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Pathogen inducible reporting in transgenic tobacco using a GFP construct

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Abstract

A model sentinel plant (phytosensor) capable of signaling pathogen attack in the field was obtained by transformation with a construct containing the green fluorescent protein (GFP) as a reporter gene, driven by the promoter of *gn1*, a tobacco β-1,3-glucanase gene. This is a first step towards the creation of sentinel plants for early disease diagnostics with an integral real-time, fluorescent-based reporting mechanism. The *gn1* promoter is responsive to pathogens and salicylic acid (SA), which is synthesized by the plant during systemic acquired resistance (SAR). Transgenic plants were sprayed with benzo-(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester (BTH), an SA analogue, at different stages of development. The presence of GFP transcript in transgenic plants after the induction with 5 mM BTH was determined. GFP was detected as early as 48 h after induction using Western blot analysis, but the fluorescence could not be robustly detected spectrally. Plants younger than 8 weeks did not express detectable levels of GFP. We show systemic induction of *gn1/mgfp5-er* by BTH in non-treated plant tissues. Time course of induction of *gn1/mgfp5-er* by BTH in transgenic plants showed that induction and GFP accumulation was slow and maximum, GFP accumulation was found between 6 and 12 days after treatment. *gn1/mgfp5-er* expression was also induced by inoculating plant leaves with the pathogen *Plectosporium tabacinum*.

Keywords: Phytosensor; Pathogen inducible promoter; Green fluorescent protein; Plant disease; Sentinel plant

1. Introduction

Crop terrorism countermeasures and precision agriculture share at least one common need: the ability to sense plant pathogens before they cause widespread damage. All of the current early warning detection technologies rely on field-level monitoring by visual observation of disease symptoms. In recent years, several groups have experimented with remote sensing of plant disease using hyperspectral images obtained from satellites or ground-based platforms [1]. These approaches suffer from detecting plant disease post-symptomatically. The goal of our research is to produce plant biosensors (or phytosensors) that can be used as early warning sentinels of disease. It is feasible to fuse the reporter gene coding for green fluorescent protein (GFP) to pathogen inducible promoters to produce disease reporting organisms. The use of GFP as a reporter gene for phytosensors has several advantages over commonly used reporter genes, such as firefly luciferase [2] and GUS [3]. It is non-destructive, does not need any substrate or cofactors, can report in real-time and can be remotely sensed. Thus GFP has become a useful reporter marker for gene expression and regulation in plants [4]. Furthermore the *mgfp5-er* variant is optimized for expression in plants [5] and, in our hands,

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proven to be the optimal whole plant visible transgenic fluorescent marker in the field [4,6].

Plants have several defense mechanisms that enable them to respond to pathogen attack. Localized defense is often manifested at the site of attack by a hypersensitive response (HR) which results in restricted lesions clearly delimited from surrounding healthy tissues [7]. HR is followed by systemic acquired resistance (SAR), which results in long-lasting immunity in non-infected tissues. It provides protection not only against the inducing pathogen but also against a spectrum of pathogens including viruses, bacteria and fungi [8]. Salicylic acid (SA) has been shown to play a central role as a signaling molecule involved in both local defense reactions and in the induction of SAR. Transgenic tobacco and *Arabidopsis thaliana* plants expressing the bacterial *nahG* gene encoding salicylate hydrogenase, an enzyme that catalyzes the conversion of SA to catechol, not only have low levels of SA, but also are unable to express SA in response to viral, fungal or bacterial pathogens [9,10]. Additionally, it was shown that the level of SA increases after pathogen inoculation and is correlated with SAR [11]. Moreover, exogenous application of SA can induce SAR [12]. Benzo-(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) mimics SA and induces SAR in a number of plants including wheat, *Arabidopsis* and tobacco [13–15].

Several pathogenesis-related (PR) genes, including those belonging to PR1 [16], PR2 [17] and PR4 [18] classes are induced during local defense and SAR. The *Nicotiana plumbaginifolia gn1* gene, encoding a β-1,3-glucanase isoform has also been described as a PR gene [19]. *gn1* was found to be expressed in roots and older leaves and is controlled by a pathogen inducible promoter. The effect of SA on the *gn1* promoter was evaluated before and after spraying transgenic tobacco plants with 5 mM SA. SA treatment induced the *gn1* promoter about 14-fold, based on increased GUS activity [19]. Analysis of GUS activity in transgenic tobacco plants containing the *gn1/gus* construct showed different GUS activity levels in different tissues. No detectable GUS activity was present in the upper parts of the transgenic plants and maximum activity was observed in older leaves and roots. The expression pattern suggested that the characterized β-glucanase played a role in plant development and in the defense against pathogen infection.

To study the feasibility of utilizing pathogen reporting phytosensors, the *gn1* promoter was fused to an *mgfp5-er* reporter gene. Transgenic plants containing the construct were produced to determine the patterns of GFP-mediated fluorescent signaling post-BTH application and/or pathogen infection.

2. Methods

2.1. Construction of gn1/mgfp5-er vector and plant transformation

The plasmid containing the *gn1* promoter was kindly provided by Marc van Montagu, Gent, Belgium [19]. A 1.6-kb fragment containing the *gn1* promoter was PCR amplified to introduce SacI restriction sites at both ends. Primers used for amplification were forward 5′-CGGCAGAGCTCGTAATCACGT-3′ and reverse 5′-GGCGGGAGCTCGGCTTTTGTT-3′. The PCR product was cloned into a pBinplusARS derivative plasmid (kindly donated by Bill Belknap) containing the *mgfp5-er* gene, an *nos* terminator and a kanamycin selection cassette. The orientation of the *gn1* fragment was confirmed by digestion with PacI/KpnI and the construct was partially sequenced to ensure the ATG initiation codon in frame with the *mgfp5-er* gene. The resulting plasmid, called pBinplus Gn1, had a molecular size of 14.5 kb.

N. tabacum cv. Xanthi seeds were sterilized by soaking in 20% Clorox for 5 min and in 70% alcohol for 2.5 min. The seeds were then washed with sterile water and grown in sterile conditions on MS basal medium [20] for 4 weeks. The plasmid pBinplus Gn1 was transformed into *Agrobacterium tumefaciens* strain GV 3850 and tobacco was transformed by leaf disc transformation method [21].

2.2. gn1/mgfp5-er induction in transgenic plants

Primary transgenic plants were grown in a growth chamber under 300 μE irradiance, 16 h photoperiod at 20 °C. The plants were then transferred to the greenhouse and the seeds were collected after 3 months. Seeds from T_0 transgenic tobacco plants were surface-sterilized and grown in Petri dishes containing MS medium supplemented with 200 μg/ml kanamycin. Plants were treated with BTH or water at different stages. Four- and eight-week-old plants were transferred to MS media containing 3 mM BTH; 12-week-old plants transferred to soil were sprayed with 5 mM BTH using about 5 ml of solution to completely wet each plant.

For inoculation with *Plectosporium tabacinum* (Van Beyma) [22], strain MEP 1353 obtained from the American Type Culture Collection (ATCC) was grown on media containing potato dextrose agar (PDA) at 24 °C for 2 weeks and conidia was harvested from the plates using 5 ml water. T_1 tobacco leaves were inoculated by placing 10 μl of 3×10^7 conidia/ml on the leaves and the plants were incubated at 22 °C in the growth chamber under cover for 48 h.

Leaves were sampled at various intervals post-induction as indicated. Protein was extracted from the leaf

samples and Western blot analysis was performed as described by Stewart et al. [23].

2.3. DNA and RNA analyses

DNA extraction from plant tissue (CTAB method) and PCR were performed as described by Stewart and Via [24]. RNA extractions were done from tissue samples taken 7 days after BTH treatment of 12-week-old plants. Leaf tissue was ground to a powder in liquid nitrogen and total RNA was extracted using RNA extraction columns according to the manufacturer's protocol (Qiagen, Valencia, CA). RNA was reverse transcribed using the SuperScript™ first-strand synthesis system for RT-PCR according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). PCR was performed on cDNA by using forward primer F1 5′-AAAAGAGCTCATCCAAGGAGATATAACAAT-3′ and reverse primer R1 5′-CCGGTTGAGCTCT-TAAAGCTCATCATGTTT-3′ to estimate the transcript levels of the *mgfp5-er* transgene in the treated and untreated plants.

2.4. Fluorescence spectroscopy

To determine the expression of GFP in response to BTH treatment, GFP activity was analyzed by fluorescence spectrophotometry in leaves of transgenic plants before and after BTH treatment. The detection of GFP fluorescence on intact tissues was attempted using a Fluoromax-2 fluorescence spectrometer (Jobin Yvon Instruments S.A., Inc., Edison, NJ). The treated and untreated leaves were excited at 385 nm and emission spectra were recorded from 400 to 550 nm according to published methods [25].

3. Results

3.1. gn1/mgfp5-er vector and transgenic plants

For the proof-of-principle of the phytosensor concept, a chimeric *gn1/mgfp5-er* reporter gene was constructed and introduced into tobacco. Forty plants resistant to kanamycin were chosen for regeneration. These plants as well as a subsample of their progenies were tested for the presence of the *mgfp5-er* gene by PCR analysis. All putative transgenic primary plants were PCR-positive (data not shown). Progeny analysis was performed to estimate the number of T-DNA loci in 20 transgenic tobacco events by analyzing the segregation of the kanamycin-resistant and -sensitive phenotypes. Of these, 75% showed ratios of 3 kan^r:1 kan^s (Chi-squared analysis, data not shown). These results indicated which events had single T-DNA locus inserts per genome. T_1 progeny from this subsample of single locus events were used for subsequent analysis.

3.2. RNA analysis of transgenic plants induced with BTH

Twelve-week-old primary transgenic plants that were grown on MS media containing kanamycin were sprayed with 5 mM BTH. Leaf samples were collected 7 days after induction. RT-PCR was performed to determine the presence of GFP transcripts and to identify high induction events. The variation of transcript levels was large among events with all events showing presence of the transcript to some degree (Fig. 1). In all cases, *gn1/mgfp5-er* transcript levels were at greatly reduced levels compared with the transgene under the control of the constitutive 35S promoter.

3.3. BTH-induced expression of a chimeric gn1/mgfp5-er gene in T_1 transgenic tobacco plants

To characterize the induction dynamics of GFP synthesis in response to BTH, 4- and 8-week-old transgenic plants resistant to kanamycin were transferred to Magenta boxes containing medium with 3 mM BTH. GFP expression was not detectable by Western blot analysis in plants treated at 4 weeks (Fig. 2A). In older plants treated at 8 weeks, GFP was not detected 48, 72 and 96 h post-induction, but was detectable 9, 11 and 18 days after BTH application. However, GFP expression was low and difficult to detect by Western blot analysis (Fig. 2B). Therefore, another set of experiments was conducted where 12-week-old plants grown in soil were sprayed with 5 mM BTH. Western blot analysis, performed on transgenic plants 48 h after induction, showed expression of GFP in most treated lines but not in water-sprayed transgenic plants (Fig. 2C).

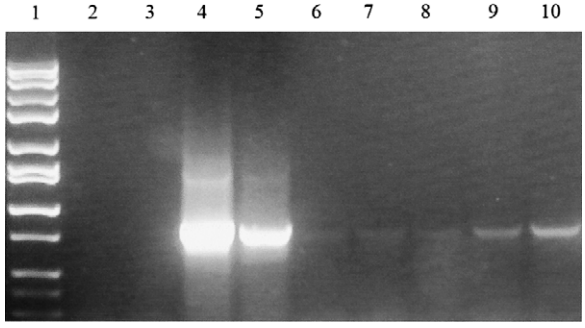

Fig. 1. Relative GFP transcript levels in *35S/gfp* and *gn1/mgfp5-er* transgenic plants. RNA was extracted from leaves sampled 7 days after treatment with 5 mM BTH and RT-PCR was performed. Lane 1: DNA marker, lane 2: water (negative control), lane 3: cDNA from non-transgenic plant, lane 4: plasmid DNA containing the *mgfp5-er* gene (positive control), lane 5: cDNA from a *35S/mgfp5-er* transgenic plant and lanes 6–10: cDNA from *gn1/mgfp5-er* plants.

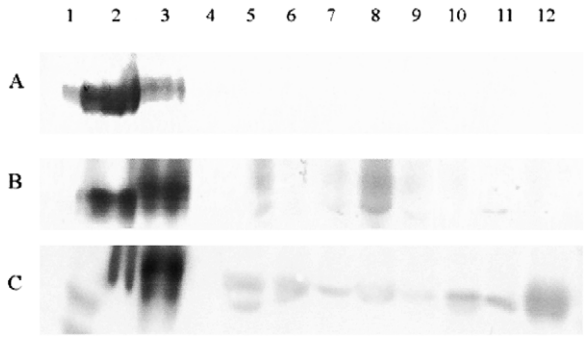

Fig. 2. Plant-age-dependent induction of *gn1/mgfp5-er*. Western blot analysis was carried out on T_1 transgenic plants induced with BTH. Four- and eight-week-old plants were grown on MS media with 3 mM BTH in Magenta boxes (A) and (B), respectively. Twelve-week-old plants grown in soil were sprayed with 5 mM BTH (C). Samples were collected from the lower leaves 7 days after the BTH treatment. Lane 1: protein marker, lane 2: 20 ng GFP standard, lane 3: positive control plant containing a *35S/mgfp5-er* fusion construct and lane 4: *gn1/mgfp5-er* transgenic plant sprayed with water (negative control). Lanes 5–12 are plants from independent *gn1/mgfp5-er* transgenic events (1, 2, 3, 4, 5, 6, 8 and 11) sprayed with 5 mM BTH.

3.4. GFP detection in transgenic plants expressing the *gn1/mgfp5-er* construct by fluorescence spectrophotometry and UV light

No constitutive expression of GFP was observed by fluorescence spectrophotometry or UV light before treatment with BTH. One week after spraying, the selected transgenic plants with 5 mM BTH, the lower leaves and roots were screened for *mgfp5-er* expression. GFP fluorescence was detectable in a single 12-week-old plant in the roots and leaves and compared with fluorescence from *35S/mgfp5-er* plants used as positive controls (Fig. 3). However, the fluorescence was very low and was not detectable by fluorescence spectrophotometry.

3.5. Systemic induction

To investigate the systemic induction of the *gn1/mgfp5-er* by BTH, GFP expression was examined after spraying plants with both BTH and water. The fourth leaf was sprayed with water whereas the rest of the plant was sprayed with BTH. We found that external application of BTH to the transgenic tobacco plants harboring the *gn1/mgfp5-er* construct led to GFP expression in treated and untreated leaves of the same plant (Fig. 4). The result showed that the BTH treatment could systemically induce the *gn1/mgfp5-er* gene.

3.6. Time course of induction of *gn1/mgfp5-er* by BTH in transgenic tobacco plants

Western blot analysis was performed on 40 transgenic events to determine the levels of GFP expression. Out of those, 19 lines expressed GFP at detectable levels after BTH treatment. Four events with high GFP expression were used for the time course analysis. These independent transgenic events (3, 6, 8 and 11) had strong signals in Western blots at different days after treatment with BTH. Plants were treated with water or BTH (5 mM) and incubated in a growth chamber. Leaf samples were collected at various periods extending to 16 days. Western blot analysis of BTH-treated transgenic plants demonstrated that GFP could be detected 48 h after treatment. Levels of GFP protein gradually increased to a maximum between 6 and 12 days and began to decrease at day 16 (Fig. 5).

3.7. *gn1/mgfp5-er* induction after fungal inoculation

Twelve-week-old transgenic tobacco plants (T_1) were inoculated with spores of the tobacco fungal pathogen *P. tabacinum*. Eight transgenic events were used for this experiment. Samples from the lower leaves close to the inoculated area were collected after 7 days and Western blot analysis was performed. GFP expression was detected by Western blot analysis upon pathogen treatment (Fig. 6). An HR was visible on the leaves and stems 10 days after inoculation.

4. Discussion

We examined the expression pattern of the *gn1/mgfp5-er* fusion and showed that the *gn1* promoter was induced locally and systemically by BTH and the fungal pathogen *P. tabacinum*. Using GUS reporter fusions, Castresana et al. [19] have previously shown induction by SA, ethylene, wounding and infection with two bacterial species, *Pseudomonas syringae* and *Erwinia carotovora*. Inducibility by a broad range of pathogens and molecules involved in different signal transduction pathways is a desirable feature when contemplating its use for the design of disease reporting phytosensors. Systemic induction is also a very useful property for such a promoter, as the output signal is not only generated at the site of infection, but also propagated throughout the plant.

Our results indicate that induction of the *gn1* promoter was dependent on plant age as GFP expression was difficult to detect in plants that were 8-week-old or younger. Castresana et al. [19] have shown a top to bottom gradient of induction, with older leaves and roots showing maximum expression of the GUS reporter gene. Spatial and developmental control of gene expression has been reported for a number of PR proteins and a relevance to various developmental processes has been suggested [26–28]. The complexity of the expression pattern of PR genes and, in general, of many plant promoters is inherent to the fact that they

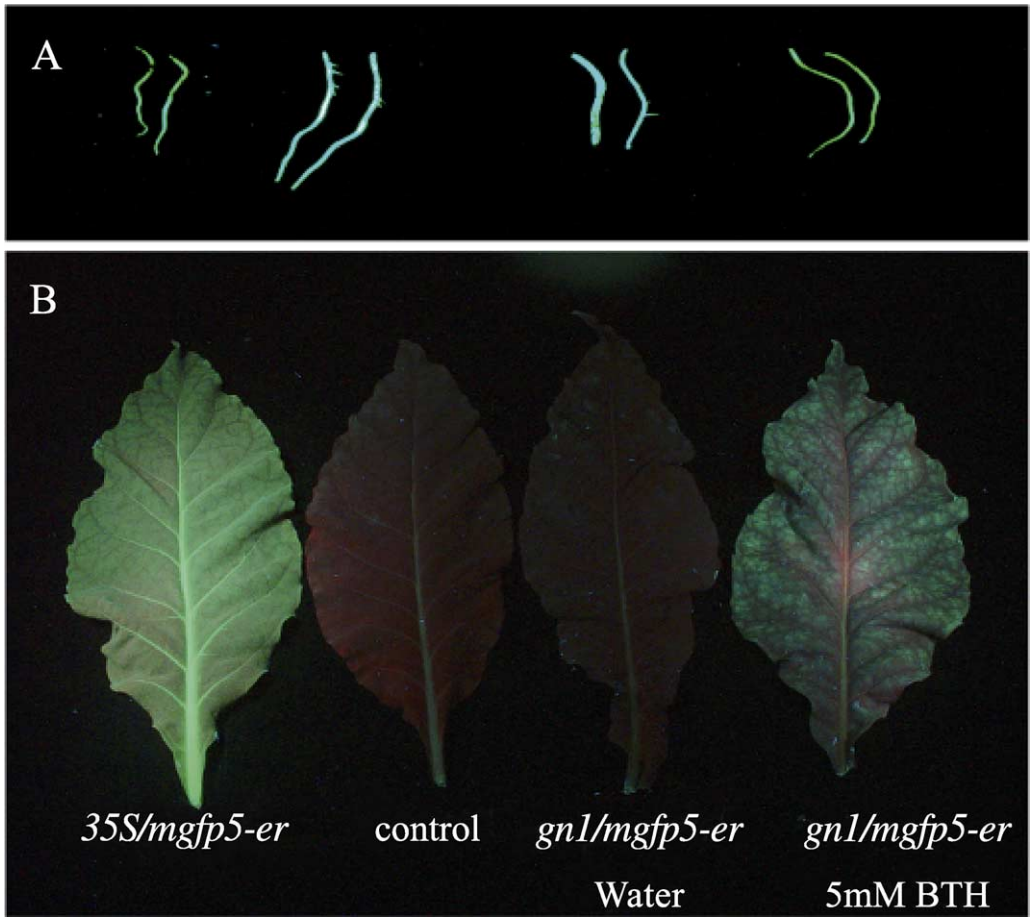

Fig. 3. GFP fluorescence detection by UV light in roots (A) and leaves (B). Fluorescence from a *gn1/mgfp5-er* line sprayed with water and 5 mM BTH was compared with fluorescence from a transgenic plant containing *35S/mgfp5-er* used as a positive control and from a non-transgenic plant used as a negative control. GFP fluorescence was slightly detectable in the *gn1/mgfp5-er* plant treated with BTH as light green in comparison to control plants.

contain several *cis*-regulatory elements, each of which can be activated or repressed by more than one transcription factor. To circumvent this limitation, synthetic promoters can be designed based on selected *cis*-regulatory motifs.

GFP was detectable by Western blot analysis in most transgenic events as early as 48 h after induction and showed maximum accumulation between 6 and 12 days. However, we were unable to detect a strong GFP signal in leaves by fluorescence spectrophotometry. Low levels of fluorescence were observed for one transgenic event upon visual inspection in the dark under UV light (Fig. 3B). High-level background fluorescence and low-level *mgfp5-er* expression in plant tissues could be the main reasons why GFP fluorescence could not be robustly optically detected. Comparing GFP and GUS as reporter markers showed that GFP might under-report promoter activity in some transgenic plants [29]. For

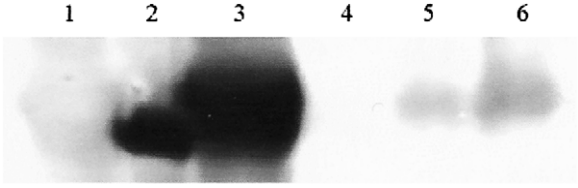

Fig. 4. Systemic induction of *gn1/mgfp5-er* gene. Twelve-week-old T_1 tobacco plants were sprayed with 5 mM BTH except for the fourth leaf which was sprayed with water. Western blot analysis was performed on leaf samples taken 4 days after treatment. Lane 1: protein marker, lane 2: 20 ng GFP standard, lane 3: *35S/mgfp5-er* transgenic plant used as a positive control, lane 4: non-transgenic plant sprayed with BTH, lane 5: fourth leaf of *gn1/mgfp5-er* transgenic plant sprayed with water and lane 6: older leaf of *gn1/mgfp5-er* transgenic plant sprayed with BTH.

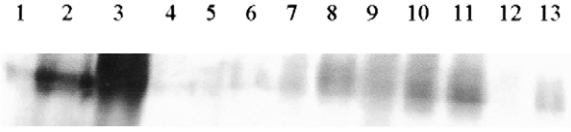

Fig. 5. Time course of GFP accumulation. Twelve-week-old T_1 transgenic plants were sprayed with 5 mM BTH and Western blot analysis was performed from lower leaf samples taken at various intervals post-induction. Lane 1: protein marker, lane 2: 20 ng GFP standard, lane 3: *35S/mgfp5-er* positive control transgenic plant, lane 4: *gn1/mgfp5-er* transgenic plant sprayed with water and lanes 5–13: *gn1/mgfp5-er* transgenic plant sprayed with BTH and samples taken 0, 2, 4, 6, 8, 10, 12, 14 and 16 days, respectively.

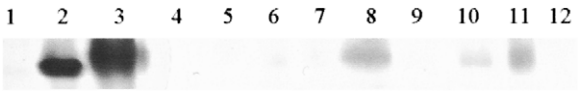

Fig. 6. Induction of *gn1/mgfp5-er* by the fungal pathogen *P. tabacinum*. Western blot analysis was performed on leaf samples collected 7 days after inoculation of T_1 tobacco plants with *P. tabacinum*. The samples were taken from the lower leaves close to the inoculated area. Lane 1: protein marker, lane 2: GFP standard (20 ng), lane 3: *35S/mgfp5-er* positive control, lane 4: non-transgenic control inoculated with water and lane 5: non-transgenic control inoculated with *P. tabacinum*. Lanes 6–12: *gn1/mgfp5-er* transgenic events; lanes 2, 5, 6, 61, 8, 14 and 18: inoculated with *P. tabacinum*.

instance, GFP expression driven by the *A. thaliana* zinc finger protein (AtZFP) promoter is difficult to distinguish from background autofluorescence present in many *Arabidopsis* tissues [29]. Since the *gn1* promoter is most induced in lower parts of plants [19], we screened the roots of independent transgenic events for GFP fluorescence after treatment with BTH. As a result of relatively less autofluorescence, we were able to detect low levels of fluorescence in roots by visual inspection under UV light (Fig. 3A). In contrast to GFP fluorescence in shoots, fluorescence in roots would not be very accessible for field-based monitoring of sentinel plants—phytosensors.

Clearly, the low GFP fluorescence in *gn1/mgfp5-er* transgenic plants is an impediment to the realization of deploying a field-level, transgenic phytosensor that could report pathogen infection in real-time. Using more advanced imaging techniques or enhancing GFP expression may overcome these problems. One possible tool to increase expression, and hence, utility, is to add an enhancer element flanking the promoter to boost the promoter. The 35S promoter of cauliflower mosaic virus (CaMV) contains complex enhancer elements that can activate or affect gene expression when present upstream or downstream of genes [30,31]. The 5′-upstream region (−343 to −46) of CaMV 35S promoter could be used in the form of tandem repeats to enhance gene expression. We will then be left with the problem of decreasing the time required between induction and detectable expression (fluorescence) to be evident. Seven days post-infection phytodetection is too long to be of practical use. Still, if expression could be significantly enhanced, a 3–4 day post-infection time to fluorescence is reasonable (see Fig. 5). Ultimately, the scheme for a real-time phytosensor with a fluorescent output may depend on the utilization of synthetic PR-type promoters with defined *cis*-regulatory elements coupled with enhancer motifs. The desired outcome will be induction to a wide range of pathogens, high expression and little lag. The application of such phytosensors may someday prove to be an important tool for early detection of routine agricultural plant diseases as well as agro-terrorism activity.

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