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Note

Development of a promoter-luciferase-based high-throughput system to monitor jasmonate-mediated defense gene expression

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Abstract Defense gene expression systems of higher plants responsible for protection against pathogen attack are predominantly regulated by salicylic acid (SA)- and jasmonic acid (JA)-mediated pathways, and control the expression of many downstream defense response genes. To monitor the regulated gene expression of SA-mediated signaling pathways, we previously described an assay system based on the bioluminescence of seedlings transformed with a promoter-luciferase fusion gene. Here, to develop a system suitable for JA-mediated gene expression monitoring, we compared the expression patterns of *Arabidopsis* gene promoters obtained from the *plant defensin 1.2* (*PDF1.2*) and *vegetative storage protein 1* (*VSP1*) genes in response to treatment with chemicals. Although both promoters responded well to treatment with JA in 3-week-old plants, only the *VSP1* promoter exhibited marked and prolonged luciferase expression in response to JA treatment in 6-day-old seedlings. The use of transgenic *Arabidopsis* seedlings harboring the *VSP1*-luciferase reporter gene construct enables multiwell plates to be used for conducting high-throughput assays for the screening of chemicals that are involved in JA-mediated signaling pathways in *Arabidopsis*.

Key words: *Arabidopsis thaliana*, firefly luciferase, high-throughput screening, jasmonic acid, *PDF1.2*, plant activator, *VSP1*.

Plant defense responses are induced by various factors associated with pathogen infection and are predominantly mediated by signal transduction pathways regulated by salicylic acid (SA) and jasmonic acid (JA) (Pieterse et al. 2009). Numerous biological and natural agents capable of triggering defense responses have been commercialized as host defense inducers (Takahashi et al. 2006). Synthetic host defense inducers, such as probenazole (PBZ) and acibenzolar-s-methyl (ASM), have also been successfully used as agrochemicals for controlling plant diseases. Such defense inducers activate SA-mediated signaling pathways and are required for systemic acquired resistance (SAR), which is mainly involved in conferring resistance to biotrophic pathogens (Lawton et al. 1996; Yoshioka et al. 2001). To date, however, no synthetic host defense inducers involved in stimulating JA-mediated signaling pathways, which control defense responses against necrotrophic pathogens, are commercially available (Thomma et al. 2001).

Compared with conventional fungicides, only a limited number of registered pesticides are classified as defense inducers, a fact that is mainly due to a lack of efficient screening and evaluation methods for the development of synthetic host defense inducers. To overcome this limitation, our group has been developing screening and evaluation methods based on the *in vivo* monitoring of defense gene expression using bioluminescence reporter gene technology (Ono et al. 2004; Tanaka et al. 2006). Recently, we established a high-throughput screening (HTS) and evaluation system for SAR inducers (Ono et al. 2011; Watakabe et al. 2011). The bioluminescence monitoring system allows us to conduct non-invasive continuous monitoring of defense gene expression levels *in planta* unavailable in previously known assay systems (Alberts et al. 2006; Narusaka et al. 2006). However, a reliable HTS system for monitoring JA-mediated gene expression has yet to be developed.

To monitor JA-signaling pathways, the *plant defensin 1.2* (*PDF1.2*) gene has been used as a marker because

Abbreviations: ABA, abscisic acid; CaMV, cauliflower mosaic virus; *EF-1a*, *elongation factor 1-alpha*; Fluc, firefly luciferase; HTS, high-throughput screening; JA, jasmonic acid; *PDF1.2*, *plant defensin 1.2*; *VSP1*, *vegetative storage protein 1*.

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of its marked induction in response to JA. The *PDF1.2* gene encodes an anti-fungal protein that is induced in plants by infection with necrotrophic fungi, including *Botrytis cinerea* and *Alternaria brassicicora*, and is also upregulated by treatment with yeast cell wall extract (Manners et al. 1998; Minami et al. 2011). Our preliminary experiments have shown that although the *PDF1.2* gene responds well to stimulus in adult plants, a *PDF1.2* promoter-luciferase (Fluc) fusion gene was not clearly induced in *Arabidopsis* seedlings in 96-well multiwell plates. In the search for JA-inducible promoters suitable for HTS, we identified the *vegetative storage protein 1* (*VSP1*) gene which encodes an acidic phosphatase that inhibits the growth of herbivorous insects and is induced upon wounding and infection by *B. cinerea* (Utsugi et al. 1998; Liu et al. 2005; Kravchuk et al. 2011). Although *VSP1* promoter-luciferase fusion construct has been successfully adopted to monitor JA/ethylene-induced gene expression for the isolation of mutants, detailed gene expression monitoring *in planta* and its application to HST have not been attempted in the previous studies (Ellin and Turner 2001). In the present study, we investigated the responsiveness of the *VSP1* promoter to JA using Fluc as a reporter gene to monitor spatio-temporal expression of the *VSP1* gene.

Toward the development of a HTS method for monitoring JA-mediated gene expression, assays capable of being performed in a multiwell plate are necessary. This requirement necessitates the use of *Arabidopsis* seedlings, which can be the cultured in the wells of a 96-multiwell plate. To test the responsiveness of the *PDF1.2* and *VSP1* genes in seedlings, we examined the mRNA induction levels of these genes in response to treatment by JA. *Arabidopsis* seeds were sown aseptically in the wells of a white 96-well plate (Greiner, Cat. No. 754074) containing 50 μl distilled water (DW). The plates were incubated in the dark at 4°C for 3 days, transferred to continuous light (70 μmol m^{-2} s^{-1}) conditions at 22°C for 5 days, and 1.6 μl of 5 mM methyl jasmonate (MeJA) dissolved in dimethyl sulfoxide (DMSO) was then added to each well together with 50 μl DW. For comparison, 3-week-old soil-grown plants maintained under a 12-h dark/12-h light (70 μmol m^{-2} s^{-1}) cycle were sprayed with 400 μl of 100 μM MeJA per plant. Total RNA samples were extracted at the indicated time points using an RNeasy Plant mini kit (Qiagen). Approximately 150 ng of total RNA was used for cDNA synthesis by AMV reverse transcriptase XL (Takara Bio, Otsu, Japan) with the oligo dT-adaptor primer in a total volume of 10 μl. PCR reactions targeting the *VSP1* and *PDF1.2* cDNAs were performed using 0.5 μl of cDNA, 0.25 μl ExTaq DNA polymerase (Takara Bio), and 0.5 μM primers in a total volume of 10 μl. The primer set *VSP1*-467-F (5′-GAA CTC TTA GAG AAA GAG GG-3′) and *VSP1*-467-R (5′-TTC TCG ACA GTG ACT TCT GA-3′) was used for the amplification of a 467-bp fragment of *VSP1* cDNA. The primer set *PDF1.2a*-US (5′-TAA GTT TGC TTC CAT CAT CAC CC-3′) and *PDF1.2a*-DS (5′-GTG CTG GGA AGA CAT AGT TGC AT-3′) was used for the amplification of a 209-bp fragment of *PDF1.2* cDNA. The primer set *EF-1a*-F (5′- ACC CTA GCC GCT ACA CAC TT-3′) and *EF-1a*-R (5′-GGT GGT CGA CTT TCC AGA AT-3′) was used for the amplification of a 128-bp fragment of *EF1A* cDNA, which served as a loading control. The PCR conditions for the *VSP1* cDNA were: denaturation at 94°C for 5 min; 27 amplification cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 50 s; and a final extension of 72°C for 4 min. For the amplification of *PDF1.2* cDNA, the primer annealing temperature was raised to 61°C and the extension time was reduced from 50 to 30 s. PCR products were separated on 3.0% agarose gels by electrophoresis and stained with 5 mg/ml ethidium bromide.

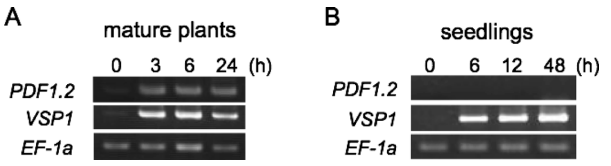

Figure 1. Comparison of responsiveness of jasmonate-inducible genes in mature *Arabidopsis* plants and seedlings by semi-quantitative RT-PCR. (A) Three-week-old *Arabidopsis* plants grown in soil at 22°C with a short photoperiod (12-h dark/12-h light) were sprayed with 100 μM MeJA at the middle of the light period and harvested at the indicated times. (B) Six-day-old *Arabidopsis* seedlings grown in water were treated with MeJA and harvested at the indicated times. RT-PCR analysis for *PDF1.2* and *VSP1* was conducted as described in the Methods. *EF-1a* was used as internal control.

In response to the MeJA treatment of soil-grown *Arabidopsis* plants, marked accumulation of *VSP1* and *PDF1.2* mRNAs was observed (Figure 1A). The induction of *VSP1* expression was also evident in seedlings cultured in the wells of 96-well plates although the clear induction of *PDF1.2* was not detected (Figure 1B). These results suggest that the expression of *VSP1,* but not *PDF1.2*, is induced in *Arabidopsis* seedlings in response to treatment with MeJA under the assay conditions used in this study. To conduct *in vivo* assays using 96-well plates, the use of seedlings is a prerequisite because the plants must be small enough to fit into the wells. Thus, we concluded that *VSP1* is a superior JA-responsive marker gene for HTS because of its high responsiveness in *Arabidopsis* seedlings.

To generate a suitable plant transformation vector for monitoring JA-responsive gene expression, we constructed and evaluated a series of plasmid vectors. A modified luciferase gene, luc2, from pGL4.10[luc2] vector (Promega) was excised as a *Xba*I–*Nhe*I-digested fragment, which was then ligated into *Sac*I–*Xba*I-digested pBI221 vector to generate pBI221-luc2. In this study, we used a 0.8 kb *VSP1* gene promoter region

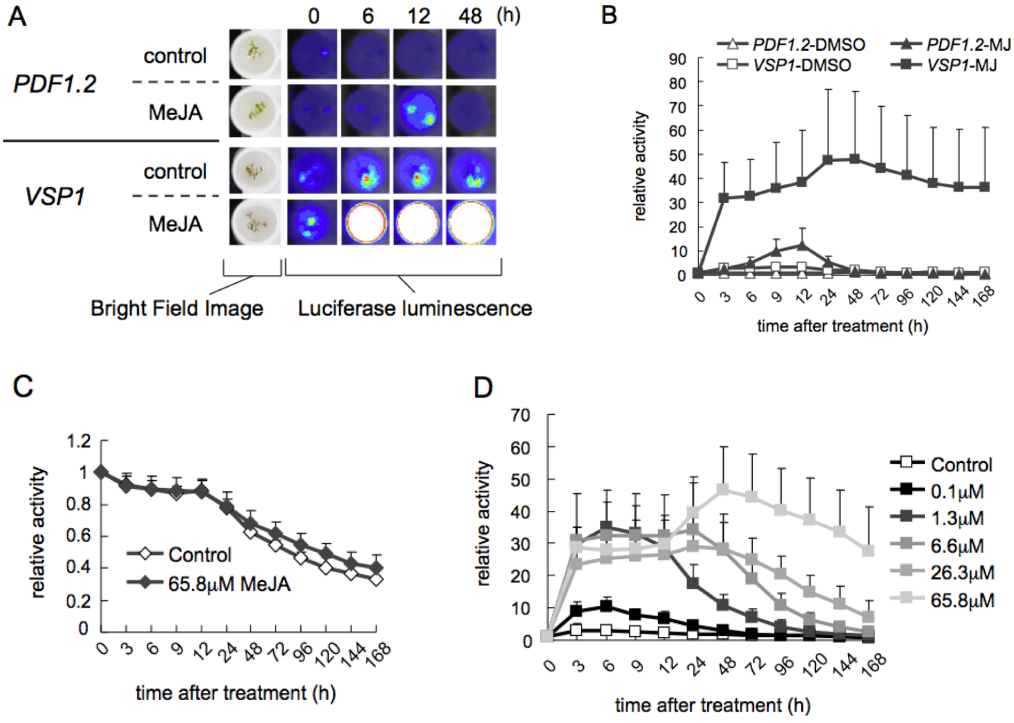

Figure 2. Monitoring of JA-responsive reporter activities in transgenic *Arabidopsis* seedlings. (A) A 96-well plate containing transgenic seedlings harboring *PDF1.2::luc2* or *VSP1::luc2* were treated with MeJA and bioluminescence images were captured at the indicated times after treatment. (B) Time–course measurement of bioluminescence levels from *PDF1.2::luc2* or *VSP1::luc2* seedlings after treatment with MeJA or DMSO (control). Values are means±SD for six independent photon counts. (C) Time–course measurement of bioluminescence levels from *35S::luc2* seedlings after treatment with MeJA or DMSO (control). Values are means±SD for six independent photon counts. (D) Time–course measurement of bioluminescence levels from *VSP1::luc2* seedlings after treatment with various concentrations of MeJA. Values are means±SD for eight independent photon counts.

based on the deletion analysis of the promoter described previously (Guerineau et al. 2003). The *VSP1* gene promoter was amplified from *Arabidopsis* genomic DNA (ecotype Columbia) by PCR using the primers *VSP1* US (*Hin*dIII), 5′-CCG TTG AAG AAA ATC AAG CTT TAA CC-3′, and *VSP1* US (*Nco*I), 5′-CCG TGA GAG GAT TTC CAT GGT TTT TTG TATGG-3′, according to the results of a previous study (Utsugi et al. 1998; Guerineau et al. 2003). The *PDF1.2* promoter which has been shown to be induced in response to inoculation with *Botrytis cinerea* and yeast cell wall extract treatment was amplified with PCR primers as described previously (Manners et al. 1998; Minami et al. 2011). The amplified promoter fragments were digested by *Hin*dIII and *Nco*I, and then ligated into the *Hin*dIII–*Nco*I sites of pBI221-luc2. The promoter-luciferase fusion genes were excised from the resulting constructs as *Hin*dIII–*Eco*RI fragments and then ligated into the *Hin*dIII–*Eco*RI sites of pBI121. The resulting plasmids, which were designated pBI121-*VSP1::luc2* and pBI121-*PDF1.2::luc2*, were then transformed into *A. thaliana* (Columbia) mediated by *Agrobacterium tumefaciens* LBA4404, as described previously (Clough and Bent 1998). After the initial selection of kanamycin-resistant seedlings, we chose transgenic lines with single locus transgene insertion by checking segregation of T3 generation and used for further studies.

To compare the responsiveness of the *PDF1.2* and *VSP1* promoters in *Arabidopsis* seedlings, seeds were sown aseptically in 96-well white plates (Greiner, Cat. No. 754074) with 50 μl DW, incubated in the dark at 4°C for 3 days, and then transferred to continuous light (70 μmol m^{-2} s^{-1}) at 22°C for 5 days. To assay for luciferase activity, 20 μl of 0.5 mM D-luciferin (Promega) solution together with 1.6 μl of chemicals dissolved in DMSO or DW and 50 μl DW were added to each well. The *in vivo* monitoring of luciferase activity was performed as described previously (Millar et al. 1992; Ono et al. 2011). Relative expression levels are shown as fold of induction of each time point versus time zero.

As shown in Figure 2A, Fluc activity in MeJA-treated seedlings was induced in transgenic plants harboring *PDF1.2::luc2* or *VSP1::luc2*. The relative activity of *VSP1::luc2* was at least 30-fold higher in response to MeJA treatment than DMSO (control) and the elevated expression level was maintained for more than 7 days. In contrast, *PDF1.2::luc2* only displayed up to 10-fold transient induction on exposure of seedlings to MeJA (Figure 2B). The expression level of the CaMV 35S promoter, which was used as a negative control, was not affected by MeJA treatment under the assay conditions used in this study (Figure 2C). To further

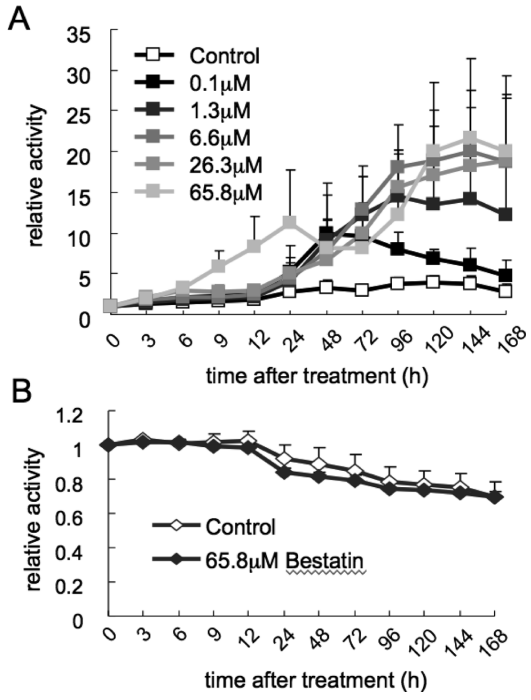

Figure 3. Monitoring of *VSP1::luc2* activities in transgenic *Arabidopsis* seedlings in response to treatment with bestatin. (A) Time–course measurement of bioluminescence levels from *VSP1::luc2* seedlings after treatment with various concentrations of bestatin. Values are means±SD for eight independent photon counts. (B) Time–course measurement of bioluminescence levels from *35S::luc2* seedlings after treatment with Bestatin. Values are means±SD for eight independent photon counts.

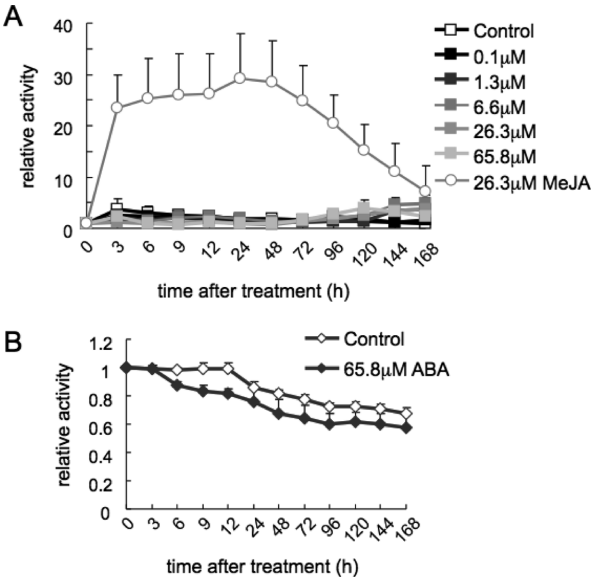

Figure 4. Monitoring of *VSP1::luc2* activities in transgenic *Arabidopsis* seedlings in response to treatment with ABA. (A) Time–course measurement of bioluminescence levels from *VSP1::luc2* seedlings after treatment with various concentrations of ABA. Values are means±SD for eight independent photon counts. (B) Time–course measurement of bioluminescence levels from *35S::luc2* seedlings after treatment with ABA. Values are means±SD for eight independent photon counts.

examine the responsiveness of *VSP1::luc2* in seedlings, we tested if the response to MeJA was concentration dependent. As shown in Figure 2D, a clear induction of luciferase activity was observed at a final concentration of approximately 1.3 μM MeJA. Interestingly, a possible biphasic response with peaks at 6 and 48 h was also observed in samples treated with higher MeJA concentrations (Figure 2B and 2D). Both promoters showed no induction in response to treatment with SAR inducers such as SA or ASM under the condition of this study (data not shown). We tested each of two independent transgenic lines randomly selected from the initial selection and obtained similar results. Taken together, these results suggest that *VSP1::luc2* is an ideal promoter-reporter gene construct to monitor the induction of gene expression in response to treatment with factors involved in JA-mediated signal transduction pathways.

To verify the utility of *VSP1::luc2* for HTS, we treated transgenic seedlings with the amino peptidase inhibitor bestatin, which is known to induce JA-mediated defense gene expression (Zheng et al. 2006). Consistent with previous results, *VSP1::luc2* was induced by the bestatin treatment of plants, but exhibited an expression profile different from that of MeJA. Specifically, the first expression peak at 6 h was absent, and the delayed induction of *VSP1::luc2* expression was observed (Figure 3A). This indicates that the mode of induction as well as the properties of chemicals such as tissue permeability can be monitored with this system. The expression levels of *luc2* under control of the CaMV 35S promoter were not affected by bestatin treatment under the conditions of this study (Figure 3B). These results suggest that the monitoring system may be applicable to not only the search for agents that induce JA-mediated defense responses, but also to the evaluation and investigation of the modes of action of factors involved in JA-mediated signaling pathways.

Similar to conventional JA-responsive genes, *VSP1* gene expression in response to JA is thought to be induced by dissociation of the JAZ repressor protein from transcription factor MYC2 upon JA-Ile binding (Boter et al. 2004; Chico et al. 2008; Shoji and Hashimoto 2011). Because MYC2 functions as a transcriptional activator of not only JA-responsive genes, but also of abscisic acid (ABA)-responsive genes such as *alcohol dehydrogenase* (Abe et al. 2003), we also investigated the induction of *VSP1::luc2* in response to the treatment of seedlings with various concentrations of ABA (Figure 4). However, no induction of *VSP1::luc2* was observed in response to ABA treatment, whereas the expression level of the CaMV 35S promoter was slightly down regulated. These results indicate that *VSP1::luc2* expression is not significantly affected by signaling pathways other than

JA-mediated responses. These results are consistent with the previous study of endogenous *VSP1* gene expression in response to treatment with ABA (Hossain et al. 2011). We therefore conclude that the specificity of the monitoring system developed here is sufficiently high to be used for the HTS of agents involved in the JA-mediated pathway. In this study, we used MeJA as sole agent for the induction experiments. To further test the utility of the bioluminescence monitoring system, induction studies using ethylene or related compounds will be necessary.

During the course of the induction experiments, we used the CaMV 35S promoter for comparison and found that its expression levels are not influenced by the treatment of plants with chemicals such as MeJA or ABA. Therefore, the use of the CaMV 35S promoter as an internal standard, possibly fused with a red-emitting luciferase reporter gene, would allow more accuratequantitative assays to be conducted using a dual-color luciferase system (Ogura et al. 2011).

Using transgenic *Arabidopsis* harboring a Fluc reporter gene fused to the tobacco *PR-1a* gene promoter, we previously developed an SA-regulated gene expression monitoring system in a 96-well plate format (Watakabe et al. 2011; Ono et al. 2011). The system enables the HTS for chemicals that induce SAR in plants and has therefore been applied to the screening and development of plant activators. In the present study, we have demonstrated that the *Arabidopsis VSP1* gene promoter is applicable to the HTS of agents that induce JA-mediated pathways. The use of this HTS system may allow the development of novel plant activators that regulate JA-mediated responses and may be effective against necrotrophic pathogens.

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