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Evaluation of two promoters for generating transgenic potato plants as salicylic acid biosensors

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Abstract

Plants are severely affected by many biotic stresses, which cause a reduction in crop quality and quantity. One of the strategies to manage biotic stresses is the generation of transgenic plant lines that can be used as biosensors. These biosensor plants can trigger an early warning upon any pathogen infection. Two promoters with β -glucuronidase reporter gene fusions were constructed. The first contained the *flagellin sensing 2* gene promoter, whereas the second contained synthetic promoter containing four repeats of *cis*-acting elements from the *pathogen-related protein 1* gene and two transcription enhancers from the 35S promoter. Transformed leaves were treated with a phytohormone salicylic acid to mimic the occurrence of biotic stress. Validation of reporter gene expression induced from both constructs in transformed potato leaves displayed an increase upon salicylic acid treatment. The results reflect that both constructs could serve in the production of potato biotic stress biosensors.

Additional key words: *FLS2* gene, pathogen infection, *PR1* gene, synthetic promoter.

Introduction

Plant metabolism must balance demands for resources to support defense *versus* requirements for cellular maintenance, growth, and reproduction (Herms and Mattson 1992, Zangerl and Berenbaum 2003, Berger *et al.* 2007a). Upon introduction of various elicitors, such as pathogen-associated molecular patterns, viral coat proteins or fatty acid conjugates in the oral secretions of insect saliva, a massive reprogramming of plant gene expression and hormonal and chemical defense responses are initiated. These processes can be costly in terms of plant growth and fitness (Tian *et al.* 2003, Zavala and Baldwin 2004). In addition to triggering defenses to fight pathogen and herbivore attack by allocating resources from growth to defense, a reduction of photosynthetic capacity in remaining leaf tissues may represent a 'hidden cost' of defense (Zangerl *et al.* 2002, Aldea *et al.* 2006, Berger *et al.* 2007b, Bilgin *et al.* 2008, Nabity *et al.* 2009).

One of the best examples for pathogen-associated

molecular pattern is the recognition of pathogens by *Arabidopsis* receptor flagellin sensing 2 (FLS2), which is specific in binding bacterial flagellin and then activate downstream defense pathways. The *FLS2* gene receptor is similar to mammals toll-like receptor (TLR) which involved in innate immunity (Bauer *et al.* 2001, Gómez-Gómez *et al.* 2001). The *FLS2* gene was characterized in both *Arabidopsis* and tomato (Xiang *et al.* 2008). Beck *et al.* (2014) generated a transgenic *Arabidopsis* with *FLS2* gene promoter upstream of the reporter gene β -glucuronidase (*GUS*). High expression of *GUS* was obtained in tissues and cells exposed to bacterial entry as stomata and hydathodes in leaves and also in lateral roots.

Among plant responses to pathogens, several genes encoding pathogen-related proteins are induced, and pathogen-related proteins accumulated significantly in plants (Peng and Kaloshian 2014, Wen *et al.* 2017). Whereas pathogen-related proteins have been described firstly in tobacco leaves, many other pathogen-related proteins from plant species of several families have been

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Abbreviations: FLS2 - flagellin sensing 2; GUS - β -glucuronidase; PR1 - pathogen-related protein 1; RT - reverse transcription; SA - salicylic acid.

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reported. The *pathogenesis-related protein 1* gene (*PR1*) is considered as a marker gene for the systemic acquired resistance (SAR) phenomena in *Arabidopsis thaliana*. Lebel *et al.* (1998) studied functional regulatory sequences in the *Arabidopsis* *PR1* gene promoter and found a putative *cis*-element that responds specifically to salicylic acid (SA). Mazarei *et al.* 2008 fused this *cis*-element to the *GUS* gene and generated transgenic tobacco plants that showed high *GUS* expression when infected with Alfalfa mosaic virus or treated with SA.

Promoters used in biotechnology applications can be grouped into four classes (Hernandez-Garcia and Finer 2014); constitutive promoters which are active in most of the tissues and developmental stages, spatio-temporal promoters which provide tissue-specific or stage-specific expression, inducible promoters which are regulated by the application of an external chemical or physical signal; and synthetic promoters which contain defined regulatory elements located adjacent to or within promoter sequences. Synthetic promoters have the merit to drive constitutive, spatio-temporal, and inducible expression patterns. It can also drive combinations of two or more expression patterns depending on the included elements.

Biosensors, as a powerful alternative to conventional analytical techniques, enable highly sensitive, real-time, and high-frequency monitoring of pollutants without extensive sample preparation (Long *et al.* 2013). Plants naturally sense biotic or abiotic changes in the environment and respond by altering biochemical and gene expression patterns (Waters *et al.* 2017, Kosová *et al.* 2018, Luo *et al.* 2019), which made them ideal to be used as biosensors (Antunes *et al.* 2006, Liu *et al.* 2011). Engineering plants by placing a reporter gene downstream of the biotic stress-inducible promoter will make them capable of releasing a signal (reporter gene protein) that can be detected and visualized upon stress recognition. This type of plant can be called phytosensors (Mazarei *et al.* 2008) which can be used for wide-area sensing from the ground or satellite. A transgenic phytosensor could be feasibly placed in any row crop as a sentinel, which will make an integrated system for real-time detection. This system could be modified to be a commercial product that will minimize losses caused by biotic stress. It will also minimize the use of pesticides which have adverse impacts on human health and the environment.

In the present study, reporter gene (*GUS*) expression obtained from two promoter/reporter gene fusions after induction with SA was evaluated. Transient transformation systems can be utilized as a rapid tool for assessing promoter function. Analysis of obtained reporter gene expression could promote the production of biosensor from potato which can detect pathogens in the early stages of infection.

Materials and methods

Plants and growth conditions: *Arabidopsis thaliana* L. (Col-0) seeds and potato (*Solanum tuberosum* L. cv. Diamond) plantlets were obtained from the Molecular

and Serological Analyses Laboratory of *AGERI*, and the Micropropagation Technology Laboratory of *ARC*, respectively, Giza, Egypt. Potato (cv. Herms) tubers were received from the brown rot project, *ARC*. According to Harrison *et al.* (2006), *Arabidopsis thaliana* Col0 seeds (~100 seeds) were sterilized and plated on autoclaved (Murashige and Skoog 1962, MS) media plates supplied with sucrose (2 %, m/v) and *Bacto Agar* (0.8 %, m/v). Thirty seeds were dispensed per standard Petri dish. All previous steps were conducted under a laminar flow hood. The plates were transferred to 4 °C in a cool room for three days to break their dormancy, enhancing germination rate and synchronization before transferring to a growth chamber set at a temperature of 23 °C, a 18-h photoperiod, and an irradiance of 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Potato tubers were planted at the same depth in pots filled with clay and sand (1:1). After that, the soil was applied to fully cover tubers and irrigation was applied when needed. Obtained plantlets were maintained in controlled greenhouse free from plant pathogens at a temperature of 22 °C, a 18-h photoperiod, and an irradiance of 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$. *In vitro* propagated potato leaves were obtained from the potato tissue culture laboratory at the Agriculture Genetic Engineering Institute, Agriculture Research Center, Giza, Egypt.

Plasmids and PCR primers used during this study were *Stratagene pBluescript SK(+)*, *Clontech pBI121*, and *pCambia1390::35S* which was kindly obtained from Dr. Pascal Ratet, the Institute of Plant Sciences, INRA, Paris, France. The pCambia vector map was drawn by *Snap Gene* (v. 2.7.3; www.snapgene.com). Amplification primers were manually designed, whereas reverse transcription PCR primers were designed using a web tool *Primer Quest* (<https://www.idtdna.com/Primerquest/Home/Index>). The sequences of the primers used in this study are in Table 1 Suppl.

Oligo annealing protocol was performed as described by Fauser *et al.* (2014) with minor modifications. Dilutions from the oligo represent top strand (Oligo PR1F) and the oligo (Oligo PR1R) represent bottom strand were made to obtain 50 μM concentrations, then 2 mm^3 from each oligo were diluted in a 0.2 cm^3 tube containing 46 mm^3 of double distilled H_2O . Then the tubes were incubated at 95 °C in a thermomixer for 5 min. After that, the thermomixer was closed, and the tubes were left to cool slowly in the device for 90 min. The result was double-stranded DNA containing a *cis*-acting element from the *Arabidopsis PR1* gene.

Extraction of DNA from *Arabidopsis* leaves was performed using a *DNeasy* plant mini kit (*Qiagen*, Hilden, Germany) following the manufacturer's instructions. During construction steps of promoter/reporter fusions, plasmid DNA was extracted using an *Invitrogen*™ *PureLink*™ *HiPure Plasmid Miniprep* kit (Carlsbad, CA, USA) following the manufacturer's instructions.

Promoter-reporter gene fusion (*FLS2/GUS*) was done by cloning the *GUS* gene (amplified from the pBI121

plasmid) inside plant expression vector pCambia 1390::35S via *Bam*HI/*Eco*RI sites. Subsequently, *FLS2* promoter (amplified from *Arabidopsis* genomic DNA) was cloned inside pCambia 1390::35S::GUS via *Hind*III/*Bam*HI sites after the removal of the 35S promoter. A synthetic promoter B4XPR1A was constructed inside the pBluescript SK(+) cloning vector. Enhancer domain A and enhancer domain B were amplified from pBI121. Enhancer domain B, synthetic *cis*-acting element *PR1*, and enhancer domain A were ligated into the vector via *Sac*III/*Not*I, *Xba*I/*Bam*HI, and *Spe*I/*Bam*HI sites, respectively. BioBrick standard assembly (Shetty *et al.* 2008) was used to produce four repeats of the *cis*-acting element linked in head to tail orientation (4X PR1). Subsequently, the synthetic promoter was amplified using En1F1 and En2R primers. After that, it was cloned inside pCambia 1390::FLS2::GUS via *Hind*III/*Bam*HI sites after the removal of the *FLS2* promoter. Final constructs were used to transform *Agrobacterium* strain LBA4404.

Transient transformation and biotic stress treatment:

The protocol of Jung *et al.* (2005) was used with *in vitro* propagated potato (cv. Diamond) with some modifications. The leaves from 8-week-old *in vitro* propagated potato were incubated with *Agrobacterium* inoculum for 10 min. After that, leaves were removed and blotted with sterile filter paper. The leaves were then plated on co-culture medium (MS medium with 20 g dm⁻³ sucrose, 500 mg dm⁻³ MES, 2 mg dm⁻³ 2,4-dichlorophenoxyacetic acid sodium salt, 0.5 mg dm⁻³ zeatin, 5 g dm⁻³ *Agargel*, pH 5.7) and incubated in the dark at 19 °C for 48 h before subjected to *GUS* histochemical assay or SA treatment.

Potato leaves were excised from *in vitro* propagated plantlets and each leaf was cut into equal three parts. Leaf parts were immersed inside medium with *Agrobacterium* (absorbance = 0.5) harboring the desired construct for two days. On the third day, *Agrobacterium* was killed by incubating leaves with 1 % (v/v) bleach followed by several double distilled H₂O washes to remove the bleach. The first parts of the leaves were immersed in either 0.5 or 1.0 or 1.5 mM salicylic acid (SA) for 24 h. The second part of the leaves was immersed in 0.1 % (v/v) ethanol for 24 h (mock treatment) since SA was dissolved in 0.1 % ethanol to generate a 100 mM stock solution. The third part of the leaf was used for immediate testing (*GUS* assay or reverse transcription (RT) PCR; 0-time read). A set of transformed leaves was specified for histochemical assay and another set was specified for RT-PCR. Each experiment was performed in three replicates. Each replicate contained three leaves.

Semi-quantitative RT-PCR: Before the RT-PCR step, RNA was purified from transformed potato leaves using an Invitrogen TRIzol™ Reagent (Carlsbad, CA, USA) according to the manufacturer's instruction. A SuperScript™ double-stranded cDNA synthesis kit (Thermo Fisher Scientific) was used for RT-PCR of *GUS* and *ACTIN* genes according to the manufacturer's instructions. All the amplification reactions in this manuscript were carried out using the following conditions:

an initial denaturation temperature of 95 °C for 3 min., then 25 cycles (denaturation at 95 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 30 s). A final extension temperature was 72 °C for 10 min, and a hold temperature was 4 °C. Amplification was conducted using *GoTaq® Flexi* DNA polymerase (Promega, Madison, WI, USA).

Histochemical assay, microscopy, and image analysis:

Potato leaf parts were soaked in staining solution (Jefferson 1987) followed by incubation at 37 °C overnight. The staining solution was removed and replaced by absolute ethanol. The mixture was incubated overnight to remove chlorophyll and clarify *GUS* resolution. The stained leaves were imaged using an epi-fluorescence microscope Olympus SZX10 (Tokyo, Japan), and the images were captured using the imaging software *Cell sense 2.56* (QImaging, Burnaby, BC, Canada). Pictures of the histochemical assay were analyzed using *Image j 1.50b* software (National Institute of Health, Bethesda, MD, USA) to obtain *GUS* reporter gene pixel intensity. Data were analyzed using a two-sample *t*-test (*P* < 0.05).

Results and discussion

Biosensors varied according to their target, biorecognition molecules, optical transducers, and signal processing (Long *et al.* 2013, Justino *et al.* 2017). In this investigation, the used models using the whole potato plant cell as a biorecognition unit to sense pathogen infection and trigger an early alarm in the form of reporter protein (signal). So, potato cells were transformed with a promoter fused to a reporter gene. During the normal conditions, transformed potato cells displayed reporter gene expression but when exposed to biotic stress, the expression of the reporter gene increased significantly. Reporter gene *GUS* can be used in the preliminary stages of the biosensor production to validate the induced expression, but when the high expression is confirmed, it can be replaced by an optical reporter like gene which causes easily detectable chlorophyll loss (Antunes *et al.* 2006).

The first biosensor model (Fig. 1A) contained a combination of the *FLS2* gene promoter and the *GUS* reporter gene. The *FLS2* gene is expressed in *Arabidopsis* leaves, but during biotic stress, it becomes highly expressed (Beck *et al.* 2014). The second model (Fig. 1B) includes synthetic promoter 4XPR1 (four repeats of a *cis*-acting element from PR1 promoter) and two transcription enhancers domains (A, B) to drive *GUS* reporter gene expression under stress condition. A synthetic promoter 4XPR1 is successful in inducing reporter gene expression during biotic stress (Rushton *et al.* 2002, Mazarei *et al.* 2008, Liu *et al.* 2011). Co-cultivation protocol (Jung *et al.* 2005) is used to transform *in vitro* propagated potato. Co-cultivation of potato leaves with *Agrobacterium* carrying 35S::GUS fusion displays reporter gene expression (Fig. 2). We also observed a high reporter gene expression in the transformed leaf petiole. Co-cultivation of potato and *Agrobacterium* is well established in many studies

(Mollaa *et al.* 2011).

Each potato leaf obtained from *in vitro* propagated potato was divided into three equal parts; one was used for the zero time readings of *GUS* pixel intensity (before SA treatment) and the other two parts were used for the treatment readings (after SA treatment or mock control). Fold changes in expression of the *GUS* reporter gene following SA treatment are presented in Fig. 3A. Semi-quantitative analysis (Fig. 4A) revealed an increase in amplified *GUS* reporter gene expression from leaf parts treated with 1.0 and 1.5 mM SA than their mock control while leaf parts treated with 0.5 mM SA displayed amplification similar to mock control. In conclusion, expression of *GUS* reporter gene in potato leaves transformed with the construct B4XPR1A::GUS displayed an increase upon treatment with higher SA concentrations. The same results were obtained from three independent experiments.

Our results were in correspondence with the results of Liu *et al.* (2011) who obtained an increase in reporter gene expression from tobacco and *Arabidopsis* infiltrated with *Agrobacterium* containing synthetic promoter B4XPR1A fused to *RFP* reporter gene. But they used only one concentration of salicylic acid (4 mM) and detected significant reporter expression after 72 h of agroinfiltration. Raventos *et al.* (1995) reported that the presence of the B_A enhancer increases the basal expression of the reporter gene but does not change the induction rate of the regulatory element. Our results also confirmed the study of Rushton *et al.* (2002) who obtained inducible expression of synthetic promoters/reporter fusions upon treatment with phytohormones. It is notable to mention that SA treatment may change plant responses to abiotic stresses, such as cold, drought, or high salinity. Additionally, the *Arabidopsis* *PR1* gene promoter was found inducible

by such stresses (Seo *et al.* 2008, Liu *et al.* 2013, Miura and Furumoto 2013). Thus, in our study, we used a *cis*-acting element sequence from the *PR1* gene promoter that responds only to pathogen infection. This biosensor system could also be utilized for temporary phytosensing without the need for deploying stable transformants, since transient expression may be much higher than that of stable transgenic plants (Janssen and Gardner 1989, Wroblewski *et al.* 2005).

Image analysis data were analyzed using paired Student *t*-test with the level of significance $P < 0.05$. Fold changes in expression of the *GUS* reporter gene following SA treatments are represented in Fig. 3B. Semi-quantitative analysis (Fig. 4B) revealed an increase (about two-fold) in amplified *GUS* reporter gene in 0.5, 1.0, and 1.5 mM SA-treated leaf parts in comparison with their mock control leaf parts. There were no significant differences between the three SA concentrations.

The expression pattern of *FLS2/GUS* fusion is expected due to the nature of the *FLS2* promoter which displays increased expression during biotic stress. According to our knowledge, no previous reports stated the transient transformation using the *FLS2* promoter. However, these results may correspond with the results of Beck *et al.* (2014) who obtained an increase in reporter gene expression from *FLS2::GUS*-transformed *Arabidopsis* plants upon inoculation with pathogens in leaves and roots treated with salicylic acid. The results also confirm that responsible elements inside *Arabidopsis* *FLS2* promoter overlap with other potato promoter elements, and *Arabidopsis* *FLS2* promoter is inducible in response to salicylic acid treatment under potato cell background. The *FLS2* orthologue may exist in the potato genome since Robatzek *et al.* (2007) characterized the tomato *FLS2* receptor gene which is considered an orthologue of *Arabidopsis* *FLS2*. In



Fig. 1. Biosensor models. A - a model containing the *flagellin sensing 2* *FLS2* promoter upstream of the β -glucuronidase (*GUS*) reporter gene and the *nopaline synthetase* (*NOS*) terminator; B - a model containing the A enhancer domain upstream of response element (RE) tetramers, the B enhancer domain, the *GUS* gene, and the *NOS* terminator.

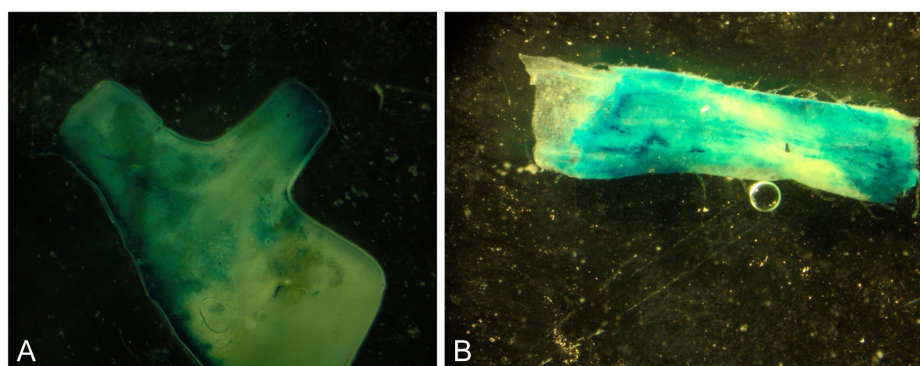


Fig. 2. Transient transformation of a potato leaf part by the co-cultivation method. A - transformed potato leaf part, B - transformed potato leaf petiole.

conclusion, this study combined the construction of native and synthetic promoter/reporter fusions, transformation techniques, and reporter gene detection system to create the foundation of a synchronized structure that will act as an indicator inside plants. Inducibility and gain-of-function analyses for both promoters were determined using *Agrobacterium*-mediated transient transformation. The results from this study are vital for promoter studies and for the further construction of stably transformed potato that can early detect pathogens.

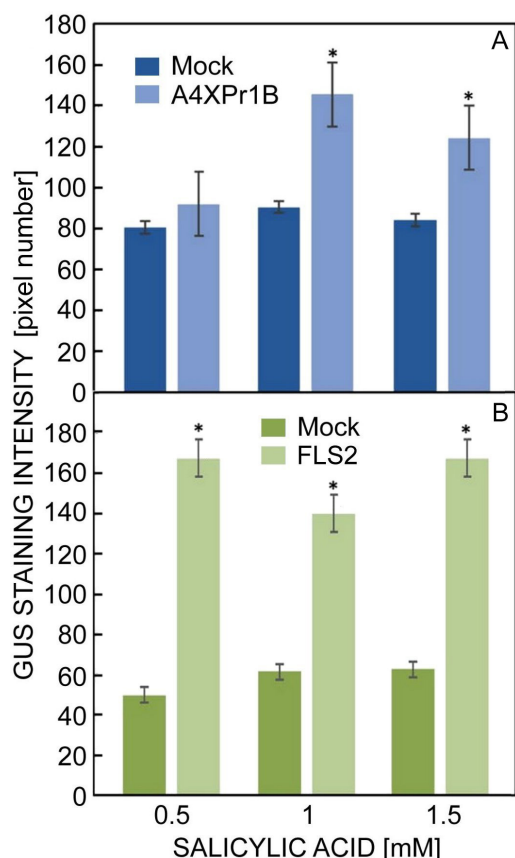


Fig. 3. Changes in expression of the β -glucuronidase (*GUS*) reporter gene from fusions B4XPR1A::GUS (A) and FLS2::GUS (B) following 0.5, 1, and 1.5 mM salicylic acid treatments. Means of GUS pixel intensity \pm SEs. Significant *GUS* expression changes (indicated by asterisks) were determined by the paired *t*-test ($P < 0.05$).

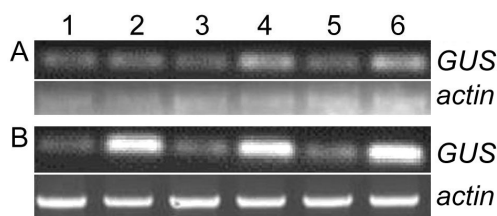


Fig. 4. Reverse transcription PCR of β -glucuronidase (*GUS*) and *actin* genes from potato leaves transformed with B4XPR1A::GUS (A) and FLS2::GUS (B) fusions. Lanes 1, 3, and 5 - mock control leaves parts; Lanes 2, 4, and 6 - leaf parts treated with salicylic acid.

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