Chitin Elicitor-Responsive Photon Emission is potentiated by Plant Activators through Priming of Salicylic Acid Signaling via OsWRKY45 in Rice

Hiroyuki Iyozumi*, Hideki Nukui and Kimihiko Kato
Shizuoka Prefectural Research Institute of Agriculture and Forestry, Tomigaoka, Iwata, Shizuoka 438-0803, Japan
*Corresponding author: Hiroyuki Iyozumi, Shizuoka Prefectural Research Institute of Agriculture and Forestry, Tomigaoka, Iwata, Shizuoka 438-0803, Japan, Tel: +81 538 36 1556; Fax: +81 538 37 8466; E-mail: hiroyuki1_iyozumi@gref.shizuoka.lg.jp

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Abstract

Priming of plant cells for faster and enhanced defense responses against pathogen attacks is a common feature of chemically or biologically induced resistance. The authors previously developed a priming detection system that detects priming as potentiation of chitin elicitor-responsive photon emission (C-ERPE) in rice cells pretreated with various types of chemical inducers of disease resistance, called plant activators. To elucidate the mechanisms underlying C-ERPE potentiation, the authors performed gene knockdown of OsWRKY45, a major regulator of salicylic acid (SA)-dependent defense responses in rice, and estimated the effects of SA isomers on C-ERPE potentiation. Plant activators induced a 200-300% increase in C-ERPE in the wild type, whereas OsWRKY45 knockdown attenuated the increase in C-ERPE to less than 60%. Native SA induced more than a 150% increase in C-ERPE, but structural isomers of SA were less effective (10-24% increase). These SA signaling-disruption experiments indicate that the potentiation of C-ERPE requires intrinsic components of hormonal signaling for defense, at least for priming by inducers of systemic acquired resistance.

Keywords Priming; Plant activator; Rice; WRKY; Ultraweak photon emission; Elicitor

Abbreviations UPE: Ultraweak Photon Emission; ERPE: Elicitor-Responsive Photon Emission; IR: Induced Resistance; SA: Salicylic Acid; SAR: Systemic Acquired Resistance; RNAi: RNA interference; PCR: Polymerase Chain Reaction; WT: Wild Type; RT-PCR: Reverse Transcription PCR; ASM: Acibenzolar-S-Methyl; TDL: Tidalin; 3HBA: 3-Hydroxy Benzoic Acid; 4HBA: 4-Hydroxybenzoic Acid; PA: Phosphatidic Acid; ROS: Reactive Oxygen Species; PLD: Phospholipase D

Introduction

Rice is a major food staple throughout the world, especially in the Asia-Pacific region, parts of South and Central America and, increasingly, in Africa. It plays a pivotal role in the food security of over half of the world’s population [1,2]. In the past half-century, despite competition for water and land use with other crops or non-agricultural purposes, rice production has continued growing around the world [3-5]. In the past few decades, especially after the mapping and sequencing of its whole genome, rice has become an important target of Omics Research and other new technologies [6-8].

Ultraweak photon emission (UPE) from plant cells is a real-time indicator of plant defense responses and can be assessed noninvasively [9,10]. The authors discovered UPEs in leaf segments and cultured cells of rice treated with microbial (fungal) elicitors including chitin [11-14] and inorganic substances such as dipotassium hydrogen phosphate or copper chloride [15], and named them elicitor-responsive photon emissions (ERPEs). Other studies of bacteria and viruses have reported UPEs in plants that are induced by pathogen-derived molecules [16,17], and ERPE is thought to be common in plant-pathogen interactions.

Induced resistance (IR) of plants against pathogens is of interest in the study of crop protection, and chemicals that induce resistance in plants, so-called “plant activators”, have been investigated [18,19]. The selection of candidates for plant activators by detecting IR-related genes, so-called pathogenesis-related genes, has been reported using microarray analysis [20] or reporter gene assays [21-23] to accelerate plant activator screening. Faster and enhanced defense responses to pathogens or elicitors are characteristic cellular events in plants treated with plant activators; this effect is called “priming” [24,25]. Priming is common in types of IR mediated by various kinds of defense-related hormones, and the key factors involved in signaling have been discovered gradually [26]. In rice, the crucial roles of the transcription factor WRKY45 (OsWRKY45) in salicylic acid (SA)-mediated systemic acquired resistance (SAR) have been reported [27-29].

Although the current knowledge-based plant activator screening process is powerful when used in the context of “Omics” databases, there may be unknown mechanisms underlying IR. In addition, the current screening methods can require invasive processes in plants, which may affect the screening results.

To address these problems, considering the previous studies of IR and ERPEs, the authors proposed a simple method for the screening of plant activators, in which enhancement of ERPE by priming could be detected [30]. This method can detect the priming effects of various kinds of plant activators or defense-related hormones [30] and may be useful for preliminary and inclusive screening of chemical libraries for plant activators. Although the mechanisms underlying ERPEs have been revealed gradually [12-14], the mechanism through which priming potentiates ERPEs has not been clarified.

Here, the authors report that the priming for enhanced C-ERPE by pretreatment of rice cells with SAR inducers requires intrinsic SA signaling components, including the transcription factor OsWRKY45.
The authors also discuss the properties of C-ERPE in rice cells as a priming detector.

Materials and Methods

Plant materials and chemicals

**Cell culture**: Rice cells (*Oryza sativa* L. cv. Kimmaze) were maintained with shaking in modified N6 liquid medium [31] containing 1 mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D) at pH 5.8, and 25°C in the dark with natural ventilation. Aliquots of 15 g (fresh weight) of cells were transferred to fresh medium every 10 days. Before plant activator and elicitor treatments, 1 ml portions of 10-day cultured cell suspensions containing 0.5 g of cells were dispensed onto plastic Petri dishes.

**OsWRKY45 gene expression knockdown by RNA interference (RNAi)**: The *OsWRKY45* (AK066225) RNAi vector was constructed using the Gateway® pENTR/D-TOPO Cloning Kit (Thermo Fisher Scientific, Waltham, MA, USA) and a pANDA vector as described [32,33]. The pANDA vector was kindly provided by Nara Institute of Science and Technology, Japan. Briefly, the 309 bp cDNA fragment of the 3′-untranslated region of *OsWRKY45* mRNA (5′-GGCACCGGGCGGGTAAACACGGTAGAAGA-3′) was amplified by polymerase chain reaction (PCR) using a primer set for directional cloning (forward 5′-ATGAGTTCCACCGTGTGTACAGAATAATGACGCGACGCCGG-3′ and reverse 5′-TCGAAGCTGCACTTACGAAAGATATGTCCTAACTGCCGTG-3′). The combination reaction of the PCR product with the pENTR/D-TOPO vector was performed according to the manufacturer’s instructions. The pANDA-OsWRKY45-RNAi vector was constructed by incubating the entry clone (pENTR-OsWRKY45), which had been pretreated with the restriction enzyme NruI, and the pANDA vector with the Gateway LR Clonase Enzyme Mix (Thermo Fisher Scientific). Rice cells (cv. Kimmaze as the wild type, WT) were transformed with the pANDA-OsWRKY45-RNAi vector (Supplemental Figure 1) by Agrobacterium-mediated transformation [34]. The transformed cell lines were selected in modified N6 liquid medium [31] containing carbenicillin (300 mg/L) and hygromycin (50 mg/L) while shaking at 100 rpm at pH 5.8, 25°C in the dark for 12 days with natural ventilation. Selection was repeated at least four times by transferring the selected lines to new medium every 12 days. In these lines, the gas linker sequence (Supplemental Figure 1) was detected by reverse transcription PCR (RT-PCR) to ascertain the triggering of double-strand RNA production [32,33]. Ten-day-old cells were used for photon counting experiments and RNA extraction.

**Plant activators and isomers**: Acibenzolar-S-methyl (ASM, synonym; benzothiadiazole: BTH), tiadinil (TDL), SA, and its inactive isomers 3-hydroxy benzoic acid (3HBA) and 4-hydroxybenzoic acid (4HBA) were purchased from Wako Pure Chemicals (Osaka, Japan). Each chemical was dissolved in the solvent (98% v/v of N, N-dimethylformamide (Wako Pure Chemicals) and 2% v/v of Tween 20 (Wako Pure Chemicals) and used as 100-fold concentrated stocks (20 mM). Aliquots of 1% v/v of the stock solutions were added to cell suspensions to adjust the plant activator concentrations to 200 μM. An equal volume of the solvent solution was used as a control treatment.

**Elicitor experiments**: N-acetylchitohexaose (Seikagaku Corporation, Tokyo, Japan) was used as a chitin elicitor of ERPE. N-acetylchitohexaose was dissolved in distilled water and adjusted to 20 μM as a 20-fold stock solution. Aliquots of 5% (v/v) of the stock solution were added to the rice cell suspensions to adjust the elicitor concentrations to 1 μM after the plant activator treatments. Equal volumes of distilled water were used as control treatments.

**Photon counting experiments**: UPE measurements were performed using a PCX-100 photon counter (Hamamatsu Photonics K.K., Hamamatsu City, Japan) as described [11]. Briefly, 10-day cultured rice cells were dispensed into 60 mm diameter plastic Petri dishes (Eiken Chemical Co. Ltd., Tokyo, Japan) and treated with each plant activator solution. The Petri dishes with cells were set in the light-tight box of the photon counter and photon counting was started. After pretreatment for 2 h, the photon counting was paused, a portion of elicitor solution was added to each dish, and the photon counting was continued. From the initial sample placement, all steps were performed in the dark to avoid external light exposure. All experiments were performed in triplicate at 26°C in an air-conditioned dark room. ERPE levels are expressed as 5 h integrated photon counts after chitin elicitor treatment and were calculated by subtracting the values for the water-treated controls (Supplemental Figure 2).

**Quantitative RT-PCR analysis**: Two hours after pretreatment with plant activators, 50 μg aliquots of cells were collected, frozen immediately in liquid nitrogen, and preserved using a mortar and pestle. Total RNA was extracted using RNeasy Plant Mini Kits (Qiagen, Hilden, Germany). Complementary DNA was obtained from 500 ng of total RNA using Quantitect Reverse Transcription Kits with a genome DNA eraser (Thermo Fisher Scientific). Quantitative PCR reactions were performed using SYBR® Premix Ex Taq (TaKaRa Bio Inc., Otsu, Japan) in an Mx3000-P Q PCR system (Agilent Technologies, Palo Alto, CA, USA). The cycling conditions were 10 s of polymerase activation at 95°C followed by 40 cycles at 95°C for 5 s and 64°C for 30 s. The expression level of *OsWRKY45* was normalized against the expression of the Rice Ubiquitin 1 gene (*Ubq1*). The expression was used as forward 5′-GAACGGAGAGGTGTTCCTTG-3′ and reverse 5′-ACGGCAGGCAGCCATGACCTTCTC-3′ for *OsWRKY45* and forward 5′-CCAGTAATGCTTCTACCATGAG-3′ and reverse 5′-GGACCAATGATTTGAGGATGAC-3′ for *Ubq1*.

Results and Discussion

**Effects of OsWRKY45 knockdown on the priming of rice cells by SAR inducers for enhanced ERPE**

The *OsWRKY45* RNAi-treated cell lines were similar to WT cells in appearance (Supplemental Figure 2) and in *Ubq1* expression level of the solvent-pretreated *OsWRKY45* RNAi cell line were almost equal to or lowers than that in the solvent-pretreated WT line (Figure 1A, white columns). The average expression rate compared with the WT was 77.6% in the ASM-pretreated *OsWRKY45*-RNAi line than in the WT (Figure 1A, gray columns). The average expression rate compared with the WT was 25.9% in the ASM-pretreated WT line (Figure 1A, white columns). OsWRKY45 gene expression knockdown on the priming of rice cells by SAR inducers for enhanced ERPE.
OsWRKY45-RNAi line #1 and 44.3% in OsWRKY45-RNAi line #2. This confirmed the knockdown of OsWRKY45.

Figure 1: ASM-induced potentiation of C-ERPE was diminished by OsWRKY45 knockdown. (A) WT and OsWRKY45-RNAi cell lines were pretreated with 200 μM ASM (gray columns) or solvent (white columns). Two hours after pretreatment, 50 mg aliquots of cells were collected for quantitative RT-PCR of OsWRKY45 and Ubq1 just before chitin elicitor treatment. The gus linker sequence (gus) was detected by RT-PCR to confirm the triggering of double-strand RNA production. (B) ERPE measurement was started immediately after pretreatment (at 2 h), paused for elicitor application (at 0 h), and then continued for 12 h. Values represent the average of triplicate experiments. Error bars represent the standard deviation.

Treatment with 1 μM chitin elicitor induces biphasic ERPE in rice cells, which peaks a few minutes and 2 h after treatment [12]. The C-ERPE was nearly similar to that of the WT in OsWRKY45-RNAi cell line #1 but was weaker in OsWRKY45-RNAi cell line #2. In these two cell lines, the second peak appeared 1 h later than that of the WT cells (Figure 1B, gray line). The effect of OsWRKY45-RNAi was more apparent during the potentiation of ERPE by pretreatment of rice cells with ASM. In WT cells, the 5 h integrated C-ERPE count increased after ASM pretreatment by 339% compared to that after solvent pretreatment (Figure 1B, black line). By contrast, the increase rate in C-ERPE by ASM pretreatment was suppressed in the OsWRKY45-RNAi lines (Figure 1B; -3% in OsWRKY45-RNAi line #1 and 57% in OsWRKY45-RNAi line #2). Taken together, these experiments confirmed the contribution of OsWRKY45 to ERPE potentiation by ASM.

TDL, which has been commercialized as a plant activator for rice disease, affects defense signaling in a similar way to ASM in tobacco [37,38]. In rice cells, TDL also induced OsWRKY45 expression in WT cells, and this was suppressed in the RNAi line #2. In these two cell lines, the second peak appeared 1 h later than that of the WT cells (Figure 1B, gray line). The effect of OsWRKY45-RNAi was more apparent during the potentiation of ERPE by pretreatment of rice cells with ASM. In WT cells, the 5 h integrated C-ERPE count increased after ASM pretreatment by 339% compared to that after solvent pretreatment (Figure 1B, black line). By contrast, the increase rate in C-ERPE by ASM pretreatment was suppressed in the OsWRKY45-RNAi lines (Figure 1B; -3% in OsWRKY45-RNAi line #1 and 57% in OsWRKY45-RNAi line #2). Taken together, these experiments confirmed the contribution of OsWRKY45 to ERPE potentiation by ASM.

Figure 2: TDL-induced potentiation of C-ERPE was diminished by OsWRKY45 knockdown. (A) WT and OsWRKY45-RNAi cell lines were pretreated with 200 μM TDL (gray columns) or solvent (white columns). Two hours after pretreatment, 50 mg aliquots of cells were collected for quantitative RT-PCR of OsWRKY45 and Ubq1 sequences just before chitin elicitor treatment. (B) ERPE measurement was started immediately after pretreatment (at 2 h), paused for elicitor application (at 0 h), and then continued for 12 h. Values represent the average of triplicate experiments. Error bars represent the standard deviation.

Contribution of SA signaling via OsWRKY45 to the potentiation of C-ERPE by priming

ASM and TDL are functional analogues of SA, and induce SAR by affecting the downstream events of SA-mediated defense signaling cascade in dicots [36,37]. In rice, the involvement of SA in induced disease resistance has not been clarified because of the constant accumulation of SA in healthy rice leaves [39]. However, Shimono et al. reported that SA application increases OsWRKY45 gene expression in rice [27]. Iwai et al. reported that SA induces resistance against rice blast fungus by exogenous application of SA on top-expanding leaves of 8th leaf-stage “adult” rice [40]. Together, these studies clearly indicate the contribution of SA to IR in rice.

As shown in Figure 3, OsWRKY45 upregulation and potentiation of C-ERPE by SA was observed in WT cells, but it was diminished in the OsWRKY45-RNAi lines. As shown in Figure 3A (gray columns), the average expression rate compared with WT cells was 55.4% in OsWRKY45-RNAi line #1. As shown in Figure 3B, the increase in the rate of C-ERPE was 179% in WT cells and 37% in OsWRKY45-RNAi line #1. As shown in Figure 4, native SA enhanced the C-ERPE (156% increase), whereas the structural isomers of SA (3HBA and 4HBA) had almost no effect on priming (increases of 24% by 3HBA and 10% by 4HBA). The isomeric effect of SA and its biologically inactive

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structural isomers has been reported in studies on SAR to show the specificity or structural requirement for SAR induction [41,42], and these effects are confirmed here. These results of experiments to interrupt SA signaling indicate that the enhancement of C-ERPE requires intrinsic hormonal signaling for defense, at least for priming by SAR inducers.

Although the authors focused on OsWRKY45 in this study, rice SA signaling branches into OsWRKY45 and NH1, the rice analogue of NPR1 in dicots [43,44]. NPR1 is the central regulator of SAR in dicots, and more than 99% of ASM (BTH)-responsive genes are regulated by NPR1 [45]. However, most ASM (BTH)-responsive upregulated genes are OsWRKY45 dependent, and most ASM-responsive downregulated genes are NH1 dependent in rice [43,44]. The precise role of both regulators in the potentiation of C-ERPE is unclear, although the participation of OsWRKY45 in the potentiation of C-ERPE is irrefutable.

Signal-disruption experiments involving several hormones were next performed to help clarify the contribution of each hormonal cascade to C-ERPE potentiation.

Properties of C-ERPE as a priming detector in rice

The biphasic intensity transition in C-ERPE, in which the first phase is shorter than the second, is a characteristic feature of this system in rice [12]. After chitin perception, phosphatidic acid (PA), a messenger phospholipid, is generated biphasically and induces a burst of reactive oxygen species (ROS) production [46,47]. The authors previously reported that suppression of the phospholipase D (PLD)-mediated second phase of PA generation induced a decrease in the second phase of C-ERPE, whereas exogenous PA application to rice cells mimicked the second phase of C-ERPE [12]. The intensity changes in C-ERPE were proportional to ROS generation, and ROS scavenging lowered the C-ERPE [12-14]. Thus, C-ERPE in rice cells is generated through PA signaling that is closely linked to ROS generation.

Zhan and Xiao [48] proposed biphasic signal amplification of the "PA-ROS-SA" module. In their proposed pathway, pathogen-associated molecular patterns or effector perceptions trigger the first phase of ROS production (0.5-2 h after perception), and this first phase potentiates the following phase (2-10 h after perception). Applications of SA or functional analogues of SA increased C-ERPE and interruptions to SA signaling attenuated this C-ERPE enhancement (See Figures 1B, 2B, and 3B for OsWRKY45-RNAi; and Figure 4 for application of inactive HBAs). This suggests that amplification driven by the PA-ROS-SA signal cascade may drive the biphasic generation of C-ERPE in rice cells.

On the one hand, C-ERPE potentiation occurs through SA signaling induced by SA or its functional analogues; on the other hand, other molecules such as methyl jasmonate and ethylene can also prime rice cells for C-ERPE amplification [30]. The chitin response in rice is accompanied by an increase in jasmonic acid synthesis [49]. To explain these complexities, the nature of PLDs and PLD-derived PAs that act as versatile signaling components by being incorporated in various kinds of hormonal signaling cascades and by functional overlapping with each other should be considered [50,51]. Further elucidation of the mechanism underlying ERPE potentiation might accelerate the development of multipurpose priming detectors for plant activators, which may be applicable to several biotic and/or abiotic stress disorders, and may help to unlock the complexities of PLD-derived PAs and ROS signaling in plants.

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Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. The structure and transformation process of pANDA-OsWRKY45-RNAi.

Supplemental Figure S2. Schema of 5 h-integrated ERPE counts after subtraction.

Supplemental Figure S3. Ten-day-old wild type (left) and OsWRKY45-RNAi cell line (right).

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