ERE and JAR element, with and without B and A1 domains of the CaMV 35S promoter, driving an OFP reporter [i.e. *pporRFP* from *P. porites*; (Alieva *et al.*, 2008)] were used in this study. Transgenic tobacco plants harbouring 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 T₁ 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) at 200-μg/mL. Homo-

zygous T₂ transgenic tobacco lines were germinated and grown in float trays (London Tobacco Market, London, KY) for at least 1 month. Plants were transplanted at the field site and allowed to establish for 7 weeks in 2012 and 6 weeks in 2013 prior to phytopathogen treatment.

Bacterial growth

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

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. Each biological sample was replicated three times, and four experimental replicates were used to calculate 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 3000 **g** for 10 min. Cells were washed two times in 10 mM MgCl₂. Final solutions of *Pto* and *Pm* were diluted to 2 × 10⁸ CFU/mL, while *Pst* solutions were diluted to 2 × 10⁷ CFU/mL (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 MgCl₂ was used for mock treatments.

Experimental design

Field site locations at ETREC in Knoxville, Tenn., USA, were used in 2012 and 2013 for our study (Figures S7 and S8). In 2012, our field site was broadcast fertilized with N (200 kg/ha), P (25 kg/ha) and K (100 kg/ha), which was carried out according to recommendations for agronomic tobacco cultivation. In 2013, urea was applied at a rate of 200 kg/ha. We used a randomized complete block design with a strip-split plot arrangement. In 2012, two biotic treatments (*Pst* and *Pto*) (Figure S7) and in 2013, three biotic treatments (*Pst*, *Pto* and *Pm*) (Figure S8) were applied to blocks. In 2012, subplots contained two independent transgenic lines harbouring 4 × SARE::*pporRFP*, 4 × PR1::*pporRFP*, 4 × ERE::*pporRFP*, 4 × JAR::*pporRFP*, enhanced version of lines and one transgenic line harbouring empty vectors -46 35S::*pporRFP*, B_A::*pporRFP*, 35S::*pporRFP* (Table 2; Figure S7). In 2013, subplots contained three transgenic lines harbouring 4 × PR1::*pporRFP* and 4 × ERE::*pporRFP*, two lines harbouring 4 × SARE::*pporRFP* and one line harbouring empty vector -46 35S::*pporRFP* and 35S::*pporRFP* (Figure S8). 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, CA) and Command (DuPont, Wilmington, DE)].

Statistical analysis

All statistical analyses were performed using R (R Development Core Team, 2005). Mixed model package 'lme4' (Bates *et al.*, 2013) was used to determine significant differences ($P < 0.05$) in OFP fluorescence. The constructs that exhibited statistically significant induction in OFP fluorescence were analysed post hoc with mean comparisons with the 'multcomp' package

(Hothorn *et al.*, 2008). 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) was used.

Fluorometric spectroscopy

A Fluorolog[®]-3 system (Jobin Yvon and Glen Spectra, Edison, NJ) was used to quantify *pporRFP* in infiltrated sections of leaves through fluorometric spectroscopy. Samples were excited with green light ($\lambda_{\text{ex}} = 530$ nm), and emission was measured from $\lambda_{\text{em}} = 560$ –605 nm to yield a spectral scan. Spectral scans were standardized as described in Millwood *et al.* (2003) to mock treat -46 35S::pporRFP plants at 24 h p.i. After normalization, the *pporRFP* peak ($\lambda = 591$ 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). Samples were excited with green light ($\lambda_{\text{ex}} = 535$ –540 nm), while images were captured through a single band-pass filter lens 593/46 nm (Semrock, Rochester, NY). 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 h p.i., kept on ice and transported back to the laboratory. Leaf tissue was flash-frozen in liquid N₂ 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) on liquid N₂. One millilitre of Tri-reagent (Molecular Research Center, Cincinnati, OH) 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) using a Take3 Micro-Volume plate (BioTek).

cDNA synthesis

RNA was treated with DNase I (Ambion, Austin, TX) prior to cDNA synthesis following manufacturer's instructions. Ten microlitres of DNase I-treated RNA extract was reverse-transcribed with a high-capacity cDNA synthesis kit (Applied Biosystems, Foster City, CA) following manufacturer's instructions. cDNA was synthesized under the following conditions: 10 min at 25 °C, 120 min at 37 °C and 5 min 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 $\approx 1.8 \pm 0.02$.

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 μ L of cDNA reaction, 2.5 μ L of 2 \times Power SYBR Green (Life Technologies) and 1.75 μ L water. A hot start began the

cycle at 95 °C for 10 min. Forty cycles of 15 s at 95 °C and 120 s at 60 °C were run while fluorescent (520 nm) measurements were taken postelongation phase. Melting curves were obtained by heating samples to 95 °C, cooling to 60 °C, followed by a 1 °C/s 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 (2010) (Table S1). Data analysis was performed using Expression Suite software (Life Technologies) using the $\Delta\Delta C_t$ method (Schmittgen and Livak, 2008).

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Supporting information

Additional Supporting information may be found in the online version of this article:

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.

Figure S2 Time-course analysis of relative *pporRFP* mRNA expression levels in stable transgenic tobacco following *Pseudomonas syringae* pv. *tomato* (*Pto*) treatment in 2012 field trial.

Figure S3 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.

Figure S4 Relative *pporRFP* expression in three transgenic lines harbouring 4 × PR1 24 h post-treatment with *Pseudomonas syringae* pv. *tomato* (*Pto*), *P. syringae* pv. *tabaci* (*Pst*) and *P. marginalis* (*Pm*) in 2013.

Figure S5 Images of the orange fluorescent protein reporter fluorescing in treated leaf tissue from 2013 field study.

Figure S6 Corresponding visual images of Figure S5.

Figure S7 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.

Figure S8 Field layout used in 2013 of a completely randomized block design at the Plant Sciences East Tennessee Research and Education Center in Knoxville, TN.

Table S1 qRT-PCR primers.