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Short Communication

In vivo bioluminescence monitoring of defense gene expression in response to treatment with yeast cell wall extract

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Abstract Previous studies indicated that Housaku Monogatari (HM), a plant activator made from yeast cell wall extract, is effective for control of various plant diseases. To investigate the effect of HM treatment on plant gene expression, we tested the expression of defense related gene induction by exploiting tobacco pathogenesis-related protein 1a (*PR-1a*) gene promoter- and the Arabidopsis plant defensin 1.2 (*PDF1.2*) gene promoter-luciferase fusions as reporter genes. Transgenic Arabidopsis plants harboring promoter-luciferase fusion genes were treated with HM and the promoter activity was monitored as changes in luciferase activity *in planta*. Results of bioluminescence monitoring assay indicated that the promoters were activated at different times after the treatment of test plants with HM. Maximum activation of the *PR-1*a promoter occurred 4 days, and of the *PDF1.2* promoter 4 h, after treatment. These results suggest that HM might contain multiple microbe-associated molecular patterns (MAMPs) that activate systemic acquired resistance and induced systemic resistance signaling pathways at different times. This may explain the mechanisms involved in the induction of defense responses against multiple plant pathogens by HM treatment.

Key words: Arabidopsis, defense gene expression, firefly luciferase, powdery mildew, yeast cell wall extract.

When plants are attacked by pathogens, tissues may respond with defense mechanisms, controlled by defense-related signaling pathways. Defense responses may be initiated by recognition of microbe-associated molecular patterns (MAMPs) from cell wall components of pathogens (Bent and Mackey 2007; Nürnberger et al. 2004). Most MAMPs are oligosaccharides, peptides, or glycopeptides. A well-known example, hepta-β-glucoside, induces soybean cotyledons to accumulate phytoalexins (Anderson-Prouty and Albersheim 1975; Sharp et al. 1984). Most yeasts including budding yeast *Saccharomyces cerevisiae*, are non-pathogenic, but a mannopeptide from yeast invertase and yeast extract are reported to function as MAMPs (Basse et al. 1993; Obara et al. 2007). However, the yeast cell wall extract (YCWE) has not been tested as a practical pesticide.

The major components of yeast cell walls are polysaccharides, such as glucan and mannan (Klis et al. 2006). These may act as MAMPs and induce defense gene expression. On the other hand, YCWE is readily available from commercial sources as a by-product of brewing process. Using YCWE as a main ingredient, Housaku Monogatari (HM) has been developed as a compound fertilizer (http://www.asahi-fh.com/hc/products/pdt09-01-1.html). HM contains YCWE which has been processed by treatment with cell wall degrading enzymes.

Plants treated with MAMPs activate two major signal transduction pathways involved in defense responses. One pathway is systemic acquired resistance (SAR) which acts against biotrophic pathogens; the second is induced systemic resistance (ISR) which acts against necrotrophic pathogens. SAR is a salicylic acid (SA)-dependent pathway, which induces expression of acidic pathogenesis-related (PR) genes including *PR-1a*. ISR is an SA-independent pathway that involves jasmonic acid (JA) and ethylene (ET) dependent signal transduction. ISR activates promoters of another group of defense genes, such as basic *PR* genes, *plant defensin 1.2 (PDF1.2)* and *thionin 2.1 (Thi2.1)*.

Our previous study indicated that HM is effective against both biotrophic and necrotrophic plant pathogens (Kitagawa et al. 2005; Minami et al. 2005). These results suggest that HM induced defense systems involved in

Abbreviations: ET, ethylene; Fluc, firefly luciferase; ISR, induced systemic resistance; JA, jasmonic acid; MAMPs, microbe-associated molecular patterns; PR, pathogenesis-related; SA, salicylic acid; SAR, systemic acquired resistance

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ISR as well as SAR. To investigate signal transduction pathways activated by HM we examined defense gene induction in response to treatment with HM using Arabidopsis as a model system.

It would be feasible to investigate how HM induces defense responses in plants by analysis of mRNA or proteins, but this process is tedious and time-consuming. An alternative method is to use a bioluminescence reporter assay system; this method is a proven technique to measure gene activity in tobacco BY-2 cells and Arabidopsis and has been used to monitor defense related gene expression sequentially and rapidly with a small amount of samples (Ono et al. 2011; Watakabe et al. 2011). To investigate the SAR induction capability of HM we exploited a bioluminescence reporter system using transgenic Arabidopsis harboring tobacco *PR-1a* promoter-luciferase fusion gene (Ono et al. 2004).

Transgenic Arabidopsis plants were grown on germination medium containing Murashige and Skoog salts (Wako, Osaka, Japan) with 1% sucrose and 0.8% agar (Wako, Osaka, Japan). All seeds were vernalized at 4°C for 2 days before transfer to growth conditions. The plants were grown for three weeks in a growth chamber at 22°C under a 16 h light (50 μmol m^{-2} s^{-1})/8 h dark cycle, then transferred to 12-well plates containing 500 μl of dH_2O and 200 μl of 1 mM luciferin (D-luciferin potassium salt, Promega, Madison, WI, USA) per well.

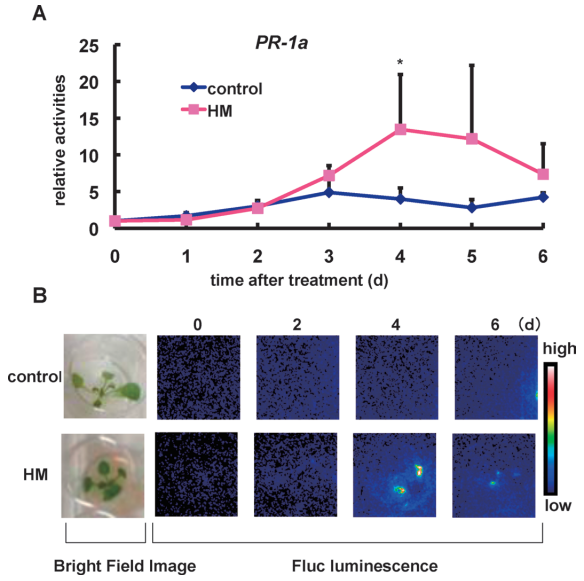

Figure 1. Induction of *PR-1a* promoter activity by treating with HM. Light emission from *PR-1a::Fluc* transgenic plants treated with 2.5% HM or dH_2O was monitored by low-intensity light video image analysis. (A) Time-course measurements of luciferase luminescence from plants by photon counting. Relative activities are shown as fold induction relative to photon counts at 0 h. Values shown are means ± SD of three samples. A single asterisk indicates significant difference at $P<0.05$ from controls by *t*-test. (B) Bright field image and the pseudo-color images of luciferase bioluminescence from the plants. Luminescence images were taken 0, 2, 4, 6 days after treatments. Fluc luminescence images were obtained after 10 min of photon collection.

Luciferin was added 24 h before the root treatment of 100 μl of HM (20% aqueous solution). Each experiment was run with at least three replicates per treatment. As shown in Figure 1, the induction of the *PR-1a* promoter was detected in 3-week-old plants by enhancement of firefly luciferase (Fluc) 4 days after treatment with HM.

Previous report of yeast extract treatment suggested that the genes involved in ISR are induced by the treatment (Obara et al. 2007). To test the ISR induction potency of HR we investigated the *PDF1.2* expression levels in response to the treatment using transgenic Arabidopsis harboring *PDF1.2::Fluc* as reporter gene. *PDF1.2* gene is used as a standard marker gene for ISR because the *PDF1.2* promoter is induced in response to treatment with JA or ET (Manners et al. 1998).

To construct the promoter-reporter gene fusion, the sequence of the Arabidopsis *PDF1.2* gene upstream promoter region (Manners et al. 1998) was amplified from the genomic DNA of *Arabidopsis thaliana* ecotype Columbia by polymerase chain reaction (PCR) using the following primers containing a restriction endonuclease recognition site (underlined); 5′-GGG ACA AGC TTT ATA TGC AGC ATG-3′ and 5′-GGC CAT GGT GAT TAT TAC TAT TTT GTT TTC AA-3′. The PCR was

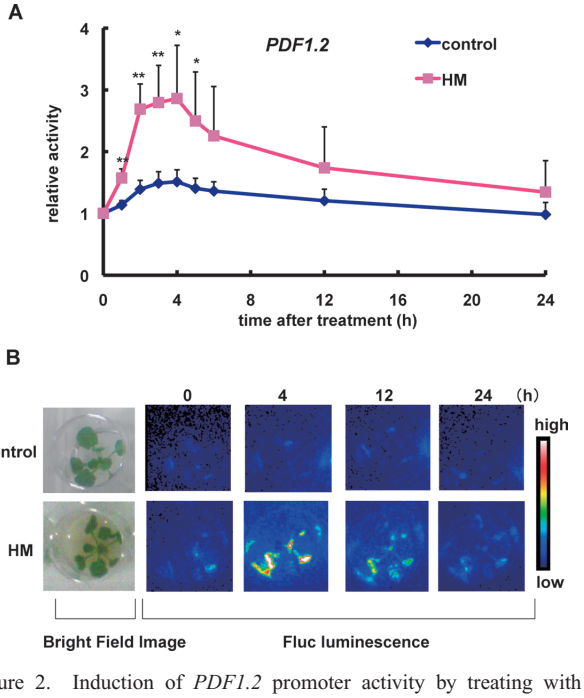

Figure 2. Induction of *PDF1.2* promoter activity by treating with HM. Light emission from *PDF1.2::Fluc* transgenic plants treated with 2.5% HM or dH_2O was monitored by low-intensity light video image analysis. (A) Time-course measurements of luciferase luminescence from plants by photon counting. Relative activities are shown as fold induction relative to photon counts at 0 h. Values shown are means ± SD of five samples. Single and double asterisks indicate significant differences at $P<0.05$ and $P<0.01$ from controls by *t*-test, respectively. (B) Bright field image and the pseudo-color images of luciferase bioluminescence from the plants. Luminescence images were taken 0, 4, 12, 24 h after treatments. Fluc luminescence images were obtained after 10 min of photon collection.

conducted by KOD-Plus DNA polymerase (Toyobo, Osaka, Japan) following manufacturer's recommendations. The reaction cycle was 94°C for 2 min, followed by 25 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The amplified 1.2-kb DNA fragments were digested with *Hind*III and *Nco*I and inserted into a *Hind*III-*Nco*I site of pBI221-luc+ (Matsuo et al. 2001). The plasmid vector for transformation was constructed by excising the *Hind*III-*Eco*RI fragment from the plasmid and cloning it into a *Hind*III-*Eco*RI site of pBI121 and introduced into Arabidopsis by *Agrobacterium*-mediated transformation as described previously (Tanaka et al. 2006). Bioluminescence detection of the transgenic Arabidopsis harboring *PDF1.2::Fluc* using 12-well plate was conducted as described above. As shown in Figure 2, *in vivo* monitoring of the *PDF1.2::Fluc* expression showed activation of *PDF1.2* promoter 2-4 h after HM treatment.

Using transgenic Arabidopsis plants harboring luciferase reporter gene, we could monitor the expression of defense related genes *in vivo*. The system enables us to conduct non-invasive detection of *PR-1a* and *PDF1.2* gene promoter expression after HM treatment. Maximum *PR-1a* promoter activation occurred 4 days after treatment, and *PDF1.2* promoter in 2 to 4 h. This difference in induction timing between two representative marker genes is consistent with previous observations of defense gene expression in Arabidopsis (Leon-Reyes et al. 2010; Ndamukong et al. 2007).

Previous studies indicated that the SAR signal transduction pathway was activated by pathogens of powdery mildew in strawberry and tomato (Hukkanen et al. 2007; Ishikawa et al. 2005). We speculate that HM suppressed powdery mildew by activating SAR, because it induced *PR-1a* gene expression. In contrast, the JA and ET dependent ISR pathway is involved in induced resistance against necrotrophic pathogens compared to SAR against biotrophic pathogens (Berrocal-Lobo et al. 2002; Thomma et al. 1998; Thomma et al. 1999; Ton et al. 2001). There appears to be antagonistic signaling of cross-talks between signal transduction pathways in Arabidopsis and tobacco (Petersen et al. 2000; Sano et al. 1996; Spoel et al. 2003; Yasuda et al. 2008). However, the results obtained from this study suggest that HM activates SAR and ISR pathways at different times, and in consequence, is potentially able to control a wide range of diseases.

On the other hand, it is interesting to note that the previous study of defense gene induction by yeast extract suggested that the SAR is not induced by the treatment (Obara et al. 2007). Although further investigation will be necessary to elucidate the mechanisms, this difference may be simply explained by the difference in the production process of the yeast extract and HM. Because HM contains various by-products from brewing processes, it is also possible that the bio-active ingredients other than yeast cell wall components play a roll in defense gene induction of plants.

The results of this study demonstrate that the transgenic plants harboring bioluminescence reporter provide a powerful tool for the study of regulatory mechanisms and expression of defense genes. Continuous monitoring of expression of marker gene is impossible with other methodologies, such as RNA assays. Moreover, the system is particularly effective when the different timescales are required, such as in this study. Also, because the non-destructive bioluminescence system exploited in this study allows us to conduct high-throughput assays, it may be possible to maximize the activity of HM by optimizing the process of yeast cell wall digestion to enhance the extraction of bioactive fractions that can activate defense gene induction.

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