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Bacterial pathogen phytosensing in transgenic tobacco and *Arabidopsis* plants

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Summary

Plants are subject to attack by a wide range of phytopathogens. Current pathogen detection methods and technologies are largely constrained to those occurring post-symptomatically. Recent efforts were made to generate plant sentinels (phytosensors) that can be used for sensing and reporting pathogen contamination in crops. Engineered phytosensors indicating the presence of plant pathogens as early-warning sentinels potentially have tremendous utility as wide-area detectors. We previously showed that synthetic promoters containing pathogen and/or defence signalling inducible *cis*-acting regulatory elements (RE) fused to a fluorescent protein (FP) reporter could detect phytopathogenic bacteria in a transient phytosensing system. Here, we further advanced this phytosensing system by developing stable transgenic tobacco and *Arabidopsis* plants containing candidate constructs. The inducibility of each synthetic promoter was examined in response to biotic (bacterial pathogens) or chemical (plant signal molecules salicylic acid, ethylene and methyl jasmonate) treatments using stably transgenic plants. The treated plants were visualized using epifluorescence microscopy and quantified using spectrofluorometry for FP synthesis upon induction. Time-course analyses of FP synthesis showed that both transgenic tobacco and *Arabidopsis* plants were capable to respond in predictable ways to pathogen and chemical treatments. These results provide insights into the potential applications of transgenic plants as phytosensors and the implementation of emerging technologies for monitoring plant disease outbreaks in agricultural fields.

Introduction

Plants are exposed to attack by a variety of phytopathogens and, in turn, have evolved multiple defence mechanisms that recognize the presence of potential pathogens and initiate effective defence responses. These defence systems are highly regulated on the transcriptional level and can be induced by chemical elicitors produced by pathogens. However, current methods and technologies are mostly limited to sense and detect the presence of plant pathogens post-symptomatically. Recently, efforts were made to produce plant sentinels (phytosensors) that can be used as reporting sensors of pathogen infection (Kooshki *et al.*, 2003; Mazarei *et al.*, 2008; Rushton *et al.*, 2002). Phytosensors are constructed by transforming plants to contain specific biotic- or abiotic-inducible promoters fused to a reporter gene to provide an early-warning signal. An ideal phytosensor construct should be highly inducible, specific and with an easily detected and measurable output signal. The output signal should be uniformly distributed throughout the plant or tissues, and its intensity should be well correlated with the concentration of inducers or severity of the conditions of interest (Liew *et al.*, 2008).

It is feasible to fuse the reporter genes for fluorescent proteins to inducible promoters for pathogen phytosensing (Kooshki *et al.*, 2003). When such transgenic plants encounter the target agent, the specific inducible promoter is triggered and subsequently drives the expression of the reporter gene, for example, one that produces a fluorescent signal for detection. However, native inducible genes typically give a weak signal (Kooshki *et al.*, 2003). We previously demonstrated the application of an orange fluorescent protein (OFP), *pporRFP* from the hard coral *Porites porites* (Alieva *et al.*, 2008; Mann *et al.*, 2012), as a suitable reporter gene for transient pathogen phytosensing (Liu *et al.*, 2011). We showed that using *Agrobacterium*-mediated transient assay, agroinfiltration, the OFP reporter gene exhibited uniform expression and real-time responsiveness following treatments with plant defence signal phytohormones or bacterial pathogens. Our previous study also demonstrated that the synthetic promoters containing *cis*-acting regulatory elements (RE) from promoter regions of pathogen inducible genes as well as those responsive to plant defence signal molecules are capable of signalling the responsiveness of the OFP reporter to a number of phytopathogenic bacteria in a transient phytosensing system (Liu *et al.*, 2011). We showed that this transient phytosensing system could be used as a rapid screening tool for *in vivo* analysis of inducible regulatory elements in a temporary pathogen phytosensing system prior to stable plant transformation, thus delimiting the candidate *cis*-regulatory elements that are used for stable transformation. Nevertheless, one cannot make absolute predictions on synthetic promoter function as there could be variables for which stable transgenic expression systems will differ from transient expression. Stable transformation involves integration of gene constructs into the plant genome, and thus, each transgenic event could show a variation in response activity due to position effects, expression levels, among others. Furthermore, although both transient and stable expression systems should exhibit congruent expression patterns, the levels of transient expression

are generally higher than that of stable transgenic plants (Janssen and Gardner, 1989; Wroblewski *et al.*, 2005).

In this study, we produced and tested transgenic plants for the purpose of early detection of pathogen infection, that is, a system that could in the near future be modified to use in commercial agriculture: deployable pathogen phytosensors. We developed stable transgenic tobacco and *Arabidopsis* plants containing the most appropriate phytosensing constructs from Liu *et al.* (2011) and examined the efficiency of these synthetic promoters for pathogen phytosensing in intact stable transgenic tobacco and *Arabidopsis* plants. These species are hosts for a wide range of pathogens that infect economically important crops. *Nicotiana tabacum*- or *Arabidopsis thaliana*–*Pseudomonas syringae* interactions are two of the model pathosystems for the study of plant–pathogen interactions. These two model pathosystems have been widely used to study the molecular mechanisms involved in both susceptible and resistant plant–pathogen interactions (Katagiri *et al.*, 2002; Krzymowska *et al.*, 2007; Quirino and Bent, 2003; Taguchi *et al.*, 2003). *Pseudomonas syringae*, a Gram-negative rod-shaped bacterium, is biotrophic or hemibiotropic (Thaler *et al.*, 2004) and host-specific, causing leaf yellow spots and necroses in host plants but inducing hypersensitive response (HR) in nonhost plants (Hirano and Upper, 1990; Table 1). For example, *P. syringae* pv. *tomato* causes HR associated with the onset of necrosis and dehydration of the tissue (resistance reaction) within 24 h post-inoculation (hpi) on nonhost tobacco (Cuppels, 1986; Taguchi *et al.*, 2003), while it infects and induces necrotic, water-soaked lesions surrounded by chlorotic halos within 48 hpi on host *Arabidopsis* (Dong *et al.*, 1991; Whalen *et al.*, 1991). *Pseudomonas syringae* pv. *tabaci* causes a normal-sensitive symptom of 'wildfire' disease associated with initial chlorosis within 72 hpi and then subsequent water-soaked and necrotic symptoms of the tissue due to the susceptible reaction with host tobacco (Taguchi *et al.*, 2003). However, on its nonhost *Arabidopsis*, it causes the development of yellow foliar spots and necrosis at low concentrations, while inducing HR at high concentrations (Ishiga *et al.*, 2005). In addition, the nonhost resistance of tobacco to *Pseudomonas marginalis* does not lead to HR reaction or evident symptom development following inoculation (Durbin, 1979), whereas the susceptible interaction between *P. marginalis* and host *Arabidopsis* causes yellow leaf spots and necrosis within 72 hpi (Achouak *et al.*, 2000). The compatible and incompatible interactions between tobacco and *Arabidopsis* with various *Pseudomonas* species are summarized in Table 1.

Here, we analysed the specificity and inducibility of synthetic promoters in transgenic tobacco and *Arabidopsis* plants in response to plant defence signal molecules salicylic acid, ethylene and jasmonic acid, or to phytopathogenic bacterial treatments. Our results demonstrated functional inducibility of synthetic promoters in transgenic tobacco and *Arabidopsis* plants with the regulation of detectable OFP reporter gene expression providing additional insights into the new technology and applications of transgenic plants as phytosensors.

Results

We engineered transgenic plants for the purpose of early detection of pathogen infection by fusing synthetic *cis*-acting regulatory elements to a reporter gene for deployable phytosensing. We previously demonstrated that the synthetic promoters containing the *cis*-acting regulatory elements, PR1 and salicylic acid responsive element (SARE), JAR and ethylene responsive element (ERE), are capable of reflecting the responsiveness of the reporter to plant defence signal molecules salicylic acid (SA), ethylene (ET) and jasmonic acid (JA), accordingly, as well as to a number of phytopathogenic bacteria in a transient phytosensing system (Liu *et al.*, 2011; Figure 1). Here, we produced stable

Table 1 Comparison of the characteristics of compatible and incompatible interactions between *Nicotiana tobacum* and *Arabidopsis thaliana* with various *Pseudomonas* species

Pathogens	*Nicotiana tabacum*	*Arabidopsis thaliana*
P. syringae pv. *tomato*	Nonhost; Hypersensitive response (HR) within 24 hpi, necrosis at OD_{600} = 0.3	Host; Necrotic, water-soaked lesions surrounded by chlorosis within 48 hpi at OD_{600} = 0.03
P. marginalis	Nonhost; Very mild or no symptom at OD_{600} = 0.3	Host; Necrosis within 72 hpi, collapse at 5–6 dpi at OD_{600} = 0.3
P. syringae pv. *tabaci*	Host; Normal-sensitive 'wildfire' symptom within 48–72 hpi at OD_{600} = 0.03	Nonhost; Yellow spot necrosis within 72 hpi at OD_{600} = 0.03

Source: Achouak *et al.* (2000), Cuppels (1986), Dong *et al.* (1991), Durbin (1979), Ishiga *et al.* (2005), Taguchi *et al.* (2003) and Whalen *et al.* (1991).

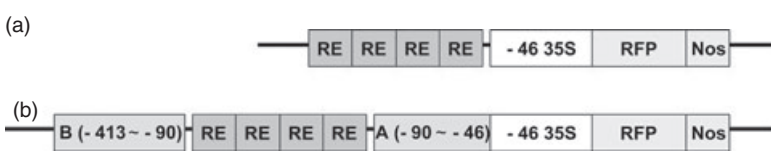

Figure 1 Scheme of synthetic promoter- *pporRFP* fusion constructs (subtracted from Liu *et al.*, 2011). (a) Scheme of synthetic promoter construct as tetramers of certain regulatory elements (4 × RE) was placed upstream of CaMV35S minimal promoter (min 35S containing the TATA box). (b) Scheme of enhanced synthetic promoter construct of 4 × RE was placed between B (−415~ −90) and A1 (−90~ −46) domains of CaMV35S promoter.

transgenic tobacco and *Arabidopsis* plants with the OFP reporter gene under the control of four copies of distinct *cis*-acting regulatory elements PR1, SARE, ERE and JAR with or without enhancer motifs B and A1 from CaMV 35S (Benfey *et al.*, 1990). Stable transgenic tobacco and *Arabidopsis* plants containing the negative controls (empty vector −46 35S RFP and B_A RFP) were also generated for measurement of the level of background expression associated with these synthetic promoters. At least two independent tobacco and *Arabidopsis* lines were selected for each synthetic promoter analysis in intact plants. These lines showed no differences in their morphology and growth characteristics with respect to nontransformed plants.

The sensitivity and inducibility of the synthetic promoters containing the distinct regulatory elements were studied by corresponding phytohormone treatments as well as bacterial pathogen infection on stable transgenic tobacco leaves. To determine the level of background OFP expression associated with the synthetic promoters, transgenic tobacco leaves containing the negative controls (empty vectors −46 35S RFP or B_A RFP) were treated with the phytohormones SA, ET and JA or bacterial pathogens *P. syringae* pv. *tomato*, *P. syringae* pv. *tabaci* and *P. marginalis*. As shown in Figures 2a,b and 3a,b, both phytohormone and bacterial pathogen treatments gave rise to similar fold changes in OFP expression in empty vectors as their corresponding mock control treatments, and no inducibility of the OFP expression was detected in either empty vector at any time point compared to their mock treatments.

Time-course analysis of expression of the OFP reporter in each synthetic promoter construct revealed that the synthetic promoter constructs exhibited significant increases in OFP expression in stable transgenic tobacco at 72 h following their corresponding phytohormone treatment (Figures 2c,d and 6).The average fold changes in expression of OFP reporter caused by their corresponding hormone treatments was up to 2.81 times higher than by their respective mock treatments at 72 h after treatments, which were statistically significant ($P < 0.05$). Among the synthetic promoter constructs, the 4 × JAR regulatory element showed the lowest induction level, while both 4 × PR1 and 4 × SARE exhibited the highest induction levels (Figures 2c,d and 6). These results demonstrated that stable transgenic tobacco plants harbouring the distinct regulatory elements are specifically responsive to and inducible by their corresponding hormone treatments.

Subsequently, the bacterial pathogens *P. syringae* pv. *tomato*, *P. marginalis* and *P. syringae* pv. *tabaci* were used to examine tobacco–pathogen interaction responses in stable transgenic tobacco plants as described in our previous study (Liu *et al.*, 2011). Time-course analysis of expression of the OFP reporter gene for each synthetic promoter construct revealed that *P. syringae* pv. *tomato* was associated with reporter gene induction in most of the stable transgenic tobacco lines within 24 hpi (Figures 3 and 6). In addition, *P. syringae* pv. *tomato* exhibited a higher inducibility of OFP expression in stable transgenic tobacco lines containing 4 × PR1 and 4 × SARE regulatory elements at time point 24 hpi (Figures 3c–f and 6). Nevertheless, the 4 × JAR motifs showed the least inducibility of OFP in response to *P. syringae* pv. *tomato* (Figures 3i,j and 6). A gradual increase in OFP expression over time following

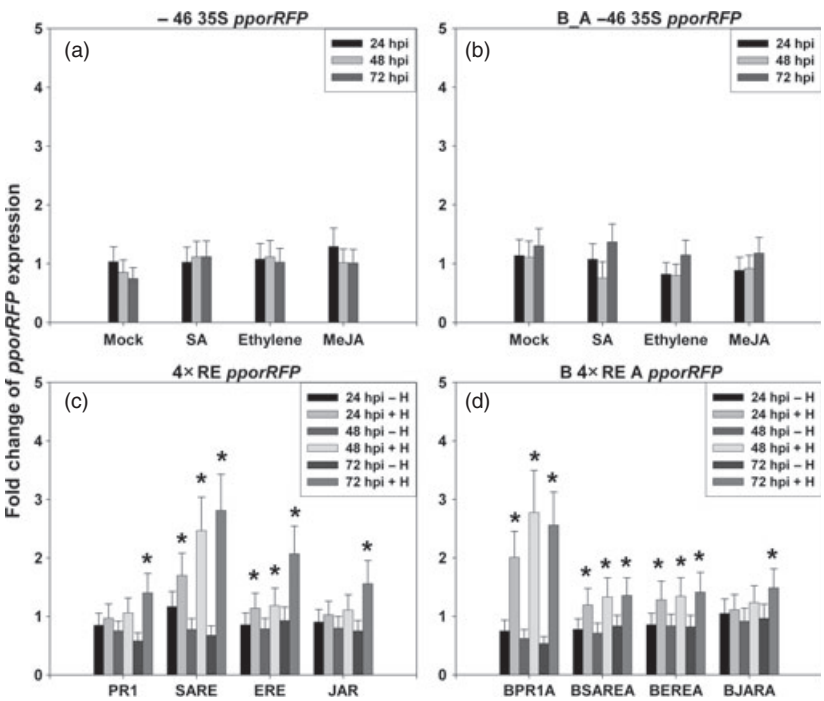

Figure 2 Fold change in expression of the *pporRFP* reporter in stable transgenic tobacco following phytohormone treatments. Stable transgenic tobacco leaves were infiltrated with 4 mM salicylic acid (SA) for PR1 and SARE, 4 mg/mL ethephon (an ethylene releasing chemical) for ERE and 100 μM methyl jasmonate (MeJA) for JAR regulatory element containing constructs. Expression of the *pporRFP* reporter was quantified using SPEX Fluorolog at time points 0 h (before treatment), 24, 48 and 72 h following treatments. The infiltrated spots were excited at 530 nm, and emission spectra were scanned and recorded from 550 to 640 nm. Intensity was measured at 591 nm in counts per second (cps). Each bar represents the mean value of the fold change in *pporRFP* expression obtained from three independent biological experiments with the standard errors of the mean noted. Significant *pporRFP* expression changes (indicated by asterisks) were determined statistically by using ANOVA PROC GLM (LSD, $P < 0.05$). H stands for hormone.

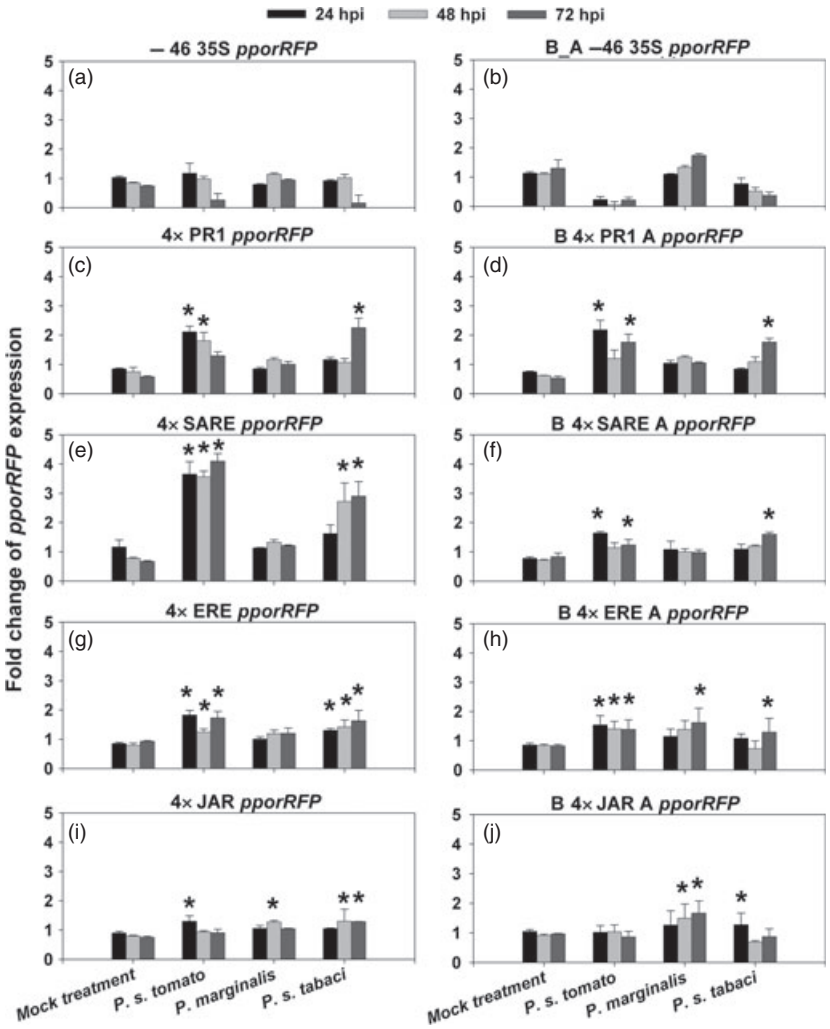

Figure 3 Fold change in expression of the *pporRFP* reporter in stable transgenic tobacco following phytopathogenic bacterial treatments. Stable transgenic tobacco leaves were infiltrated with mock, *Pseudomonas syringae* pv. *tomato* (OD_{600} = 0.3), *Pseudomonas marginalis* (OD_{600} = 0.3) or *P. syringae* pv. *tabaci* (OD_{600} = 0.03). Expression of *pporRFP* was quantified using a SPEX Fluorolog at 0, 24, 48 and 72 h post-inoculation (hpi). The infiltrated spots were excited at 530 nm, and emission spectra were scanned and recorded from 550 to 640 nm. Intensity was measured at 591 nm in counts per second (cps). Each bar represents the mean value of *pporRFP* expression obtained from three independent biological experiments with the standard errors of the mean noted. Significant *pporRFP* expression changes (indicated by asterisks) were determined statistically by using ANOVA PROC GLM (LSD, $P < 0.05$).

P. syringae pv. *tabaci* inoculation was monitored in transgenic tobacco harbouring most, if not all, synthetic constructs (Figure 3). The first significant increase was documented in either 48 or 72 hpi (Figure 3). *P. marginalis*, a 'soft rot' disease causing pathogen, gave rise to little induction of OFP expression in all the transgenic lines harbouring different regulatory elements at all the time points (Figure 3). The only exception came from the transgenic lines harbouring the ERE regulatory element in which *P. marginalis* caused a similar level of induction in OFP as the other two pathogens (Figure 3g,h), which was also observed in the transient phytosensing system (Liu *et al.*, 2011). In addition, different regulatory elements exhibited variable increases in OFP expression over time caused by different pathogens. For example, both *P. syringae* pv. *tomato* and *P. syringae* pv. *tabaci* caused higher induction in OFP expression in transgenic tobacco lines containing PR1 and SARE regulatory elements (Figure 3c–f), while all three pathogens showed similar inducibility in OFP expression in transgenic lines harbouring ERE and JAR elements (Figure 3g–j).

To better understand the application potentials of these synthetic promoters *in planta* for pathogen phytosensing, we generated stable transgenic *Arabidopsis* plants with the distinct synthetic promoter constructs. Both empty vectors (−46 35S RFP or B_A RFP) conferred similar fold changes in reporter gene expression in transgenic *Arabidopsis* lines in response to phytohormone treatments or bacterial pathogens as their corresponding mock control treatments (Figures 4a,b and 5a,b). No induction of OFP expression was detected in transgenic *Arabidopsis* lines harbouring either empty vector at any time point compared to their mock treatments.

We tested whether these stable transgenic *Arabidopsis* lines containing the regulatory elements were responsive to and inducible by their corresponding phytohormone treatments. Surprisingly, almost all the synthetic promoters exhibited a significant increase in OFP expression only at 72 h following their corresponding phytohormone treatment (Figure 4c,d). This is different from what was observed in stable transgenic tobacco lines, where the synthetic promoters harbouring different regulatory elements showed a significant increase at much earlier time points (24 hpi; Figure 2c,d). Another discrepancy between transgenic *Arabidopsis* lines and tobacco lines was the average fold change in expression of the OFP reporter caused by their corresponding hormone treatments, which in transgenic *Arabidopsis* was up to 10.14 times higher than their respective mock treatments at 72 h after treatment (statistically significant at $P < 0.05$). The inducibility of expression of OFP in transgenic *Arabidopsis* was ~3 times higher than in transgenic tobacco. The 4 × JAR regulatory element exhibited the lowest induction level, which was also observed in transgenic tobacco (Figures 2c,d and 4c,d). Stable transgenic *Arabidopsis* lines were specifically responsive to and inducible by their corresponding hormone treatments.

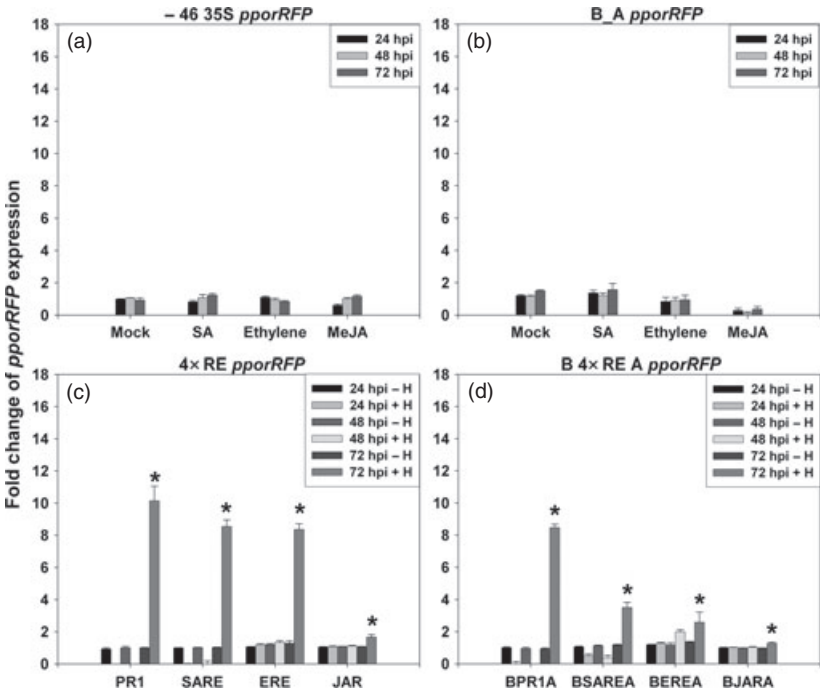

Figure 4 Fold change in expression of the *pporRFP* reporter in stable transgenic *Arabidopsis* following phytohormone treatments. Stable transgenic tobacco leaves were infiltrated with 4 mM salicylic acid (SA) for PR1 and SARE, 4 mg/mL ethephon (an ethylene releasing chemical) for ERE and 100 μM methyl jasmonate (MeJA) for JAR regulatory element containing constructs. Expression of the *pporRFP* reporter was quantified using SPEX Fluorolog at 0 h (before treatment), 24, 48 and 72 h following treatments. The infiltrated spots were excited at 530 nm, and emission spectra were scanned and recorded from 550 to 640 nm. Intensity was measured at 591 nm in counts per second (cps). Each bar represents the mean value of the fold change in *pporRFP* expression obtained from three independent biological experiments with the standard errors of the mean noted. Significant *pporRFP* expression changes (indicated by asterisks) were determined statistically by using ANOVA PROC GLM (LSD, $P < 0.05$). H stands for hormone.

The *Arabidopsis*–bacterial pathogen interaction was studied by using the above-mentioned three bacterial pathogens to inoculate leaves of the stable transgenic *Arabidopsis* plants containing the regulatory elements. Time-course analysis of OFP expression in each synthetic promoter revealed a gradual increase in OFP expression in all stable transgenic *Arabidopsis* lines in response to *P. syringae* pv. *tomato* (Figure 5c–j). However, the first significant increase in OFP expression was observed at 48 hpi (Figure 5c–j), much later than the 24 hpi response observed in transgenic tobacco (Figure 3c–j). The inoculation of *P. syringae* pv. *tabaci* on its nonhost transgenic *Arabidopsis* lines also gave rise to a gradual increase in OFP expression even though the first significant increase appeared at 48 hpi (Figure 5c–j) instead of either 24 hpi or 72 hpi as observed in transgenic tobacco (Figure 3c–j). This increase was not documented in transgenic tobacco lines harbouring the same elements (Figure 3c–f). The most striking finding came from the inducibility of synthetic promoters containing the JAR element in transgenic *Arabidopsis* (Figures 5i,j and 6). The inducibility of JAR element in transgenic *Arabidopsis* was much higher than in transgenic tobacco (Figure 3i,j) as well as in transient expression in tobacco leaves (Liu *et al.*, 2011) following the three bacterial pathogen treatments.

Discussion

The three phytohormones—SA, JA and ethylene—play key roles in regulating plant defence pathways against pathogens, pests and abiotic stresses (Balbi and Devoto, 2008; Bari and Jones, 2009; Broekaert *et al.*, 2006; Fujita *et al.*, 2006; Glazebrook, 2005; Loake and Grant, 2007; Lorenzo and Solano, 2005). Substantial evidence demonstrated that pathogen infections result in increase in SA or JA–ethylene levels, and exogenous applications of each hormone give rise to activation of their specific defence responses. In this study, we engineered stable transgenic plants with four distinct hormone responsive elements, which are specifically responsive to SA, JA or ET. With the phytohormone treatments of these stably transformed plants, we demonstrated that these regulatory elements are responsive to, and inducible by, their corresponding hormones treatments, as revealed by *Agrobacterium*-mediated transient assay (Liu *et al.*, 2011). However, the two model plants exhibited up to three times difference in induction levels and highly different timing of responsiveness to phytohormone treatments [24 hpi (Figure 2c,d) versus 72 hpi (Figure 4c,d)]. These differences might arise from the difference of the biosynthetic pathways and/or regulation of defence responses of the three hormones between the two model species. For example, different precursors of SA may cause different timing of defence responses in the two species, with benzoic acid/glucosyl benzoate or isochorismate being the SA precursors in tobacco (Catinet *et al.*, 2008) or *Arabidopsis* (Nawrath and Metraux, 1999), respectively.

Moreover, SA is mainly involved in resistance to biotrophic pathogens and JA–ET associated with the responses to necrotrophic pathogens and chewing insects, even though the SA and JA–ET hormone pathways also act synergistically (Dodds and Rathjen, 2010; Glazebrook, 2005). The bacteria pathogen *P. syringae* is often considered a hemi-biotroph (Thaler *et al.*,

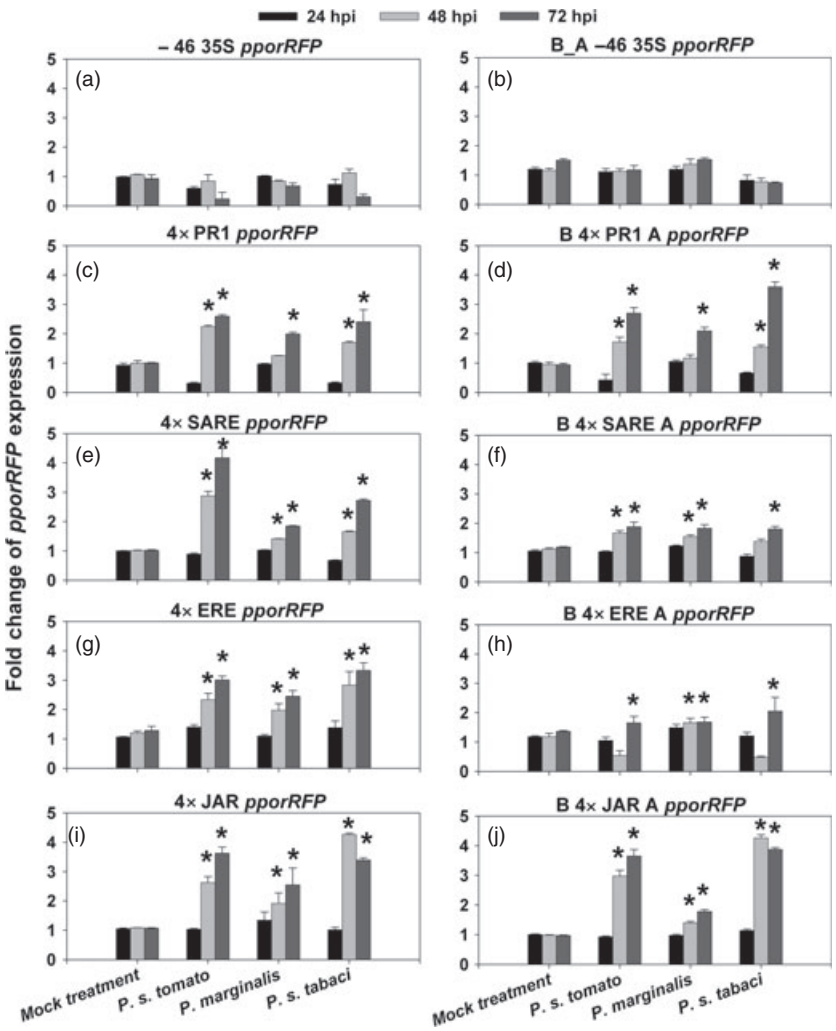

Figure 5 Fold change in expression of the *pporRFP* reporter in stable transgenic *Arabidopsis* following phytopathogenic bacterial treatments. Stable transgenic tobacco leaves were infiltrated with mock, *Pseudomonas syringae* pv. *tomato* (OD_{600} = 0.03), *Pseudomonas marginalis* (OD_{600} = 0.3) or *P. syringae* pv. *tabaci* (OD_{600} = 0.03). Expression of *pporRFP* was quantified using a SPEX Fluorolog at 0, 24, 48 and 72 h post-inoculation (hpi). The infiltrated spots were excited at 530 nm, and emission spectra were scanned and recorded from 550 to 640 nm. Intensity was measured at 591 nm in counts per second (cps). Each bar represents the mean value of *pporRFP* expression obtained from three independent biological experiments with the standard errors of the mean noted. Significant *pporRFP* expression changes (indicated by asterisks) were determined statistically by using ANOVA PROC GLM (LSD, $P < 0.05$).

2004). It fails to cause disease in nonhosts due to the gene-for-gene resistance, which always triggers the activation of SA-dependent signalling. But, in its hosts, this pathogen is virulent and always gives rise to host tissue chlorosis and necrosis (Glazebrook, 2005). In this project, we demonstrated that our pathogen phytosensing systems perfectly reflect the difference between HR and gradual disease development and necrosis following different bacterial pathogen infections. The quick response of regulatory elements in transgenic tobacco by *P. syringae* pv. *tomato* infection appeared as early as 24 hpi followed by gradual decrease in expression of the OFP reporter (Figure 3), which matched the growth trend of this pathogen on tobacco leaves (Liu *et al.*, 2011) and exactly reflects the rapid plant cell collapse and necrosis followed by location and propagation of the pathogen at the inoculation site (Cuppels, 1986; Taguchi *et al.*, 2003). Nevertheless, when infected with *P. syringae* pv. *tomato*, the first significant increase in the expression of OFP reporter in transgenic *Arabidopsis* plants emerged at 48 hpi followed by further increase in the expression of OFP reporter at 72 hpi (Figure 5). Disease symptoms in *Arabidopsis* develop gradually (Dong *et al.*, 1991; Whalen *et al.*, 1991) and commensurate with the gradual increase in bacterial population size over the time period after its infection (data not shown).

Pseudomonas syringae pv. *tabaci* induced disease symptoms in its host tobacco as well as nonhost *Arabidopsis* at low concentration (OD_{600} = 0.03) (Table 1; Ishiga *et al.*, 2005; Taguchi *et al.*, 2003). Time-course analysis of expression of OFP reporter demonstrated a gradual increase in the inducibility of the synthetic promoters in both species (Figures 3 and 5). The inoculation of *P. syringae* pv. *tabaci* on its nonhost transgenic *Arabidopsis* lines also gave rise to a gradual increase in OFP expression even though the first significant increase appeared at 48 hpi (Figure 5c–j) instead of either 24 hpi or 72 hpi as observed in transgenic tobacco (Figure 3c–j). This observation may be due to the difference between the incompatible *Arabidopsis–P. syringae* pv. *tabaci* interaction (Ishiga *et al.*, 2005) and the compatible tobacco–*P. syringae* pv. *tabaci* interaction (Taguchi *et al.*, 2003).

We also demonstrated that *P. marginalis*, which causes necrosis (collapses 5–6 dpi) in its host *Arabidopsis* (Achouak *et al.*, 2000), rendered significant increases in OFP expression in transgenic *Arabidopsis* lines containing PR1 and SARE elements (Figure 5c–f). This increase was not documented in transgenic tobacco lines harbouring the same elements (Figure 3c–f). This discrepancy may come from the different defence mechanisms in the two plant species against *P. marginalis* infection. Moreover, *P. marginalis* caused a similar level of induction in OFP expression in transgenic lines harbouring the ERE regulatory element as the other two pathogens did (Figure 3g,h), which was also observed in the transient phytosensing system (Liu

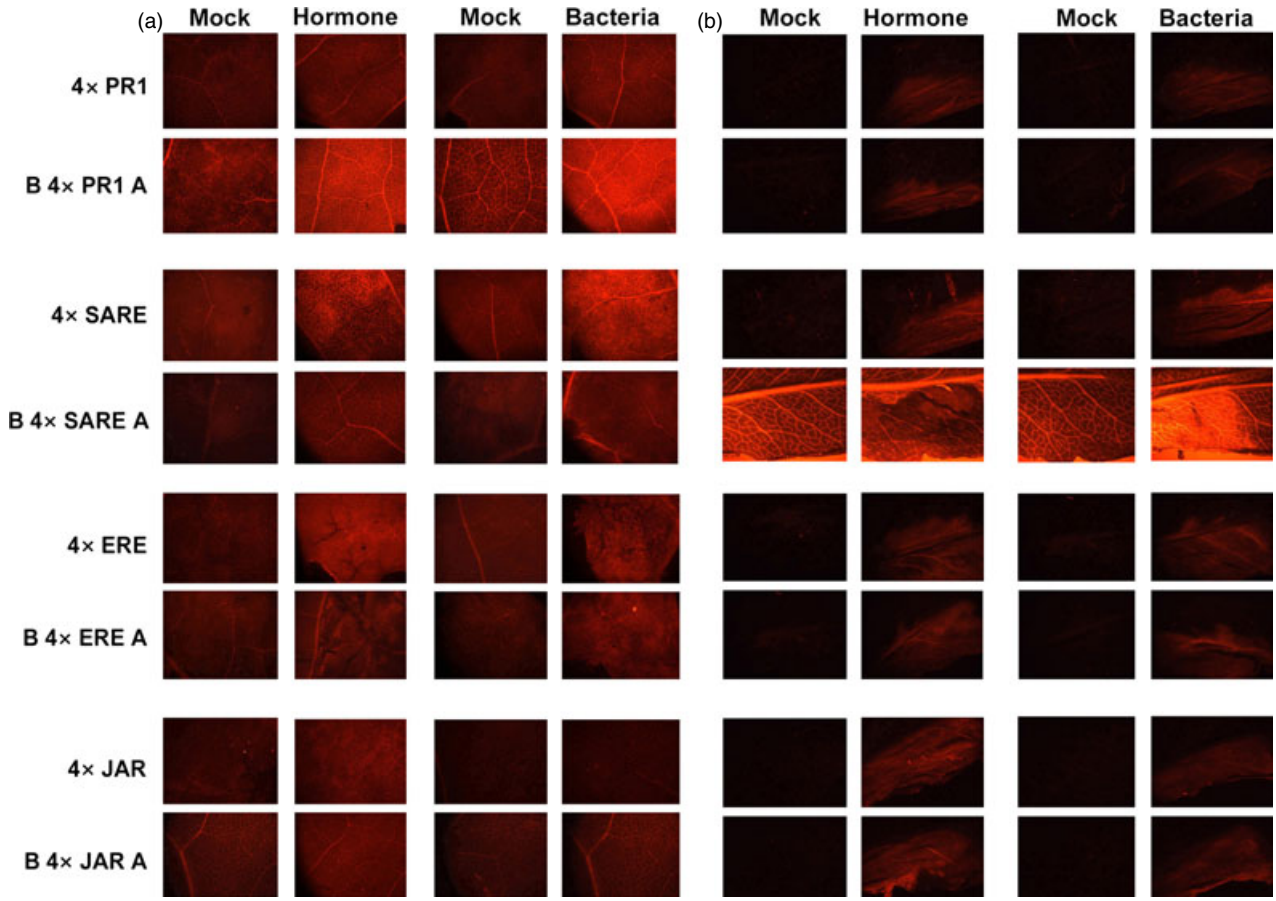

Figure 6 Visual images (representative) of expression of the *pporRFP* reporter on stable transgenic tobacco and *Arabidopsis* leaves following phytohormone or phytopathogenic bacterial treatments. Stable transgenic tobacco (a) and *Arabidopsis* (b) leaves were infiltrated with salicylic acid for PR1 and SARE, ethephon (an ethylene releasing chemical) for ERE and methyl jasmonate for JAR regulatory element containing constructs or with *Pseudomonas syringae* pv. *tomato*. Expression of the *pporRFP* reporter was visualized 72 h following the treatment using an epifluorescent microscope (Olympus SZX12) and a tdTomato filter set: 535/30 nm excitation and 600/50 nm band pass emission, and the images were captured using imaging software QCapture 2.56. The exposure time was 30 s.

et al., 2011). This may indicate that defence against *P. marginalis* infection in tobacco might mainly rely on ethylene-related defense pathway.

In addition, following the three bacterial pathogen treatments, the inducibility of JAR element in transgenic *Arabidopsis* was much higher than in transgenic tobacco (Figures 3i,j and 5i,j) as well as in transient expression in tobacco leaves (Liu *et al.*, 2011). This may imply that the low inducibility of JAR element in tobacco comes from the less involvement of JA defence pathway in tobacco against the bacterial pathogens.

Developing suitable phytosensors that permit accurate, real-time, rapid and sensitive analysis would be beneficial for monitoring the distribution of pathogens (Byrne *et al.*, 2009; Kovalchuk and Kovalchuk, 2008). It is a desirable feature that pathogen phytosensors could be inducible by a broad range of pathogens involved in different signal transduction pathways when contemplating its use for the design of disease reporting phytosensors (Kooshki *et al.*, 2003). As expected, these stable transgenic tobacco and *Arabidopsis* harbouring distinct inducible regulatory elements were capable of not only reflecting the differences among the three hormone-specific defence pathways, but also differentiating the responsiveness of plants to different pathogens. Their induced responses were highly specific, easily measurable and monitored. These stable transformed plants allow *in situ* monitoring of biological properties during their immediate contact with pathogens. They could be used for stable phytosensing for early detection and rapid report of bacterial pathogen infection. These results provide insights into the potential *in planta* application of transgenic plants as phytosensors in crops and the implementation of emerging technologies for monitoring plant diseases.

The use of stable transgenic plants as bacterial pathogen phytosensors has several important attributes. Stable transgenic plants permit accurate and real-time sensing of pathogen infection. They allow easy scoring and monitoring of the early responses. They are also cheap in cost and simple in use. However, it is noted that each stable transformation line could be different in synthetic promoter response activities because stable transformation involves integration into the plant nuclear genome. Transgenics could also be generated to be more sensitive to a specific pathogen using a more specific motif, which renders the specific inducible motif discovery being the first priority to achieve the goal of making an ideal phytosensor (Venter, 2007). The combination and multimerization of different *cis*-regulatory elements (in this case 4 × REs) remains a feasible strategy for synthetic promoter design, and the diversity of REs

should be further investigated for improved and enhanced spectrum of sensitivity. Increasing the number of *cis*-elements in the promoter increases strength but may also increase background, as we see between 4 × RE and B 4 × RE A. Furthermore, an increase in motif copy number might not necessarily enhance promoter inducibility. The results of varying the numbers of different *cis*-elements cannot be easily predicted and will have to be determined experimentally. Refinement of phytosensors for field-level implementation in agriculture will likely involve testing the arrangements of different *cis*-motifs in different motif copy numbers (with/or without enhancer regions) in combination with biotic and/or abiotic dose–response and time-course analysis. There needs to be a fine balance in synthetic promoter design, as for a biotech tool, what we need is a robust system that is highly specific and sensitive enough to provide rapid information.

It is expected that this strategy of making stable transgenics could be applied in creating phytosensors in economically important crops for phytosensing, even though there is a concern about the gene flow from transgenic phytosensing plants to nontransgenic crops (Moon *et al.*, 2011). Because the two model species studied in this article responded highly differently to phytohormone or bacterial pathogen treatments, the phytosensors would have to be generated in the crops themselves so that they could pinpoint the real-time defence responses occurring within the crops. If these stable transgenic tobacco or *Arabidopsis* plants are used in crop field for pathogen phytosensing, they may report the onset of pathogenesis much later than the crops themselves. Due to the fact that these regulatory elements are inducible by the defence signals, it is reasonable to expect that they would work in most, if not all, crops even though the timing and expression level of reporter gene might be highly different in plants. Before creating transgenic crops with these regulatory elements for stable phytosensing, it is also worthwhile to test the phytosensing effects of these stable transgenic tobacco and *Arabidopsis* plants in the field with hormone and bacterial pathogen treatments.

Experimental procedures

Plasmid construction

Details of the construction of synthetic promoter—*pporRFP* reporter fusion cassettes in pZP222 binary vector plasmids with or without enhancer domains B and A1 from CaMV 35S have been provided previously (Liu *et al.*, 2011). The pZP222 synthetic promoter constructs contained four head-to-tail copies of distinct *cis*-acting regulatory elements of pathogenesis-related (PR1), SARE, ERE or jasmonic acid responsive element (JAR). The sequences of *cis*-acting regulatory elements PR1, SARE, ERE and JAR were reported previously (Mazarei *et al.*, 2008; Table 2). The pZP222 synthetic promoter constructs consisting of the regulatory elements were named as pZP (4 × PR1), pZP (4 × SARE), pZP (4 × ERE), pZP (4 × JAR), pZP (B 4 × PR1 A), pZP (B 4 × SARE A), pZP (B 4 × ERE A), pZP (B 4 × JAR A). Construction of negative control vectors (empty vectors pZP −46 35S RFP and pZP B_A RFP) were described in the study by Liu *et al.* (2011).

Plant transformation

Arabidopsis thaliana (ecotype Columbia) and *N. tabacum* (cv Xanthi) plants were transformed with each plasmid construct using *Agrobacterium tumefaciens* strain GV3850. *Arabidopsis* transformation was performed by the floral dip method (Clough and Bent, 1998), and tobacco transformation, by the leaf disc transformation method (Horsch *et al.*, 1985). Transgenic *Arabidopsis* was selected on Murashige and Skoog (MS) media containing 100 mg/L gentamicin (Sigma, St. Louis, MO), and transgenic tobacco plants were selected on MS media with 200 mg/L gentamicin.

Transgenic plants

Transgenic tobacco plants were grown in a growth chamber at 25 °C under fluorescent white light in a 16 : 8 h light/dark cycle for 3 weeks and then allowed to self-fertilize in a greenhouse in a 16 : 8 h light/dark cycle at 27–30 °C. Transgenic *A. thaliana* plants were grown in a growth chamber at 22 °C under fluorescent white light in a 16 : 8 h light/dark cycle and allowed to self-fertilize. For phytosensing experiments, nontransgenic and transgenic tobacco plants were grown under the same growth chamber conditions as above for 6–7 weeks, while the nontransgenic and transgenic *Arabidopsis* plants were grown in a growth chamber at 18 °C under 8-h light and 22 °C in 16-h darkness for 7 weeks.

Biotic and abiotic treatments

At least two independent transgenic lines were selected from T1 tobacco or T3 *Arabidopsis* lines for biotic and chemical treatments. For chemical treatments, 4 mM salicylic acid (SA), 4 mg/mL ethephon (an ethylene releasing chemical) or 100 μM methyl jasmonic acid (MeJA) (all from Sigma, St. Louis, MO) was infiltrated to plant leaves as described in the study by Liu *et al.*, 2011;. Leaves were infiltrated with water for mock control treatments. For bacterial pathogen treatments, *P. syringae* pv. *tomato*, *P. marginalis* and *P. syringae* pv. *tabaci* were prepared and infiltrated into plant leaves as described in the study by Liu *et al.*, 2011;. For mock control treatments, leaves were infiltrated with 10 mM $MgCl_2$. The bacteria populations were estimated on leaf discs as described in the study by Liu *et al.*, 2011. Three biological replicates (i.e. three plants) were used.

Table 2 Sequence information of the 4 *cis*-acting regulatory elements (RE) used in this research (subtracted from Mazarei *et al.*, 2008). In each RE, the core sequences are in bold

RE	Sequence (5′→3′)	Original source	Defence pathway	Reference
PR1	**ACGTCA**TAGATGT**GGCGGC**ATATATT CTTCA**GGACTTTTC**	Arabidopsis *PR1*	Salicylic acid	Lebel *et al.* (1998)
SARE	**TTCGACCTCC**AAAGAGGACCCAGAAT	Tobacco *PR2-d*	Salicylic acid	Shah and Klessig (1996)
JAR	CAACGA**CACGCCAAAT**TCTAATTTAGC ACAGTCTC**ACGTG**	Arabidopsis *VSP1*	Jasmomic acid	Guerineau *et al.* (2003)
ERE	**CAGCCGCC**AAAGAGGACCCAGAAT	Tobacco chitinase	Ethylene	Brown *et al.*(2003), Ohme-Takagi and Shinshi (1995) and Rushton *et al.* (2002)

Determination of pporRFP expression

Expression of *pporRFP* was visualized with an epifluorescent microscope (Olympus stereo microscope model SZX12, Olympus America, Center Valley, PA) using a tdTomato filter set: 535/30 nm excitation and 600/50 nm band pass emission and QCapture 2.56 imaging software. Spectrofluorometry was performed as described in the study by Millwood *et al.* (2003) but using an updated Fluorolog®-3 system (Jobin Yvon and Glen Spectra, Edison, NJ). The infiltrated spots were excited at 530 nm, and emission spectra were scanned and recorded from 550 to 640 nm. Intensity was measured at 591 nm in counts per second (cps). Time-course analyses of the expression of *pporRFP* reporter were conducted at time points 0 (before treatment), 24, 48 and 72 h after phytohormone or bacterial pathogen treatment.

Data analysis

Data normalization was conducted according to Millwood *et al.* (2003). Background subtraction was applied to each measurement of *pporRFP* expression using measurements from non-transgenic tobacco or *Arabidopsis* (ecotype Columbia) as background expression when treated with the biotic and chemical treatments. Fold change in the expression of *pporRFP* reporter was calculated by using the normalized and subtracted data at different time points 24, 48 or 72 h post-treatments divided by the normalized data at time point 0 h. Analysis of variance (ANOVA) using PROC GLM was used for statistical analyses ($P < 0.05$) (SAS 9.2 for Windows; SAS Institute Inc, Cary, NC).

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