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

Masahiro Kusama1, Nobuaki Urata1, Rieko Ogura2, Shin-ichi Ogata1, Kazuyuki Hiratsuka1,*

1Graduate School of Environment and Information Sciences, Yokohama National University, Yokohama, Kanagawa 240-8501, Japan; 2Venture Business Laboratory, Yokohama National University, Yokohama, Kanagawa 240-8501, Japan
*E-mail: hiratsk@ynu.ac.jp Tel & Fax: +81-45-339-4413

Received August 29, 2012; accepted October 18, 2012 (Edited by H. Shimada)

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...
Development of HTS for JA-dependent gene expression

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 brassicicola, 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 (Utugi 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 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 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 cycle were sprayed with 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 primer set PDF1.2a-UTGCTC TTGAGATACTG 5'-TAA GTTTTGCATCATCACCC-3') and PDF1.2a-DSDGCTG GGAAGA CATAGTTGCATAT-3') was used for the amplification of a 209-bp fragment of PDF1.2 cDNA. The primer set EF-1a-F 5'- ACCCTGAGGCTACCTT-3' and EF-1a-R 5'-GCTCGA CCTTGGAGAT-3') was used for the amplification of a 128-bp fragment of EF1α 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% agarose gels by electrophoresis and stained with 5 µg/ml ethidium bromide.

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 XbaI–NheI-digested fragment, which was then ligated into SacI–XbaI-digested pBl221 vector to generate pBl221-luc2. In this study, we used a 0.8 kb VSP1 gene promoter region

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.
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 (HindIII), 5′-CCG TTG AAG AAA ATC AAG CTT TAA CC-3′, and VSP1 US (NcoI), 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 HindIII and NcoI, and then ligated into the HindIII–NcoI sites of pBI221-luc2. The promoter-luciferase fusion genes were excised from the resulting constructs as HindIII–EcoRI fragments and then ligated into the HindIII–EcoRI 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 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 various concentrations of MeJA. 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 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 accurate quantitative 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 a 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.

Acknowledgements

This study was supported in part by an Industrial Technology Research Grant Program in 2004 from the New Energy and Industrial Technology Development Organization (NEDO) of Japan and by a grant from Kanagawa Academy of Science and Technology (KAST).

References


Shoji T, Hashimoto T (2011) Tobacco MYC2 regulates jasmonate-inducible nicotine biosynthesis genes directly and by way of the NIC2-locus ERF genes. Plant Cell Physiol 52: 1117–1130


reporter assay system to monitor Arabidopsis MPK3 gene expression in response to infection by Botrytis cinerea. J Gen Plant Pathol 72: 1–5