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Development of a High-Throughput Screening Method Using a Cell-Based, Lawn Format Assay for the Identification of Novel Plant Defense Activators from Combinatorial Peptide Libraries

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Plants respond to attack by pathogens through various defense mechanisms. These defense responses are triggered by a variety of molecules derived from pathogenic microorganisms as well as host plants. In this study, we developed a high-throughput screening method using a cell-based lawn format assay for the identification of novel peptides that can induce plant defense responses from combinatorial peptide libraries. Solid-phase peptide libraries were synthesized using a photocleavable linker and immobilized using agarose gel. The peptides were partially cleaved from beads, and the agarose gel was layered on the tobacco cells. The defense response was then observed by detecting the generated H_2O_2 using a sensitive H_2O_2 indicator dye, *N*-(carboxymethylaminocarbonyl)-4,4′-bis(dimethylamino)diphenylamine sodium salt (DA-64). Using this assay format, a 6859-member peptide library based on the sequence of flagellin-derived peptides was screened, and several structural features important for the activity were obtained.

KEYWORDS: Plant defense; combinatorial library; cell-based assay; H_2O_2 generation; flagellin-derived peptide

INTRODUCTION

Plants respond to attack by pathogens through various defense mechanisms including cell wall strengthening, phytoalexin production, oxidative burst, ethylene biosynthesis, expression of pathogenesis-related proteins, and hypersensitive cell death (*1*, *2*). These defense responses are triggered by a variety of molecules derived from pathogenic microorganisms as well as host plants, which are referred to as elicitors (*3*, *4*). Recently, several peptides that are derived from microbial proteins have been shown to act as elicitors (*5*−*8*). A 15-amino acid peptide derived from the common bacterial protein flagellin, the building block of the flagella, induces plant defense responses in many plants including tomato, tobacco, and *Arabidopsis* (*7*, *9*). The perception of flagellin peptides by *Arabidopsis* was shown to depend on FLS2, a transmembrane receptor kinase protein with an extracellular leucine-rich repeat (leucine-rich repeat receptor kinase, LRR-RK) (*10*). This perception system in plants for the flagellin peptides has striking similarities to the innate immune system in animals, and flagellin has been recognized to act as a pathogen-associated molecular pattern (PAMP) in both plants and animals (*11*). The existence of other unknown PAMPs is anticipated in the plant−pathogen interaction. In the *Arabidopsis* genome, there are more than 200 LRR-RK genes, although the biological roles of them as well as their ligands are unknown. It is speculated that some of these LRR-RK genes might be additional PAMP receptors (*12*).

Combinatorial chemistry has become an important tool in various biological studies because of its ability to rapidly synthesize thousands to millions of compounds (*13*). These libraries have been tested to identify novel ligands for biological targets such as enzymes or receptors. In particular, combinatorial peptide libraries have been widely used because of their ease of synthesis as well as structural identification after obtaining a hit. Millions of individual peptides can be readily produced by means of split-and-mix synthesis (*14*). To effectively identify active compounds from libraries containing a large number of compounds, however, the development of high-throughput screening methods is essential. In the split-and-mix approach, each compound is synthesized on a single bead (one-bead one-compound combinatorial library) and tested without cleavage in a solid-phase assay or after being partially released from beads in a solution-phase assay (*15*). Although positive beads can be easily identified in the solid-phase assay by inspection of a color change caused by the binding of target molecules to which enzymes or fluorescence probes are attached, these target molecules must be available in the isolated form in these assays. However, in many biological events including plant defense responses, it is difficult to obtain these biological targets in the isolated form because the targets are often transmembrane proteins or even unknown. Therefore, cell-based functional assays in the solution phase must be employed for those cases. The cell-based assays for solid-phase libraries are often carried

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out using a microtiter plate format, and beads in which compounds are attached via chemically or photochemically cleavable linkers are dispensed into each well of the 96-well filtration plates (*16*). In this assay format, compounds are partially cleaved and filtered into a 96-well plate placed beneath the filtration plate to measure the biological activity. After measurement of the activity in each well, beads inducing a positive response are recovered from wells of the filtration plate. However, handling of the beads for distribution and recovery is laborious and time-consuming in this assay format and sometimes requires a robotic workstation to facilitate the efficiency. In addition, because the concentration of individual peptides cleaved into 96-well plates is relatively low (<1 μM), it is difficult to detect peptides having weak activity in this assay format. To overcome these disadvantages, the lawn or well-less screening format has been constructed (*17*, *18*). In this assay format, beads are immobilized in the agar or agarose gel. This thin agarose gel layer is then put in contact with the biological matrix such as bacterial (*19*) or *Xenopus laevis* melanophore cells (*20*). After compounds are partially cleaved from the beads, biological responses are monitored. Positive beads can be easily identified by inspection of a color change induced on the biological matrix in the vicinity of the beads. Since the beads can be directly recovered from the agarose layer, the handling of the beads is much easier than that in the 96-well plate format. The lawn format assay also has an advantage that the concentration of the compound obtained in the biological matrix is much higher (>1 μM) than that in the 96-well plate format (*15*). This enabled the lawn format assay to be sensitive enough to identify compounds that have EC_{50} or IC_{50} values in the micromolar range.

In this study, we developed a high-throughput screening method using a cell-based lawn format assay for the identification of novel peptides that can induce plant defense responses from combinatorial peptide libraries. We first prepared known active peptides attached with beads via a photocleavable linker as a positive control and assayed using tobacco suspension cells. H_2O_2 generation was monitored as an indicator of plant defense responses. A 6859-member peptide library, which was prepared based on the amino acid sequence of known active peptides, was screened to demonstrate the effectiveness of this assay system. To further validate the screening results, several analogues were synthesized, and their elicitor activity was measured.

MATERIALS AND METHODS

Chemicals. *N*-(Carboxymethylaminocarbonyl)-4,4′-bis(dimethylamino)-diphenylamine sodium salt (DA-64) was obtained from Wako Pure Chemical Industries (Osaka, Japan). Hydroxyethyl-Photolinker NovaSyn TG resin and NovaSyn TGA resin were obtained from NovaBiochem (San Diego, CA). All other chemicals were commercially available.

Plant Cell Culture. Tobacco suspension cultured cells (*Nicotiana tabacum* cv Xanthi) were kindly provided by Prof. Fumihiko Sato of Kyoto University. Cells were grown in LS medium at pH 5.8 supplemented with 10 μM 1-naphthalenacetic acid, 1 μM 5-benzyladenine, and 3% sucrose. The cells were subcultured every 2 weeks and used for assays 8−10 days after subculture.

Cell-Based Lawn Format Assay for Detection of H_2O_2 Generation as a Defense Response. Aliquots of tobacco suspension cultured cells were placed in a cell culture dish (60 mm × 15 mm, Corning, Corning, NY). After addition of the assay buffer (175 mM mannitol, 0.5 mM $CaCl_2$, 0.5 mM K_2SO_2, and 2 mM MES, pH 5.8), most of the medium was removed by a micropipette from the edge of the dish. The resultant lawn of the cells (1−2 mm thick) was incubated at 25 °C for 1 h in the dark. Solid-phase peptide libraries, in which peptides were linked to beads via a photocleavable linker, were used in this assay. The peptide library beads (approximately 1000 beads per dish) were added to 2.5 mL of molten 0.8% agarose in the assay buffer in a Petri dish (45 mm diameter). After the agarose was solidified by cooling at room temperature, the gel was irradiated with UV (365 nm) at an intensity of 10 mW/cm^2 (UVP, Upland, CA) for 1−3 min to partially release the peptides from the beads. The agarose gel containing peptide beads was immediately removed from the dish and placed on the lawn of the tobacco cells. A solution of DA-64, an H_2O_2 indicator, in the assay buffer (1 mg/mL) was added onto the agarose gel and incubated for 15 min. The active beads were identified by visual inspection of a color change in the vicinity of the beads, indicating that H_2O_2 was generated from the tobacco cells as a defense response. The gel exhibiting a color change (approximately 5 mm diameter) was excised by a scalpel, and the agarose was removed by filtration after melting with 6 M NaI. All beads included in the excised gel were manually isolated using a needle under the microscope. Typically, one to 20 beads were recovered from the excised gel and subjected to the second round assay to identify and confirm a single positive bead. Amino acid sequences of the positive beads were analyzed by an automated Edman sequencer (Procise 491-HT, Applied Biosystems, Forster City, CA).

Synthesis of a Solid-Phase Peptide Library Using a Photocleavable Linker. A combinatorial peptide library, containing XXXSAKDDAAGLQ in which X denotes one of 19 natural L-amino acids excluding Cys, was prepared by a split-and-mix synthesis method using a photocleavable linker. Hydroxyethyl-Photolinker NovaSyn TG resin was used for the library synthesis. Coupling of amino acids at the C-terminal fixed sequence (i.e., SAKDDAAGLQ) was carried out using a standard Fmoc peptide synthesis method. The C-terminal amino acid, Fmoc-Gln(Trt), was attached to the resins by a symmetrical anhydride method catalyzed by 4-dimethylaminopyridine. After the Fmoc group was deprotected with 20% piperidine in DMF, Fmoc-protected amino acids were coupled to the resins with a diisopropylcarbodiimide (DIC)/1-hydroxybenzotriazole (HOBt) method. This cycle was repeated until coupling of Fmoc-Ser(tBu) was finished. The randomized N-terminal sequence of the library was constructed according to the literature (*21*). Briefly, after the Fmoc group was deprotected, the resins were equally distributed into 19 polypropylene reaction columns. The solution of each Fmoc-protected amino acid/HOBt in DMF and DIC at a 3-fold molar excess was added to the resins and agitated for 1.5 h at room temperature. The reaction was monitored by a Kaiser test. After the reaction was completed, all resins were combined in a reaction column and washed with DMF. The deprotection of the Fmoc group, resin distribution, coupling of each amino acid, and resin mixing was repeated for two more cycles. After the last coupling, resins were not combined so that N-terminal residues in each of the 19 pools were already known before screening. Side-chain-protecting groups were removed by treatment of the resins with TFA/*m*-cresol/water/thioanisole/ethandithiol [82.5/5/5/5/2.5 (v/v)] for 6 h at room temperature. The resins were washed successively with DMF, methanol, chloroform, and DMF again and stored in the assay buffer containing 0.05% sodium azide at 5 °C in the dark.

Synthesis of Flagellin-Derived Peptide Analogues for Validation of Screening Results. For validation of the screening results, peptides were resynthesized using a solid-phase Fmoc peptide synthesis method. NovaSyn TGA resin was used for the synthesis. Attachment of a C-terminal amino acid to the resin, deprotection of Fmoc groups, and coupling of Fmoc-protected amino acids were conducted in the same manner as described previously. Removal of side-chain protecting groups and cleavage of the peptide from the resin was carried out with TFA/triisopropylsilane/water [95/2.5/2.5 (v/v)] for 2 h at room temperature. The cleaved peptides were precipitated in cold diethyl ether, washed 2 times with diethyl ether, and dried in vacuo. Crude peptides were purified by preparative PR-HPLC (Cosmosil 5C18-AR-II 20 × 250 mm, Nakarai Tesque, Kyoto, Japan) and lyophilized. Electrospray ionization quadrupole mass spectrometry (ESI-MS, LCMS-2010, Shimadzu, Kyoto, Japan) was employed to confirm the molecular weight of the desired products.

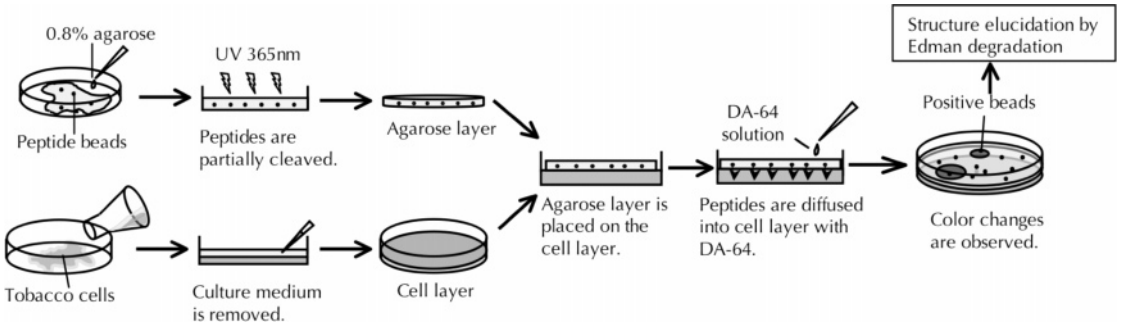

Figure 1. Scheme of the cell-based lawn format assay for identification of peptides that induce plant defense responses from combinatorial peptide libraries.

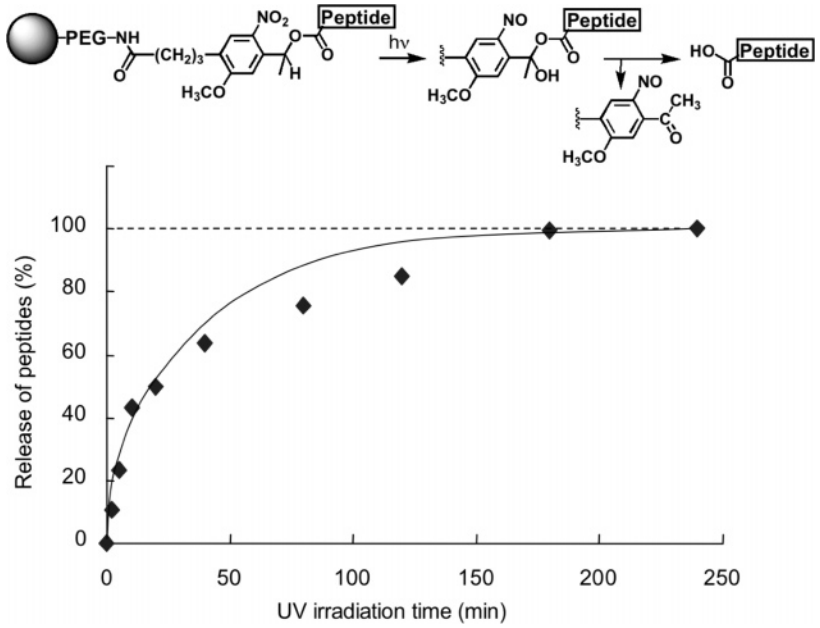

Figure 2. Structure of the photocleavable linker and reaction scheme of peptide cleavage by UV irradiation. An aliquot of beads to which known peptides were attached was suspended in 1 mL of the assay buffer and irradiated with UV light (365 nm, 10 mW/cm^2). After various incubation times (0–250 min), 50 μL of the solution was removed, and the amount of peptides was quantified by LC/ESI-MS (LCMS-2010, Shimadzu, Kyoto, Japan). The release rate was calculated from the ratio of the amount of peptides detected after each incubation time to that after 250 min. Data points are the mean of triplicate measurements.

Measurement of H_2O_2 Generating Activity of Flagellin-Derived Peptide Analogues (22). Tobacco suspension cultured cells were collected by filtration through Miracloth (Calbiochem, San Diego, CA). These cells (0.2 g of fresh weight) were resuspended in 1 mL of the assay buffer (175 mM mannitol, 0.5 mM $CaCl_2$, 0.5 mM K_2SO_4, and 2.0 mM MES, pH 5.75) in a microfuge tube and incubated for 1 h at 25 °C using a tube rotator (Biospin MBS-1, Tokyo Rikakikai, Tokyo, Japan). Ten microliters of sample solutions was added to the cells and incubated for 14 min at 25 °C using the tube rotator. The cell suspension was then allowed to stand for 1 min so that the cells were sedimented. From the upper portion of the cell suspension, 60 μL of the assay buffer was removed and mixed with 140 μL of DA-64 solution (100 μM) containing 1 unit/mL of POD and 100 mM PIPES (pH 7.0) in a 96-well microplate. After incubating for 10 min at 37 °C, the absorbance at 727 nm (Abs_{sample}) was measured by a microplate reader (Benchmark, Bio-Rad Laboratories, Hercules, CA). For a negative control measurement, the assay buffer containing 0.1% BSA was added to the cells instead of the sample solution. For various concentrations of samples, H_2O_2 generation rates were measured, and the concentration that gave 50% of the maximum response (EC_{50}) was calculated using a statistical software PRISM (GraphPad Software, San Diego, CA) and used as an index of activity. Each experiment was carried out in triplicate and repeated at least 3 times.

RESULTS AND DISCUSSION

Principle of the Cell-Based Lawn Format Assay for Identification of Plant Defense Activators. A scheme of the cell-based lawn format assay for identification of peptides that can induce plant defense responses developed in this study is illustrated in **Figure 1**. The lawn of tobacco suspension cells is prepared simply by removing the majority of the medium without any fixation of the cells by agar or agarose. Peptides are attached to beads via a photocleavable linker (**Figure 2**) (*23*). The photocleavable linker has the following advantages over chemically cleavable ones. First, reaction conditions for photocleavage are mild enough to permit the release of peptides directly in the assay medium without contamination by cleavage reagents. Second, the amount of released peptides can be readily controlled by the duration of UV irradiation as shown in **Figure 2**. Library beads are embedded in the agarose gel, and peptides are partially released from the beads by exposure to UV light (365 mm) for 1−3 min. Under these conditions, approximately 10% of the peptides is released from each bead. H_2O_2 generation, which is well-known to be one of the earliest events in plant defense responses (*24*), was monitored in this assay method. The H_2O_2 generation can be visualized by the addition

Table 1. Amino Acid Sequences of Flagellin-Derived Peptides Used as a Positive Control and Their H_2O_2 Generating Activity Measured by the Solution-Phase Assay

Peptide	Amino acid sequence	EC_{50} (μM)
flg15	RINSAKDDAAGLQIA	0.087 ± 0.030
flg15-Δ2	RINSAKDDAAGLQ	1.2 ± 0.28
flg14-Δ2	INSAKDDAAGLQ	9.7 ± 3.9

Figure 3. Detection of H_2O_2 generation by the cell-based lawn format assay. Regions at which beads containing positive control peptides (flg15) were placed are indicated by dashed circles and arrows.

of H_2O_2 indicators such as diaminobenzidine (DAB) (*25*). In this study, we used DA-64 for the detection of H_2O_2 because the detection by DA-64 is highly sensitive as shown previously (*22*). DA-64 is oxidized to a highly chromogenic compound (Bindschedler's Green) by H_2O_2 in the presence of peroxidases, the color of which can be easily observed by visual inspection. In this assay method, endogenous peroxidase activity in the plant was sufficient enough to catalyze the oxidation of DA-64. The addition of horseradish peroxidase had no effect on the detection of H_2O_2 (data not shown). The DA-64 solution was permeated into the cell layer through the agarose gel, which was accompanied by the delivery of the peptides released from the beads to the cell layer. Active beads can be simply identified by visual inspection of a color change caused by H_2O_2 generation on the cell layer and recovered by excision of the agarose gel just above the region showing a color change. After the agarose was removed by filtration after melting by NaI, the beads were subjected to the second round assay to specify a single positive bead. The amino acid sequence of peptides attached to the positive beads was analyzed by an automated Edman sequencer.

Validation of the Assay by Testing a Positive Control. To validate this assay system, a known active peptide attached to the beads via a photocleavable linker was prepared and tested. Flagellin-derived peptide has been known to induce plant defense responses in a wide range of plants including tobacco (*7*). A peptide, referred to as flg15 (RINSAKDDAAGLQIA), was reported to induce H_2O_2 generation in tobacco cells with an EC_{50} value of 0.087 μM (**Table 1**) and was used in this study as a positive control (*22*). The beads bearing flg15 were immobilized by the agarose gel and exposed to UV light (365 mm) for 3 min. After the agarose gel was placed on the tobacco cell layer, a DA-64 solution was applied to the gel. The cells below the positive bead began to exhibit a blue-green color caused by H_2O_2 generation after 10 min, and a positive spot became most obvious after 15−30 min (**Figure 3**). To examine the effect of the photolysis period on the sensitivity, the gel

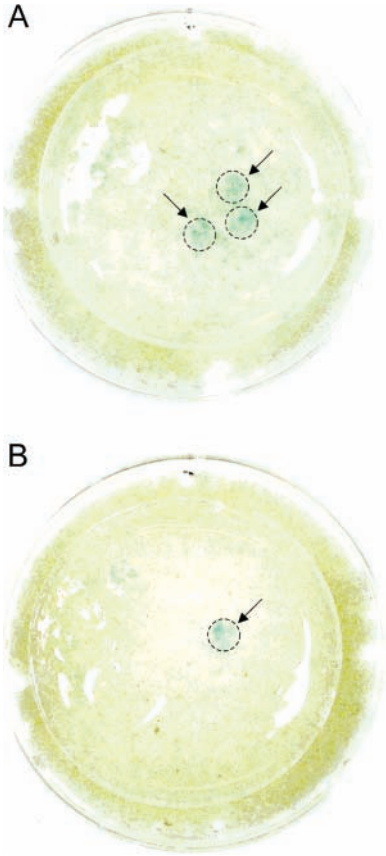

Figure 4. Representative results of first-round (**A**) and second-round screening (**B**). Regions at which positive beads were obtained are indicated by dashed circles and arrows.

was exposed to UV light for 1, 3, 5, or 10 min and then placed on the tobacco cell layer, followed by the application of DA-64 solution. The clear positive spots were observed for all conditions tested (data not shown), indicating that the photolysis even for 1 min is sufficient for peptides that have a 0.1 μM level of EC_{50}. To further evaluate the sensitivity of this assay method, two flg15 analogs, flg15-Δ2 (RINSAKDDAAGLQ) and flg14-Δ2 (INSAKDDAAGLQ), which have EC_{50} values of 1.2 and 9.9 μM (**Table 1**), respectively, were prepared on the bead with a photocleavable linker as in the case of flg15 and subjected to the assay. When photolysis was performed for 3 min, formation of clear positive spots was observed for flg15-Δ2. Positive spots were also induced by flg14-Δ2 occasionally, although the spots were less clear than that induced by flg15-Δ2. These results indicate that peptides having an EC_{50} of roughly <10 μM are detectable in this assay when photolysis was performed for 3 min. The sizes of positive spots were weakly correlated with the potencies of the peptide analogues. The differences in the cell conditions as well as fluctuations in diffusion rate of peptides from the agarose to the cell layer among assays have affected the relationship between the potencies of the peptides and the response intensities.

To demonstrate that the positive beads can be successfully recovered from a pool of beads, several beads containing flg15 were mixed with approximately 1000 blank beads that contained only the photocleavable linker. The pool of mixed beads was immobilized in the agarose gel, photolyzed, and placed on the cells. Several clear positive spots appeared after the addition of the DA-64 solution (**Figure 4A**). One of the positive spots was excised, and the isolated beads (typically one to ∼20 beads) were subjected to the second round assay to specify a single positive bead. A positive spot was also observed in the second

Table 2. Amino Acid Sequences of Active Peptides Detected by Screening

$X_1X_2X_3$SAKDDAAGLQ

$X_1X_2X_3$	$X_1X_2X_3$	$X_1X_2X_3$
A I N	L M N	R V N
A V N	M I N	S I N
D V N	N L M	T I N
E V N	P V N	T P N
F V N	Q I N	V V N
G I N	Q L N	W L N
H V N	Q V N	Y L N
I I N	R L N	
K I N	R M S	

Table 3. Amino Acid Sequences of Flagellin-Derived Peptide Analogues and Their H_2O_2 Generating Activity Measured by the Solution-Phase Assay

No.	Peptide	Amino acid sequence	EC_{50} (μM)
1	flg15-$\Delta2_{R\rightarrow A}$	AINSAKDDAAGLQ	4.2 ± 1.8
2	flg15-$\Delta2_{I\rightarrow F}$	RFNSAKDDAAGLQ	16 ± 3.1
3	flg15-$\Delta2_{N\rightarrow A}$	RIASAKDDAAGLQ	24 ± 7.9

round assay, and a single bead could be recovered from the gel of the positive spot (**Figure 4B**). Edman sequencing analysis of this positive bead revealed the presence of flg15 on it. This result represents that positive beads can be readily isolated from a pool of ∼1000 beads.

Screening of a Combinatorial Peptide Library for a Structure−Activity Relationship Study. To demonstrate the efficacy of the assay system, a combinatorial peptide library was prepared and subjected to the screening. The library was constructed based on the sequence of flg15-Δ2, and the three residues at the N-terminal portion of the peptide, which was reported to be particularly important for receptor binding, were randomized by a split-and-mix method ($X_1X_2X_3$SAKDDAAGLQ; X_n = 19 natural amino acids excluding Cys), resulting in a library size of 6859 peptides. Approximately 19 000 beads (∼1000 beads × 19 assays) were screened to cover almost 3 times the number of all possible peptide sequences. As a result of the screening, 25 positive beads were obtained. Amino acid sequences of peptides in the positive beads are shown in **Table 2**. No definite tendency was observed for amino acids at the X_1 position of active peptides. This suggests that the chemical properties of side-chain structure at the X_1 position have no critical effect on elicitor activity. On the other hand, at the X_2 position, all active peptides contained hydrophobic amino acids, particularly those with a branched alkyl side chain (Leu, Ile, and Val). Asn was observed at the X_3 position of all active peptides, except the one having Ser at this position. These results were consistent with the structural characteristics of the original peptide sequence (flg15-Δ2), in which Ile and Asn are located at the X_2 and X_3 positions, respectively. Thus, the validity of the screening method was demonstrated.

To further validate the screening results, three analogues were designed based on the screening results, and their H_2O_2 generating activity was quantitatively measured (**Table 3**). Analogue peptide **1** (flg15-$\Delta2_{R\rightarrow A}$) was synthesized to examine the effect of substitution of Arg at the X_1 position to Ala. The EC_{50} value of analogue **1** (4.2 ± 1.8 μM) was about 3.5-fold weaker than that of flg15-Δ2 but still in the detectable range of this assay method (EC_{50} of <10 μM). In contrast, analogue **2**, in which Ile at the X_2 position was substituted with Phe (flg15-$\Delta2_{I\rightarrow F}$), and analogue **3**, in which Asn at the X_3 position was substituted with Ala (flg15-$\Delta2_{N\rightarrow A}$), showed 16-fold (EC_{50} = 16 ± 3.1 μM) and 24-fold (EC_{50} = 24 ± 7.9 μM) weaker activity, respectively. The activity of analogues of these levels of EC_{50} was below the detectable limit of the assay, as mentioned previously. These results were all consistent with the structure−activity relationship obtained by the screening and clearly demonstrate that the screening method developed is highly reliable. In this screening, we used the peptide library that is based on the sequence of flg15-Δ2 having an EC_{50} value in the micromolar range and randomized only three residues at the N-terminal portion. It may seem difficult to obtain potent peptides having EC_{50} values in the low nanomolar range from this library, but the chance of finding highly potent peptides will be increased by increasing the number of amino acid residues to be randomized as well as by introducing structural constraints such as cyclization into the library peptides. Under the experimental conditions used in this screening, all peptides having EC_{50} values below 10 μM are detected, while it is difficult to distinguish potent peptides having EC_{50} values in the nanomolar range from those with moderate activity by visually inspecting the size of the active spots. To obtain only highly potent peptides, the EC_{50} threshold should be set lower, which is achieved simply by reducing the amount of released peptides with shortening the UV irradiation time.

Several important structural features of the N-terminal portion of flagellin-derived peptides for elicitor activity can be deduced from the results of this study. At the X_1 position of flg15-Δ2, substitution of an Arg residue with Ala had only a small effect on activity. But, removal of the Arg residue of flg15-Δ2 (i.e., flg14-Δ2) resulted in much lower activity (**Table 1**), indicating that the presence of some kind of amino acid at the X_1 position is important. This suggests that the main chain structure at the X_1 position is involved in the interaction with the receptor or necessary for the stabilization of the secondary structure of the peptide. Whereas the branched alkyl side chain at the X_2 position was important for the activity, substitution of the Ile residue of flg15-Δ2 with Phe, a hydrophobic but aromatic side-chain structure, caused a significant decrease in the activity. It is therefore likely that the bulkiness of the side chain rather than the hydrophobicity is more important for activity. The Asn residue at the X_3 position was the most important among the three N-terminal residues. The amide group in the side chain of Asn might be involved in the hydrogen bond formation with the receptor.

In summary, a high-throughput screening method using a cell-based lawn format assay for identification of peptides that induces plant defense responses was developed in this study. Solid-phase peptide libraries were synthesized using a photocleavable linker and immobilized with the agarose gel. After the agarose gel in which peptides were partially cleaved from beads was layered on the tobacco cells, the plant defense response was observed by detecting the generated H_2O_2 using a sensitive H_2O_2 indicator dye, DA-64. Using this assay format, approximately 1000 beads were readily screened in a single assay. This assay has several advantages over those using the microtiter plate format. First, the handling of the beads is much easier, requiring no robotic workstation. Second, concentrations of peptides released from the library beads are high enough to detect compounds having a weak activity. The screening method developed in this study will be useful not only for efficient structure−activity relationship studies but also for the discovery of novel peptides that induce plant defense responses from the random peptide libraries. Plants appear to have distinct, diverse

PAMP receptors to recognize invasion of a wide variety of pathogens. The peptides that will be discovered by this screening method should provide valuable probes for investigation of the pathogen recognition system in plants including PAMP receptors.

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