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Cyclic lipopeptide iturin A structure-dependently induces defense response in *Arabidopsis* plants by activating SA and JA signaling pathways

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ABSTRACT

Iturin A is the most well studied antifungal cyclic lipopeptide produced by *Bacillus* species that are frequently utilized as biological control agents. Iturin A not only shows strong antifungal activity against phytopathogens but also induces defense response in plants, thereby reducing plant disease severity. Here we report the defense signaling pathways triggered by iturin A in *Arabidopsis* salicylic acid (SA) or jasmonic acid (JA)-insensitive mutants. Iturin A activated the transcription of defense genes *PR1* and *PDF1.2* through the SA and JA signaling pathways, respectively. The role of iturin A as an elicitor was dependent on the cyclization of the seven amino acids and/or the β-hydroxy fatty acid chain. The iturin A derivative peptide, NH_2-(L-Asn)-(D-Tyr)-(D-Asn)-(L-Gln)-(L-Pro)-(D-Asn)-(L-Ser)-COOH, completely suppressed *PR1* and *PDF1.2* gene expression in wild *Arabidopsis* plants. The identification of target molecules binding to iturin A and its derivative peptide is expected to shed new light on defense response in plants through the SA and JA signaling pathways.

1. Introduction

Iturin A is the most well studied broad-spectrum antifungal cyclic lipopeptide (a heptapeptide with a β-hydroxy fatty acid chain) produced by *Bacillus* species [1]. It is thought to be the principal antifungal substance responsible for the biological control activity of *Bacillus* species against fungal pathogens [2,3]. On the other hand, iturin A triggers systemic acquired resistance (SAR), which is a whole-plant defense response to an earlier and localized exposure to pathogens, in strawberry plants, thereby resulting in the suppression of strawberry anthracnose disease by accumulating pathogenesis-related (PR) proteins [4]. Accordingly, iturin A produced by biological control agents plays a role in the suppression of plant diseases by acting as a bifunctional molecule through its antifungal activity and the activation of plant defense systems.

How does iturin A trigger plant defense response? No evidence of the mechanisms is available so far. The simplest mode would be the direct interaction of iturin A with specific receptors on plant cells to activate signal transduction pathways. Typically, plant cells recognize both avirulence factors produced by specific pathogens and non-specific elicitors constitutively present in pathogens, thereby inducing local acquired resistance (LAR) in the localized tissue [5]. Through the recognition, signals are transmitted to nuclei in plant cells to result in the induction of defense-related proteins, including PR proteins [6]. Salicylic acid (SA) synthesized in plant cells functions as a long-distance SAR signal [7]. Plant cells receiving SA signals accumulate PR proteins. A study of the mechanism of SAR demonstrated that the positive regulator protein NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1) is transported to the nucleus in response to SA where it activates the expression of defense genes, including PR proteins PR1 and PR2 [8]. On the other hand, jasmonic acid (JA) also plays a role in the acquisition of plant disease resistance as a signaling molecule [9]. The JA signaling pathway is positively regulated by the nuclear-localized helix-loop-helix-leucine zipper-type transcription factor MYC2 and induces plant defense related proteins, such as PDF1.2

Abbreviations: JA, jasmonic acid; LAR, local acquired resistance; NPR1, NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES1; PAMP, pathogen-associated molecular pattern; SA, salicylic acid; SAR, systemic acquired resistance.

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[10]. Overall, the SA and/or JA signaling pathways are presumed to be involved in plant defense response triggered by iturin A, as iturin A induced SAR in strawberry plants [4].

Here we report the defense signaling pathways triggered by iturin A. We demonstrate that both SA and JA signaling pathways are involved in the defense response triggered by iturin A. Moreover, we show that the activity of iturin A as an elicitor may be depend on its structure.

2. Materials and methods

2.1. Plant materials

Arabidopsis thaliana wild-type (Col-0), SA-insensitive mutant *npr1-5* (CS3724) [11], and JA-insensitive mutant *atmyc2* (SALK_039235) [12] were used. *Arabidopsis* seeds were sown on rockwool blocks (2.5 cm × 2.5 cm × 3.8 cm) and incubated at 22 °C in an incubator (11.8 Wm^{-2}/16 h/d).

2.2. Treatment of Arabidopsis plants with iturin A or iturin A derivative peptides

Iturin A was purchased from Sigma (Tokyo, Japan). Iturin A derivative peptides, NH_2-(L-Asn)-(D-Tyr)-(D-Asn)-(L-Gln)-(L-Pro)-(D-Asn)-(L-Ser)-COOH (ATAGPAS), NH_2-(L-Asn)-(D-Tyr)-(D-Asn)-(L-Gln)-COOH (ATAG), and NH_2-(D-Asn)-(L-Gln)-(L-Pro)-(D-Asn)-COOH (AGPA), were synthesized by Greiner Bio-One (Tokyo, Japan). The concentrations of iturin A and the derivative peptides were adjusted to 0.01 mg/ml with 0.02% Tween 20. Each solution or water was applied to a rosette leaf of 30-day *Arabidopsis* seedlings by spraying (1 ml each). The seedlings were incubated at 25 °C for the indicated periods in an incubator (11.8 Wm^{-2}/14 h/d).

2.3. RNA isolation

Rosette leaves treated or untreated with iturin A or the derivative peptides were collected at 0, 6, 12, 24, 72 or 144 h after the treatment. The leaves were homogenized with an SK mill (SK-200, Tokken, Kashiwa, Japan) according to the manufacturer's instructions. Total RNA isolation from the pulverized samples was performed with a NucleoSpin RNA Plant (Takara, Otsu, Japan).

2.4. Real-time RT-PCR analysis

cDNA was synthesized from total RNA using a PrimeScript RT Reagent Kit with gDNA Eraser (Takara), and then real-time RT-PCR was performed using an SYBR Premix Ex Taq II (Takara). The conditions for real-time RT-PCR were as follows: 37 °C for 15 min and 85 °C for 5 s for cDNA synthesis, and then 40 cycles at 95 °C for 5 s and at 60 °C for 30 s for PCR amplification. Nucleotide sequences of the primers used in this study were as follows: AtPR1 primers (5′-CCTGGGGTAGCGGTGACTT-3′ and 5′-CGTGTTCGCAGCGTAGTTGT-3′, *A. thaliana* pathogenesis-related protein 1 mRNA, GenBank accession no. NM_127025), AtPDF1.2 primers (5′-TCACCCT-TATCTTCGCTGCTC-3′ and 5′-ACCATGTCCCACTTGGCTTC-3′, *A. thaliana* putative antifungal protein PDF1.2 mRNA, GenBank accession no. AY063779), and AtACT primers (5′-GCCGACA-GAATGAGCAAAGAG-3′ and 5′-AGGTACTGAGGGAGGCCAAGA-3′, *A. thaliana* actin 1 mRNA, GenBank accession no. NM_179953). Actin was used for normalization. Dissociation curves were analyzed to verify the specificity of the amplification reaction using the Thermal Cycler Dice Real Time System Single Software ver. 3.00 (Takara). The expression level of each gene was determined as the number of amplification cycles needed to reach a fixed threshold using the standard curve method, and then expressed as a relative value.

2.5. Antagonistic activity of iturin A derivative peptides

Mycelial discs of *Colletotrichum gloeosporioides*, which causes strawberry anthracnose and grape ripe rot diseases, were placed on potato dextrose agar plates. At the same time, sterilized paper discs were placed on the plates and then inoculated with 50 μL of 0.1 mg/ml iturin A or iturin A derivative peptides. The plates were incubated at 25 °C for 5 days. The antagonistic effects of the derivative peptides toward *C. gloeosporioides* mycelial growth were evaluated by measuring the growth inhibition zones formed on the plates.

2.6. Statistics

Data are presented as means ± standard deviations. Statistical analysis was performed by Tukey's test using Excel statistics software 2012 (Social Survey Research Information, Tokyo, Japan).

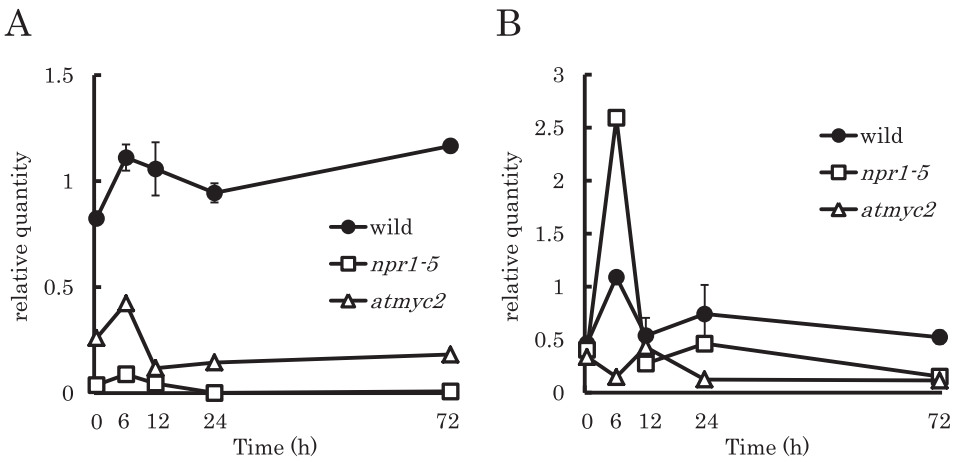

Fig. 1. Expression of *PR1* and *PDF1.2* genes in iturin A treated leaves. Transcription levels of *PR1* (A) and *PDF1.2* (B) in iturin A treated rosette leaves of wild Col-0, *npr1-5* mutant, and *atmyc2* mutant were estimated by real-time RT-PCR. β-Actin was used as internal control. Data were calculated as gene expression relative to β-actin gene expression. Data indicate means ± standard deviations of independent triplicate experiments.

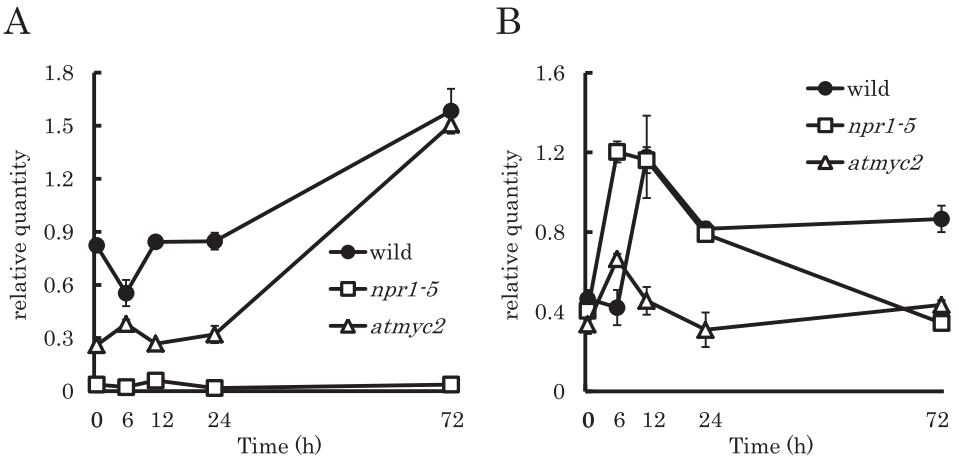

Fig. 2. Expression of *PR1* and *PDF1.2* genes in iturin A untreated leaves. Transcription levels of *PR1* (A) and *PDF1.2* (B) in untreated rosette leaves of wild Col-0, *npr1-5* mutant, and *atmyc2* mutant exposed to iturin A were estimated by real-time RT-PCR. β-Actin was used as internal control. Data were calculated as gene expression relative to β-actin gene expression. Data indicate means ± standard deviations of independent triplicate experiments.

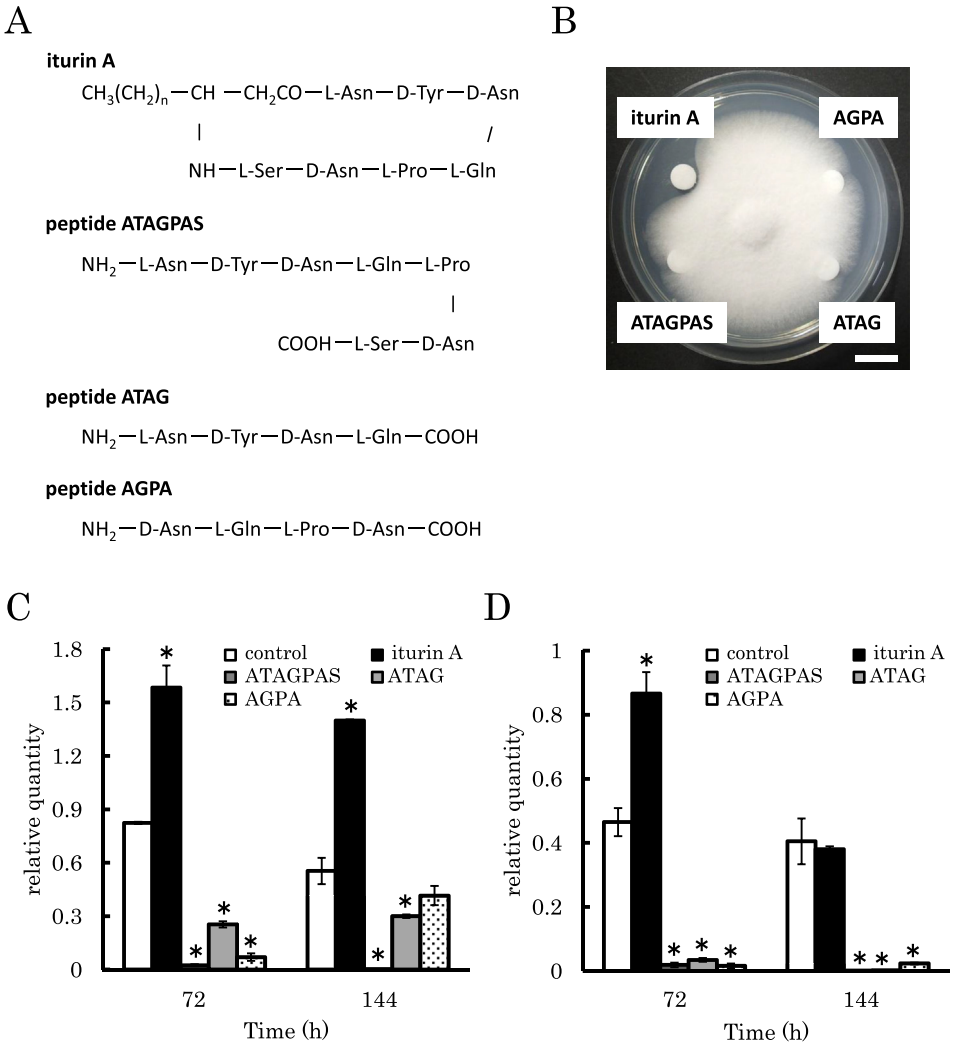

Fig. 3. Iturin A structure-dependently induces defense response in *Arabidopsis* plants. (A) Structures of iturin A and its derivative peptides. (B) Antagonistic activity of the derivative peptides. Growth inhibition zones of *C. gloeosporioides* mycelia were formed in response to iturin A, but not the derivative peptides. Bar, 1 cm. (C) Transcription levels of *PR1* in untreated rosette leaves of wild Col-0 exposed to iturin A were estimated by real-time RT-PCR. (D) Transcription levels of *PDF1.2* in untreated rosette leaves of wild Col-0 exposed to iturin A were estimated by real-time RT-PCR. β-Actin was used as internal control. Data were calculated as gene expression relative to β-actin gene expression. Bars indicate means ± standard deviations of independent triplicate experiments. Mean values statistically different from control ($p < 0.05$) are indicated by asterisks.

3. Results

3.1. Iturin A induces plant defense response through SA and JA signaling pathways

PR1 and *PDF1.2* gene expression was induced in the iturin A treated leaves of wild plants (Fig. 1), suggesting that iturin A induced LAR. *PR1* (Fig. 1A) and *PDF1.2* (Fig. 1B) gene expression was acutely increased 6 h after treatment of the leaves with iturin A. Similarly, the transient upregulation of *PR1* gene expression was confirmed in the iturin A treated leaves of *atmyc2* mutant. In *npr1-5* mutant, *PR1* gene expression was not upregulated in response to the iturin A treatment. In contrast, *PDF1.2* gene expression was upregulated 6 h after iturin A treatment of the leaves of *npr1-5* mutant, but this upregulation was not observed in *atmyc2* mutant.

In our previous study, we demonstrated that iturin A induced SAR in strawberry plants, conferring defense resistance to fungal pathogens [4]. In the present study, we confirmed that iturin A induced SAR in *Arabidopsis* plants (Fig. 2). PR1 gene expression was increased in iturin A untreated leaves of wild plants 72 h after exposure to iturin A (Fig. 2A). A similar upregulation of *PR1* gene expression was observed in the untreated leaves of *atmyc2* mutant.

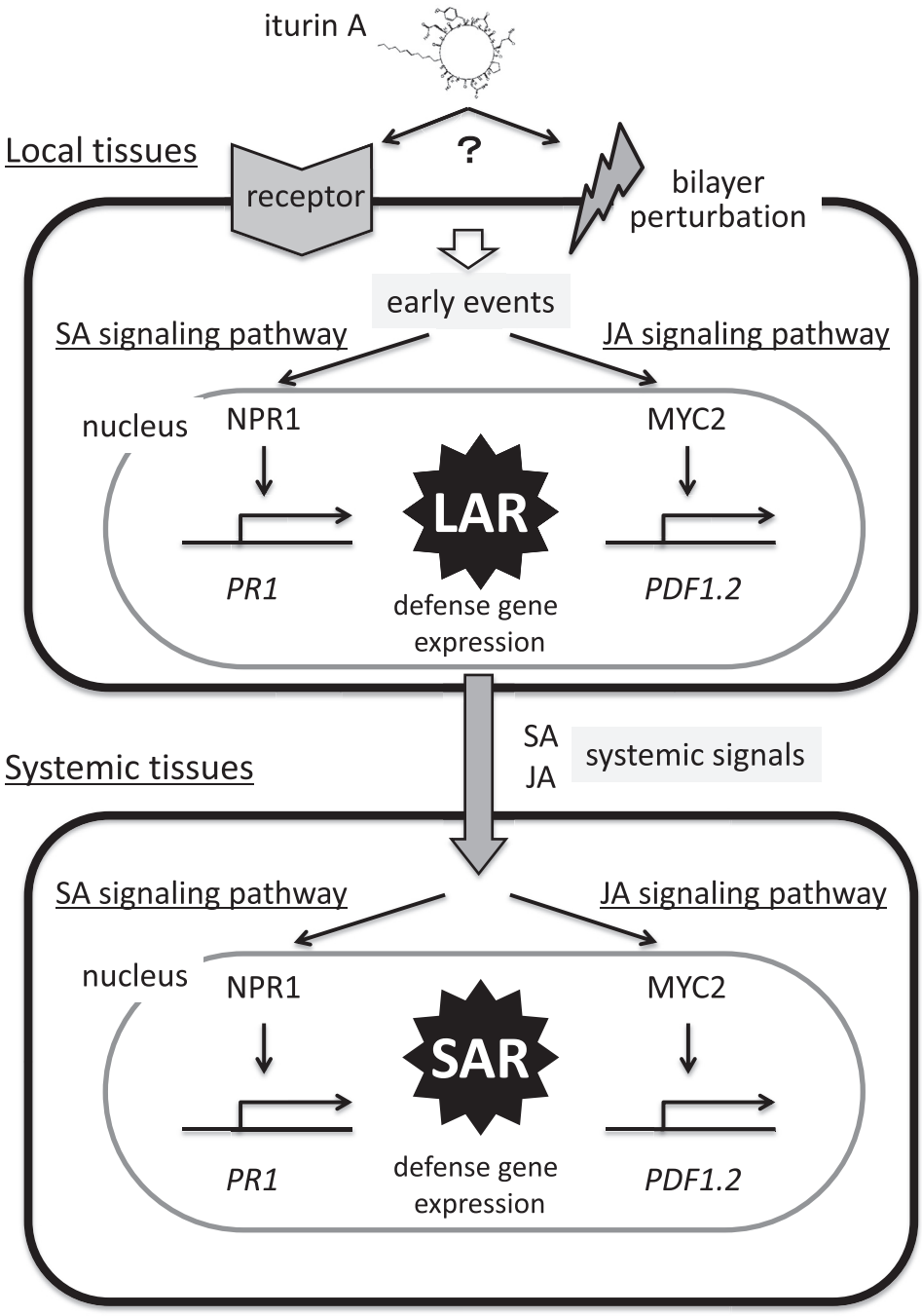

Fig. 4. Predicted signaling pathways of LAR and SAR in *Arabidopsis* plants exposed to iturin A.

In *npr1-5* mutant exposed to iturin A, *PR1* gene expression was not upregulated in the untreated leaves. Iturin A also upregulated *PDF1.2* gene expression in iturin A untreated leaves of wild plants 12 h after exposure to iturin A (Fig. 2B). The upregulation was maintained up to 72 h after exposure to iturin A. A similar upregulation of *PDF1.2* gene expression was confirmed in the untreated leaves of *npr1-5* mutant after exposure to iturin A, but not in those of *atmyc2* mutant, although *PDF1.2* transcripts were transiently increased in the untreated leaves of *atmyc2* mutant 6 h after exposure to iturin A.

Taken together, the results suggest that iturin A induces *PR1* and *PDF1.2* gene expression in both local and systemic tissues through the SA and JA signaling pathways, respectively.

3.2. Structure-dependent activity of iturin A in the induction of plant defense response

To determine whether plant defense response induction by iturin A is structure-dependent, we synthesized three iturin A derivative peptides (Fig. 3A). Iturin A inhibited the mycelial growth of fungal plant pathogen *C. gloeosporioides*, whereas all the derivative peptides lost their antagonistic effect on the fungus (Fig. 3B).

As described above, iturin A induced *PR1* and *PDF1.2* gene expression in the untreated leaves of wild plants (Fig. 3C and D). When wild plants were exposed to the three iturin A derivative peptides, *PR1* and *PDF1.2* gene expression was not systemically upregulated in the untreated leaves. The ATAGPA peptide, in particular, drastically decreased the expression of both genes in the untreated leaves compared with control plants (Fig. 3D).

4. Discussion

The predicted signaling pathways of LAR and SAR triggered by iturin A are shown in Fig. 4. Plant cells recognize iturin A through an unknown mechanism, probably through receptor recognition and/or bilayer perturbation of plasma membranes. The recognition guides the cells to early events triggering plant defense response. When the SA signaling pathway is activated, NPR1 is transported to the nucleus in response to SA [8], where it upregulates defense gene expression, such as *PR1*. The JA signaling pathway is positively regulated by the recognition, and MYC2 induces defense gene expression, such as *PDF1.2* [10]. Consequently, LAR is induced in local tissues exposed to iturin A. SA and JA signals are transported in the phloem and then to systemic tissues from the phloem. Similar to local tissues, in iturin A untreated cells, SA and JA induce defense gene expression through NPR1-and MYC2-related signal transduction, respectively. Finally, SAR is systemically expressed in distant tissues.

The perception that iturin A acts as an elicitor of plant defense response has yielded contradictory results. Some reports demonstrated that iturin is not the major determinant of induced defense response in tobacco [13] and melon [14]. On the other hand, some studies showed that iturin A activated plant defense response in pepper [15] and strawberry [4]. This contradiction might be due to the perception that iturin A acts as an elicitor only in specific plant species. In the present study, we observed the elicitor activity of iturin A in *Arabidopsis* plants. Therefore, we concluded that iturin A, at least in part, can systemically induce plant defense response by activating both SA and JA signaling pathways in *Arabidopsis* plants.

Why does iturin A act as an elicitor in specific plant species? To date, it is not clear whether specific receptor proteins binding lipopeptide antibiotics exist in plant cells. It is perceived that surfactin disturbs lipid compartmentalization through surfactin-lipid bilayer binding [16], although the existence of low-affinity receptors cannot be completely ruled out. Surfactin does not have specific affinity to plant sterols, whereas iturins strongly bind to plant sterols [1]. The permeability of ions, such as $K+$, is greatly increased in target cells exposed to iturins through the formation of ion-conducting pores using the β-hydroxy fatty acid chain of iturins [1]. Therefore, the ability of iturin A to act as an elicitor in specific plant species may depend on the lipid composition of plant membrane that binds the β-hydroxy fatty acid chain of iturins. In fact, iturin A was inactivated when it was transformed into a linear structure without the β-hydroxy fatty acid chain, as shown in the iturin A derivative peptide ATAGPA (Fig. 3).

On the other hand, it is plausible that the seven amino acids composing the iturin A structure do not function as elicitors themselves. Plant cells possess receptors that recognize pathogen-associated molecular patterns (PAMPs) [17]. Peptides flg22 and elf18 are well-studied PAMPs from phytopathogenic bacteria; they bind to the leucine-rich repeat receptor kinase flagellin-sensitive 2 [18] and elongation factor Tu receptor [19], respectively. The biological activity of iturin A is modulated by the primary structure of the cyclic peptide. For example, the inversion between the adjacent L-Ser and D-Asn in the cyclic peptide enhanced the antifungal activity of iturin A [1]. Fig. 3 demonstrated that the cyclization of the seven amino acids and/or the β-hydroxy fatty acid chain might be required for the induction of defense response. Accordingly, we speculated that (1) the peptides of iturin A are not molecular components categorized in PAMPs, (2) iturin A specific receptor (protein) does not exist in plant cells, and (3) bilayer perturbation in plant cells exposed to iturin A is essential for the induction of plant defense response.

Surprisingly, the three iturin A derivative peptides suppressed *PR1* and *PDF1.2* gene expression (Fig. 3). Phytopathogenic bacteria infecting plants deliver 'effector' proteins into plant cells. The effectors block PAMP-triggered plant immunity, resulting in compatibility between the bacteria and the plants. The effectors directly target PAMP-recognition receptors and their associated accessory proteins [20]. As both SA and JA signaling pathways were inactivated by iturin A derivative peptides, those peptides might inhibit the upstream signaling pathways probably common to the SA and JA signaling pathways. Although further studies of the function of iturin A derivative peptides as effector peptides that suppress plant defense response are required, exploration of target molecules binding to the peptides would shed new light on plant defense response through the SA and JA signaling pathways.

Conflict of interest

None.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.143.

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