

Studies on regulated expression of plant defense genes

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1. Introduction

To ward off pathogen infection, plants rely on various disease responses at the cell/tissue level as part of their immune system and have long been attractive topics for research. Extensive research has been conducted on the interaction between host plants and pathogens and on plant recognition of pathogen infection and the signal transduction system as plant molecular biology has advanced (Ohashi 1996). Later, genes involved in disease responses in plants were discovered and studied using molecular biological methods from the 1980s, and since the 1990s, salicylic acid and jasmonic acid have been recognized as plant hormones related to the control of defense responses. Work on these molecules has contributed to the elucidation of regulatory mechanisms involved in the expression of defense-response genes in higher plants. Research on the regulation of gene expression in higher plants has progressed dramatically, and *cis*-regulatory elements and cognate sequence-specific DNA-binding proteins have been identified as *trans*-regulators through the analysis of the regulatory mechanisms of gene promoters. Research on the practical application of disease-responsive gene promoters and the evaluation and search for resistance inducers to use in the field has also flourished. In this paper, I will introduce related studies in which I have been involved, mainly on transcription factors and applied research using the bioluminescence reporter method.

2. Transcriptional regulators involved in defense response gene expression

Research on gene expression regulation in the 1980s progressed from functional analysis of expression control regions to identification of cis-regulatory elements within the promoter sequence. Subsequently, research was conducted on sequence-specific DNA-binding proteins as trans-regulators that bind to cis-regulatory elements. Many DNA-binding proteins as trans-regulators were isolated and identified as transcription-factors using a search method that uses binding activity to specific DNA sequences and a method based on DNA sequence homology (Hiratsuka and Chua 1997; Ramachandran et al. 1994).

GT-1, which was first identified as a type of plant-specific transcription factor trihelix protein, was initially identified from a nuclear extract of tobacco as a DNA-binding protein that binds to a light-inducible gene promoter. A tobacco cDNA was isolated with a method using the target sequence as a probe (Gilmartin et al. 1992). The identification of a homologous gene in *Arabidopsis thaliana* led to the further characterization of GT-1, including detailed binding characteristics to cis-regulatory factors, nuclear translocation ability, and regulation of binding activity by phosphorylation (Hiratsuka et al. 1994; Maréchal et al. 1999). Subsequent studies showed that it also functions as a binding factor for the tobacco *PR-1a* promoter, which is a typical disease-responsive gene promoter, and showed a relationship between light-responsive gene control and defense-responsive gene expression at the transcriptional level (Buchel et al. 1999). Extensive research was also carried out on linking cis-regulators to signal transduction systems, and new findings on signal transduction systems and cis-elements mediated by calcium and cyclic GMP were discovered (Neuhaus et al. 1997; Wu et al. 1996).

A large-scale analysis of genes highly expressed in germ cells of the trumpet lily (*Lilium longiflorum*) was carried out, focusing on the genes , which show remarkable antimicrobial activity in its floral organs (Morohashi

et al. 2000). One of the genes highly expressed in reproductive organs was discovered to encode LISCL (*Lilium longiflorum* Scarecrow-like), one of the novel GRAS proteins, and investigated in detail. The GRAS proteins are a group of regulator of higher plants and their mechanism of action was unknown. The LISCL was shown to be localized in the nucleus and function as a transcriptional activator (Morohashi et al. 2003). Subsequent studies showed that the LISCL homologous gene in *Arabidopsis* is involved in the defense response to saprophytic pathogens such as *Botrytis cinerea*, and the LISCL group of genes are regarded as transcriptional regulators involved in defense responses specific to higher plants (Fode et al. 2008).

3. Firefly luciferase assay as a tool to study regulation of defense-response gene expression

We worked on the technological development of a bioluminescence reporter using firefly luciferase that enables continuous monitoring of gene expression control in plants (Hiratsuka 1997; Millar et al. 1992). The luciferase reporter assay was originally used in studies targeting light-inducible gene promoters. For developing luciferase to study defense gene expression in higher plants, first we exploited the tobacco *PR-1a* gene promoter using a promoter sequence obtained from the genomic DNA of tobacco BY-2 cells. We made a fusion gene between the firefly luciferase gene and the *PR-1a* promoter sequence of about 1600 base pairs, introduced it into BY-2 cells and tobacco plants to assess the activity of the *PR-1a* promoter as a change in luminescence activity. The results showed that salicylic acid treatment significantly increased the bioluminescence intensity and that samples could be continuously observed nondestructively (Hiratsuka 1997, Watakabe et al. 2011). We also constructed a fusion gene between the firefly luciferase gene and the promoter region of *Arabidopsis thaliana* *MPK3* gene, which encodes a protein kinase involved in signal transduction of defense responses. Using these transgenic *Arabidopsis* plants harboring the bioluminescence reporter gene, we could analyze expression profiles of the

MPK3 promoter in response to infection by *B. cinerea* (Tanaka et al. 2006).

We also developed a transient expression assay system using a microprojectile bombardment method. Using the *Renilla* luciferase gene as an internal standard, we established a dual luciferase assay system for the accurate quantitative evaluation of gene expression. The experimental method is now widely used for transient assay in plant cells (Matsuo et al. 2001). The transient assay system was used to show transcriptional activation of *PR-1a* after salicylate treatment or simultaneous introduction of *NPR-1* gene (Ono et al. 2004).

In addition, studies on multicolor luciferase to develop a highly accurate bioluminescence reporter gene system in plants. The two types of bioluminescent genes derived from the click beetle encoding green-luminescent and red-luminescent luciferases, respectively, use luciferin as a luminescent substrate, the same substrate for firefly luciferase. Therefore, the expression levels of two independent genes can be monitored simultaneously by measuring green emission and red emission independently using a spectral filter, which is called a dual color luciferase assay. We were the first to develop this assay system for use in plant tissues, and showed that it is effective for finding and analyzing defense gene inducers (Kusama et al. 2009; Ogura et al. 2005, 2011).

In parallel, we worked on the development of a high-throughput system to screen and evaluate defense gene inducers (plant activator). Arabidopsis seeds harboring a bioluminescence reporter are dispensed into a multi-well plate, then treated with chemicals to be tested. The system allows us to continuously observe many samples nondestructively and is an ideal method for screening chemical libraries or the like for novel compounds that can control plant pathogens. In particular, the fusion gene of the tobacco *PR-1a* promoter and firefly luciferase showed better responsiveness to induction stimulation than the endogenous gene of Arabidopsis, and was shown to be suitable for search and evaluation system for defense gene inducers (Ono et al. 2011; Watakabe et al. 2001). In addition, we prepared a fusion gene

between firefly luciferase and the promoter region of the *Vegetative storage protein 1* gene, a responsive gene of jasmonic acid system of Arabidopsis. Using Arabidopsis seedlings harboring the fusion gene and multi-well plates, we demonstrated that the system can be applicable to a high-throughput system for monitoring jasmonate-regulated defense response genes (Kusama et al. 2012).

These systems are also used to evaluate the physiological activity of existing chemicals and the like. We used the system to detect defense gene induction activity from yeast cell wall extracts and suggested that the disease control activity resulted from treatment with yeast cell wall extracts may be due to induction of the defense gene expression (Minami et al. 2011). We also found that tolprocarb, a fungicide for rice blast that acts as a melanin synthesis inhibitor, clearly has resistance-inducing activity and it is also effective in controlling various bacterial diseases (Hagiwara et al. 2020).

The screening system functions effectively as a high-throughput screening system and is utilized for discovering new compounds that activate defense genes of higher plants. In particular, a group of methyl jasmonate agonists discovered using the high-throughput system has no structural similarity to jasmonates and showed a novel type of physiological activity (Tomita et al. 2019).

4. Social implementation of research results

We established Yokohama Biotechnology Co., Ltd., a venture company originating from Yokohama National University, for the purpose of disclosing and utilizing a series of research results on regulating defense-response gene expression and as a contract service for evaluating substances for expression induction of plant-defense-response genes (<http://ybt.co.jp/>). In particular, the assay system using a bioluminescence reporter has been well-received because it can quantitatively evaluate inducing activity of the substances. So far, contract research for the development of plant defense inducers has been completed for more than 10 domestic companies.

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Compliance with ethical standards

Conflicts of interest The author has no conflicts of interest to declare.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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