

**Elucidation of the plant immune system by using the elicitor
peptide PIP-1 as a chemical probe**

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Abbreviations

ACS	1-Amino-cyclopropane-1-carboxylic acid synthase
Anp	3-Amino-3-(2-nitrophenyl)propionic acid
AOC	Allene oxide cyclase
AOPP	L-2-aminooxy-3-phenylpropionic acid
Ape	5-Aminopentanoic acid
AtPEPR	AtPEP1 receptor
CAT	Catalase
CCoAOMT	Caffeoyl-CoA <i>O</i> -methyltransferase
CHX	Cycloheximide
COMT	Caffeic acid <i>O</i> -methyltransferase
DA-64	(Carboxymethylaminocarbonyl)-4,4'-bis(dimethylamino)- diphenylamine sodium salt
DIPEA	<i>N,N</i> -Diisopropylethylamine
DMF	<i>N,N</i> -Dimethylformamide
DPI	Diphenyleneiodonium
EAS	5- <i>epi</i> -aristolochene synthase
EF	Elongation factor
EFR	EF-Tu receptor
ERF	Ethylene response factor
ETI	Effector-triggered immunity
FLS	Flagellin sensing
Fmoc	<i>N</i> -(9-Fluorenyl)methoxycarbonyl
FPP	Farnesyl pyrophosphate
HBTU	<i>O</i> -(Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HMGR	3-Hydroxy-3-methylglutaryl CoA reductase
HOBt	1-Hydroxybenzotriazole
HR	Hypersensitive response
LOX	Lipoxygenase

LS	Linsmaier and Skoog
MAP	Mitogen-activated protein
MAPKK	MAP kinase kinase
MAPKKK	MAPKK kinase
MES	2-(<i>N</i> -Morpholino)ethanesulfonic acid
MPK	MAP Kinase in <i>Arabidopsis</i>
NB-LRR	Nucleotide binding and leucine-rich repeat
Npg	(2-Nitro)phenylglycine
PAL	Phenylalanine ammonia-lyase
PAMP	Pathogen-associated molecular pattern
PDF	Plant defensin
PI	Protease inhibitor
PIP-1	Plant immunity-activating peptide 1
PIPES	Piperazine- <i>N,N'</i> -bis(2-ethanesulfonic acid)
PR	Pathogenesis-related
PRR	Pattern-recognition receptor
PTI	PAMP-triggered immunity
RLK	Receptor-like kinase
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulfate
SIPK	Salicylic acid-induced protein kinase
SS	Squalene synthase
TFA	Trifluoroacetic acid
TGase	Transglutaminase
TIPS	Triisopropylsilane
WIPK	Wound-induced protein kinase
WRKY	Transcription factor, WRKYGQK conserved

Research Background

Plant immunity and MAP kinase cascade

In the natural environment, plants have been growing together with a variety of microorganisms, such as bacteria, fungi, and virus; however some of them penetrate plants to obtain essential nutrients for survival, thus named ‘pathogen’. Against pathogen penetration, plants have evolved unique immune systems (Ausubel, 2005; Nürnberger et al., 2004). In a first step, PRRs, a kind of membrane-bound proteins in plant, play a role in sensing a part of pathogens, such as pathogen-derived polysaccharide, protein and peptide, named ‘PAMP’, which subsequently induce a kind of immune response like PTI (Boller and Felix, 2009). On the other hand, pathogens also have gradually evolved their pathogenicity to weaken PTI using injection of PTI suppressor proteins named ‘effector’ into host plant cells by a type III secretion system. In response to suppression of PTI by the effector, however, plants have developed a highly specific *R* gene-based resistance protein, such as NB-LRR receptor kinases, which directly or indirectly interact with pathogen effector protein, and then plants present more vigorous immune responses referred to as ETI (Beth Mudgett, 2005; Boller and Felix, 2009) (Figure 1). Both the PAMPs and effectors are broadly referred to as ‘elicitor’, in that they trigger similar plant immune responses. Practically, PTI and ETI show the similar series of immune responses, such as influx of calcium ions, induction of oxidative burst, defense-related gene expressions, cell wall reinforcement, phytohormone regulation, phytoalexin accumulation and HR-like cell death, those are closely associated with initiation of MAP kinases signaling induced by elicitors (Meng and Zhang, 2013), in spite of their differential intensity reported (Jones and Dangl, 2006; Tsuda et al., 2013).

In the initial immune responses, activation of MAP kinase cascades is well-known as the earliest signaling event, which is immediately activated after recognition of elicitors via sequential phosphorylation from their upstream kinases: MAP kinase is phosphorylated by MAPKK which is also phosphorylated by MAPKKK (Meng and Zhang, 2013), eventually leading to activation of transcription factors belonging to defense-related genes and/or other substrates to initiate immune responses. In the *Arabidopsis*, activation of MPK3 and MPK6 are induced by flagellin, a protein constituting bacterial flagellum, and flg 22, a 22-residues peptide derived from flagellin, leading to phosphorylation of WRKY-type transcription factors that induce defense-related gene expressions (Asai et al., 2002). Specifically, MPK6

phosphorylates ERF104, a ethylene response factor, that activates defensin gene family, *PDF1.2a* and *PDF1.2b* (Bethke et al., 2009). Recently, Li et al. (2012) demonstrated that WRKY33, phosphorylated by MPK3 and MPK6, directly binds to promoters of both *ACS2* and *ACS6*, which regulate the ethylene production after the pathogen infection. Also, activation of MPK3 and MPK6 is associated with biosynthesis of camalexin, a representative phytoalexin in *Arabidopsis*, by up-regulating the camalexin biosynthesis-related genes (Ren et al., 2008). In tobacco, SIPK and WIPK phosphorylate WRKY8, which activate transcription of *HMGR2*, an elicitor-inducible *HMGR* isogene involved in the biosynthesis of capsidiol, a representative isoprenoid phytoalexin in tobacco (Ishihama et al., 2011). Although it has been known that MAP kinase cascades are closely implicated in the signal transduction leading to the initiation of plant immune responses, most of their precise substrates still remain unclear.

MAP kinase cascades are thought to be a point of convergence of signaling because a variety of elicitors recognized by different types of receptor proteins eventually induce similar immune responses via MAP kinase cascades. Thus, its kinetics or magnitudes is likely

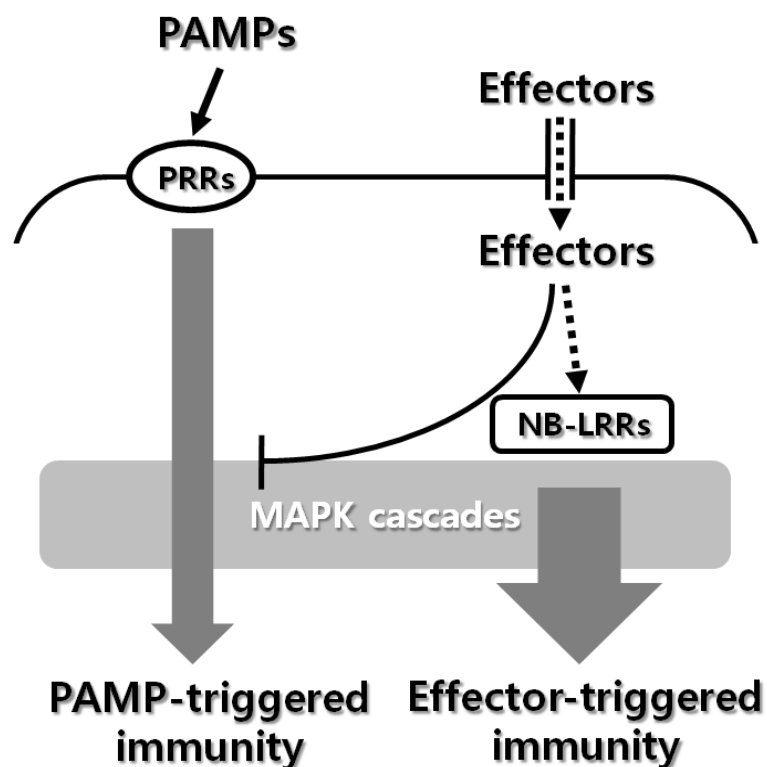


Figure 1. The model of plant immune systems

to influence the formation of downstream immune responses, depending on the duration of elicitor recognition. Kadota et al. (2006) reported that a continuous recognition of cryptogein, a proteinous elicitor derived from *Phytophthora cryptogea*, for several hours led to a prolonged activation of both SIPK and WIPK in tobacco cells, which is associated with the induction of a full set of immune responses, such as HR-like cell death and long-lasting production of reactive oxygen species. These data clearly indicated that the duration of elicitor recognition is one of the critical factors to determine either the types and/or magnitude of immune responses, via MAP kinase cascades.

Peptide elicitors and PIP-1

Plants recognize various types of elicitor molecules such as proteins, peptides, oligosaccharides, and lipopolysaccharides derived from microbes and/or plants (Zhao et al., 2005). Among the various chemical types of elicitors, peptides are one of the most-well studied elicitors. For example, flg22 is the most representative bacterial-derived peptide elicitor, consisting of 22-amino acid residues highly conserved in bacterial flagellin, and recognized by FLS2, a receptor kinase bound on plasma membrane in *Arabidopsis* (Table 1) (Felix et al., 1999; Gómez-Gómez et al., 2001). After recognition of flg22 by FLS2, *Arabidopsis* displays common defense responses, such as induction of extracellular alkalization, oxidative burst, callose deposition, defense-related gene expression and MAP kinase activation. (Asai et al., 2002; Felix et al., 1999; Gómez-Gómez et al., 1999). Another well-known peptide elicitor is Pep-13, a 13-amino acid peptide, derived from TGases in *Phytophthora sojae* and highly conserved in the genus *phytophthora* TGases (Brunner et al., 2002; Nürnberger et al., 1994). Pep-13 induces defense responses, such as oxidative burst, Ca^{2+} influx, defense-related gene expression and phytoalexin synthesis, when applied to parsley cells at low nanomolar levels (Brunner et al., 2002; Nürnberger et al., 1994); however, its receptor protein has not been identified yet. Elf18 is also one of microbe-derived peptide elicitors, comprised of 18-amino acid residues derived from N-terminus of EF-Tu, which is the most conserved and abundant protein among the bacteria like *Escherichia coli* (Kunze et al., 2004). As a receptor of elf18, EFR was identified in *Arabidopsis* (Zipfel et al., 2006). Elf18 display several defense responses, such as induction of extracellular alkalization, oxidative burst, and ethylene biosynthesis, similar to those induced by other peptide elicitors

(Kunze et al., 2004; Zipfel et al., 2006). In addition to those microbe-derived peptide elicitors, several plant endogenous peptide elicitors have been identified, released when plants are wounded and infected. The most well-known example is the systemin, a 18-amino acid peptide, derived from prosystemin in tomato leaves (Pearce et al., 1991). Systemin have been considered to be immediately released from prosystemin in cytoplasmic region after wound and recognized by the receptor protein in neighboring cells, leading to induction of proteinase inhibitor I and II via jasmonic acid signaling (Li et al., 2001; Pearce et al., 1991). AtPep1, a 23-amino acid peptide, was firstly identified in *Arabidopsis*, derived from the 92-amino acid cytoplasmic precursor protein (Huffaker et al., 2006). AtPep 1 is recognized by two receptor proteins named AtPEPR1 and AtPEPR2, leading to extracellular alkalization and expression of defense-related genes in *Arabidopsis* (Huffaker et al., 2006; Yamaguchi and Huffaker, 2011; Yamaguchi et al., 2006). ZmPep1, an ortholog of AtPep1, is isolated from maize, and it also induce jasmonic acid and ethylene accumulation and defense-related gene expression (Huffaker et al., 2011). Recently, GmPep914, an 8-amino acid peptide, was isolated from soybean leaves, displaying induction of extracellular alkalization and defense-related gene expression when applied to suspension-cultured soybean cells at low nanomolar concentrations (Yamaguchi et al., 2011).

Recently, the peptide elicitor PIP-1 (YGIH⁺TH-NH₂) was discovered by the screening of a synthetic combinatorial random hexapeptide library, which is known as the shortest peptide elicitor identified to date (Miyashita et al., 2011). PIP-1 induces typical immune responses, such as oxidative burst, phytoalexin biosynthesis, and defense-related gene expression, via jasmonic acid signaling, when applied to suspension-cultured tobacco cells at low micromolar levels. Although its receptor protein has not been identified so far, the

Table 1. Identified peptide elicitors

Elicitor	Sequence	Elicitor source	Reference
Flg22	QRLSTGSRINSAKDDAAGLQIA	<i>Pseudomonas aeruginosa</i>	Felix et al. 1999
Pep-13	VWNQPVRGFKVYE	<i>Phytophthora sojae</i>	Nürnberg et al. 1994
Elf18	SKEKFERTKPHVNVGTIG	<i>Escherichia coli</i>	Kunze et al. 2004
Systemin	AVQSKPPSKRDPPKMQTD	Tomato	Pearce et al. 1991
AtPep1	ATKVKAKQRGKEKVSSGRPGQHN	<i>Arabidopsis</i>	Huffaker et al. 2006
ZmPep1	VRRRP ⁺ TTTPGRPREGSGGNGGNHH	Maize	Huffaker et al. 2011
GmPep914	DHPRGGNY	Soybean	Yamaguchi et al. 2011
PIP-1	YGIH ⁺ TH-NH ₂	Combinatorial hexapeptide library	Miyashita et al. 2011

structure-activity relationship study revealed that the N-terminus 4 residues of PIP-1 are essential elements to induce the immune responses. Interestingly, PIP-1 induced distinctive immune responses in tobacco cells, in which types of immune responses induced by PIP-1 were determined by its concentration. For example, induction of the maximum level of oxidative burst, an early-phase immune response, was observed immediately after treatment with PIP-1 at the 10 μ M concentration; however, 10-fold higher concentrations was required to induce late-phase immune responses like production of capsidiol, a major phytoalexin in tobacco. It has been unclear why a higher concentration of PIP-1 is needed to initiate capsidiol production. Capsidiol production becomes detectable at least 6 h after the PIP-1 treatment. In this respect, it is possible that long-term recognition of elicitor may be necessary for capsidiol biosynthesis like the induction of HR-like cell death as mentioned above (Kadota et al., 2006). Although the exact fate of applied PIP-1 after the treatment in the culture medium of tobacco cells has not been examined yet, the amount of PIP-1 is likely to decrease due to enzymatic degradation in the incubation medium. Thus, a higher concentration of PIP-1 might be sufficient for initiation of capsidiol biosynthesis to stimulate the immune system for longer time, compared to low concentrations of PIP-1. However, the effect of elicitor stability on plant immune system has been rarely discussed even if it has a possibility that the types and magnitudes of plant immune system could be altered by whether elicitor is stable or not.

Based on the this possibility, this study focused on the relationship between the degradation of peptide elicitor PIP-1 and induction of plant immune systems, and investigated temporal regulation factors to initiate late-phase immune responses depending on elicitor stability using PIP-1 as a chemical probe.

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Chapter 1

Design of Degradation-Resistant PIP-1 Analogs

Introduction

Plants grow together with a variety of their potential enemies such as bacteria, fungi, and virus. In order to maintain their growth and subsequent reproduction against above enemies, plants evolved their own unique immune systems (Ausubel, 2005; Nürnberger et al., 2004). When plants are sensing an invasion of their potential enemies, they induce various defense responses, including oxidative burst, reinforcement of cell wall, phytoalexin synthesis, and hypersensitive cell death to protect themselves (Glazebrook, 2005; Hammond-Kosack and Jones, 1996; Király et al., 2007; Meng and Zhang, 2013). Plants recognize various molecules derived from the enemies, referred to as elicitors or PAMPs to start a series of defense responses.

Recently, a peptide elicitor PIP-1 (YGIHTH-NH₂) was discovered by the screening of a synthetic combinatorial random hexapeptide library (Miyashita et al., 2011). PIP-1 induces typical defense responses, such as oxidative burst, phytoalexin biosynthesis, and defense-related gene expressions, in tobacco cells via a jasmonic acid signaling pathway. The structure-activity relationship study revealed that the N-terminal 4 residues are essential to induce these immune responses. Interestingly, types of defense responses induced by PIP-1 were different based on its concentration: oxidative burst (an early phase immune response) was induced at low μM concentrations, whereas to induce phytoalexin biosynthesis (a late phase immune response) approximately 10- to 50-fold higher concentrations than those required for oxidative burst were necessary. Since PIP-1 is a linear short peptide, it is possible to readily undergo enzymatic degradation in and/or on the surface of the plant cells. Although the exact fate of applied PIP-1 after treatment in the culture media of tobacco cells has not been known, a decrease in the concentration of the elicitor by degradation may well affect the persistence of its stimulation to the plant cells, leading to failure to initiate late-stage defense responses such as phytoalexin biosynthesis.

The author hypothesized that prevention of enzymatic degradation of PIP-1 can alter the profile of immune responses induced, in particular, with respect to the phytoalexin production. Therefore, in this Chapter, a degradation-resistant PIP-1 analog was newly

designed based on the degradation profile of PIP-1 using several peptide modification strategies, and tested their elicitor activity for subsequent studies.

Materials and Methods

Plant cell culture

Tobacco cells (*Nicotiana tabacum* cv. Xanthi) were maintained as a suspension culture in a 100 mL Erlenmeyer flask with 25 mL of liquid LS medium (pH 5.75) containing 10 μ M 1-naphthalenetic acid, 1 μ M 5-benzyladenine, and 3% (w/v) sucrose. The cell suspensions were grown on a rotary shaker (100 rpm) at 25°C under continuous illumination with subculture every 2 weeks, and used at 9 and 10 days after subculture for experiments.

Chemicals

Fmoc protected-amino acids, Fmoc-NH-SAL resin, and Cl-Trt(2-Cl) resin were purchased from Watanabe Chemical Industries (Hiroshima, Japan). DA-64 was obtained from Wako Pure Chemical Industries (Osaka, Japan). The peptides, YGIH⁺TH-NH₂, YGIH⁺TH-OH and D-(YGIH⁺TH)-NH₂, were previously synthesized in the laboratory.

Synthesis of peptide analogs

PIP-1 analogs (YGIHT-OH, YGIH-OH, YGIH⁺TH(Ape)-NH₂, YGIHTD-OH, and YGIHTmeA-NH₂) were synthesized manually using the standard Fmoc-based solid-phase peptide synthesis technique (Figure 1-1). For the synthesis of YGIHT-OH, YGIH-OH, and YGIHTD-OH, Cl-Trt(2-Cl) resin was used, and for the synthesis of YGIH⁺TH(Ape)-NH₂ and YGIHTmeA-NH₂, Fmoc-NH-SAL resin was used. Fmoc was removed with 20% piperidine in DMF three times for 3, 3, and 20 min. Each Fmoc-protected amino acid (3 eq) was coupled to the resin in the presence of HBTU (3 eq), HOBt (3 eq), and DIPEA (6 eq) in DMF. The completion of coupling reaction was verified by Kaiser test (Kaiser et al., 1970). After all coupling reactions were completed, the resin was sequentially washed with DMF, diethyl ether, and methanol, and dried *in vacuo*. To detach the peptide from the resin and remove all side-chain protecting groups, the solution A [trifluoroacetic acid (TFA)/triisopropylsilane (TIPS)/dimethoxybenzene (92.5: 2.5: 5, v/v/v)] for YGIH⁺TH(Ape)-NH₂, and YGIHTmeA-NH₂ and solution B [(TFA/TIPS/distilled water (95: 2.5: 2.5, v/v/v)] for YGIHT-OH, YGIH-

OH, and YGIHTD-OH were reacted with each resin for 2 h at room temperature. Then, the reaction mixture was filtered into cold diethyl ether to precipitate the peptide. The precipitated peptide was dried *in vacuo* overnight. The purification of the peptide was carried out using HPLC equipped with a semipreparative column (250 mm × 10 mm i.d., 5 μm, Protein and peptide C18, Grace, Columbia, MD), and lyophilized. The peptides were identified by ESI-LC/MS on a Shimadzu LCMS-2010 (Kyoto, Japan).

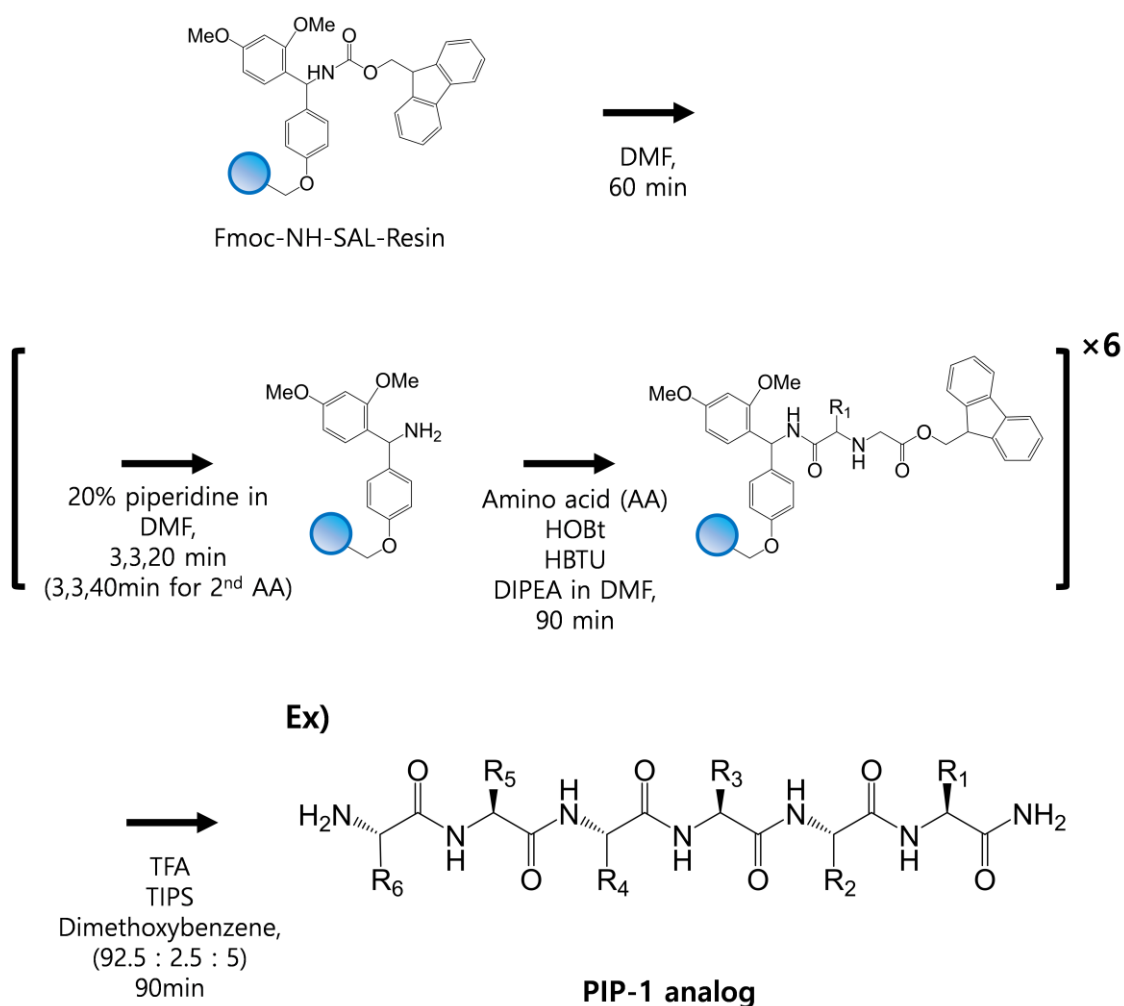


Figure 1-1. General procedure of the Fmoc-based solid-phase peptide synthesis

Analysis of degradation profile of PIP-1 and its analogs

Tobacco cells were collected by filtration using miracloth (Calbiochem, San Diego, CA). The cells (0.2 g) were re-suspended in a microtube containing 1 mL of LS medium, and incubated for 1 h at 25°C using a tube rotator. The solution of PIP-1 or its analogs in distilled water (final concentration of 10 μ M) was added to the cells. After incubation for a given time period (0-24 h) at 25°C, the media were collected and filtered using syringe filters (0.45 μ m pore size). The filtrates were immediately frozen in liquid nitrogen and stored at -80°C to prevent further peptide degradation. Each sample was subjected to ESI-LC/MS analysis using an LCMS-2020 (Shimadzu, Kyoto, Japan). Degradation products were identified by comparison of their *m/z* values and retention times on ESI-LC/MS analysis with those of the synthesized standard peptides. Quantitation of PIP-1 and its analogs was carried out by HPLC under the following condition (Table 1-1). The amount of peptides in the medium was quantitated based on a calibration curve constructed using the synthesized standard peptides.

Table 1-1. HPLC analysis condition

Column	Discovery BIO Wide Pore C18 column 150 mm $\times$ 2.1 mm i.d., 3 μ m, (Supelco, St. Louis, MO)
Column oven	40°C
Flow rate	0.2 mL/min
Mobile phase A	0.1% TFA in H ₂ O
Mobile phase B	0.1% TFA in acetonitrile
Gradient	10-30% solvent B for 20 min
UV detection	215 nm

Measurement of H₂O₂ generation

H₂O₂ generation was measured as described previously (Miyata et al., 2006). Briefly, tobacco cells (0.2 g) re-suspended in 1 mL of assay buffer (175 mM mannitol, 0.5 mM CaCl₂, 0.5 mM K₂SO₄, 2.0 mM MES, pH 5.75) were incubated for 1 h at 25°C using a tube rotator. After incubation, the cells were treated with PIP-1 analogs (10 μ L) and incubated for 15 min

including 2 min to allow cell sedimentation. The supernatants (60 μ L) were mixed with the H₂O₂ indicator solution (140 μ L; 100 mM DA-64, 1 U/mL horse radish peroxidase, 100 mM PIPES, pH 7.0) in a 96-well microplate, and incubated for 10 min at 37°C. The absorbance at 727 nm was monitored with a Benchmark microplate reader (Bio-Rad, Hercules, CA). As an index of activity, a concentration required for half-maximal responses (EC₅₀) was calculated with PRISM (GraphPad Software, La Jolla, CA).

Results and Discussion

Degradation of PIP-1 by tobacco cells

The degradation profile of PIP-1 in the presence of tobacco cells was first investigated. The extracellular medium after treatment with 10 μ M of PIP-1 was analyzed by ESI-LC/MS. PIP-1 (m/z observed for $[M+2H]^{2+}$: 363.8), was detected at a retention time of 13.8 min immediately after treatment with PIP-1 (Figure 1-2). A new peak with m/z of 364.3 at a retention time of 14.9 min was detected 15 min after treatment with PIP-1. The observed molecular mass was consistent with that of C-terminally deamidated PIP-1 (YGIH⁺TH-OH, m/z calculated for $[M+2H]^{2+}$: 364.2), which was shown to exhibit significant elicitor activity (Table 1-2) (Miyashita et al., 2011). Two other peaks at retention times of 15.2 min (m/z 245.3) and 17.4 min (m/z 295.7) were detected 30 min after treatment with PIP-1. Molecular masses of these peaks correspond to those of YGIH-OH (m/z calculated for $[M+2H]^{2+}$: 245.1) and YGIHT-OH (m/z calculated for $[M+2H]^{2+}$: 295.7), which showed no measurable or weak activity, respectively (Table 1-2). The identity of these peaks was further confirmed by using authentic standards. PIP-1 and these degraded products were further degraded and could not be detected 3 h after treatment. This indicated that active degraded PIP-1 products were not present at that time. When the initial concentration of PIP-1 was raised to 100 μ M, PIP-1 was detected for an extended time, but disappeared 6 h after the treatment (Figure 1-2). In this case, however, elicitor-active YGIH⁺TH-OH remained even after 6 h at a concentration that could be enough for induction of capsidiol. In this way, PIP-1 (10 μ M) is rapidly hydrolyzed from its C-terminal residue probably by proteases secreted from tobacco cells because several proteases have been identified from the extracellular space (Delannoy et al., 2008), some of which are induced by pathogen infection (Swaroop Rani and Podile, 2014).

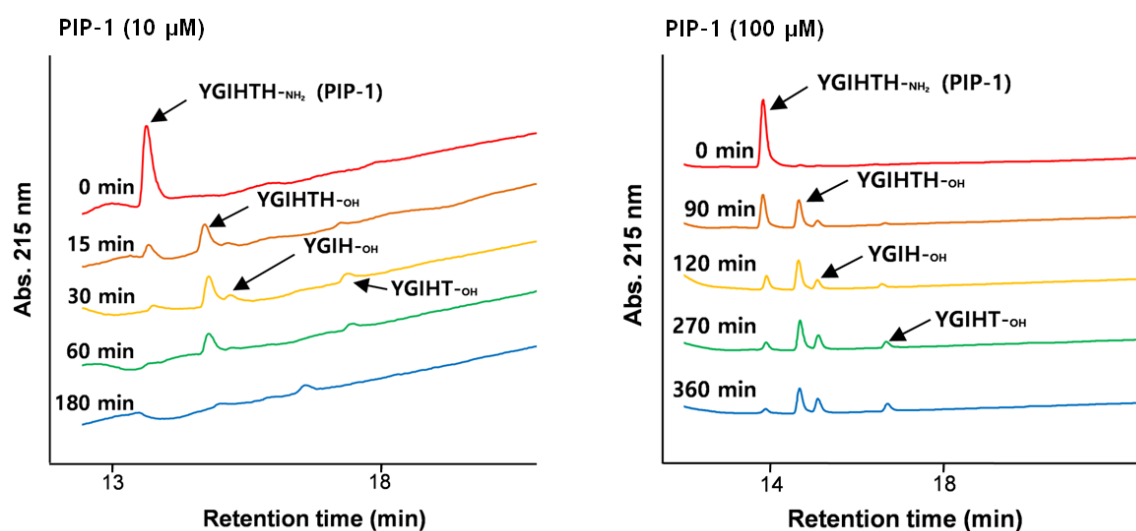


Figure 1-2. Degradation profile of PIP-1 in suspension-cultured tobacco cells

Table 1-2. H₂O₂ generation activity of PIP-1 analogs

No.		Sequence	EC ₅₀ (μM)	Reference
1	PIP-1	YGIHTH-NH ₂	2.2 ± 0.9	Miyashita et al. 2011
2	PIP-1-OH	YGIHTH-OH	0.27 ± 0.13	Miyashita et al. 2011
3	[Δ ⁶] PIP-1	YGIHT-OH	13 ± 5.3	
4	[Δ ^{5,6}] PIP-1	YGIH-OH	>100	
5	[Ape ⁷]PIP-1	YGIHTH(Ape)-NH ₂	6.9 ± 2.3	
6	[Asp ⁶]PIP-1	YGIHTD-OH	0.78 ± 0.22	
7	(MePIP-1)	YGIHT meA -NH ₂	0.63 ± 0.32	

Design of degradation-resistant PIP-1

Several peptide modification strategies were known to improve its bioavailability (Sato et al., 2006). Specifically, substitution with natural or unnatural amino acids and methylation of amide bond has been often utilized to enhance stability against enzymatic degradation (Biron et al., 2008; Cody et al., 1997; Gentilucci et al., 2010; Powell et al., 1993; Sato et al., 2006).

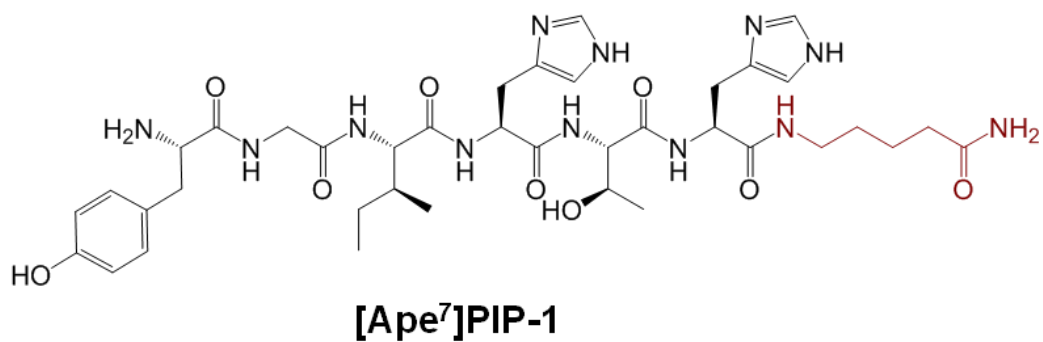
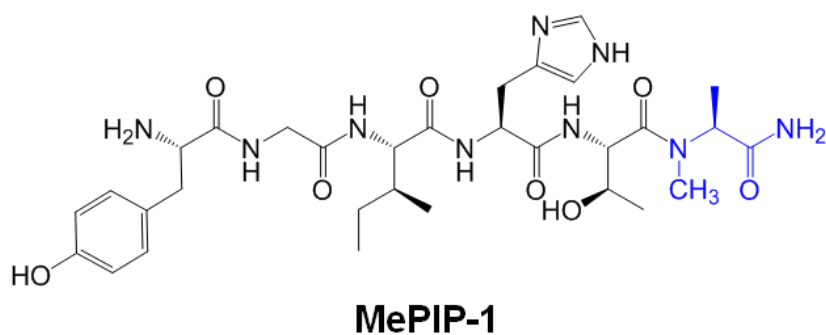
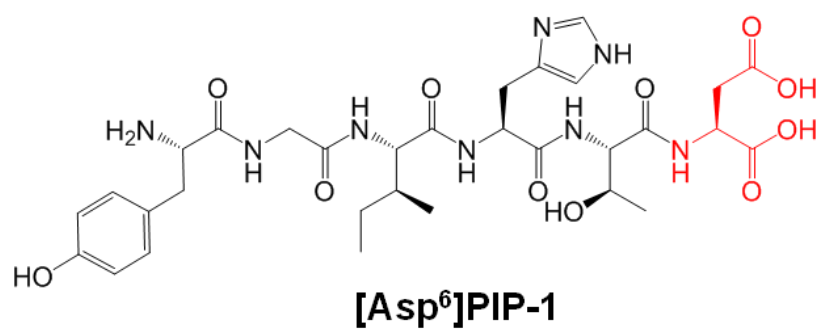
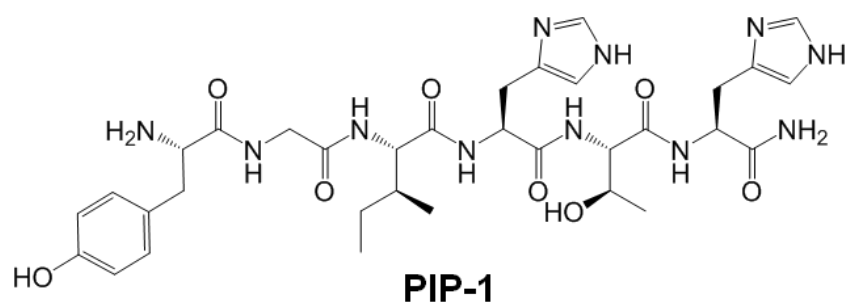


Figure 1-3. The structure of PIP-1 and its analogs tested for degradation-resistance in this study

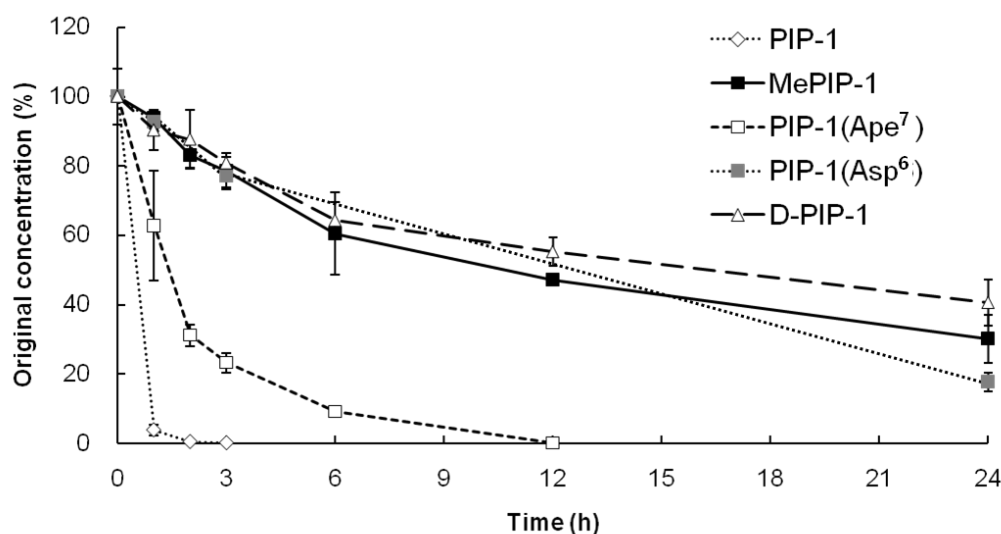


Figure 1-4. Comparison of degradation profile of PIP-1 (10 μ M) and degradation-resistant analogs (10 μ M) in the incubation medium of tobacco cells. Vertical bars indicate $\pm$ S.D. three independent replicates.

Taking into account that the hydrolysis of PIP-1 was occurred from the C-terminal end, the C-terminal His residue might be responsible for the recognition by some protease secreted from tobacco cells. Therefore, the author designed three PIP-1 analogs to search for degradation-resistant analogs (Figure 1-3). The C-terminal His residue was substituted with an acidic amino acid, Asp, to obtain [Asp⁶]PIP-1, or with N-methylated Ala to obtain MePIP-1. In the case of [Asp⁶]PIP-1, the C-terminal amide group was replaced by a carboxylate group. For the synthesis of [Ape⁷]PIP-1, an unnatural amino acid, Ape, was attached to the C-terminal side of the His residue. To investigate the stability of these analogs against the enzymatic degradation in the presence of tobacco cells, each analog (10 μ M) was incubated with suspension-cultured tobacco cells, and their concentrations were quantitated by HPLC. The concentration of PIP-1 rapidly decreased to 3% of the initial level 1 h after treatment, and completely disappeared after 3 h (Figure 1-2 and 1-4). On the other hand, 76%, 78% and 23% of the initial concentrations were detected for [Asp⁶]PIP-1, MePIP-1 and [Ape⁷]PIP-1, respectively, 3 h after treatment. [Asp⁶]PIP-1 and MePIP-1 were still detectable at 17% and 30% of the initial levels, respectively, even 24 h after treatment (Figure 1-4). In the case of [Asp⁶]PIP-1, YGIH-OH was observed as a degradation product 24 h after treatment (Figure 1-5), suggesting that the basicity of the His residue could be important for recognition by proteases. On the other hand, since no degradation products of MePIP-1 were detected 24 h after the treatment (Figure 1-5), a decrease in its concentration was likely due to its

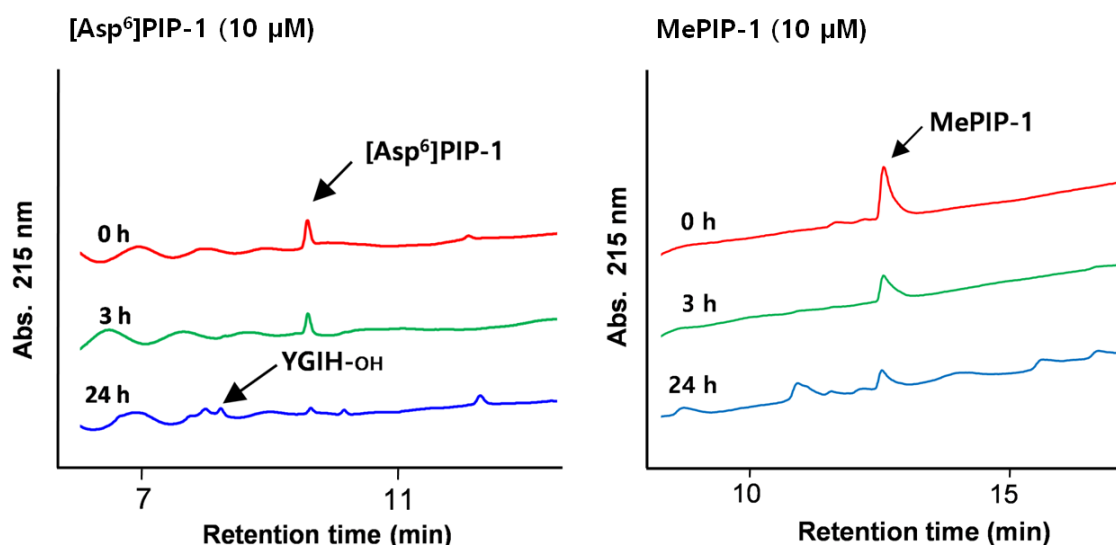


Figure 1-5. Degradation profile of MePIP-1 and [Asp⁶]PIP-1 in suspension-cultured tobacco cells

adsorption to tobacco cell surfaces rather than degradation. To confirm this, the author evaluated the effect of adsorption using elicitor-inactive D-PIP-1, which is composed of all D-forms of amino acid residues (Miyashita et al., 2011) and resistant to enzymatic degradation. As expected, concentrations of D-PIP-1 decreased in a similar manner to MePIP-1 through 24 h incubation period. This result clearly indicates that MePIP-1 is the most resistant to enzymatic degradation among three analogs tested in this study.

Regarding the elicitor activity, [Asp⁶]PIP-1 and MePIP-1 exhibited rather higher H₂O₂ generation activity than PIP-1 (Table 1-2). It is possible that the side chain of the Asp residue in [Asp⁶]PIP-1 and a conformational change caused by N-methylation at the C-terminal residue of MePIP-1 allow more favorable interaction with the receptor, leading to relatively higher activity (Biron et al., 2008; Chatterjee et al., 2007). On the other hand, [Ape⁷]PIP-1 showed rather lower activity than PIP-1 probably because of the steric hindrance of the Ape residue. As a consequence, [Asp⁶]PIP-1 and MePIP-1 are suitable for further experiments that investigate the mechanisms of immune response induction by PIP-1 in terms of the degradation-resistance property and the intensity of biological activity.

In conclusion, newly synthesized PIP-1 analogs, in which the C-terminal His residue was substituted with an Asp or an N-methylated Ala residue, exhibited a higher degradation resistance as compared with PIP-1, and also showed significant biological activity. The fact

that substitution of the His residue with Asp enhanced the enzymatic stability suggests that proteases secreted from tobacco cells recognizes basic residues of PIP-1 although further investigations are needed. As a consequence, due to its high stability and elicitor activity, MePIP-1 was found to be suitable for further experiments in Chapter 2, in which the effect of elicitor stability on initiation of late-stage immune responses was investigated.

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Chapter 2

Effects of Degradation-Resistance of the PIP-1 Analog on Plant Immune Response Induction

Introduction

Plant immune system is activated by the compounds of various chemical types, which referred to as elicitors. These compounds are useful as a tool to investigate the mechanisms of pathogen recognition and immune response induction. Peptides are one of the most well-studied elicitors, including flg22: a 22-amino acid peptide from bacterial flagellin; elf18: a 18-amino acid peptide from bacterial elongation factor Tu; and Pep13: a 13-amino acid peptide from 42kD glycoprotein of *Phytophthora sojae* (Felix et al., 1999; Kunze et al., 2004; Nürnberger et al., 1994). In addition to these elicitors, several endogenous peptide elicitors released from host plants upon wounding and/or infection have been identified. For example, a 23-amino acid peptide AtPep1 has been isolated from *Arabidopsis*, and its ortholog ZmPep1 from maize (Huffaker et al., 2011; Huffaker et al., 2006). These elicitors are mainly perceived by distinct RLKs. RLKs trigger activation of downstream signaling networks controlled by MAP kinase- and/or calcium-dependent protein kinase-cascades, which further regulate the production and the activity of transcription factors, enzymes, hormones, and phytoalexins (Meng and Zhang, 2013).

Each elicitor presents either similar or different type of immune responses (Zhao et al., 2005). For example, flg22 can induce oxidative burst, defense-related gene expressions and MAP kinase activations, but not HR-like cell death and phytoalexin synthesis. (Asai et al., 2002; Felix et al., 1999; Gómez-Gómez et al., 1999); however, Pep13 induces HR-like cell death and phytoalexin synthesis as well as oxidative burst, Ca^{2+} influx, defense-related gene expressions (Halim et al., 2004; Jabs et al., 1997). It is still unclear what have made those differences of immune response induced. In the study of PIP-1, it is suggested that a decrease in the concentration of the elicitor by degradation affects the initiation of late-stage defense responses such as phytoalexin biosynthesis. In this regard, plant may respond differently depending on the stimulation period by the elicitor.

In this Chapter, to investigate the effect of PIP-1 degradation on induction of different types of immune response, the author compared the immune responses induced by PIP-1 with

that by the degradation-resistant analog (MePIP-1), and investigated regulating factors important for the induction of late-phase immune response such as HR-like cell death and phytoalexin production

Materials and Methods

Plant cell culture

Tobacco cells (*Nicotiana tabacum* cv. Xanthi) were maintained as a suspension culture as described in Chapter 1, and used at 9 and 10 days after subculture for experiments.

Chemicals

DA-64 was obtained from Wako Pure Chemical Industries (Osaka, Japan). K252 was purchased from LC labs (Woburn, MA). The peptides, YGIHTH-NH₂ (PIP-1) and YGIHTmeA-NH₂ (MePIP-1) were synthesized as described in Chapter 1.

Measurement of H₂O₂ generation

H₂O₂ generation was measured as described previously (Miyata et al., 2006). Tobacco cells (0.2 g) suspended in 1 mL of assay buffer (175 mM mannitol, 0.5 mM CaCl₂, 0.5 mM K₂SO₄, 2.0 mM MES, pH 5.75) were incubated for 1 h at 25°C using a tube rotator. After incubation, the cells were treated with PIP-1 analogs (10 µL) and incubated for 0-12 h. After 2 min to allow cell sedimentation, the supernatants (60 µL) were mixed with the H₂O₂ indicator solution (140 µL; 100 mM DA-64, 1 U/mL horse radish peroxidase, 100 mM PIPES, pH 7.0) in a 96-well microplate, and incubated for 10 min at 37°C. The absorbance at 727 nm was monitored with a microplate reader (Benchmark, Bio-Rad, Hercules, CA). The amount of H₂O₂ was calculated using a calibration curve constructed with pure H₂O₂.

Cell death assay

Cell death assay was performed as described previously with modifications (Kadota et al., 2005). Tobacco cells (0.2 g) suspended in 1 mL of LS medium were incubated for 1 h. The cells were treated with PIP-1 and MePIP-1, and incubated for 24 h at 25°C using a tube rotator. After removal of medium, the cells were collected and transferred into the solution of Evans blue (0.01%). The cells incubated for 15 min were washed 3 times with fresh distilled

water, and transferred into 50% methanol containing 1% SDS to extract absorbed dye in dead cells. After incubation at 60°C for 1 h, cells were centrifuged for 15 min at $1,900 \times g$. Absorbance at 595 nm of the supernatant was measured using a microplate reader (Benchmark, Bio-Rad).

Quantitation of capsidiol

Capsidiol extraction and quantitation from tobacco cells or leaves was conducted as described previously (Huchelmann et al., 2014; Miyashita et al., 2011). Tobacco cells (0.6 g) suspended in 3 mL of LS medium were incubated for 1 h. The cells were treated with PIP-1 and MePIP-1 and incubated for 24 h at 25°C on a rotary shaker under continuous illumination. Capsidiol was extracted from the culture medium twice with 4 mL of ethyl acetate, and vacuum dried. The residue was dissolved in 1 mL of ethyl acetate to use GC/MS analysis. For extraction from tobacco leaves, leaf discs (1.5 cm diameter) were punched from tobacco leaves 6-week after sowing, and the adaxial side of the leaves was placed upward on petri dishes (one disc per dish) containing LS medium. The leaf discs were treated with PIP-1 and MePIP-1, and incubated for 24 h at 25°C. Capsidiol secreted from leaf discs into the LS medium was extracted twice with 2 mL of ethyl acetate, and vacuum dried. The residue was dissolved in 0.5 mL of ethyl acetate to use GC/MS analysis. The extracts were subjected to GC/MS analysis. Capsidiol quantitation was performed by GC/MS (QP-2010, Shimadzu, Kyoto, Japan) equipped with a DB-5MS capillary column (30 m×0.25 mm 0.25 µm phase thickness, J & W scientific, Folsom, CA). For GC separation, helium was used as a carrier gas (100 kPa), and splitless injections were carried out at 220°C. The column temperature was initially set at 100°C for 2 min, and then increased up to 270°C for 42.5 min (a gradient of 4°C/min), and held at 270°C for 2 min. The interface and ion source was set at 270°C and 200°C, respectively. Spectra were acquired using electron ionization mode with a scan range of m/z 45–500. Identification of capsidiol was done by comparison of spectra and retention times on GC/MS analysis with pure standards extracted from *Capsicum annuum* fruits (Whitehead et al., 1987). The amount of capsidiol was quantified from peak areas of the total ion chromatogram, based on a calibration curve constructed with pure standards.

Analysis of defense-related gene expression

Tobacco cells (0.6 g) suspended in 3 mL of LS medium were incubated for 1 h at 25°C. The cells were treated with PIP-1 and MePIP-1 for a given time period (0-18 h). After

removal of the medium by filtration, cells were ground in liquid nitrogen using a mortar and pestle. Total RNA was extracted with Trizol reagent (Life technologies, Carlsbad, CA), according to the manufacturer's instructions. The RNA was incubated with 10 U of RQ1 RNase-Free DNase (Promega, Madison, WI) for 30 min at 37°C to degrade DNA. Resultant RNA (1 µg) was reverse transcribed to cDNA in a final volume of 20 µL using a PrimeScript RT reagent kit (Takara Bio, Otsu, Japan) according to the manufacturer's instructions. For semi-quantitative reverse RT-PCR, 1 µL of the cDNA template was used for PCR in a solution (final volume of 20 µL) containing 0.5 U of TaKaRa *Ex Taq* (Takara Bio), 200 µM each of dNTP mixture, and the 300 nM each of forward and reverse primers (Table 2-1). PCR was performed by T100 Thermal Cycler (Bio-Rad) using following conditions; 95°C for 3 min (first cycle), 95°C for 30s (denaturation), 53-56°C for 30s (annealing), 72°C for 30 s (extension; 20-28 cycles), and 72°C for 5 min (last cycle). PCR products were separated on a 1 or 2% agarose gel, and stained by EtBr (0.5 µg/L) for visualization. For quantitative RT-PCR, 2 µL of the cDNA template was amplified by using Thermal Cycler Dice (Takara Bio) in the presence of amplification buffer (25 µL of final volume) containing SYBR Premix *Ex Taq* II (Takara Bio) and 0.4 µM each of following forward and reverse primers (Table 2-1). The relative quantities were determined using *Actin* as a reference gene (Table 2-1).

Table 2-1. List of primer sequences used for semi-quantitative RT-PCR and quantitative RT-PCR

Gene	Primer sequence (5'-3')		Reference
	Forward	Reverse	
<i>PR1a</i>	5'-GCTTCCCAATTGGCTGCAG-3'	5'-ATCCTCCATTGTTACTGAAC-3'	van Verk et al. 2008
<i>PR3b</i>	5'-GCCATAGGAGTGGACCTGCTAAAC-3'	5'-AAAAGACCTCTGGTTGCCGC-3'	Shinya et al. 2006
<i>PI II</i>	5'-ATGCTAAACTCTGTAATGTGG--3'	5'-CATTAATTCCTTGGGCATGTCCTTG-3'	Takabatake et al. 2007
<i>PAL</i>	5'-TTACGCCCTCAGAACATCACCC-3'	5'-GCTTGGATTCCCTCCTGCTGTC-3'	Shinya et al. 2006
<i>AOC</i>	5'-TGCTGCTCTTAGAACCAATT-3'	5'-AGTCAAGTACGATCCCTGAA-3'	Wang 2009
<i>LOX</i>	5'-TTGGAGTTCAGGAGCATT-3'	5'-CGCAATGTGTCTGGTGTTC-3'	Cacas et al. 2005
<i>SIPK</i>	5'-GATGTCTGATCGGGGGCGG-3'	5'-GTGGCTCAACGTCGCCGAA-3'	
<i>WIPK</i>	5'-CGGTGGAGGTCAATCCCTG-3'	5'-CATTTACAAAAGGTTGCTC-3'	Seo et al. 2007
<i>HMGR2</i>	5'-GAGGGGAGAGGAAAGTCTGTAG-3'	5'-TCTCTATGTTCTGAGCTGGGTC-3'	
<i>EAS</i>	5'-GTAAGGACTCATGCTGACGA-3'	5'-TCCACCACCTTGATACTTCG-3'	
<i>SS</i>	5'-AAACTGTTCCATGCCTCTGG-3'	5'-CATATTCACGGGGCCAGAA-3'	
<i>Actin</i>	5'-CCATTGGCTCAGAGAGGTTC-3'	5'-GTTGGAAGGTGCTGAGAG-3'	Wang et al. 2006

In-gel kinase assay

In-gel kinase assay were conducted as described previously with modifications (Romeis et al., 1999). Tobacco cells (0.6 g) suspended in 3 mL of LS medium were incubated for 1 h at 25°C. The cells were treated with PIP-1 and MePIP-1 for given time period (0-9 h). After removal of medium, tobacco cells were ground in liquid nitrogen using mortar and pestle. Powdered tobacco cells were homogenized with 1.5 mL of extraction buffer [50 mM HEPES, pH 7.4, 5 mM EDTA, 5 mM EGTA, 5 mM DTT, 10 mM NaF, 10 mM Na₃VO₄, 50 mM β-glycerophosphate, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 2 μg/mL antipain, 2 μg/mL aprotinin, and 2 μg/mL leupeptin], and centrifuged at 16,000 × *g* for 30 min at 4°C. Supernatants were quickly frozen in liquid nitrogen and stored at -80°C. Extracts containing 10 μg of proteins were electrophoresed on 12% SDS-polyacrylamide gels embedded with 0.25 mg/mL myelin basic protein (MBP, Sigma, St. Louis, MO) as a substrate for kinases. After electrophoresis, the gel was washed with washing buffer (20% 2-propanol, 50 mM Tris, pH 8.0) three times for 20 min each at room temperature to remove SDS, and incubated with buffer A (50 mM Tris, pH 8.0, 5 mM DTT) for 1 h. The proteins in the gel were denatured in denaturation buffer (50 mM Tris-HCl, pH 8.0, 6 M guanidine•HCl, 20 mM DTT, 2 mM EDTA) twice for 30 min each. The kinases were allowed to renature on incubation with buffer A containing 0.04% of tween 20 overnight at 4°C with five changes of buffer. After equilibrated with assay buffer (50 mM HEPES, pH 8.0, 2.0 mM DTT, 0.1 mM EGTA, and 20 mM MgCl₂) for 30 min at room temperature, the gel was incubated in 20 mL of assay buffer containing 30 μM ATP and 1.85 MBq of [γ-³²P]-ATP (111 TBq/mmol; PerkinElmer, Waltham, MA) for 1 h at room temperature. The reaction was stopped by transferring the gel into a solution containing 10% trichloroacetic acid (w/v) and 1% sodium phosphate (w/v). The gel was washed five times with the same solution for 20 min each to remove unincorporated [γ-³²P]-ATP, and dried on filter paper using a vacuum gel-dryer. The dried gel was exposed to imaging plates (BAS-MS 2040, Fujifilm, Tokyo, Japan) overnight and analyzed using BAS-2500 (Fujifilm, Tokyo, Japan). Prestained size markers (Nacalai Tesque, Kyoto, Japan) were used to estimate the size of the kinases.

Results and Discussion

H₂O₂ generation and defense-related gene expressions induced by MePIP-1

In order to determine whether prevention of PIP-1 degradation affects induction of

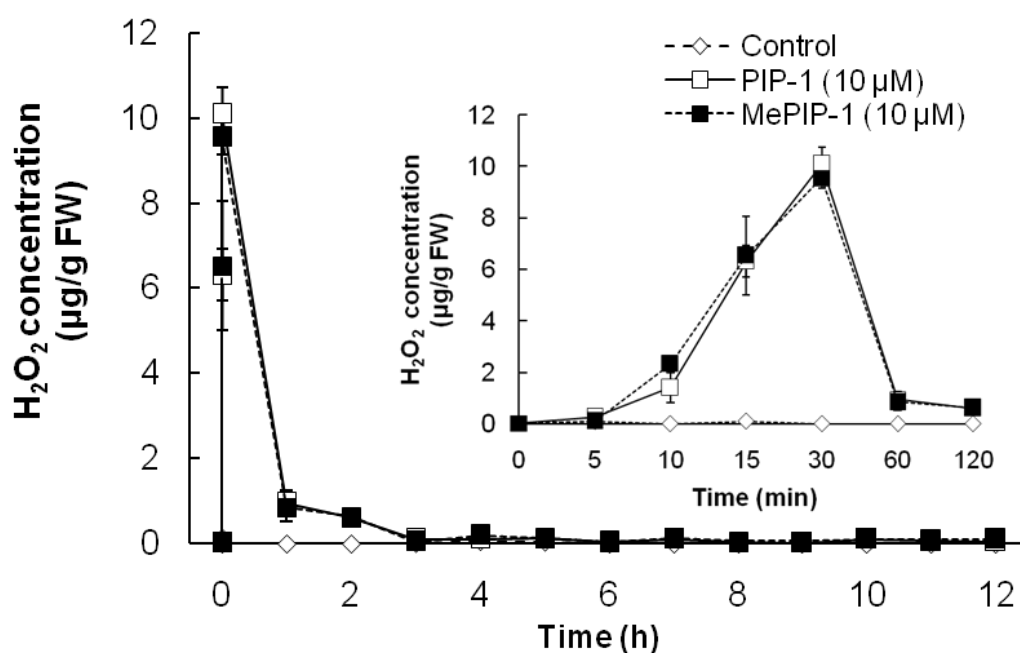


Figure 2-1. Time-course of H_2O_2 generation induced by PIP-1 and MePIP-1. Inset graph exhibits the enlarged view for 120 min after elicitation. Vertical bars indicate $\pm$ S.D. of three independent replicates.

immune responses, firstly, a time course analysis of H_2O_2 generation was performed. As a result, both PIP-1 and MePIP-1 induced similar responses at a concentration of 10 μM where H_2O_2 production reached its maximum at 30 min after the treatment and decreased to the basal level at 2 h. Thus, no significant difference was observed in oxidative burst induction between PIP-1 and MePIP-1 (Figure 2-1).

Next, expression of the defense-related genes was compared between PIP-1 and MePIP-1. Previously, PIP-1 induced the accumulation of defense-related genes, such as *PR-1a*, *PI-II*, *LOX*, *AOC* and *PAL* (Miyashita et al., 2011). In this study, MePIP-1 (10 μM) induced the gene expression in a similar manner to PIP-1 (10 μM) over a time course of 6 h, in which maximum transcript levels of *PR-3b* were observed 6 h after the treatment, and *PR-1a*, *PI II*, *LOX*, *AOC* and *PAL* were 3 h after the treatment (Figure 2-2). These results indicate that degradation resistant property of MePIP-1 has no effect on the induction of H_2O_2 generation and defense-related gene expression. This also suggests that the induction of these immune responses is initiated through short-term stimulation with PIP-1.

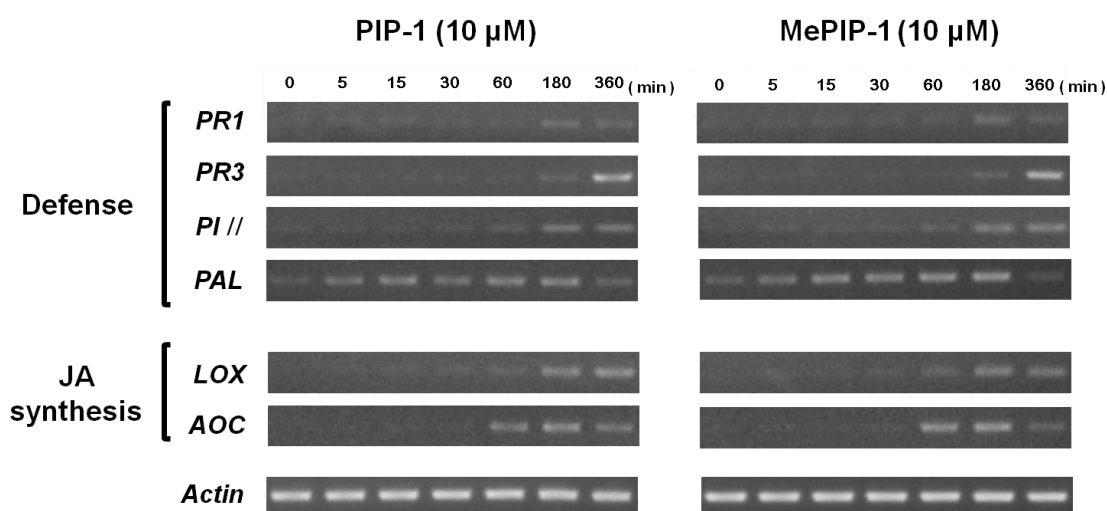


Figure 2-2. Relative expression patterns of defense-related genes induced by PIP-1 and MePIP-1. Gene expressions were determined by semi-quantitative RT-PCR after treatment with PIP-1 or MePIP-1 at 10 μ M. *Actin* was used as a control.

HR-like cell death and phytoalexin production induced by MePIP-1

Hypersensitive response (HR) has a crucial role in plant immune responses to restrict pathogen growth by inducing death of plant cells, and is initiated approximately 6 h after elicitor treatment (Coll et al., 2011; Durrant and Dong, 2004). To elucidate the effect of degradation-resistance of MePIP-1 on cell death, the author compared the cell death-inducing activity of MePIP-1 with that of PIP-1. At a concentration of 100 μ M, a similar degree of cell death was induced both by PIP-1 and MePIP-1 (Figure 2-3A). On the other hand, significant difference was observed between the two elicitors when treated at 10 μ M: MePIP-1 induced cell death at a similar level to that induced at 100 μ M, whereas the number of dead cells induced by PIP-1 was about half of that induced at 100 μ M. Since the treatment at 10 μ M was sufficient enough to induce the maximum response of oxidative burst both for PIP-1 and MePIP-1, it is possible that this difference is associated with the degradation-resistance of MePIP-1.

Capsidiol is a major phytoalexin accumulated in the extracellular medium of tobacco cells after elicitor treatment (Chappell and Nable, 1987; Zhao et al., 2005). Previous report showed that treatment with 100 μ M of PIP-1 induced capsidiol biosynthesis in tobacco cells (Miyashita et al., 2011). However, only marginal levels of capsidiol production were observed upon treatment with 10 μ M of PIP-1 (Figure 2-3B). In contrast, a significant level

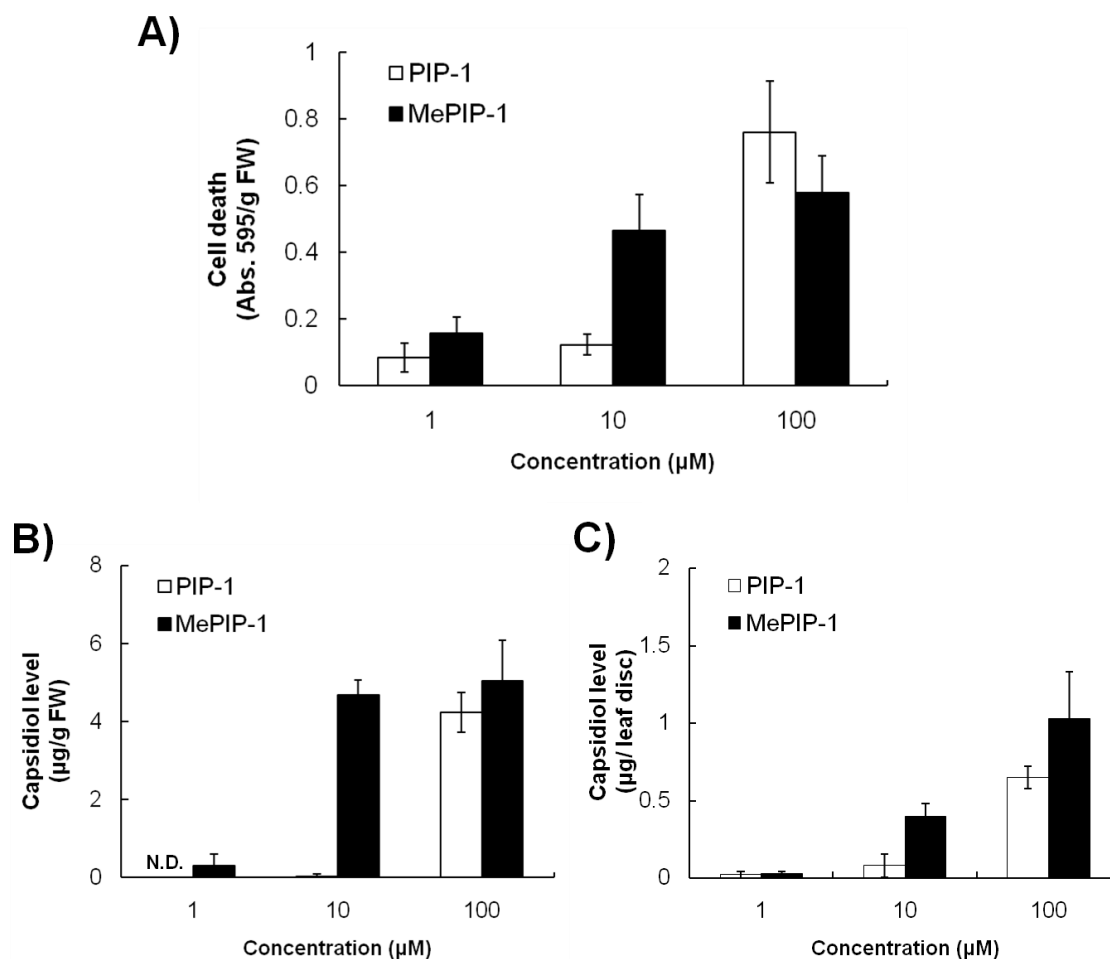


Figure 2-3. Comparison of late-phase immune responses induced by PIP-1 and MePIP-1. A) HR-like cell death. B) Capsidiol production induced in suspension-cultured cells. C) Capsidiol production induced in leaf discs. Vertical bars indicate $\pm$ S.D. of three independent replicates. N.D.: not detected.

of capsidiol accumulated ($4.7 \mu\text{g/g FW}$) upon treatment with $10 \mu\text{M}$ of MePIP-1, which is comparable to that induced by $100 \mu\text{M}$ of PIP-1 (Figure 2-3B). This enhancement in capsidiol production by MePIP-1 was also observed in tobacco leaves using leaf disc floating assay (Figure 2-3C). To confirm that this difference was due to the degradation-resistance of MePIP-1, the effect of cell washing to remove treated MePIP-1 on capsidiol production was examined. Consequently, no capsidiol accumulation was observed when MePIP-1 was removed from the medium by cell washing 1 or 3 h after treatment with MePIP-1 (Figure 2-4). On the other hand, the removal of MePIP-1 by cell washing 6 or 9 h after the treatment induced a significant amount of capsidiol, although the amount was relatively low probably

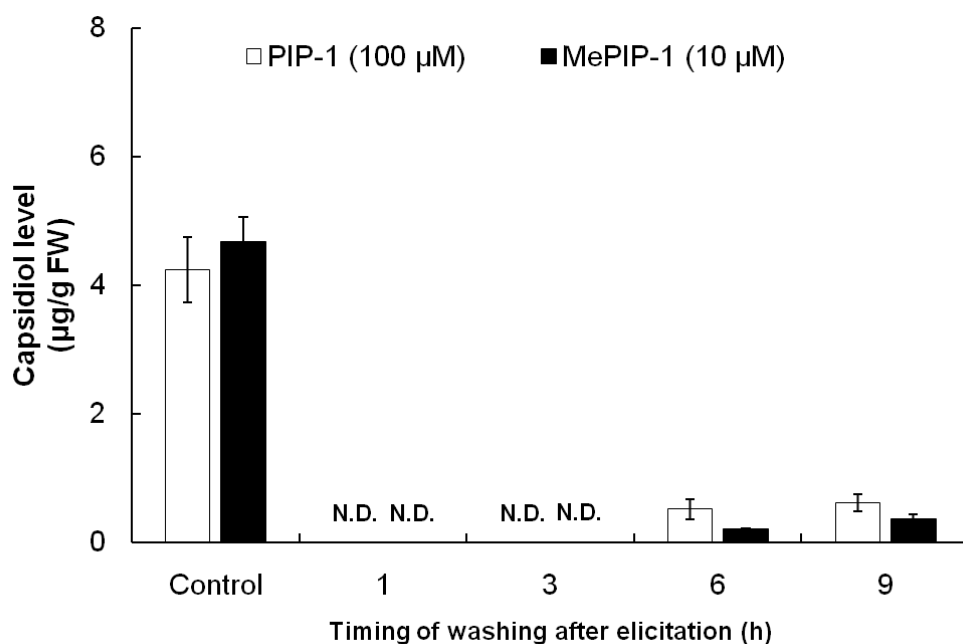


Figure 2-4. Effect of cell washing on capsidiol production. Amount of capsidiol were quantitated 24 h after elicitation. Vertical bars indicate $\pm$ S.D. of three independent replicates. N.D.: not detected.

due to the concomitant removal of accumulated capsidiol by washing. This result indicates that the degradation-resistance critically contributes to the higher potency of MePIP-1 in inducing capsidiol production, and that a continuous stimulation by the elicitor for at least 3 to 6 h is required for the induction of phytoalexin in tobacco cells. This also implies that the phytoalexin biosynthesis is not directly related to H_2O_2 generation in tobacco cells, as reported before (Nürnberg et al., 1994).

Effects of MePIP-1 on MAP kinase activity

MAP kinase cascades play a crucial role in early signaling event associated with induction of defense responses against pathogen infection and elicitor recognition (Meng and Zhang, 2013). It was reported that activation of SIPK and WIPK, representative MAP kinases in tobacco, is closely involved in elicitor-mediated immune responses (Zhang et al., 1998; Zhang et al., 2000). To investigate the possible involvement of MAP kinases in PIP-1 induced defense responses, effect of kinase inhibitor on capsidiol production was firstly examined using tobacco cells. As a consequence of pre-treatment with K252a, a general protein kinase inhibitor, capsidiol production induced by MePIP-1 was suppressed in a concentration-dependent manner (Figure 2-5). This result suggested that activation of MAP kinases could

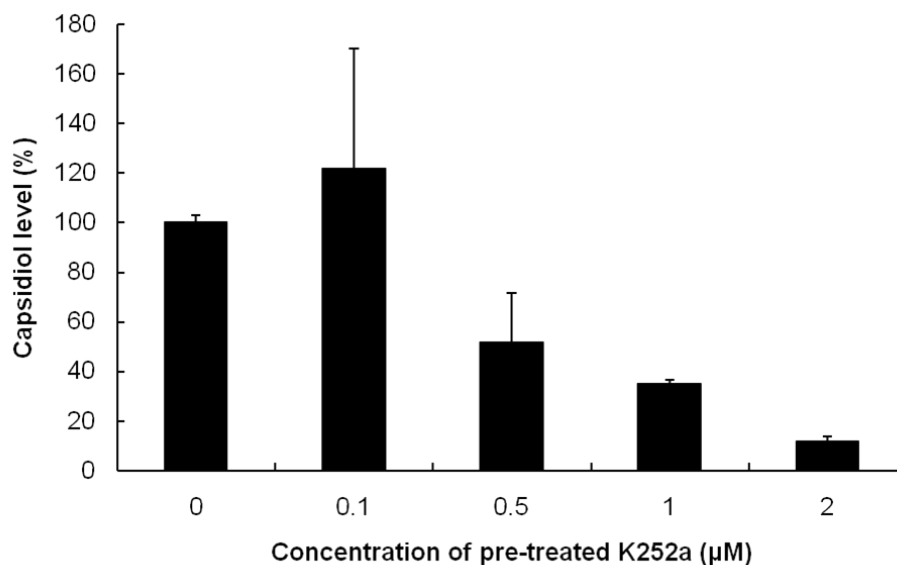


Figure 2-5. Inhibitory effect of K252a on capsidiol production mediated by PIP-1. A mount of capsidiol was quantitated 24 h after treatment with MePIP-1. Capsidiol production without K252a was set at 100%. Vertical bars indicate $\pm$ S.D. of three independent replicates.

be associated with capsidiol production induced by MePIP-1. Thus, to clarify the relationship between continuous elicitor stimulation and MAP kinase activation for capsidiol production, the expression of genes encoding *SIPK* and *WIPK* was examined. *SIPK* transcripts were expressed constantly in tobacco cells, and no effect of treatment both of PIP-1 and MePIP-1 was observed (Figure 2-6A). On the other hand, both PIP-1 and MePIP-1 induced an increase in *WIPK* transcript levels, reaching a maximum 1 h after the treatment, although no difference in the expression pattern was observed between PIP-1 and MePIP-1 over a time course of 6 h. Since it is known that the activity of these MAP kinases is regulated by post-translationally with sequential phosphorylation, not by an increase in transcript or protein levels (Zhang et al., 1998; Zhang et al., 2000), the author then investigated whether these MAP kinases are differently activated by PIP-1 and MePIP-1 using in-gel kinase assay. Upon treatment of either PIP-1 or MePIP-1, a significant activation of a 48-kD kinase, which corresponds to SIPK, was observed (Figure 2-6B). However, the time-dependent changes of SIPK activation were significantly different between PIP-1 and MePIP-1. SIPK was activated 1 h after the treatment with PIP-1, but rapidly decreased to a basal level after 6 h. In contrast, MePIP-1 induced a long-lasting activation of SIPK even up to 9 h after the treatment. When MePIP-1 was removed from the medium 1 h after the treatment by cell washing, SIPK

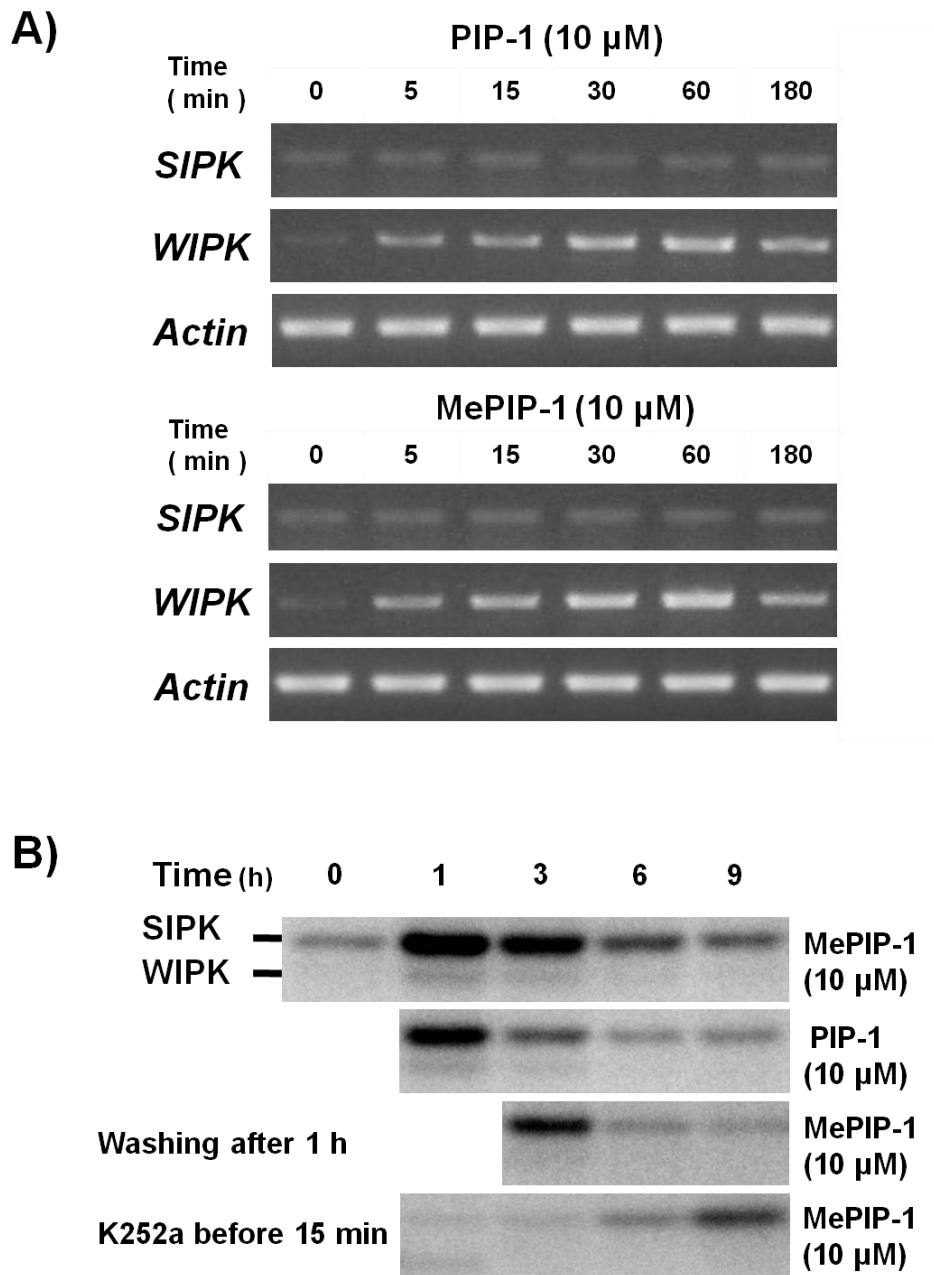


Figure 2-6. Effect of PIP-1 and MePIP-1 on activation of MAP kinases. A) Accumulation of *SIPK* and *WIPK* transcripts in response to PIP-1 and MePIP-1. B) Activation of *SIPK* and *WIPK* after treatment with PIP-1 and MePIP-1.

activity was decreased to a basal level 6 h after the treatment, which was analogous to the observation when treated with PIP-1. In addition, *SIPK* was not activated when K252a was pretreated. This result clearly indicates that a continuous stimulation by MePIP-1 induced a prolonged *SIPK* activation, which might be necessary for the activation of downstream signaling events that are associated with capsidiol production. Little *WIPK* (44-kDa kinase)

activation was observed both for PIP-1 and MePIP-1 treatments in spite of its increase in transcript levels.

In this study, it is demonstrated that the continuous stimulation by MePIP-1 for 3-6 h is required for the accumulation of capsidiol in tobacco cells. Of the two MAP kinases examined, this continuous stimulation induced a prolonged SIPK activation, whereas WIPK was only slightly activated in spite of accumulation of the *WIPK* transcript. This indicates that prolonged SIPK activation alone is required for induction of phytoalexin accumulation by MePIP-1. Involvement of SIPK and/or WIPK in elicitor-induced defense responses has been reported previously. A continuous stimulation for several hours by cryptogein, a proteinaceous elicitor derived from *Phytophthora cryptogea*, caused a prolonged activation of both SIPK and WIPK, leading to the induction of a full set of immune responses, such as HR-like cell death and long-lasting production of reactive oxygen species, in tobacco suspension cells (Kadota et al., 2006). In the case of removing cryptogein from the incubation medium 0.5 h after treatment, HR-like cell death was not observed, which coincided with a decrease in SIPK and WIPK activity. Meanwhile, it also has been reported that an activation of SIPK alone is sufficient to induce HR-like cell death, and that WIPK activation could play a role in accelerating the cell death process (Liu et al., 2003). In the present study, activation of SIPK alone was associated with the induction of phytoalexin production in tobacco cells. Although little is known about the relationship between phytoalexin biosynthesis and MAP kinases to date, the activation of SIPK alone is likely to be the prerequisite for the phytoalexin biosynthesis in tobacco, and the WIPK activation may play a role in accelerating its production. As shown in the case of fungal cell wall elicitor, it appears that a transient activation of SIPK is not sufficient enough for inducing the late-phase responses. Fungal cell wall was shown to selectively activate SIPK in a transient manner, but no cell death was induced (Zhang et al., 1998). Although the reason why the fungal cell wall elicitor could not induce the prolonged SIPK activation is unknown at present, it is likely that the enzymatic degradation of the elicitor in the media as well as on the surface of plant cells after the treatment is responsible for the failure in the induction of late-phase responses, like in the case of PIP-1.

Capsidiol biosynthesis-related gene expression induced by MePIP-1

Capsidiol is synthesized through the mevalonate pathway in plants (Figure 2-7) (Kirby and Keasling, 2009; Vögeli and Chappell, 1988). In this pathway, HMGR is

considered to be a key enzyme (Chappell and Nable, 1987), which catalyzes the rate-limiting step in the biosynthesis of all isoprenoid compounds, including capsidiol. Transcription levels of this gene are known to be regulated by elicitor recognition (Choi et al., 1992; Takahashi et al., 2004). FPP is synthesized from mevalonate in several steps, and is located at a final branch point for sesquiterpene and sterol synthesis. FPP is converted to capsidiol in several steps including the reaction catalyzed by EAS, which is demonstrated to be induced in response to elicitor treatment (Vögeli and Chappell, 1988). FPP is also an important precursor for the sterol biosynthesis, where SS plays a major regulatory role, and its enzyme activity is known to be suppressed by elicitor treatment (Vögeli and Chappell, 1988). To evaluate whether the degradation-resistance of MePIP-1 affects transcription levels of these capsidiol biosynthesis-related genes, the gene expression of *HMGR* 2, *EAS* and *SS* after the treatment either with PIP-1 or MePIP-1 was examined using quantitative RT-PCR.

The time-course analysis for up to 18 h revealed that both PIP-1 and MePIP-1 (10 μ M) increased the expression levels of *HMGR*2 and *EAS* 3 h after the treatment, but induced a continuous decrease in *SS* expression for 18 h (Figure 2-8). However, no significant difference in these expression profiles was observed between PIP-1 and MePIP-1. Since continuous activation by MePIP-1 for 3 to 6 h was shown to be required for capsidiol biosynthesis in this study, the author postulated that the gene expression profile might be different at some point in the period from 3 to 6 h after the treatment between PIP-1 and MePIP-1, so expression of *HMGR*2 and *EAS* transcripts were further analyzed 4 h after the treatment with PIP-1 or MePIP-1. Contrary to expectation, however, no statistically

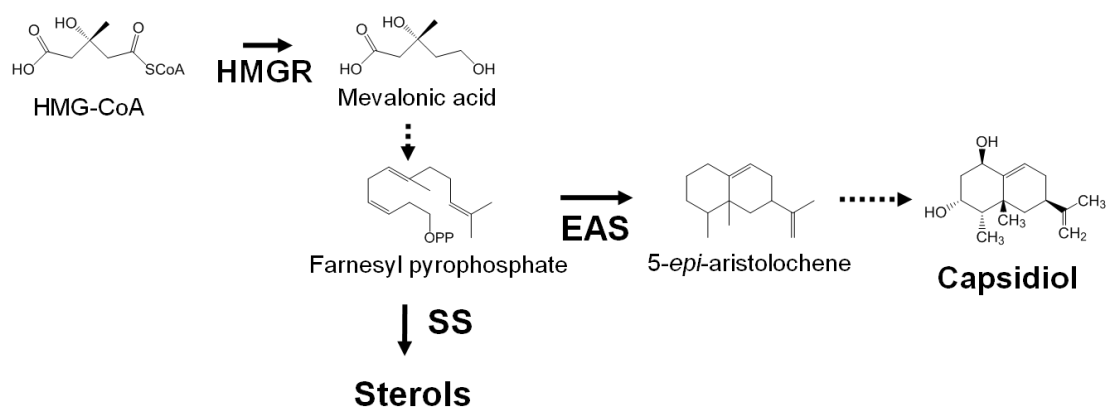


Figure 2-7. Isoprenoid pathway of plants for capsidiol biosynthesis

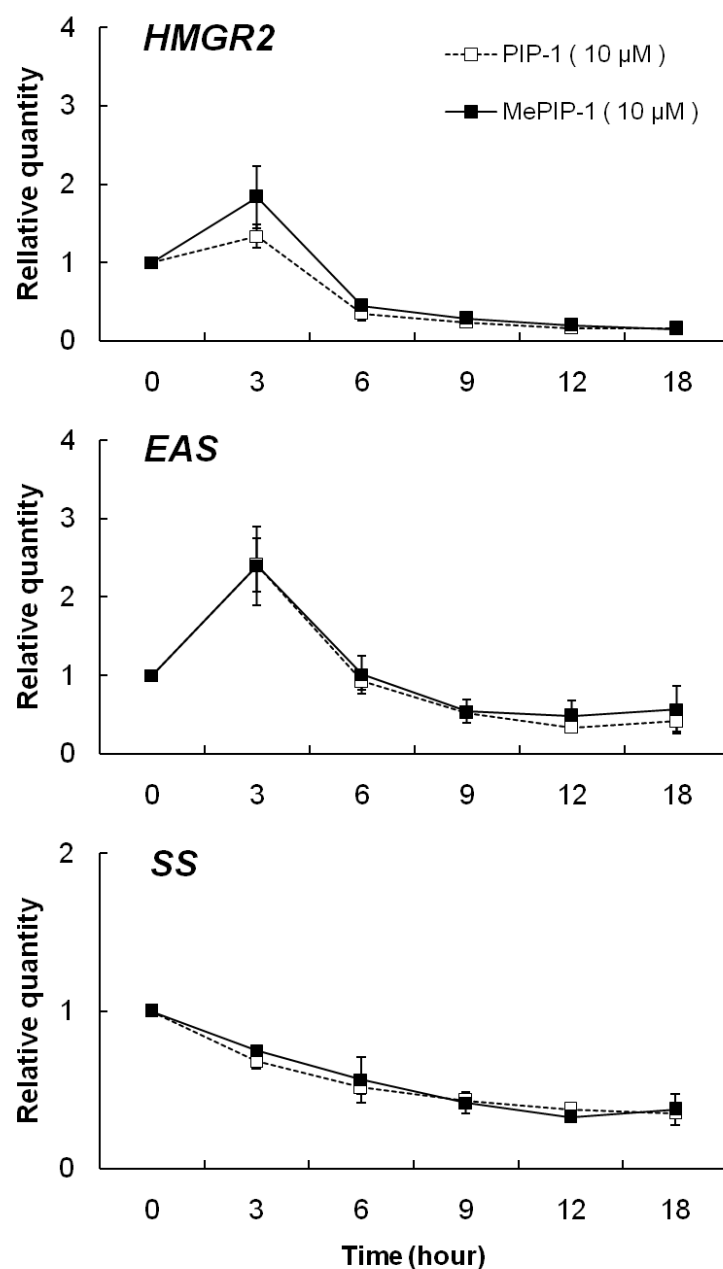


Figure 2-8. Time-course analysis of *HMGR2*, *EAS* and *SS* expression in response to PIP-1 and MePIP-1. Tobacco cells were treated with PIP-1 or MePIP-1 at 10 μ M. Vertical bars indicate $\pm$ S.D. of three independent replicates.

significant difference was observed between PIP-1 and MePIP-1 treatment (Figure 2-9). On the other hand, no induction of these gene expressions was observed in the case of tobacco cells pretreated with K252a, indicating that the transient SIPK activation induced by early short stimulation by PIP-1 is sufficient for induction of expression of *HMGR2* and *EAS* genes.

These results suggest that the capsidiol production is likely to be regulated post-translationally by SIPK activity at the late phase, which is observed only for MePIP-1.

HMGR2 is reported to be elicitor-responsive among the *HMGR* isogenes, and SIPK activity has been shown to be significantly involved in its transcriptional up-regulation (Choi et al., 1992; Ishihama et al., 2011; Yoshioka et al., 2001; Zhang and Liu, 2001). In the present study, *HMGR2* was accumulated after treatment with PIP-1, even at the concentration where PIP-1 failed to induce capsidiol production. This indicates that an early short-term recognition of PIP-1 is sufficient for the induction of *HMGR2*. Likewise, no significant differences were observed in the transcription levels of *EAS* and *SS* between PIP-1 and MePIP-1. It seems likely that capsidiol production induced by PIP-1 is not regulated at the transcription level of biosynthesis-related genes. Thus, post-transcriptional and/or post-translational regulation systems might play a more important role in the phytoalexin production. Alternatively, the biosynthesis of phytoalexin could be regulated by some factor(s) present downstream of MAP kinase cascades.

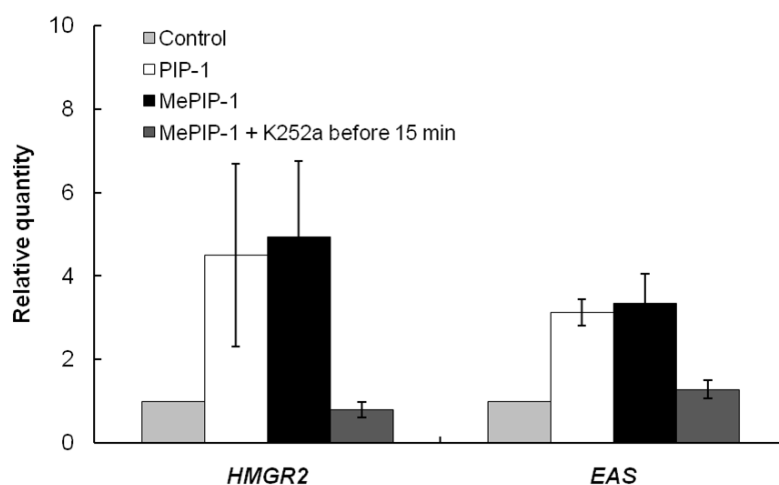


Figure 2-9. Relative transcription levels of *HMGR2* and *EAS* in response to PIP-1 and MePIP-1 at 10 μ M. Vertical bars indicate $\pm$ S.D. of three independent replicates.

In conclusion, MePIP-1 induced H_2O_2 generation in a time-course analysis similar to that by PIP-1, whereas significant differences were seen between PIP-1 and MePIP-1 in the late-phase immune responses such as HR-like cell death and capsidiol production. Using the degradation-resistant PIP-1 analog, it is demonstrated that continuous stimulation of the immune system by PIP-1 is necessary for the induction of late-stage immune responses, and

whether the late-stage responses are induced or not may depend on the duration of SIPK activation caused by the continuous elicitor stimulation.

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Chapter 3

Investigation of Temporal Factors Important for Phytoalexin Biosynthesis Induced by PIP-1 Using a Photocleavable PIP-1 Analog

Introduction

PIP-1 induces various immune responses, but types of the responses depend on the concentration of PIP-1. For example, phytoalexin production (a late-phase response) requires about 10- to 50-fold higher concentrations than those required for oxidative burst (an early-phase response). It was found that this difference is ascribed to a rapid decrease in the concentration of PIP-1 due to the enzymatic hydrolysis, implying that a continuous stimulation with PIP-1 for 3-6 h is necessary for the induction of phytoalexin biosynthesis, as shown in Chapter 2. In that study, to estimate the stimulation period required for phytoalexin biosynthesis, treated PIP-1 was removed from the culture medium at various time points by cell washing. However, the precise control of stimulation period was difficult in this method, and the washing procedure could have more or less mechanically stressed the cells to induce some additional responses, making the results affected by unwanted and/or unknown factors. Therefore, quicker and yet milder ways of “switching off” of the activity of PIP-1 were desired to analyze more precisely and conveniently their time-dependent actions on the plant immune system.

Over the past two decades, the integration of photo-sensitive substructure into a biologically active molecule has been attempted to design a probe whose activity is capable of being controlled in time simply by UV-irradiation (England et al., 1997; Lawrence, 2005; Toebe et al., 2006; Umezawa et al., 2011). In the case of peptides, photocleavable amino acids such as Anp and Npg have been successfully introduced (Figure 3-1A). They undergo conformational change upon UV-irradiation, followed by a rapid degradation to modify the activity of the “built-in” peptide (Rodenko et al., 2006). In particular, Anp was effectively utilized by Parker et al. (2006) in the study of yeast peptide pheromone α -factor (Figure 3-1B). A photocleavable α -factor analog integrated with an Anp residue was active in arresting the cell cycle at G1 phase, and was rapidly degraded into inactive form by UV-irradiation. This released the arrested state of the cells in a homogeneous and synchronous manner, allowing the detailed analysis of the cell cycle progress.

Materials and Methods

Plant cell culture

Tobacco cells (*Nicotiana tabacum* cv. Xanthi) were maintained as a suspension culture described in Chapter 1, and used at 9 and 10 days after subculture for experiments.

Chemicals

Fmoc protected-amino acids, Fmoc-NH SAL resin, and Cl-Trt(2-Cl) resin were obtained from Watanabe Chemical Industries (Hiroshima, Japan). Fmoc-(R)-3-amino-3-(2-nitrophenyl)propionic acid was purchased from PepTech Corporation (Bedford, MA) and Npg was synthesized as described by Rodenko et al. (2006). DA-64 was obtained from Wako Pure Chemical Industries (Osaka, Japan). CHX was purchased from Nacalai Tesque (Kyoto, Japan).

Synthesis of photocleavable analogs

The standard Fmoc solid-phase peptide synthesis strategy was used for synthesis of photocleavable analogs as described in Chapter 1. In the case of analogs containing Npg, dichloromethane was used instead of DMF in all steps of the procedure.

Measurement of H₂O₂ generation

Tobacco cells (0.2 g) suspended in 1 mL of assay buffer (175 mM mannitol, 0.5 mM CaCl₂, 0.5 mM K₂SO₄, 2.0 mM MES, pH 5.75) were incubated for 1 h at 25°C using a tube rotator. After incubation, the cells were treated with photocleavable analogs (10 µL) and incubated for 15 min including 2 min to allow cell sedimentation. H₂O₂ generation was measured as described in Chapter 2. The amount of H₂O₂ was calculated using a calibration curve constructed using pure H₂O₂.

Photolysis of peptides

The photocleavable analog was dissolved in distilled water at a final concentration of 100 µM. The solution was irradiated by UV light at 365 nm by Blak-ray B-100A UV-lamp (UVP, Upland, CA). The photolyzed products were analyzed on LCMS-2020 (Shimadzu) equipped with a reverse phase column (150 mm × 2.1 mm i.d., 3.6 µm, Aeris PEPTIDE XB-C18, Phenomenex, Torrance, CA).

Cell death assay

Cell death assay was performed as described in Chapter 2.

Quantitation of capsidiol

Capsidiol extraction from tobacco cells and quantitation was conducted as described in Chapter 2.

In-gel kinase assay

Tobacco cells (0.6 g) suspended in 3 mL of LS medium were incubated for 1 h at 25°C. The cells were treated with a photocleavable analog for 3 h. After removal of the medium by filtration, cells were collected and stored at -80°C. Subsequent protein extraction and in-gel kinase assay were performed as described in Chapter 2.

Analysis of defense-related gene expression

Tobacco cells (0.6 g) suspended in 3 mL of LS medium were incubated for 1 h at 25°C. The cells were treated with a photocleavable analog for 4 h. After removal of the medium by filtration, cells were collected and stored at -80°C. Subsequent total RNA extraction and quantitative RT-PCR were performed as described in Chapter 2.

Measurement of HMGR activity

3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) activity was measured as described previously (Mansouri et al., 2009). Briefly, tobacco cells (0.6 g) treated with samples for 4 h were used for crude enzyme extraction. The tobacco cells were ground in a mortar and pestle with liquid nitrogen and homogenized in the extraction buffer (50 mM Tris-HCl, pH 7.5, 10 mM β -mercaptoethanol, 1% (w/v) PVP). The mixture was centrifuged at $16,000 \times g$ for 30 min at 4°C, and the supernatant was used for measurement of HMGR activity after determination of protein concentrations using the Bio-Rad protein assay reagent. The crude extract was added to the assay buffer (50 mM Tris-HCl, pH 7.0, 0.3 mM HMG-CoA, 0.2 mM NADPH, and 4 mM DTT), and oxidation of NADPH was measured by monitoring a decrease in absorbance at 340 nm. The solution of free HMG-CoA was used as a blank.

Results and Discussion

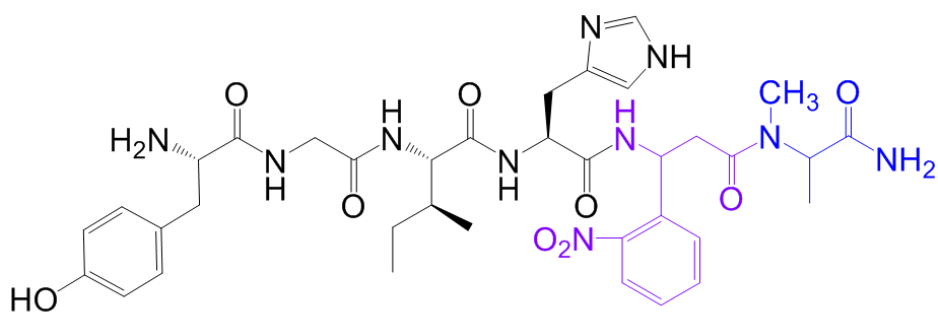
Design of photocleavable PIP-1 analogs

PIP-1 is rapidly degraded from its C-terminal end in the presence of tobacco cells. To obtain photocleavable PIP-1 analogs whose elicitor activity can be controlled by UV-irradiation, the structure of analogs must be stable against enzymatic degradation without UV-irradiation. In this regard, degradation-resistant analogs obtained in Chapter 1, such as MePIP-1, [Asp⁶]PIP-1, and [Ape⁷]PIP-1, are suitable as a lead compound for development of photocleavable PIP-1 analogs (Figure 3-2).

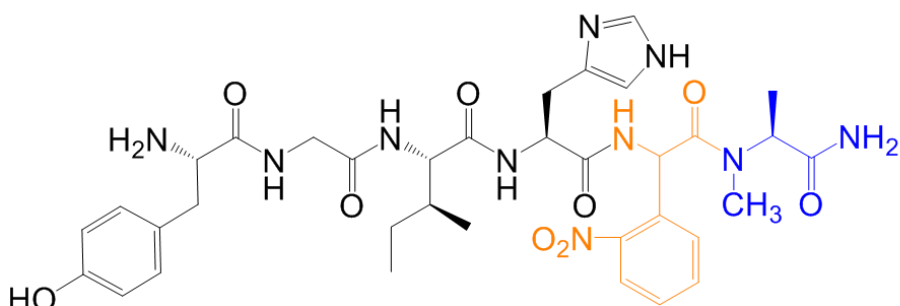
The N-terminal four residues in PIP-1 have been shown to be essential for its elicitor activity (Miyashita et al., 2011). Therefore, the Thr residue of MePIP-1 was substituted with Anp (Figure 3-2; analog 1), which is expected to lose the activity by UV-irradiation to cleave the amide bond between His and Anp. Unfortunately, this analog without UV-irradiation showed no measurable activity (Table 3-1). Since Anp is a beta-amino acid that gives an additional carbon atom in the peptide main chain, it could have caused an unfavorable structural change in the peptide for the interaction with the receptor. The author then tried the introduction of a photocleavable alpha-amino acid (Npg) for the substitution of Thr (Figure 3-2; analog 2). However, this substitution also resulted in a loss of biological activity (Table 3-1). It is speculated that the steric interaction between the 2-nitrobenzyl group of Npg and

Table 3-1. H₂O₂ generation activity of PIP-1 analogs

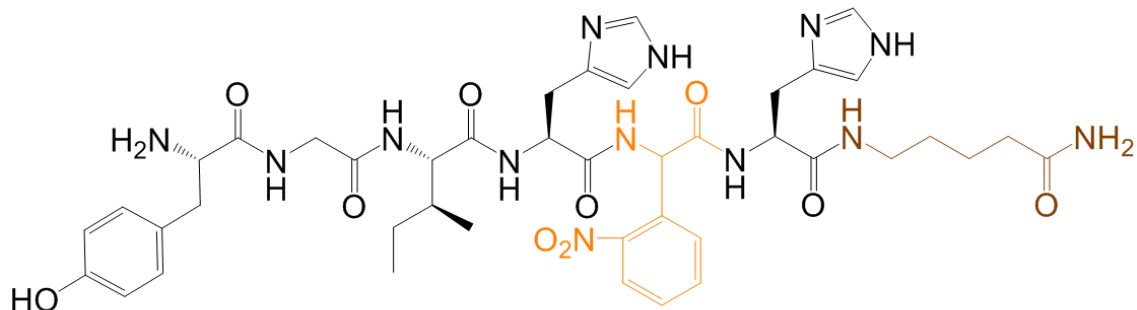
No.		Sequence	EC ₅₀ (μM)	Reference
1	PIP-1	YGIHTH-NH ₂	2.2 ± 0.9	Miyashita et al. 2011
2	MePIP-1	YGIHTmeA-NH ₂	0.63 ± 0.32	
3	[Ape ⁷]PIP-1	YGIHTH(Ape)-NH ₂	6.9 ± 2.3	
4	[Asp ⁶]PIP-1	YGIHTD-OH	0.78 ± 0.22	
5		YGIH-NH ₂	>100	
6		YGIH(Anp)meA-NH ₂	>100	
7		YGIH(Npg)meA-NH ₂	>100	
8		YGIH(Npg)HApe-NH ₂	>100	
9	PcPIP-1	YGIH(Anp) D -OH	30.5 ± 2.9	



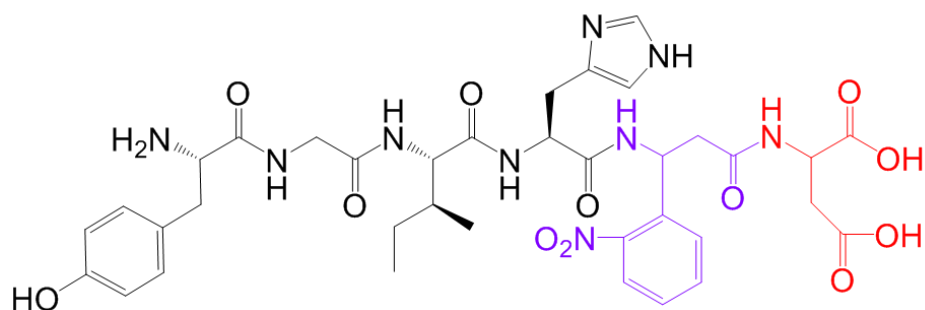
Analog 1



Analog 2



Analog 3



PcPIP-1

Figure 3-2. Synthesized PIP-1 analogs containing UV-cleavable amino acids

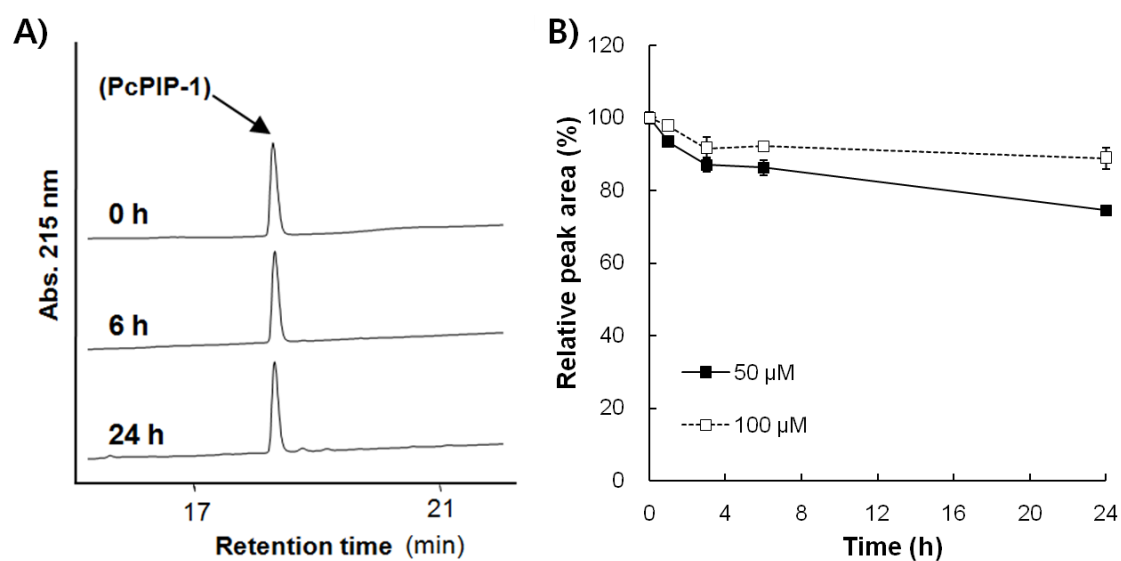


Figure 3-3. The degradation resistance of PcPIP-1 in the presence of tobacco cells. A) LC/UV chromatogram of PcPIP-1 in extracellular fluid of tobacco cells after treatment. B) Relative amounts of PcPIP-1 in the cell culture medium after treatment are plotted (n=2).

the neighboring N-methyl group of Ala somehow changed the overall molecular conformation into inactive form and had a deleterious effect on the activity. To evade the deleterious effect caused by this steric interaction, other degradation-resistant analogs were used as a lead compound. First, the Thr residue of [Ape⁷]PIP-1 was substituted with Npg (Figure 3-2; analog 3). However, analog 3 also showed no measurable activity, probably because the 2-nitrobenzyl group of Npg affects the neighboring structure, such as the His side chain, to lead to unfavorable conformation. Next, [Asp⁶]PIP-1 was used as a lead compound. Since this analog has the Asp residue at the C-terminus, it is expected that the steric interaction between side chains less affects the elicitor activity, compared with analogs containing MeAla or His as a neighboring residue. As expected, substitution of the Thr residue of [Asp⁶]PIP-1 with Anp (Figure 3-2; PcPIP-1) retained the elicitor activity although it was 14-fold less active than PIP-1 (Table 3-1). In addition, PcPIP-1 showed the high degradation-resistance (Figure 3-3). In the following sections, the author examined whether the photocontrol of the elicitor activity of this analog is possible.

Photolysis of the photocleavable PIP-1 analog

Reactivity of PcPIP-1 in aqueous solution under UV-irradiation was evaluated by monitoring the degradation products using ESI-LC/MS. When the PcPIP-1 (m/z observed for $[M+2H]^{2+}$: 398.68, retention time: 15.1 min) was irradiated at 365 nm, about 90% of the initial amount disappeared after 5 min to give several new peaks in the chromatogram (Figure 3-4A). The molecular masses of two peaks at the retention times of 3.7 (m/z 488.27 for $[M+H]^+$) and 6.3 min (m/z 489.26 for $[M+H]^+$) were consistent with those of YGIH-NH₂ and

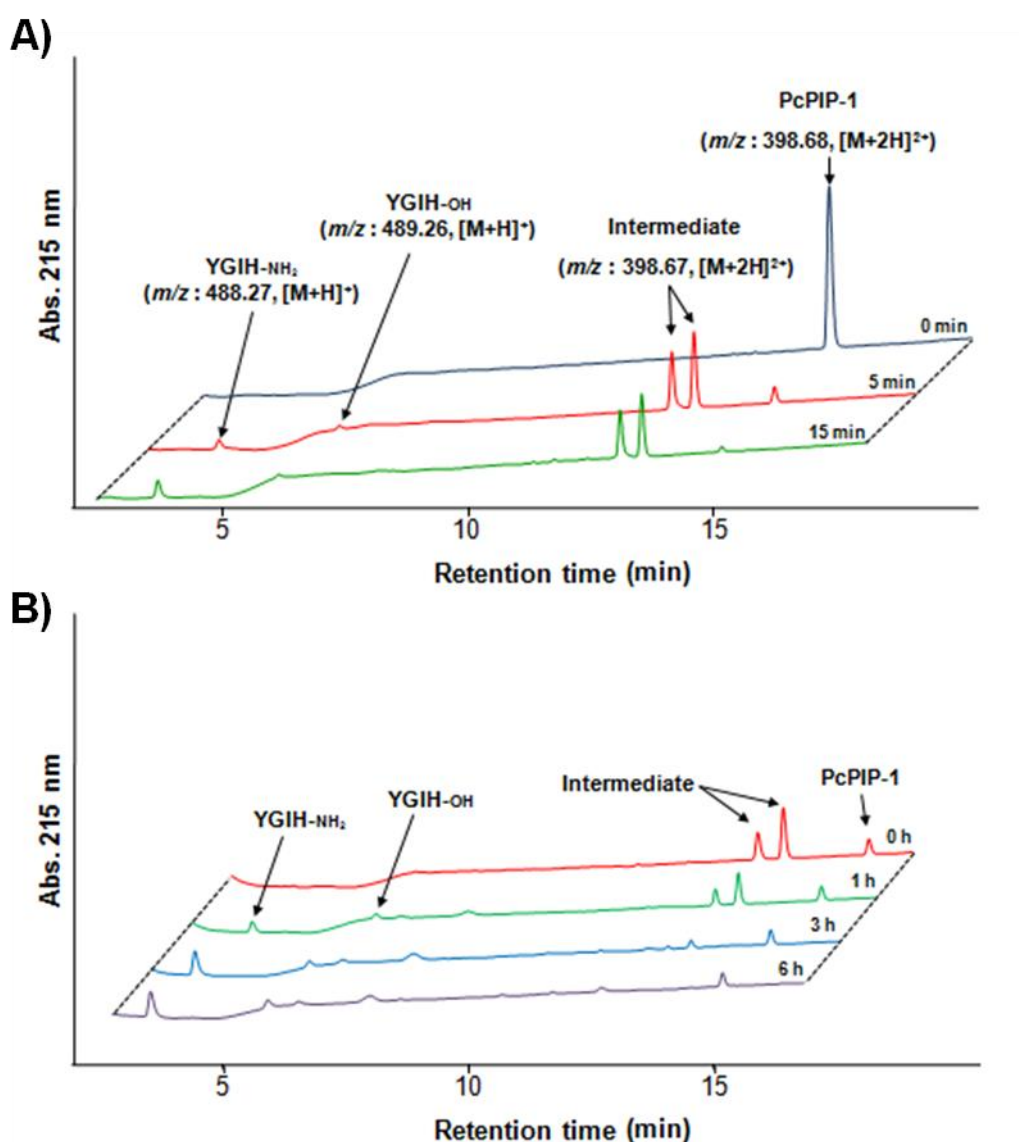
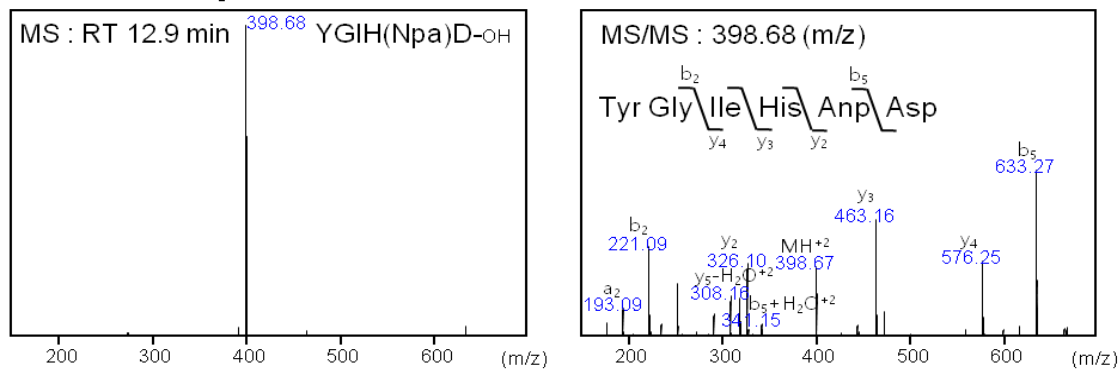
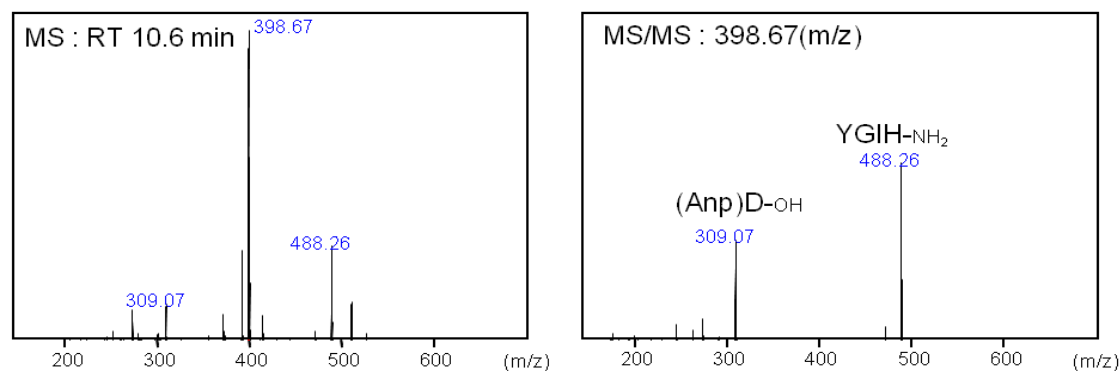


Figure 3-4. HPLC profile of photolyzed products of PcPIP-1 at 100 μ M. A) LC/UV chromatogram of PcPIP-1 in aqueous solution with UV-irradiation. B) LC/UV chromatogram of extracellular fluid of tobacco cell after treatment with PcPIP-1 UV-irradiated for 5 min.

YGIH(Anp)D-OH, m/z 398.68 [M+2H]²⁺



Unknown peak 1, m/z 398.67 [M+2H]²⁺



Unknown peak 2, m/z 398.67 [M+2H]²⁺

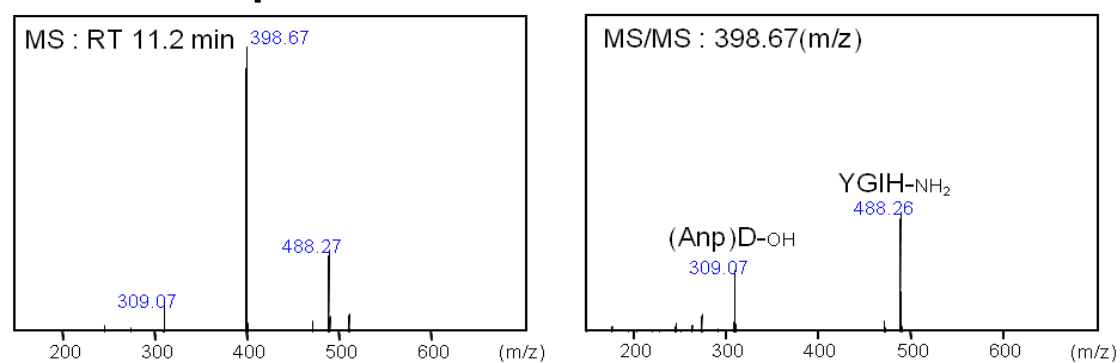
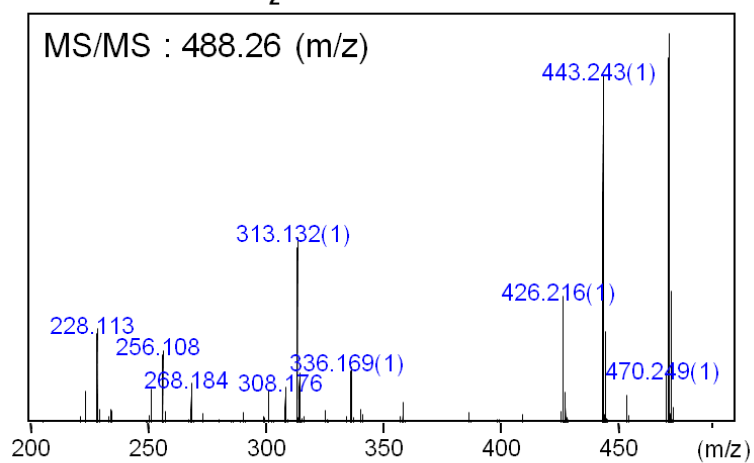
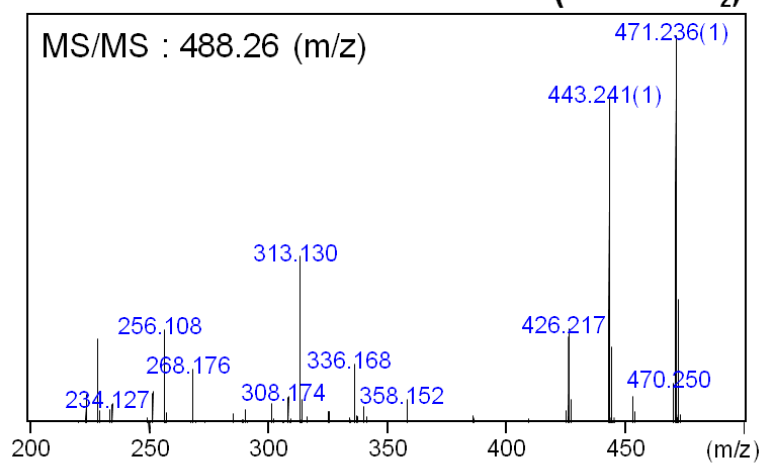


Figure 3-5. MS/MS spectrum of PcPIP-1 and its unknown products after UV-irradiation. MS/MS spectrum was obtained using LCMS-IT-TOF (Shimadzu, Kyoto, Japan).

STD YGIH-NH₂



Cleaved PcPIP-1: m/z 488.26 (YGIH-NH₂)



Unknown peak1 m/z 398.67 → 488.26

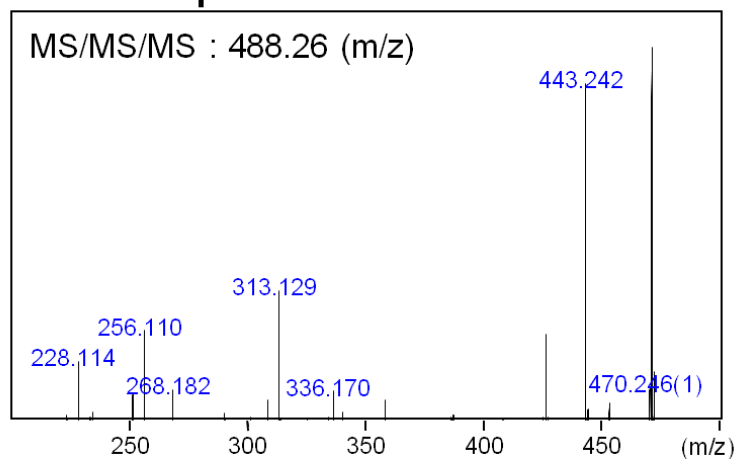


Figure 3-6. MS³ spectrum of standard YGIH-NH₂ and degradation product of PcPIP-1 after UV-irradiation. MS/MS spectrum was obtained using LCMS-IT-TOF (Shimadzu).

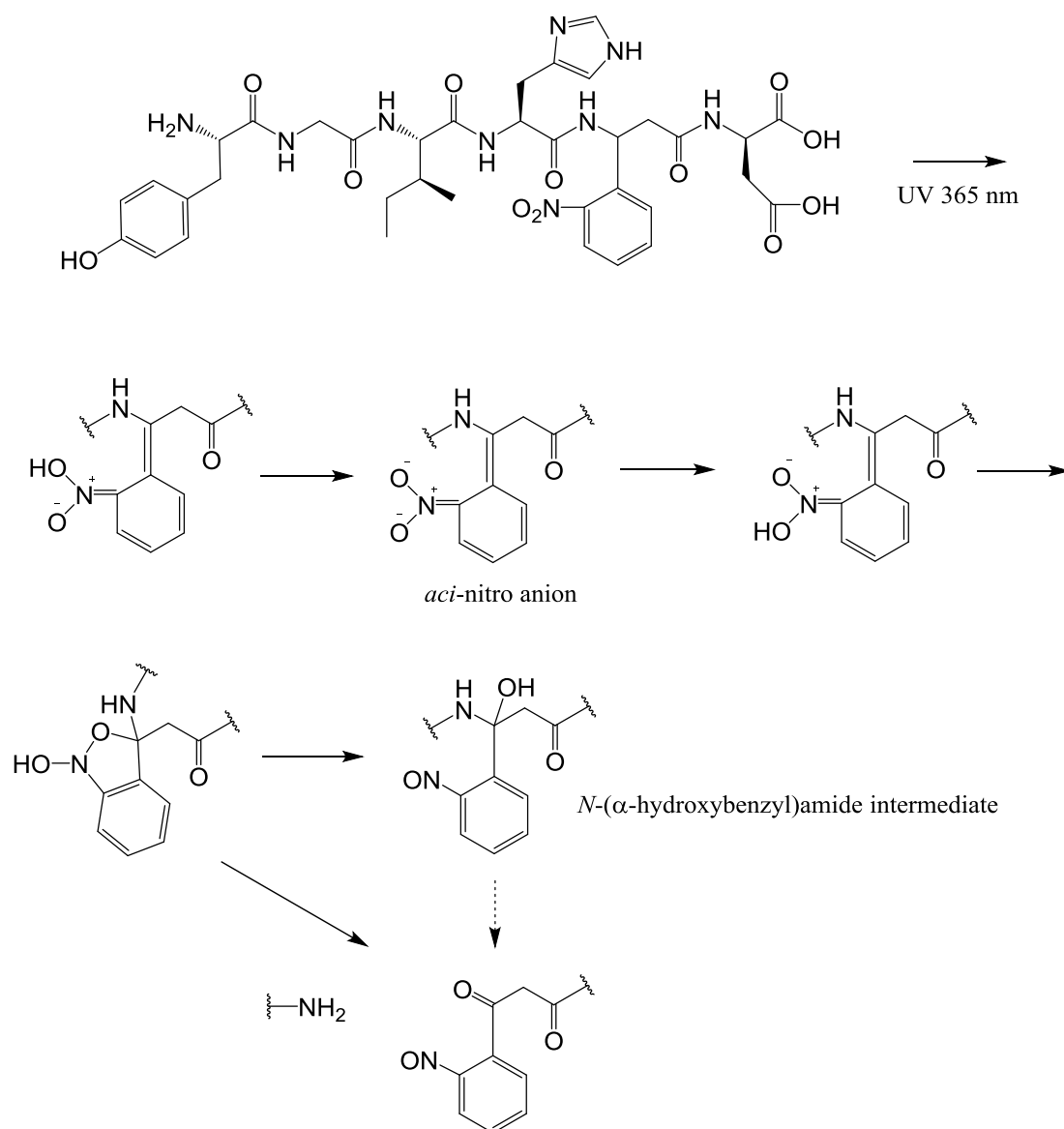


Figure 3-7. Possible photolysis process of PcPIP-1 and its long-lived intermediate by UV-irradiation in the aqueous solution

YGIH-OH, respectively, which were identified by comparing with the synthesized standards. Both of these peptides were considered to be produced by the photolysis of the Anp residue, although the formation mechanism of the latter was not clear. Two other peaks at the retention times of 13.0 and 13.5 min had the same molecular mass as the original peptide (m/z observed: 398.67, calculated for $[\text{M}+2\text{H}]^{2+}$: 398.66). These peaks were still detected at the time point 15 min after UV-irradiation, when only a marginal level of the original peptide was present. Based on the result of MS/MS analysis, both of these two peaks only gave two major

fragment ions (m/z 309.07 and 488.26), while the amino acid sequence-related fragment ions were regularly observed for the case of the original peptide (Figure 3-5). The MS³ analysis suggested that these two fragment ions corresponded with YGIH-NH₂ and 3-(2-nitrosophenyl)-3-oxopropanoyl aspartic acid (Figure 3-6), the expected photolysis products of PcPIP-1. Based on these results, the author speculated that these two unknown peaks are the structural isomers of PcPIP-1 produced in the process of photolysis. It was proposed that the photolysis of 2-nitrobenzyl compounds proceeds in several steps, of which the decay of *aci*-nitro intermediate (Figure 3-7) is considered to be rate-limiting (McCray and Trentham, 1989; Yu et al., 2010). In several cases, the photolysis of 2-nitrobenzyl compounds undergoes via the formation of relatively long-lived alpha-hydroxybenzyl intermediates (Corrie et al., 2003; Peyser and Flechtner, 1987; Salerno et al., 2000), so it seems likely that the unknown peaks are associated with the 3-hydroxy-3-(2-nitrosophenyl) intermediate produced in the manner shown in Figure 3-7. The possible epimerization of this intermediate can account for the occurrence of two peaks with the same molecular mass. On the other hand, when the aqueous solution containing UV-irradiated PcPIP-1 for 5 min was incubated in the presence of tobacco cells, two unknown peaks gradually decreased in incubation medium for 3 h coincided with increase of YGIH-NH₂ (Figure 3-4B), indicating that putative intermediates is structurally unstable in the presence of tobacco cells.

Photocontrol of immune responses induced by PcPIP-1

To confirm that PcPIP-1 is inactivated by UV-irradiation, different types of elicitor activity of intact PcPIP-1 was compared with that of UV-irradiated one for 15 min prior to treatment. Regarding the early-phase responses, treatment of the cells with intact PcPIP-1 induced a significant level of H₂O₂ generation at 50 or 100 μM (Figure 3-8A). UV-irradiation of PcPIP-1 markedly lowered the elicitor activity. The amounts of H₂O₂ generation were reduced by 6- and 2-fold, when the cells were treated with UV-irradiated preparations of PcPIP-1 at 50 and 100 μM, respectively. The effect of UV-irradiation was also significant on the induction of the late-phase immune responses such as HR-like cell death and phytoalexin production (Figure 3-8B). The levels of cell death induced by the UV-irradiated PcPIP-1 were evidently lower, compared to intact PcPIP-1. More obvious difference was observed with respect to the production of capsidiol, the major phytoalexin in tobacco, where the UV-irradiation almost cancelled the activity of PcPIP-1 at 50 or 100 μM (Figure 3-8B). These results indicated that the UV-irradiated PcPIP-1, a mixture of the degradation products and

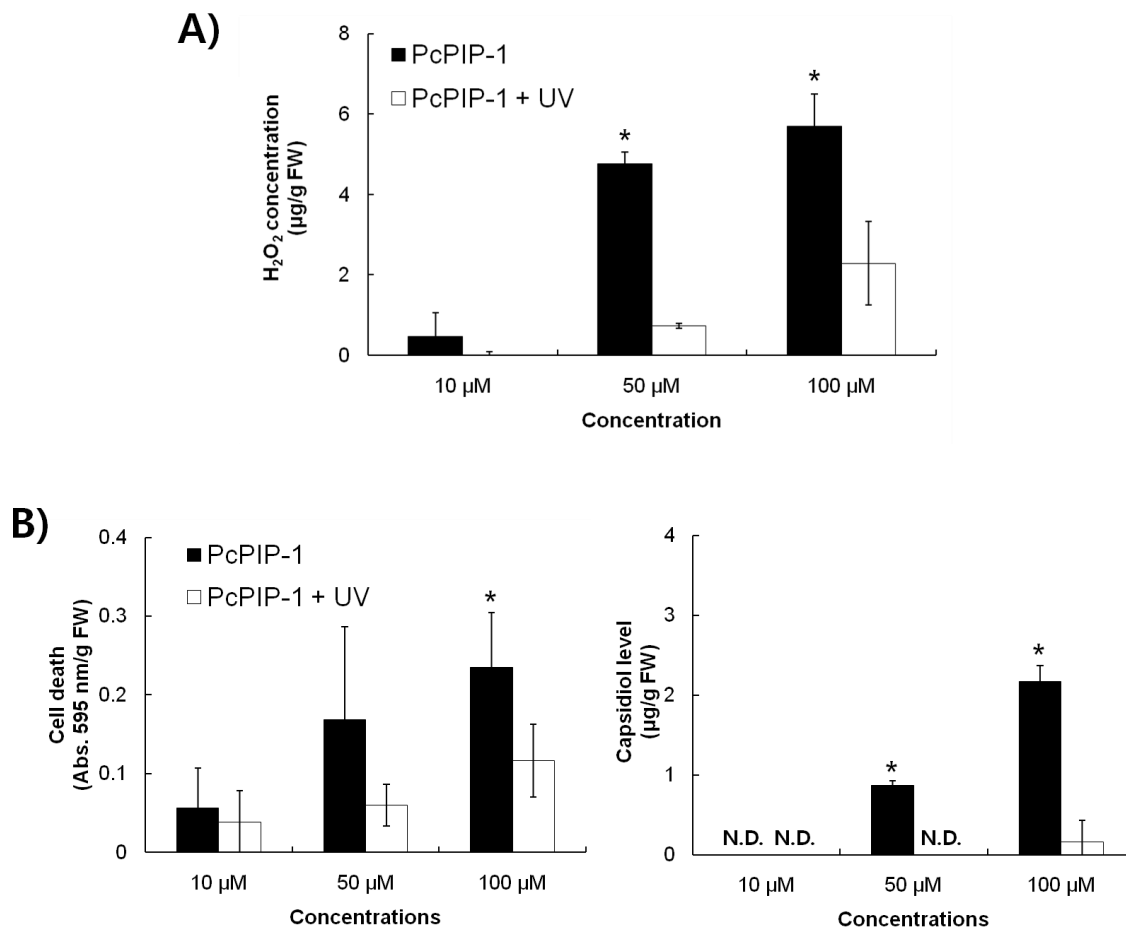


Figure 3-8. Comparisons of immune responses induced by PcPIP-1 between with and without UV-irradiation in the presence of tobacco cells. (A) H₂O₂ generation. (B) HR-like cell death and capsidiol production. Asterisk indicates significant difference at $P < 0.05$ according to the Student's paired *t*-test. Vertical bars indicate $\pm$ S.D. of three independent replicates. N.D.: not detected.

the degradation intermediates, has a sufficiently lower elicitor activity than the intact PcPIP-1.

We then examined whether the PcPIP-1 is also inactivated by UV-irradiation in the presence of tobacco cells for capsidiol production. As shown in Figure 3-9A, when the tobacco cells were UV-irradiated for 5, 10 or 15 min following the treatment with 100 μM of PcPIP-1 for 1 h, the capsidiol production at 24 h after the initiation of elicitation was completely abolished by the UV-irradiation for 15 min, indicating that photolysis of PcPIP-1 occurred in a same manner as in the aqueous solution without the cells. This was consistent with the result of Chapter 2 in which the elimination of PIP-1 by washing the cells 1 h after the treatment resulted in no capsidiol production. Therefore, it was confirmed that

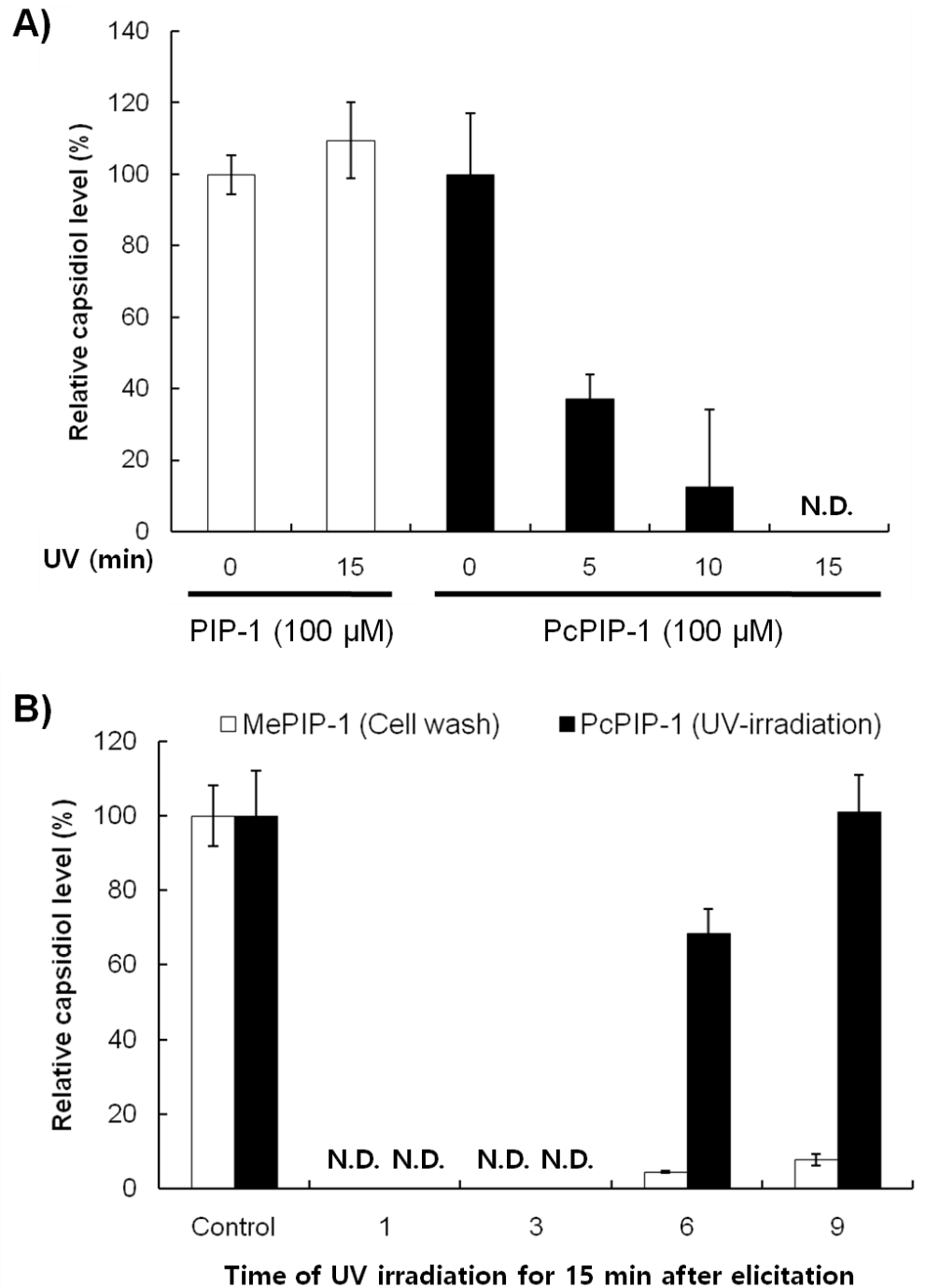


Figure 3-9. Effect of UV-irradiation on capsidiol production after treatment with PcPIP-1 at 100 μ M. (A) Effect of UV-irradiation period. UV-irradiation for 0-15 min was applied 1 h after sample treatment. (B) Effect of activation period. UV-irradiation (15 min) was applied 1-9 h after sample treatment. Cell washing was conducted at same time point. Vertical bars indicate $\pm$ S.D. of three independent replicates. N.D.: not detected.

the UV-irradiation for 15 min effectively worked to terminate the action of PcPIP-1 on the tobacco cells. The capsidiol production after the treatment with 100 μ M of PIP-1 was not affected by the UV-irradiation for 15 min, indicating that UV-irradiation itself had no negative effect on the tobacco cells regarding the phytoalexin production.

Next, the stimulation period required for initiating capsidiol production in the tobacco cells using the PcPIP-1 was investigated, and those results were compared with that by washing cell. Tobacco cells were treated with PcPIP-1 at 100 μ M, and irradiated with UV light for 15 min after 1, 3, 6 and 9 h. When the produced capsidiol was quantitated at 24 h after the initiation of elicitation, significant levels were detected in the tobacco cells irradiated with UV at 6 and 9 h. By contrast, no capsidiol was observed in tobacco cells irradiated with UV at 1 and 3 h after the elicitation (Figure 3-9B). This indicates that a continuous stimulation by the elicitor at least for 6 h is required for the phytoalexin induction. The requirement of a continuous stimulation for 3-6 h for the capsidiol production was also demonstrated in Chapter 2, in which the cell-washing method was used to remove treated PIP-1. In the present study, however, the amount of the produced capsidiol was much larger than that observed using the cell-washing method (Figure 3-9B). This is probably due to the difference in the degree of stresses in tobacco cells. Cell-washing is more likely to damage cells than irradiation with UV light, possibly affecting capsidiol biosynthesis negatively. Moreover, capsidiol accumulated in the extracellular medium could be lost by cell-washing, resulting in an underestimation of capsidiol production. Thus, these results showed that photocleavable PcPIP-1 presents several advantages as a tool to study phytoalexin induction mechanisms by elicitors, because of its ability to stop the stimulation of the immune system rapidly and not aggressively, without damaging the cells or losing the capsidiol accumulated in the medium.

Identification of factors important for capsidiol production induced by PIP-1

MAP kinase cascades are involved in the signaling pathway activated by the recognition of pathogen infections or elicitors, to invoke a variety of late-phase immune responses (Meng and Zhang, 2013). As shown in Chapter 2, PIP-1 induced activation of 48-kDa kinase in tobacco cells, which corresponds to SIPK. From the study on the relationship between SIPK activation and capsidiol production, it was concluded that a prolonged SIPK activation induced by continuous stimulation with PIP-1 was likely to be involved in the activation of capsidiol biosynthesis. However, which key factors directly control capsidiol

production remains unknown.

It has been shown that 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) acts as upstream rate-limiting enzyme in the mevalonate pathway involved in the biosynthesis of isoprenoids including capsidiol (Vranova et al., 2013). In addition, the inhibition of HMGR activity significantly suppresses capsidiol biosynthesis in tobacco cells induced by elicitors (Chappell and Nable, 1987). However, no capsidiol was detected following a short stimulation with PIP-1, even though the treatment did induce the accumulation of the *HMGR2* transcript, an elicitor-inducible *HMGR* isogene in Solanaceae plants (Choi et al., 1992; Yoshioka et al., 2001). The author hypothesized that some translational and/or post-translational regulation could be associated with the expression of HMGR activity to initiate the capsidiol accumulation in response to the continuous stimulation with PIP-1. Therefore, the author examined the relationship between the elicitor activation period and the induction of HMGR activity as well as of SIPK activity by using PcPIP-1.

First, the relationship between the activation period and the induction of SIPK activity was examined. As shown in Figure 3-10A, a significant level of SIPK activity was observed 3 h after the treatment with the PcPIP-1. On the other hand, when the cells were UV-irradiated to halt the activation by PcPIP-1 at 1 h after the elicitation, SIPK activity 3 h after the treatment was markedly diminished. This was consistent with the observation in Chapter 2, in which SIPK activity decreased after the removal of MePIP-1 by the cell-washing method. However, a decrease in activity was much sharper in the present study using the UV-cleavage method, compared to the cell-washing method. In the latter, somewhat high level of SIPK activity was retained after the removal of the elicitor, probably due to the physical stress or damage caused by the washing operation. Thus, the method using the photocleavable analog has the greater advantage of being able to investigate temporal factors regulating late-phase immune responses, such as phytoalexin production, without undesired side effects.

Next, the relationship between the activation period and the induction of *HMGR2* activity was examined using the photocleavable analog. When the tobacco cells were treated either with PcPIP-1, a significant level *HMGR2* transcripts were detected regardless of UV-irradiation (Figure 3-10B), indicating that a short-term elicitation was enough for the expression of *HMGR2* as observed before. On the other hand, the induction of HMGR activity was significantly affected by stimulation period controlled by the UV-irradiation. An increase in the HMGR activity in the tobacco cells at 4 h after the treatment with PcPIP-1 was canceled when PcPIP-1 was inactivated by UV-irradiation at 1 h after elicitation. This

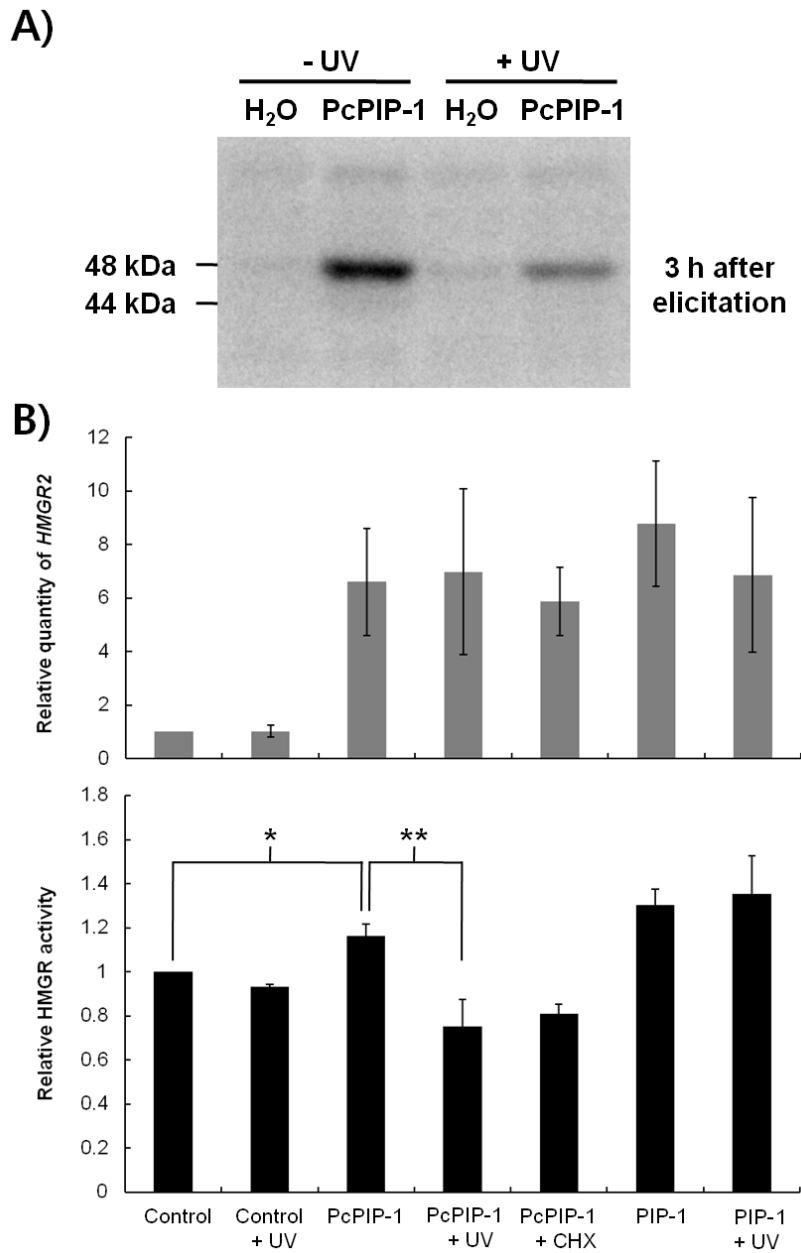


Figure 3-10. Effect of UV-irradiation on MAP kinase and HMGR activation. (A) Comparison of MAP kinase activity induced by PcPIP-1 (100 μ M) between with and without UV-irradiation. UV-irradiation (15 min) was applied 1 h after treatment with PcPIP-1. The activity of MAP kinase was measured 3 h after sample treatment. (B) Comparison of relative quantity of *HMGR2* transcript and relative activity of HMGR. Suspension cultured tobacco cells were treated with 100 μ M of PIP-1 or PcPIP-1. Distilled water was used as a control. +UV: UV-irradiation for 15 min at 1 h after elicitation, +CHX: cycloheximide (10 μ g/mL) treatment. The total RNA and crude enzyme were extracted 4 h after elicitation. Vertical bars indicate $\pm$ S.D. of three independent replicates. Asterisks indicate significant differences according to the Student's paired t-test (* P < 0.05; ** P < 0.01).

reduction was not observed when a sufficiently high concentration of PIP-1 was treated to stimulate the cells for a longer period for the induction of capsidiol production. These results suggest that a long-term elicitation is required to enhance the HMGR activity. It is likely that the HMGR activity is regulated by the translational or post-translational mechanisms, and that capsidiol biosynthesis induced by PIP-1 is, at least partly, associated with this enhancement in the HMGR activity. As to the regulation of HMGR activity, however, it remains unknown how the constitutive level in the control cells is maintained and why the protein synthesis inhibitor, CHX, had almost no effect on it. Although it is possible to assume that the regulation of capsidiol biosynthesis is specifically associated with the induced HMGR, and that the constitutive HMGR plays a role in synthesizing a variety of isoprenoids essential for growth and development (Vranova et al., 2013), involvement of other factors regulating the capsidiol biosynthesis cannot be ruled out.

In Chapter 2, it was shown that transient activation of SIPK only induced the accumulation of *HMGR2* transcript without capsidiol production. Capsidiol production required an elevation of SIPK activity for more than 3 h. In the present study, increase in HMGR activity was also associated with the prolonged activation of SIPK activity. Therefore, it is possible that SIPK has a dual role in the signaling for the immune responses: induction of defense-related genes at an early phase, and translational and/or post-translational activation of enzyme activity, such as HMGR, at a late phase. Actually, it has been demonstrated in *Nicotiana benthamiana* that the expression of *HMGR2* is regulated by MAP kinase-mediated phosphorylation of the WRKY8 transcription factor in response to pathogen inoculation (Ishihama et al., 2011). As to the translational or post-translational regulation of HMGR activity, it has been shown in recombinant HMGR1 from *Arabidopsis* that HMGR1 is inactivated by the phosphorylation of serine residue in the catalytic domain by *Brassica oleracea* HMGR kinase A in vitro (Dale et al., 1995). Besides, HMGR activity has been demonstrated to be negatively regulated by protein phosphatase 2A, which is considered to be a post-translational regulator of HMGR activity under the stressed conditions (Leivar et al., 2011). Taking these finding into account, the author suggests that the continuous stimulation with PIP-1 affects the phosphorylation state of these regulatory proteins via kinase cascades including SIPK, in order to control the initiation of capsidiol biosynthesis. Further investigations on the changes in the phosphorylation states of regulatory proteins by the continuous stimulation with PIP-1 would help clarify the regulatory mechanisms regarding the phytoalexin biosynthesis in response to elicitors.

In conclusion, PcPIP-1 containing 3-amino-3-(2-nitrophenyl)propionic acid as the photocleavable residue exhibited the significant elicitor activity to induce both early- and late-phase immune responses. This analog was highly resistant to enzymatic degradation by tobacco cells, but easily degraded into inactive forms upon UV-irradiation. In this Chapter, it was found using PcPIP-1 that the prolonged elevation of SIPK activity by the continuous action of PIP-1 caused an increase in HMGR activity, which is likely to be importantly associated with the “turning on” of the capsidiol production.

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Chapter 4

Elucidation of Signaling Factors Important for Acetosyringone Accumulation in Tobacco Cells Induced by PIP-1

Introduction

Phenolic compounds are well-known secondary metabolites produced in plants, primarily accumulated in response to the pathogen attack and/or wounding. They play crucial roles in the plant defense system as a material for plant cell wall re-enforcement, phytoalexin, and antioxidant (Baker et al., 2002; Dixon and Paiva, 1995; Nicholson and Hammerschmidt, 1992). Acetosyringone have been considered to be one of the representative extracellular phenolics in suspension-cultured tobacco cells induced by wounding or elicitor treatment. Although biological roles of this molecule have not been fully understood, involvement in the plant-pathogen interaction was reported as a compound triggering activation of virulence genes in *Agrobacterium tumefaciens* or as a redox control agent that affects pathogen infection (Baker et al., 2005a; Baker et al., 2005b; Bolton et al., 1986; Lee et al., 1995).

A precise biosynthetic pathway of acetosyringone has not been elucidated yet; however, several evidences suggested that acetosyringone is synthesized via the phenylpropanoid pathway, where COMT and/or CCoAOMT play pivotal roles in its biosynthesis (Maury et al., 2010). In addition, Negrel and his colleague recently reported that acetosyringone was synthesized from feruloyl-CoA via acetovanillone *in vitro* (Negrel and Javelle, 2010; Negrel et al., 2014). Thus, the biosynthetic route of acetosyringone has been gradually uncovered, but signaling factors important for induction of acetosyringone accumulation remains unclear.

PIP-1 is a hexapeptide elicitor identified from the combinatorial random hexapeptide library, which can induce oxidative burst and capsidiol production in tobacco cells (Miyashita et al., 2011). In Chapter 2, it was demonstrated that initiation of capsidiol production is closely associated with long-lasting activation of SIPK, one of the MAP kinases in tobacco, induced after continuous stimulation with PIP-1. In those experiments, a large amount of acetosyringone was observed concurrently with capsidiol in the incubation medium of tobacco cells treated with PIP-1 (Figure 4-1). However, a relatively high level of

acetosyringone was produced even after treatment with a low concentration (10 μ M) of PIP-1, where only marginal levels of capsidiol were observed. These results suggest that a decrease in the PIP-1 concentration due to degradation within 3-6 h does not affect the acetosyringone accumulation, implying that early short stimulation by PIP-1 is sufficient to induce acetosyringone production. Thus, the author hypothesized that signaling events leading to acetosyringone production may be different from those to capsidiol production.

In this Chapter, therefore, effects of PIP-1 degradation on acetosyringone accumulation were examined using the degradation-resistant analog MePIP-1. Then, early signaling events involved in acetosyringone production were investigated using various inhibitors of MAP kinases, Ca^{+} influx, oxidative burst and biosynthesis enzymes. In addition, biological roles of acetosyringone were discussed.

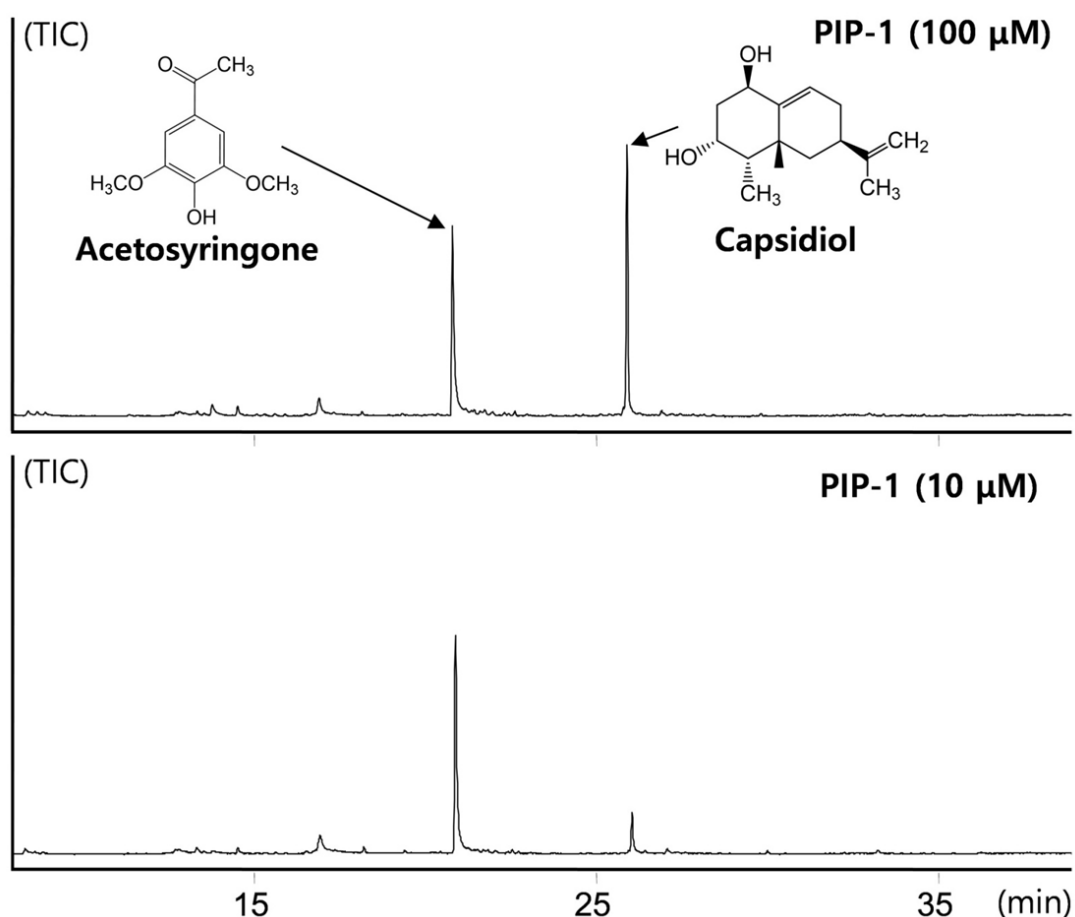


Figure 4-1. GC/MS chromatograms of the organic extract from the incubation medium of tobacco cells treated with PIP-1

Materials and Methods

Plant cell culture

Tobacco cells (*Nicotiana tabacum* cv. Xanthi) were maintained as a suspension culture as described in Chapter 1. The cells were used for experiments 9 and 10 days after subculture.

Chemicals

The peptides, YGIHTH-NH₂ (PIP-1) and YGIHTmeA-NH₂ (MePIP-1), were synthesized as described in Chapter 1. DA-64 was purchased from WakoPure Chemical Industries (Osaka, Japan). All inhibitors, K252 (LC labs, Woburn, MA), DPI (Alexis, San Diego, CA), AOPP (WakoPure Chemical Industries), and CHX (Nacalai Tesque, Kyoto, Japan), were commercially available. CAT (EC 1.11.1.6) were obtained from WakoPure Chemical Industries

Measurement of H₂O₂ generation

Tobacco cells (0.2 g) suspended in 1 mL of assay buffer (175 mM mannitol, 0.5 mM CaCl₂, 0.5 mM K₂SO₄, 2.0 mM MES, pH 5.75) were incubated for 1 h at 25°C using a tube rotator. After incubation, the cells were treated with PIP-1 (10 µL) and incubated for 15 min including 2 min to allow cell sedimentation. H₂O₂ generation was measured as described in Chapter 2. The amount of H₂O₂ was calculated using a calibration curve based on pure H₂O₂.

Quantitation of acetosyringone

Extraction and quantitation of acetosyringone from suspension-cultured tobacco cells was conducted as described in Chapter 2.

Analysis of defense-related gene expression

Tobacco cells (0.6 g) suspended in 3 mL of LS medium were incubated for 1 h at 25°C. The cells were treated with PIP-1 for 4 h. After removal of the medium by filtration, cells were collected and stored at -80°C to use subsequent total RNA extraction. The RNA extraction and quantitative RT-PCR were performed as described in Chapter 2 using forward and reverse primers (Table 4-1).

Table 4-1. List of primer sequences used for quantitative RT-PCR

Gene	Primer sequence (5'-3')		Reference
	Forward	Reverse	
<i>PAL</i>	5'-TTACGCCCTCAGAACATCACCC-3'	5'-GCTTGGATTCTTCCTGCTGTC-3'	Shinya et al. 2006
<i>COMT</i>	5'-CCTGCAAATGGGAAGGTGAT-3'	5'-CAGTCCTTTCTTTGCCTCCT-3'	Amelot et al. 2011
<i>CCoAOMT</i>	5'-ACACCCTATGGAATGGATCA-3'	5'-CCTTGTTGAGTTCCAATACGA-3'	Amelot et al. 2011
<i>Actin</i>	5'-CCATTGGCTCAGAGAGGTTC-3'	5'-GTTGGAAGGTGCTGAGAG-3'	Wang et al. 2006

PAL activity

PAL activity was determined as described previously with modifications (Gao et al., 2012). Tobacco cells (0.6 g) were suspended in 3 mL of LS medium and incubated for 1 h at 25°C. The cells were treated with PIP-1 for 4 h and collected by filtration with miracloth for subsequent enzyme extraction. The cells were ground using a mortar and pestle with liquid nitrogen and homogenized in 2 mL of 0.1 M borate buffer (pH 8.8) containing 2% (w/v) polyvinylpyrrolidone, 5 mM β -mercaptoethanol, and 2 mM EDTA. The homogenized mixture was centrifuged at $14,000 \times g$ for 15 min at 4°C, and supernatant was used as a crude enzyme extract to measure PAL activity. The crude extract was added to 0.1 M borate buffer (pH 8.0) containing 3 mM L-phenylalanine, and the formation of trans-cinnamate was measured by monitoring the increase in absorbance at 290 nm. PAL activity was expressed as a change in absorbance at 290 nm for 1 h per mg of protein. Total protein concentration in all of crude extracts was determined using a protein assay reagent (Bio-Rad).

H₂O₂ scavenging activity

Tobacco cells (0.6 g) suspended in 3 mL of LS medium were incubated for 1 h at 25°C. PIP-1 (final concentration of 10 μ M) was added to the cells. Distilled water was used as a control. After incubation for a given time period (0-12 h) at 25°C, the media were collected and filtered using syringe filters (0.45 μ m pore size). The filtrates were immediately frozen in liquid nitrogen and stored at -80°C to use subsequent assay. H₂O₂ (final concentration of 200 μ M) was added to the sample (300 μ L), and the mixture was incubated for 1 h using a tube rotator. The mixture (60 μ L) was mixed with the H₂O₂ indicator solution

(140 μ L; 100 mM DA-64, 1 U/mL horse radish peroxidase, 100 mM PIPES, pH 7.0) in a 96-well microplate, and incubated for 10 min at 37°C. The absorbance at 727 nm was measured with a Benchmark microplate reader (Bio-Rad, Hercules, CA). The amount of H₂O₂ was calculated using a calibration curve based on pure H₂O₂. H₂O₂ scavenging activity was estimated using the following equation.

$$\text{H}_2\text{O}_2 \text{ scavenging rate \%} = 100 - \left(\frac{\text{Quantity of H}_2\text{O}_2 \text{ in incubated sample}}{\text{Quantity of H}_2\text{O}_2 \text{ in water}} \times 100 \right)$$

Determination of total phenol content

Tobacco cells (0.6 g) suspended in 3 mL of LS medium were incubated for 1 h at 25°C. PIP-1 (final concentration of 10 μ M) was added to the cells, and distilled water was used as a control. After incubation for a given time period (0-12 h) at 25°C, the media were collected and filtered using syringe filters (0.45 μ m pore size). The medium was re-incubated with H₂O₂ (final concentration of 200 μ M) or distilled water as a control. The sample (100 μ L) was added to distilled water (440 μ L), and mixed with 60 μ L of a Folin-Ciocalteu's reagent (1.8 N, Nacalai Tesque). After incubated for 3 min, and the mixture was mixed with 400 μ L of 1 M Na₂CO₃ and further incubated for 1 h using a tube rotator. The absorbance at 724 nm was measured by a UV/Vis spectrophotometer (UV-1600, Shimadzu). The amount of total phenol was calculated using a calibration curve based on authentic 4-hydroxycinnamic acid.

Results and Discussion

Effect of PIP-1 degradation on acetosyringone accumulation

PIP-1 is a peptide elicitor, which is rapidly hydrolyzed from the C-terminal residue in the presence of tobacco cells. A decrease in concentration due to degradation significantly affects the induction of phytoalexin production that requires continuous stimulation by the elicitor. As shown in Figure 4-1, PIP-1 at a concentration of 100 μ M induced a large amount of capsidiol, but only marginal level of capsidiol was observed after treatment of PIP-1 at 10 μ M. On the other hand, the similar level of acetosyringone was detected in both cases. Thus, it is speculated that acetosyringone accumulation may not be affected by a concentration change due to PIP-1 degradation. This may also imply that acetosyringone production is

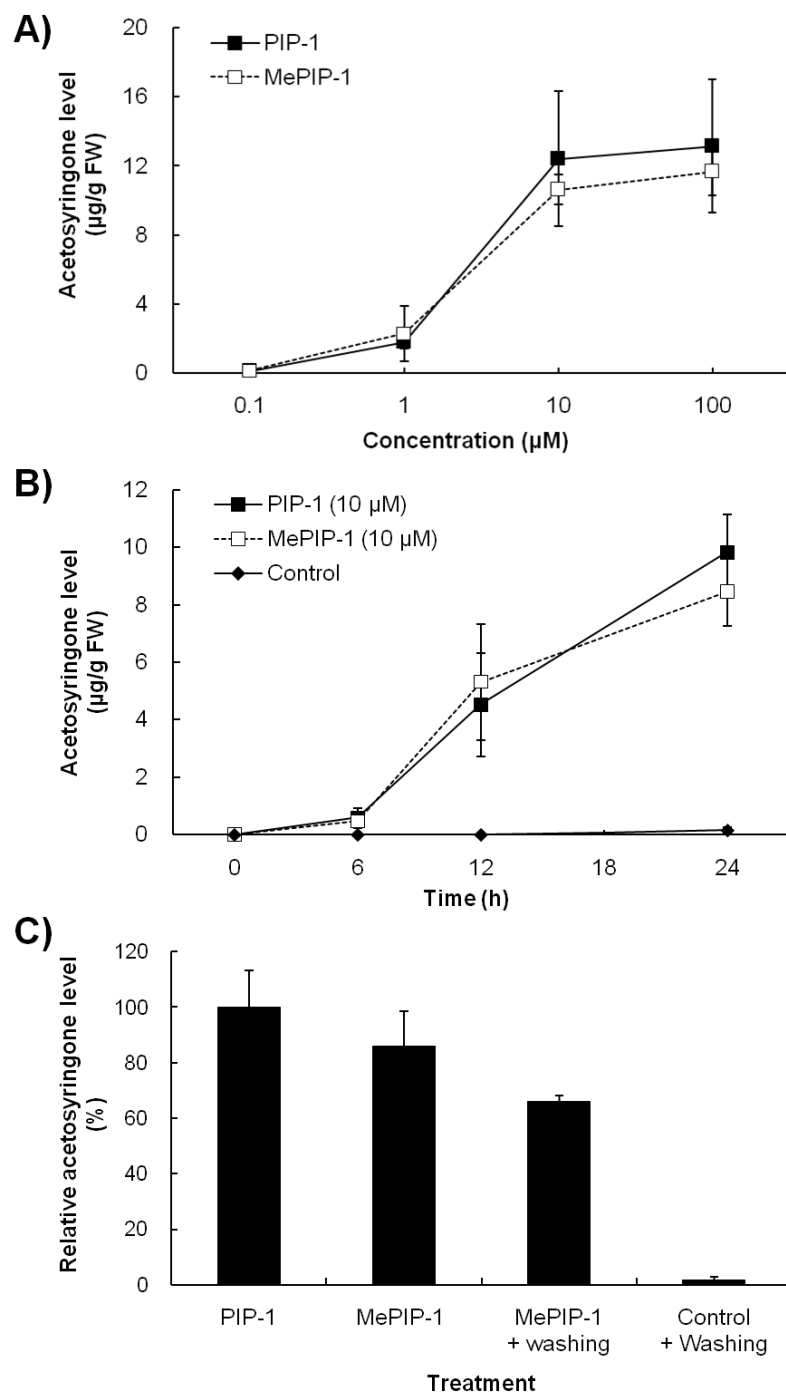


Figure 4-2. Acetosyringone accumulation induced by PIP-1 and MePIP-1. A) Comparison of concentration-dependent accumulation of acetosyringone. B) Comparison of time-dependent accumulation of acetosyringone. C) Effect of treatment period on acetosyringone accumulation. Tobacco cells were treated with 10 µM of MePIP-1, and washed 1 h after elicitation to remove MePIP-1. The acetosyringone was extracted 24 h after initiation of elicitation with PIP-1 and MePIP-1. The amount of acetosyringone induced by PIP-1 was set to 100%. Vertical bars indicate $\pm$ S.D. of three independent experiments.

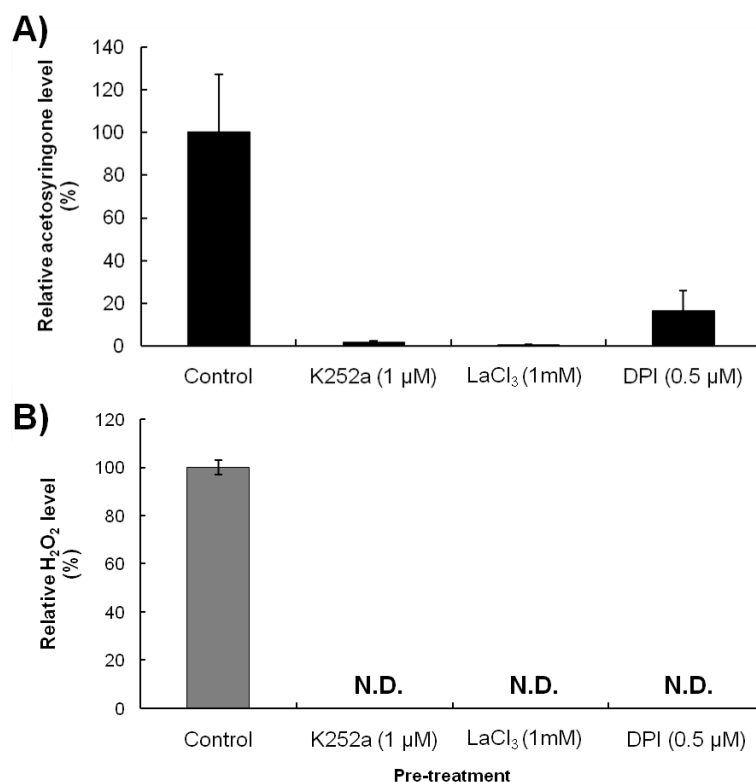


Figure 4-3. Inhibitory effect of several inhibitors involved in the early signaling event A) Relative acetosyringone level and B) H₂O₂ generation induced by 10 μM of PIP-1. Inhibitors were applied 20 min prior to treatment with PIP-1. 10 μM of PIP-1 treatment without inhibitor was used as a control, and set to 100%. Vertical bars indicate ± S.D. of three independent experiments. N.D.: not detected.

induced after early short stimulation by PIP-1. To examine this hypothesis, acetosyringone accumulation profiles were compared between PIP-1 and its degradation-resistant analog MePIP-1.

As a result, both PIP-1 and MePIP-1 showed similar accumulation profiles in terms of concentration- and time-dependencies (Figure 4-2A and 4-2B). In addition, a significant level of acetosyringone was detected even when MePIP-1 was removed by washing cells 1 h after elicitation (Figure 4-2C). These results indicated that early short-term elicitor stimulation is sufficient for acetosyringone accumulation.

Early signaling event involved in acetosyringone accumulation

In response to elicitor, plants quickly initiate early immune responses, such as activation of MAP kinase cascade, Ca²⁺ influx, and oxidative burst, which lead to

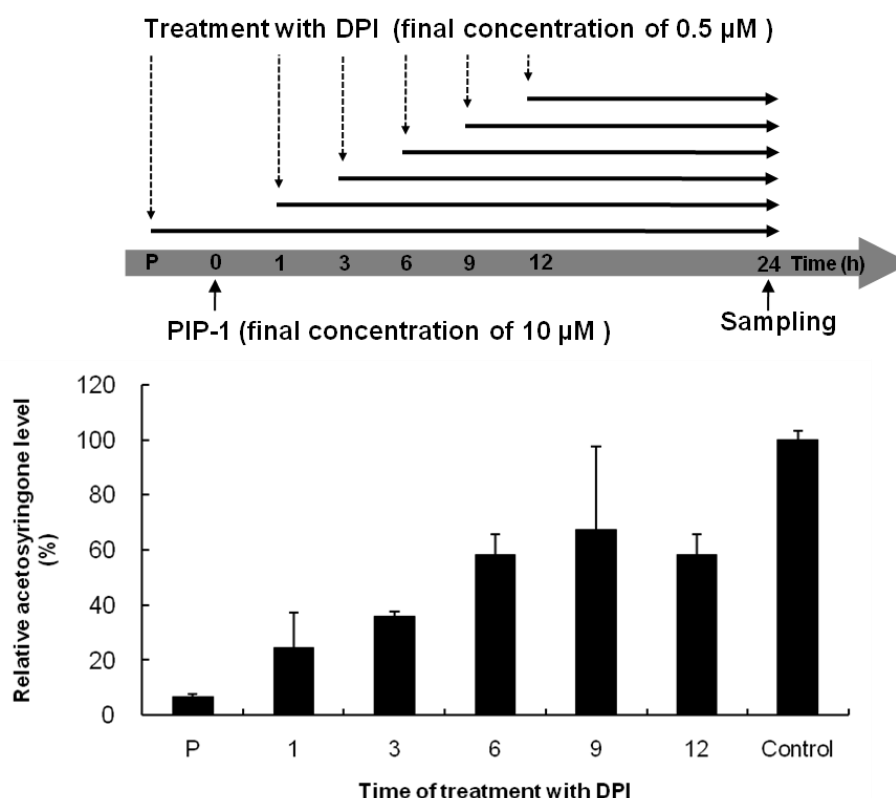


Figure 4-4. Inhibitory effect of DPI on acetosyringone accumulation. DPI was applied to tobacco cells either 20 min prior to treatment with PIP-1 or 1, 3, 6, 9, and 12 h after treatment with PIP-1, and all samples were collected at 24 h after treatment with PIP-1. 10 μ M of PIP-1 treatment without inhibitor was used as a control, and set to 100%. Vertical bars indicate $\pm$ S.D. of three independent experiments.

establishment of down-stream defense responses (Zhao et al., 2005). To investigate early signaling events important for acetosyringone accumulation, effects of several inhibitors on acetosyringone accumulation were examined. Pre-treatment with K252a; a general inhibitor of kinases, LaCl₃; a calcium channel blocker, and DPI; an inhibitor of oxidative burst via inhibition of NADPH oxidase, efficiently suppressed the PIP-1-induced acetosyringone accumulation (Figure 4-3A). These inhibitors also suppressed the H₂O₂ generation, one of ROS produced during oxidative burst, induced by PIP-1 (Figure 4-3B). ROS are generated by various environmental stimuli including pathogens or elicitors (Apel and Hirt, 2004; Baxter et al., 2014; Mehdy, 1994). NADPH oxidase-dependent ROS are considered to be a major source of ROS in plant defense responses (Marino et al., 2012). NADPH oxidases release a superoxide via reduction of oxygen into the extracellular space, and then superoxide is converted to H₂O₂ following enzyme-catalyzed dismutation (Lamb and Dixon, 1997).

Alternatively, peroxidase-derived H_2O_2 is proposed to be the other source of H_2O_2 during oxidative burst (Apel and Hirt, 2004). However, pre-treatment with KCN, an inhibitor of peroxidases, did not suppress the H_2O_2 generation at all (data not shown), and H_2O_2 generation was completely suppressed by pre-treatment with DPI, indicating that the ROS generated in this study is mostly mediated by the NADPH oxidase. Since activation of NADPH oxidases is the down-stream event after MAP kinase activation and Ca^{2+} influx, NADPH oxidase activity might be closely associated with acetosyringone accumulation.

Next, inhibitory effect of DPI on acetosyringone accumulation was more intensively examined. In this experiment, DPI was applied to tobacco cells either 20 min prior to treatment with PIP-1 or 1, 3, 6, 9, and 12 h after treatment with PIP-1, and all samples were collected at 24 h after initiation of elicitation with PIP-1. As shown in Figure 4-4, inhibitory effect of DPI was observed up to 12 h time point, although its effects were gradually declined. This indicated that NADPH oxidase-mediated oxidative burst affects relatively longer time after elicitation to accumulate acetosyringone despite the fact that short-term stimulation with PIP-1 is enough for the induction. As shown in Chapter 2, H_2O_2 generation induced by PIP-1 was not detected after 3 h, suggesting that H_2O_2 may not be a signal molecule to induce acetosyringone accumulation.

Assessment of the importance of H_2O_2 on acetosyringone accumulation

PIP-1 induced a monophasic oxidative burst in suspension-cultured tobacco cell, in which the level of H_2O_2 was reached a maximal level after 30 min and gradually decreased to the basal level 3 h after treatment, as described in Chapter 2. To assess the importance of extracellular H_2O_2 in acetosyringone accumulation induced by PIP-1, the author investigated the effect of exogenous addition of H_2O_2 and CAT on acetosyringone accumulation. CAT converts H_2O_2 into H_2O . Incubation with exogenous H_2O_2 alone for 24 h did not induce acetosyringone accumulation in the incubation medium of tobacco cells (Figure 4-5). Co-treatment with PIP-1 and H_2O_2 also had no effect on the level of acetosyringone accumulation. Likewise, no significant change in the level of acetosyringone accumulation induced by PIP-1 was observed when CAT was added to the medium to remove endogenous H_2O_2 (Figure 4-S1A). When both PIP-1 and H_2O_2 was concurrently added to the tobacco cells pre-treated with DPI, which inhibits H_2O_2 generation induced by PIP-1, no acetosyringone accumulation was observed. These data indicated that acetosyringone accumulation is not affected by the presence of H_2O_2 in the extracellular space during defense responses, and that

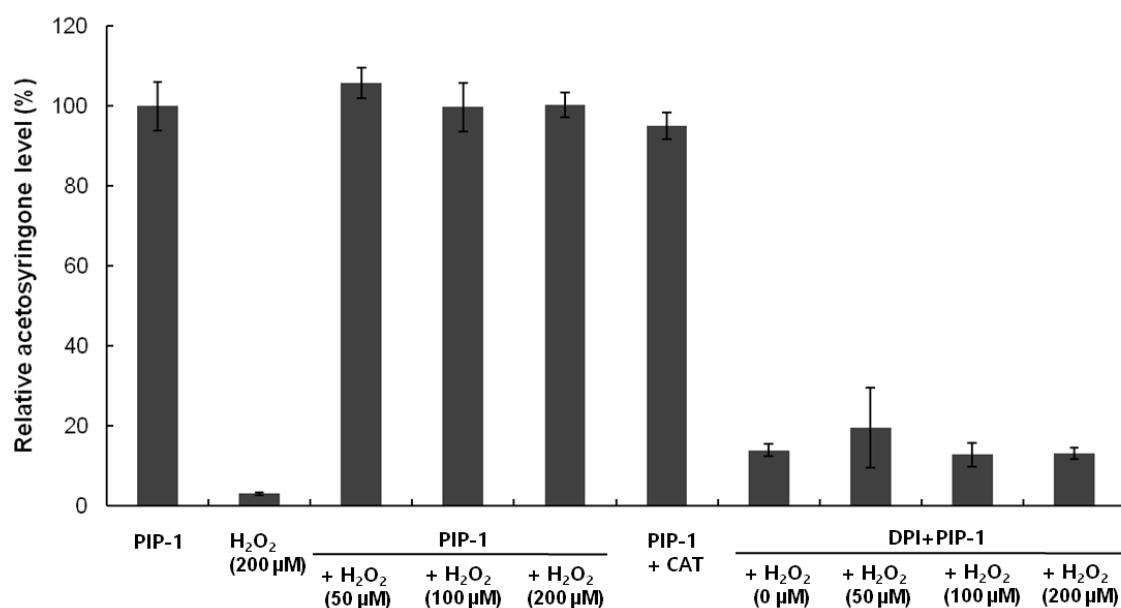


Figure 4-5. Effect of extracellular H₂O₂ status on acetosyringone accumulation induced by PIP-1 in incubation medium of tobacco cells. PIP-1 (10 μM) was added into suspension-cultured tobacco cells with H₂O₂ (50, 100, and 200 μM), and CAT (100 U/mL). DPI (0.5 μM) was added into suspension-cultured tobacco cells 20 min prior to treatment with PIP-1. Vertical bars indicate ± S.D. of three independent experiments.

H₂O₂ does not play a role as a second messenger in signaling events to induce accumulation of acetosyringone following recognition of PIP-1.

Among ROS generated during oxidative burst, H₂O₂ is a diffusible element across the plasma membrane and was reported to play a role as a signal molecule in the intracellular space to activate plant defense responses (Apel and Hirt, 2004; Desikan et al., 1999; Levine et al., 1994; Neill et al., 2002). In suspension-cultured *Arabidopsis* cells, exogenous H₂O₂ was reported to induce expression of *PAL* and *glutathione S-transferase* genes (Desikan et al., 1998). In tomato, H₂O₂ was shown to be important for induction of expression of late defense-related genes (Orozco-Cárdenas et al., 2001). In the present study, however, exogenous H₂O₂ did not induce acetosyringone accumulation, and co-treatment with PIP-1 and H₂O₂ also did not show any enhancement in acetosyringone accumulation. Although H₂O₂ has been implicated either as an inducer or as a signal molecule in the plant defense responses, it is not likely to be directly linked to the signaling pathway leading to acetosyringone accumulation in tobacco cells. Several reports demonstrated that the superoxide generated during oxidative burst induces defense responses such as phytoalexin

synthesis (Jabs et al., 1997; Perrone et al., 2003). It is possible that accumulation of acetosyringone induced by PIP-1 is activated via ROS signaling, in which superoxide acts as a signal molecule rather than H₂O₂ (Jabs et al., 1997; Tang and Smith, 2001; Xu and Dong, 2005).

Regulation of acetosyringone production through phenylpropanoid pathway

To investigate the regulation mechanism of acetosyringone accumulation, the significance of PAL, which catalyzes the first step of the phenylpropanoid pathway, in acetosyringone accumulation was evaluated. Effects of a PAL inhibitor, AOPP, and a protein synthesis inhibitor, CHX, on acetosyringone accumulation induced by PIP-1 were examined. Acetosyringone accumulation induced by PIP-1 was significantly suppressed by pre-treatment with both AOPP and CHX (Figure 4-6A). This suggests that acetosyringone is synthesized via the phenylpropanoid pathway. Next, effects of inhibitors including DPI, K252a, AOPP and CHX on the expression of *PAL*, *COMT*, and *CCoAOMT* genes were examined. Expression levels of *PAL*, *COMT*, and *CCoAOMT* transcripts were increased in response to PIP-1. Pre-treatment with DPI, AOPP, and CHX except K252a failed to suppress the expression of these genes (Figure 4-6B). The fact that K252a showed the inhibitory effect suggests that the MAP kinase cascade is associated with the regulation of the expression of these genes. Then, effects of inhibitors on PAL activity were examined. PIP-1 induced the significant level of PAL activity, but pre-treatment with DPI as well as the other inhibitors suppressed the activity to near basal levels (Figure 4-6C). This suggests that PAL activity is regulated downstream of NADPH oxidase.

In plants, phenylpropanoid biosynthetic pathway comprises an enormous pool of phenolic compounds involved in secondary metabolisms in response to biotic and abiotic stress (Vogt, 2010). It is initiated from the enzyme named PAL catalyzing phenylalanine into cinnamic acid (Dixon and Paiva, 1995), and transcription and enzyme activity of PAL is sensitively up-regulated in response to various elicitors (Amelot et al., 2011; El Modafar et al., 2001; Gao et al., 2012; Rajasekhar et al., 1999; Tanaka et al., 2003; Xu and Dong, 2005). In the present study, pre-treatment with AOPP and CHX significantly suppressed the acetosyringone accumulation induced by PIP-1 (Figure 4-6A), suggesting that PAL activity regulates the acetosyringone biosynthesis.

The COMT and CCoAOMT have been known as a key enzyme involved in methylation step of their substrates to ultimately synthesize monolignols in plants, in which

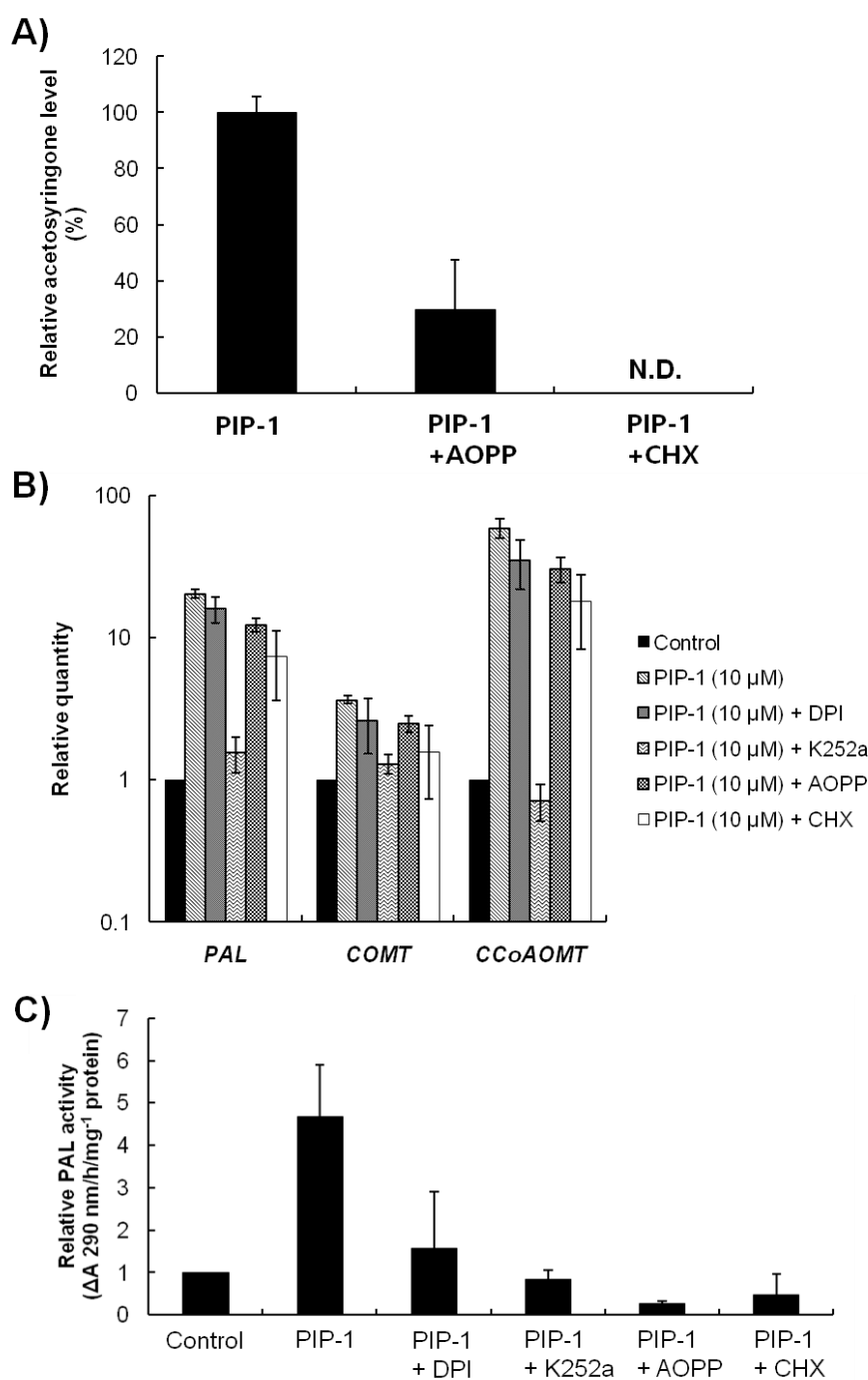


Figure 4-6. Phenylpropanoid pathway induced by PIP-1. A) Inhibitory effect of AOPP and CHX on acetosyringone accumulation. B) Relative transcript levels of *PAL*, *COMT*, and *CCoAOMT* in tobacco cells 4 h after treatment with PIP-1. C) PAL activity in tobacco cells 4 h after treatment with PIP-1. All of inhibitors (0.5 μM of DPI, 1 μM of K252a, 100 μM of AOPP, and 10 μg/mL of CHX) were applied 20 min prior to treatment with PIP-1(10 μM). Vertical bars indicate $\pm$ S.D. of three independent experiments. N.D.; not detected.

caffeoyl-CoA is a one of substrates to these enzymes, and converted into feruloyl-CoA (Boerjan et al., 2003). Up-regulation of *COMT* and *CCoAOMT* was observed in tobacco leaves infected by pathogen, coincided with those protein accumulations and elevation of activity (Maury et al., 1999), and inhibition of these gene expression suppressed acetosyringone accumulation after co-treatment with both wound and pathogen infection in tobacco (Maury et al., 2010). These reports was supported by the fact that acetosyringone biosynthesis is started from feruloyl-CoA as a distal precursor *in vitro* (Negrel and Javelle, 2010). In the present study, *COMT* and *CCoAOMT* were up-regulated by PIP-1. However, their transcription was significantly inhibited by pre-treatment with K252a, but not with DPI even though acetosyringone accumulation was inhibited by both K252a and DPI. It is suggested that transcription of *COMT* and *CCoAOMT* could be regulated by MAP kinase cascades, but not by oxidative burst. Although the effect of NADPH oxidase-derived ROS on enzymatic *O*-methylation step remains to be elucidated, it is likely that that PAL could be rate-limiting step in biosynthesis of acetosyringone, which is regulated via NADPH oxidase-derived oxidative burst.

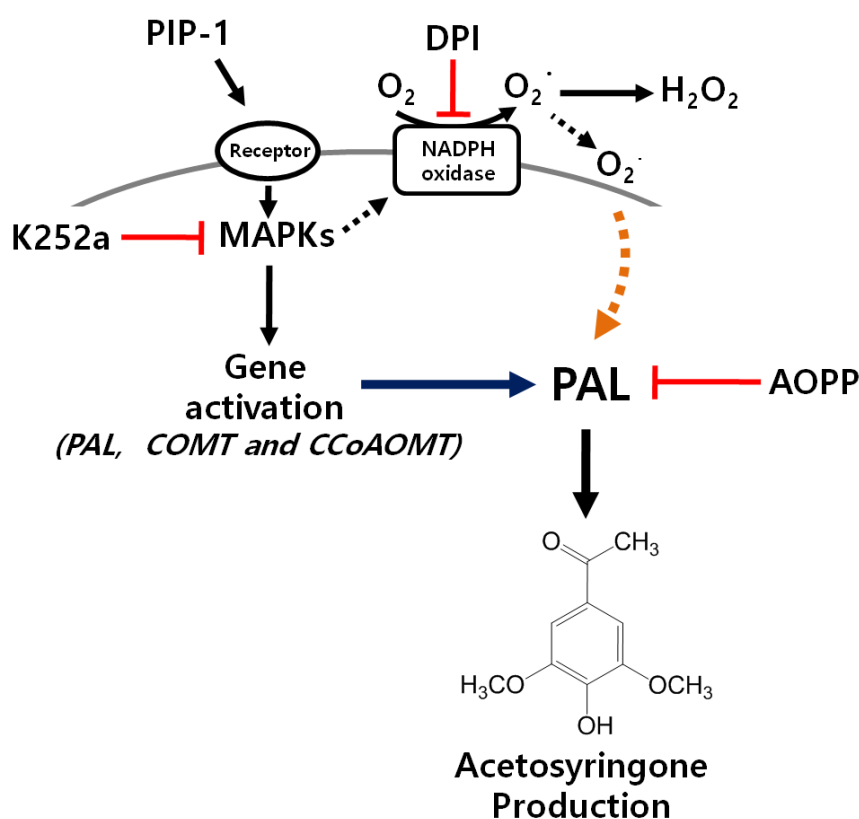


Figure 4-7. Hypothesized model for induction mechanism of acetosyringone accumulation

Based on the results obtained in this study, the author proposes the hypothetic model of the induction mechanism of acetosyringone production (Figure 4-7). Initiation of acetosyringone accumulation requires elicitor-induced early signaling events such as activation of MAP kinase cascades and oxidative burst, which depend on short-term elicitor stimulation. Specifically, NADPH oxidase-derived ROS are likely to play a crucial role in activation of the first enzymatic step, PAL, in the phenylpropanoid pathway toward acetosyringone biosynthesis.

Acetosyringone as an antioxidant

Excess level of extracellular H_2O_2 may have a deleterious effect on plant cell growth even if it plays an important role in defense responses. In this regard, acetosyringone is suggested to act as a reductant in peroxidase-mediated H_2O_2 scavenging system. To assess the importance of acetosyringone as a redox control agent, the relationship between the H_2O_2 scavenging capacity of the incubation medium and the amount of acetosyringone was investigated. The amount of acetosyringone accumulated in the incubation medium 24 h after treatment with PIP-1 was decreased after incubation with exogenous H_2O_2 for 1 h in the absence of tobacco cells (Figure 4-S1B). This is consistent with the previous results reported by Baker et al. (2014). The H_2O_2 scavenging capacity of the extracellular fluid gradually increased following longer incubation with PIP-1 (Figure 4-S1C), whereas the amount of phenolic compounds derived from tobacco cells decreased (Figure 4-S1D). Taking these data into consideration, it is possible that accumulated acetosyringone in early phase could be used as an antioxidant to scavenge H_2O_2 .

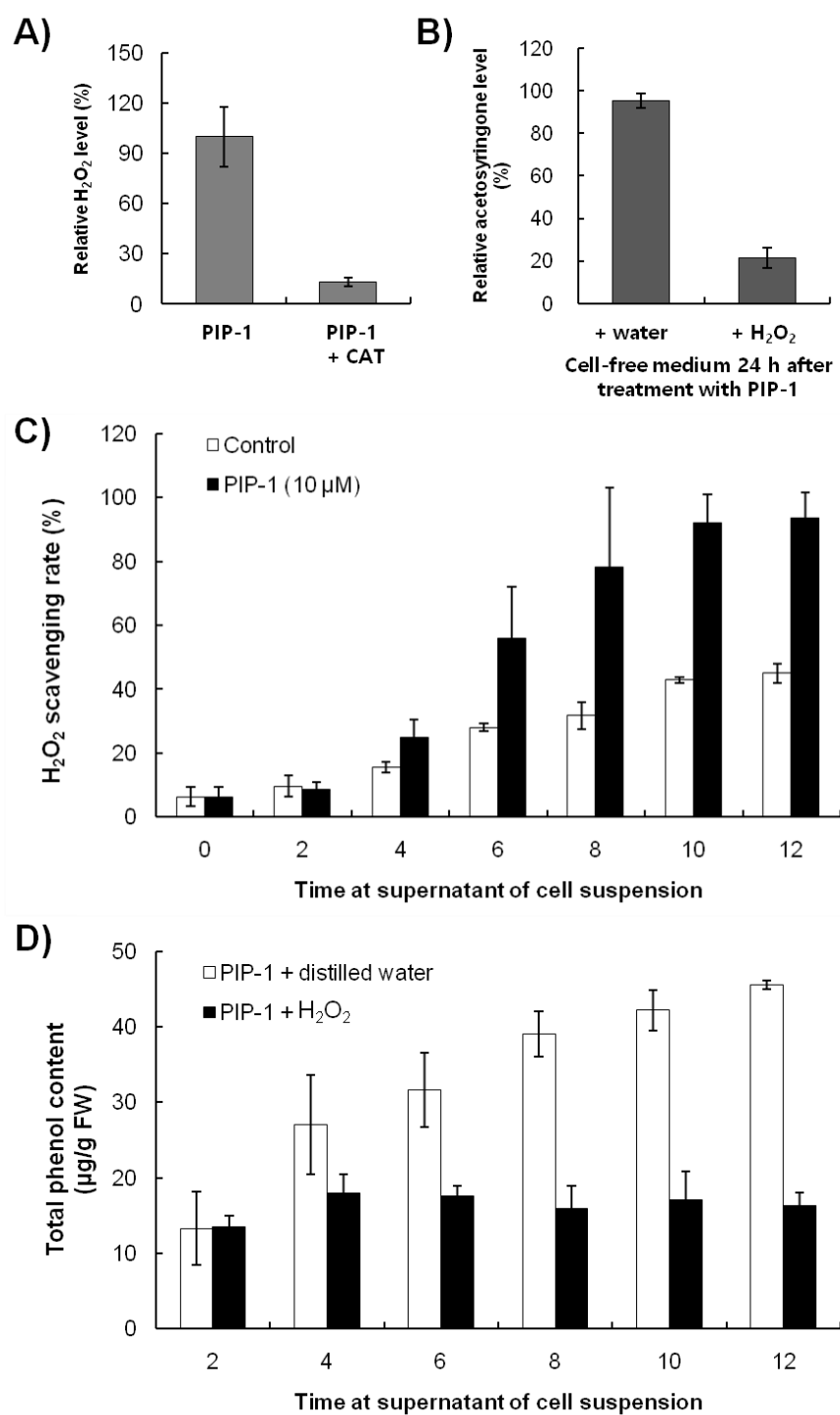


Figure 4-S1. A) H₂O₂ generation induced by 10 μM of PIP-1 in the presence of CAT. B) Effect of H₂O₂ on acetosyringone accumulated in incubation medium. The medium was additionally incubated for 1 h with exogenous H₂O₂ in the absence of tobacco cells. C) H₂O₂ scavenging activity of extracellular medium. D) Effect of exogenous H₂O₂ on total phenol content in the extracellular medium after treatment with PIP-1 (10 μM). Vertical bars indicate ± S.D. of three independent experiments.

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Summary

Chapter 1

Peptide elicitor PIP-1 induces various immune responses in tobacco cells. However, types of immune responses induced are different based on the concentration of PIP-1. Since PIP-1 was rapidly hydrolyzed from its C-terminal end in the incubation medium, it was speculated that a decrease in its concentration due to degradation is likely to be a primary reason for this difference. To examine the potential impact of such degradation on induction of late-phase immune responses, degradation-resistant PIP-1 analogs were designed using several peptide modification strategies: substitution of the C-terminal residue, N-methylation of the amide bond, and introduction of an additional residue at the C-terminus. The newly synthesized PIP-1 analogs exhibited outstanding degradation resistance in the presence of tobacco cells without loss of biological activity. This result indicated that the C-terminal His residue is recognized by proteases secreted from tobacco cells, and that enzymatic stability of PIP-1 is effectively improved by modification at the C-terminal residue.

Chapter 2

To evaluate the effect of PIP-1 degradation on plant immune response induction, the responses induced by PIP-1 were compared with those by the degradation-resistant analog MePIP-1. H₂O₂ generation and defense-related gene expressions in tobacco cells induced by MePIP-1 were similar to those induced by PIP-1. However, significant differences were observed between PIP-1 and MePIP-1 in the late-phase immune responses, such as capsidiol production and HR-like cell death, in which the concentration of MePIP-1 10-times lower than that of PIP-1 was enough to induce those responses. Further investigation of the mechanism of action of MePIP-1 showed that continuous elicitor stimulation for 3-6 h was required to initiate capsidiol production, which coincided with long-lasting MAP kinase activation. Thus, the capsidiol production is likely to be regulated either by post-translational regulation or changes in metabolic flux, which is associated with long-lasting MAP kinase activity induced by continuous stimulation with MePIP-1. On the other hand, an expression pattern of phytoalexin biosynthesis-related genes were similar between MePIP-1 and PIP-1.

Up-regulation of these gene expressions was not observed when tobacco cells were pretreated with kinase inhibitor, suggesting that the transient MAP kinase activation induced by early short stimulation with PIP-1 may be sufficient for transcription of these genes.

Chapter 3

To investigate temporal factors governing the induction of capsidiol production, the activity of the PIP-1 must be “switched off” at specific time points. In the experiments performed in Chapter 2, the cells were thoroughly washed with fresh media to halt the stimulation by PIP-1, which may provide undesired effects on the cells due to mechanical stress. In this chapter, a photocleavable PIP-1 analog (PcPIP-1) was synthesized using substitution of the Thr residue of another degradation-resistant analog [Asp⁶]PIP-1 with a photocleavable amino acid to “switch off” the activity by UV-irradiation at any time point. PcPIP-1 was highly resistant to enzymatic degradation by tobacco cells and induced a significant level of phytoalexin capsidiol production unless UV-irradiated. This response was effectively diminished after cleavage of the analog by UV-irradiation, and no negative effects of UV-irradiation were observed. Using this photocleavable analog, it was found that the prolonged activation of a MAP kinase, induced by the continuous action of PIP-1 caused an elevation of HMGR activity, which is likely to be associated with the initiation of the capsidiol production.

Chapter 4

Acetosyringone, one of the representative extracellular phenolics in suspension-cultured tobacco cells, was accumulated in the incubation medium after PIP-1 treatment at the concentration where no capsidiol production was induced. When PIP-1 was removed by washing cells 1 h after elicitation, a significant level of acetosyringone was still induced, indicating that short-term stimulation is sufficient for the initiation of acetosyringone production. Based on the inhibitor studies, it was found that early signaling events induced by PIP-1, such as activation of kinase cascades and induction of oxidative burst, are closely associated with initiation of acetosyringone production. On the other hand, it was revealed that acetosyringone accumulation was involved in the phenylpropanoid synthetic pathway

where some of the biosynthesis-related enzymes seem to be activated by the elicitation with PIP-1. In particular, the activity of PAL, an initial enzyme in the phenylpropanoid synthetic pathway, was shown to be regulated by NADPH oxidase-mediated ROS signals. These results suggested that NADPH oxidase-mediated oxidative burst plays a crucial role in acetosyringone accumulation through activation of PAL in the phenylpropanoid pathway.

List of Publications

1. **Yonghyun Kim**, Eriko Komoda, Masahiro Miyashita, and Hisashi Miyagawa, Continuous stimulation of the plant immune system by the peptide elicitor PIP-1 is required for phytoalexin biosynthesis in tobacco cells, *J. Agric. Food Chem.* 62: 5781–5788, 2014

- Chapter 1 (in part) and Chapter 2

2. **Yonghyun Kim**, Masahiro Miyashita, and Hisashi Miyagawa, Investigation of temporal factors regulating phytoalexin biosynthesis in tobacco cells using a photocleavable PIP-1 analog (*in preparation*)

- Chapter 1 (in part) and Chapter 3

3. **Yonghyun Kim**, Masahiro Miyashita, and Hisashi Miyagawa, Elucidation of signaling factors important for acetosyringone accumulation induced by peptide elicitor PIP-1 (*in preparation*)

- Chapter 4

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