Acquired resistance to the rice blast in transgenic rice accumulating the antimicrobial peptide thanatin

Tomohiro Imamura[a,b,f], Michiko Yasuda[c], Hiroaki Kusano[a], Hideo Nakashita[c], Yuko Ohno[e], Takashi Kamakura[e], Seiichi Taguchi[d] and Hiroaki Shimada[a,b,*]

[a]*Department of Biological Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda 278-8510, Japan*

[b]*Research Institute of Science and Technology, Tokyo University of Science, 2641 Yamazaki, Noda 278-8510, Japan*

[c]*Plant Acquired Immunity Research Unit, Advanced Science Institute, RIKEN, Wako, Saitama 351-0198, Japan*

[d]*Division of Biotechnology and Macromolecular Chemistry, Graduate School of Engineering, Hokkaido University, Sapporo 060-0810, Japan*

[e]*Department of Applied Biological Science, Tokyo University of Science, 2641 Yamazaki, Noda 278-8510, Japan*

[f]*Present address: Iwate Biotechnology Research Center, Kitakami 024-003, Japan*

[*]Corresponding author. Fax: +81 4 7125 1841.

E-mail address: shimadah@rs.noda.tus.ac.jp (H.Shimada).

Abstract

Thanatin is an antimicrobial peptide with a strong and wide-ranging antimicrobial spectrum, including certain species of fungi and Gram-negative and -positive bacteria. To evaluate the application of thanatin to the generation of disease-resistant plants, we introduced a synthetic thanatin gene into rice. Several transformants that expressed the introduced gene showed significant level of antimicrobial activity. The substances showing antimicrobial activity were partially purified from these transformants and their properties were determined. The molecule with characteristics similar to those of native thanatin on the elution pattern in HPLC analysis had an identical molecular mass to that of native molecule. It should also be noted that the transformant acquired a sufficient level of resistance to the rice blast fungus, *Magnaporthe oryzae*, presumably due to the repressive activity of thanatin to its initial stage of infection. This result demonstrates that thanatin has antifungal activity for *M. oryzae* and that the introduction of the thanatin gene into rice is effective in generating a plant resistant to rice blast disease.

Keywords: Antimicrobial peptide; Thanatin; *Oryza sativa*; Rice blast; Disease resistance; Molecular farming

INTRODUCTION

Antimicrobial peptides (AMPs) are widely spread throughout a variety of multicellular organisms, including primitive invertebrates, higher plants and animals, and are considered important major components of their natural immunity against invading pathogens (Hancock and Lehrer 1998). At present, more than 880 AMPs have been identified in many kinds of insects, mammals and plants (Brogden 2005). The antimicrobial mechanism has been proposed on many AMPs. Their peculiar characteristics allow them to attach to and insert into membrane layers to form pores. Formation of transmembrane pores is not the only mechanism of microbicidal activity but also causing alteration of cytoplasmic membrane septum formation, or inhibition of cell-wall synthesis, nucleic-acid synthesis, protein synthesis and enzymatic activity (Brogden 2005; Marcos et al. 2008; Zasloff 2002). These antimicrobial mechanisms are quite different from those of currently distributed conventional antibiotics. Therefore, it is expected that AMPs will be sources of new type of antibiotics.

Every AMP consists of a short peptide, which is translated from the corresponding gene, and AMPs are considered to be easily produced with recombinant DNA techniques. However, the production of AMPs is quite difficult in recombinant bacteria, because AMPs are extremely harmful to any bacterial host strain that is commonly used as the hosts for industrial production. Therefore, AMPs are currently produced in a modified and inactive form in recombinant bacteria (Taguchi et al. 2000).

Plants are becoming a common platform for production of recombinant proteins such as industrial enzymes and pharmaceuticals (Daniell et al. 2001; Kusnadi et al. 1997). A production system using recombinant plants potentially offers great advantages over those based on microorganisms or animal cells when foreign gene products are produced, because it can be applied to traditional agricultural system at low cost (Twyman et al. 2003). In

addition, plant system can produce molecules that cannot be produced in bacterial systems, such as antibiotics and AMPs. Furthermore, the production of AMP in plant is expected to provide an acquired resistance to plant disease caused by fungi or bacteria.

Thanatin, produced in the stinkbug, the hemipteran insect *Podisus maculiventris*, is an AMP consisting of 21 amino acid residues (GSKKPVPIIYCNRRTGKCQRM) with a molecular weight of 2.4 kDa (Fehlbaum et al. 1996). Thanatin is categorized as a β-hairpin-type of AMP, which contains an internal disulfide bond. The disulfide bond forms a loop rich in cationic amino acid residues and resultant secondary structure is important for the antimicrobial activity of thanatin (Taguchi et al. 2000).

Rice blast disease, caused by *Magnaporthe oryzae* B. Couch (anamorph *Pyricularia oryzae* Cavara), is regarded as a worldwide problem because it often results in the serious deterioration of crop yield and quality (Baker et al. 1997; George 1997). To protect rice from a pathogen attack, a genetically engineered plant containing a disease-resistance gene is proposed as a tool for directional improvement. In the last decade, acquired resistance to plant disease caused by a various pathogens, including fungi and bacteria, can be achieved by the introduction of AMPs into transgenic plants (Coca et al. 2006; Mitsuhara et al. 2000; Osusky et al. 2004; Sharma et al. 2000; Yevtushenko et al. 2007). Thanatin has a wide-ranging antimicrobial spectrum, which includes many kinds of bacteria and fungi (Fehlbaum et al. 1996). It is expected that thanatin can be used as a powerful antimicrobial substance in this system. Here, we report the generation of transgenic rice with acquired resistance to blast disease by the introduction of a recombinant thanatin gene.

Materials and Methods

Plasmid construction

The thanatin gene, consisting of the native nucleotide sequence, has been chemically synthesized and cloned in a plasmid, pU-tan (Taguchi et al. 2000). The coding region of the thanatin gene was prepared as an amplified fragment by polymerase chain reaction (PCR) from pU-tan using the primers 5′-GAATCCATGGGCTCCAAGAA-3′ and 5′-GTAAAACGACGGCCAGT-3′, which are located in the region that includes the initiation codon and the region downstream from the termination codon, respectively. An *Nco*I site was generated at the initiation codon in the amplified fragment and a *Bam*HI site was placed downstream from the termination codon. The amplified fragment was digested with *Nco*I and *Bam*HI, then inserted into the *Nco*I and *Bam*HI sites of the plasmid, pBSM61, which was modified from pBluescript II KS+ (Toyobo, Osaka, Japan) by insertion of an *Nco*I site between the *EcoR*I and *Hind*III sites. A fragment corresponding to the thanatin gene was prepared from the resultant plasmid by its digestion with *Nco*I and *BamH*I, and was then introduced into the *Nco*I and *Bgl*II sites downstream from the cauliflower mosaic virus 35S (CaMV35S) promoter of the plant expression plasmid pCAMBIA1302 (CAMBIA, Canberra, Australia). The resultant plasmid contained a non-coding sequence downstream the termination codon of the thanatin gene prior to the Nos terminator (Fig. 1A).

Plant materials and growth conditions

Oryza sativa L. cv. Nipponbare was used as the wild-type plant. Seeds were germinated at 30°C in a dark chamber. The seedlings were grown under continuous light (13 μmol m^{-1} s^{-1})

for three days. The rice plants were cultivated in a growth chamber with 80% humidity under a long day condition, with a cycle of 14 h light (30°C) (270 μmol m^{-1} s^{-1}) and 10 h dark (25°C).

Plant transformation

Rice (*Oryza sativa* L. Cv. Nipponbare) was transformed with the *Agrobacterium*-mediated method (Hiei et al. 1994). Transformants were grown on 1/2 Murashige-Skoog plates (Murashige and Skoog 1962) supplemented with 50 mg L^{-1} hygromycin B (Wako Pure Chemicals, Osaka, Japan) to screen for hygromycin-resistant lines before thier cultivation in soil. The plants were grown in a growth cabinet or green chamber. The gene introduced into the transformants was confirmed by PCR using the primer set 5′-GAATCCATGGGCTCCAAGAA-3′ (P1) and 5′-ATGTCATCCATGCCATGTGT-3′ (P2) for *thanatin* gene. Genomic DNA was extracted according to the method of Murray and Thompson (1980). The transgene was detected by DNA blot analysis according to the method as previously described (Ausubel et al. 1987).

RNA isolation and semiquantitative reverse transcription (RT)-PCR

Total RNA was extracted from rice leaves as described previously (Imamura et al. 2007). The first-strand cDNA was synthesized from 1 μg of the total RNA using a ReverTra-Ace cDNA synthesis kit (Toyobo, Osaka, Japan) with an oligo-dT(20) primer. Semiquantitative RT-PCR was performed using the GeneAmp® PCR system 9700 (Applied Biosystems, CA, USA). The procedure for amplification of the thanatin transcript comprised initial denaturation at 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 30 s. The

amplification protocol for *Actin I* (acc. no. X16280) transcript included initial denaturation at 94°C for 2min followed by 30 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 30 s. The *thanatin* mRNA was quantified relative to the amount of *Actin I* mRNA. The primers used for semiquantitative RT-PCR were 5′-CCCTCCTGAAAGGAAGTACAGTGT-3′ and 5′-GTCCGAAGAATTAGAAGCATTTCC-3′ for *Actin I,* and 5′-GAATCCATGGGCTCCAAGAA-3′ (P1) and 5′-ATGTCATCCATGCCATGTGT-3′ (P2) for *thanatin*.

Bioassay of antimicrobial activity

Antimicrobial activity was determined as previously described, using *Micrococcus luteus* in 96-well microtiter plate, to which were added 10 μg of crude plant extract or 10 μl of the high-performance liquid chromatography (HPLC) fraction and 90 μl of assay medium (1% Bacto tryptone [Difco, Detroit, USA], 0.2% Bacto yeast extract [Difco], 0.5% NaCl, 0.5% glucose, and 1% beef extract [Kyokuto Pharmaceutical Industrial, Tokyo, Japan]) (Imamura et al. 2008). They were diluted by the assay medium as necessary. Approximately 300 cells of *M. Luteus* were added to every well for each assay, and the cells were incubated at 30°C for two days. The antimicrobial activity was estimated as the degree of growth inhibition of *M. Luteus* (Fehlbaum et al. 1996).

Purification and identification of thanatin in the transformants

Crude extract was prepared from rice leaves (1 g fresh weight) by grounding them to fine powder in liquid nitrogen, followed by sonication in extraction buffer (50 mM Tris-HCl [pH 7.5], 1 mM EDTA [pH 7.5], 5 mM β-mercaptoethanol, 400 mM NaCl, 15% glycerol, 1%

Protease Inhibitor Cocktail [SIGMA, St. Louis, MO, USA]). The resultant cell extracts were centrifuged at 17,400 × g for 30 min at 4°C, and the collected supernatant was filtered through a 0.45 μm filter (Millipore, MA, USA) to remove insoluble materials. The thanatin was then purified by cation exchange chromatography using HiTrap-CM FF (GE Healthcare), followed by reversed-phase HPLC on a 5C18-ARII reversed-phase column (Nacalai tesque, Kyoto, Japan). Stepwise elution from the cation exchange column was performed with sequential concentrations of 0 mM, 100 mM, 250 mM, and 1000 mM NaCl in 50 mM phosphate buffer. After each fraction was desalted by ultrafiltration, the antimicrobial activity was measured with the bioassay described above. The fractions containing antimicrobial substances were condensed in a centrifugal concentrator (CC-105, Tomy Seiko Inc., Tokyo, Japan). The antimicrobial materials were separated by HPLC on a reversed-phase column, with a combined solution of solvent A (0.1% trifluoroacetic acid [TFA] in H_2O) and solvent B (0.1% TFA in acetonitrile) as the mobile phase. The gradient system of these solvents was: 0% to 20% solvent B (5 min), then 20% to 30% solvent B (50 min), and 30% to 100% solvent B (5 min). The flow rate was 0.5 mL/min and the elution was monitored by the absorbance at 225 nm. The fraction were collected every 3 min between 36 min and 60 min during the HPLC program. The antimicrobial activity of each fraction was analyzed against *M. luteus*. The fraction with the antimicrobial activity was concentrated with a centrifugal concentrator, then resolved in water. The structure of the antimicrobial substance was determined by electrospray ionization-mass spectrometry (ESI-MS) (QuattroII, Micromass, Manchester, UK) according to the manufacturer's protocols.

Determination of antifungal activity to *M. oryzae*

Antifugal activity of thanatin to *M. oryzae* was determined according to the method described

previously (Kamakura et al. 2002). *M. oryzae* strain P2, a Japanese rice-pathogenic isolate, and chemically-synthesized thanatin (Imamura et al. 2008) were used. Conidiation was induced on an oatmeal agar (Difco) plate under illumination by a 365 nm light for 5 days. Conidia formed were brushed off and suspended with distilled water. Polycarbonate plates were used for the appressorium formation assay. After the adjustment of the concentration of conidia to 1 x 10^6 conidia/ml, appropriate concentration of thanatin solution dissolved in sterile water was applied into the suspension of conidia of *M. oryzae*. The 20 µl each of conidial suspensions were dropped on the plates and incubated for 6 hours in a moistened transparent box under constant light. Appressorium formation was observed and counted. The data were statistically analyzed by using student t-test.

Pathogen-resistance assay

All the transformants and wild-type plant were cultured in pots containing 20 cm^3 of soil (5 plants per pot) in a greenhouse. The youngest leaf of the five-leaf-stage plant was detached and inoculated with *M. oryzae* (race 007) by applying 3 µl of conidial suspension (10^5 spores ml^{-1}) onto the surface. The inoculated leaves were kept under saturated humidity for seven days in a greenhouse (25°C). The disease index (0-4) was expressed in terms of the severity of the lesions (n=24) using a standard evaluation system (SES) by International Rice Research Institute (IRRI; http://www.knowledgebank.irri.org/SES.htm): 0, no lesion; 1, yellow lesion < 1 mm; 2, yellow lesion 1-3 mm; 3, yellow lesion 3-5 mm; 4, yellow lesion > 5 mm. The data were statistically analyzed by using student t-test.

Results

Generation of transgenic rice carrying the thanatin gene

The synthetic thanatin gene expressed under the CaMV35S promoter was introduced into rice (*Oryza sativa* L. *japonica* cv. Nipponbare). More than 30 transformants were obtained. DNA blot hybridization analysis showed that each of these transformants contained the transgene in their genomes (data not shown). Genomic PCR confirmed introduction of the desired fragment into the transformants (Fig. 1B). Semiquantitative RT-PCR indicated that the introduced *thanatin* gene was expressed in these transformants (Fig. 1C), and some transformants showed strong expression of the transgene. In this experiment, we detected an additional transcript, smaller than the predicted one, in addition to the expected (Fig. 1C). Nucleotide sequence analysis revealed that the smaller fragment was also derived from the transgene, and included the entire cording sequence of the thanatin gene. Since genomic PCR detected the single fragment corresponding to the introduced thanatin gene, it was suggested that unexpected splicing occurred in the downstream region of the thanatin-coding region. Therefore, it was suggested that the entire thanatin peptide was translated from both transcripts.

These transformants grew normally without any apparent morphological changes (Fig. 1D), and set sufficient amounts of seeds as likely on the vector control plant transformed by pCAMBIA1302 (Fig. 1E). From these, we chose several transformants that showed a high level of expression of the thanatin gene, and established T3 homozygous lines from their progenies.

Analysis of thanatin produced in the transformants

We detected an apparent antimicrobial activity against *M. luteus* in crude extracts of the

transformants (5, 9, 16, 21 and 26) (Fig 2A). We partially purified the antimicrobial substances in the progenies of the rice transformants (lines 5-1 and 9-5, the progenies of lines 5 and 9, respectively) by the cation exchange chromatography. The antimicrobial substances were then further purified by C18-reverse phase HPLC. An apparent anti-microbial activity against *M. luteus* was detected in several HPLC fractions (Fig. 2B, C). The retention time of the peak for fraction 7 (54-57 min) in the HPLC analysis coincided with that of authentic thanatin. The fraction 5 (retention time: 48-51 min.) also showed an antimicrobial activity. Since none of the antimicrobial substance was detected in the corresponding fraction of the vector control, it was suggested that there was an unknown antimicrobial material being a presumable derivative of thanatin. The antimicrobial substance corresponding to the peak for fraction 7 was isolated by preparative HPLC and was subjected to the mass-spectroscopic analysis. As shown in Fig. 3, the signal for this substance was essentially identical to that for authentic thanatin. The molecular mass of the purified thanatin was 2436, which was considered to correspond to the calculated molecular size (2434). These results suggest that the recombinant thanatin produced by these transformants has the same structure as the native form. The yield of thanatin from the leaves of the transformant was estimated by HPLC analysis to be approximately 1 μg/g fresh cell weight.

Acquired resistance of the transformant to rice blast

It has not previously been reported that thanatin has antifungal activity against the rice blast pathogen *M. oryzae*, although this was expected because thanatin has wide-ranging activity against many kinds of fungi (Fehlbaum *et al*. 1996). As shown in Fig. 4, *in vitro* assay detected repression of appressorium formation of *M. oryzae* was observed in the presence of thanatin. This result indicates that thanatin has an antifungal activity to *M. oryzae*. Thanatin

also caused some inhibitory effects to the morphology on normal conidial germination. As shown in Fig. 4C, we observed the malformation on emerging germ tubes in inignorable frequency. Therefore, we performed a pathogen-inoculation assay to examine whether the transformant was resistant to the rice blast disease using the young leaves of the five-leaf-stage plants to test this assumption. Although disease areas were observed on both the transgenic lines and the wild-type plants, the sizes of the diseased areas on the transformants were significantly smaller than those on the wild-type plants (Fig. 5A). Transformants 9-5, in particular, showed strong anti-blast activity, inhibiting 50% of disease progression compared with that in the wild type (Fig. 5B). Thus, the areas affected by the symptom of rice blast were clearly reduced in the transformant. These results indicate that these transformants had the acquired resistances, which efficiently prohibited the development of pathogenicity of *M. oryzae*, although it did not fully protect the plants from pathogen infection.

Discussion

Rice blast causes white to gray lesions with dark green to brown borders, surrounded by yellowish halo on the leaves (George 1997). Thanatin is attractive AMP because of its strong and wide-ranging target spectrum, which includes species of fungi. In this study, we focus on the creation of plants resistant to fungus-triggered blast diseases in rice, which cause a serious reduction in farming activity. We transferred the thanatin gene into rice to produce resistance to rice blast. Our results indicated that transformants carrying the thanatin gene showed biological activity against *M. luteus* and a consequent resistance to blast disease.

The antifungal activity of thanatin against *M. oryzae* is a novel finding. Since our

transformants produced an active thanatin with the same structure of the authentic one, it is suggested that inhibitory effects on the conidial germination of *M. oryzae* may be derived from the product of the transgene in the transformants. The fact that the effective concentration was relatively high (more than 50 μM) is due to the *in vitro* assay condition taken in this study, because some other defensive reactions may occur *in vivo* on the surface of rice plants. Our results also suggest that thanatin is applicable to the generation of acquired resistance to blast disease in rice, because we have demonstrated that the introduction of *thanatin* gene into rice is effective in conferring an efficient tolerance to rice blast disease. Previously, many strategies have been proposed to improve plant disease resistance with recombinant DNA techniques. However, some transformants, in which the salicylic acid biosynthesis pathway or the ethylene and jasmonic acid signal pathways were enhanced, have often showed abnormal feature and severe reduction in their growth (Lorenzo et al. 2003; Mauch et al. 2001). Our strategy shows that transformant producing thanatin can effectively inhibit the growth of *M. oryzae* without any morphological changes in the transformants.

Until now, no β-hairpin-type AMP has been used to generate a disease resistant plant. Our findings indicate that thanatin, a β-hairpin-type AMP, is useful and powerful tool with which to create a plant tolerant to blast disease as easily as that previously reported for other types of AMPs, such as Sarcotoxin IA, MsrA3, MsrA2, temporinA and cecropin B (Coca et al. 2006; Mitsuhara et al. 2000; Osusky et al. 2004; Sharma et a.l 2000; Yevtushenko et al. 2007). The primary structure of the thanatin produced in the rice transformants appeared to be identical to the native form (Fig. 3). In conclusion, we achieved that a resistant rice plant to blast disease was created by introduction of the thanatin gene expression. It is evident that thanatin has very few harmful effects no host plant cells. We suggest that the introduction of an AMP gene into a plant is an attractive and effective way to establish a disease-resistant plant and to facilitate the molecular farming of that AMP in the transformant.

Acknowledgments We thank Ms. Yumiko Nakamura (Ebara Research Co., Ltd., Japan) and Dr. Kazunori Taguchi (RIKEN, Japan) for ESI-MS analysis. We also thank Dr. Ken′ichiro Matsumoto and our colleagues for many fruitful discussions.

References

Ausubel, F. M. *et al.* (1987) Current Protocols in Molecular Biology. John Wiley & Sons, Inc., New York.

Baker B, Zanmbryski P, Staskawicz B, Dinesh-Kumer SP (1997) Signaling in plant-microbe interactions. Science 276:726–733. doi: 10.1126/science.276.5313.726

Brogden KA (2005) Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat Rev Microbiol. 3:238-250. doi:10.1038/nrmicro1098

Coca M, Peñas G, Gómez J, Campo S, Bortolotti C, Messeguer J, Segundo BS (2006) Enhanced resistance to the rice blast fungus *Magnaporthe grisea* conferred by expression of a cecropin A gene in transgenic rice. Planta 223:392–406. doi:10.1007/s00425-005-0069-z

Daniell H, Streatfield SJ, Wycoff K (2001) Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. Trends Plant Sci 6:219–226. doi:10.1016/S1360-1385(01)01922-7

Fehlbaum P, Bulet P, Chemysh S, Briand JP, Roussel JP, Letellier L, Hetru C, Hoffmann JA (1996) Structure-activity analysis of thanatin, a 21-residue inducible insect defense peptide with sequence homology to frog antimicrobial peptides. Proc Natl Acad Sci USA 93:1221–1225

George NA (1997) Plant Disases Caused by Fungi. in: George NA (Ed.) Plant Pathology, 4th edn. Academic Press, San Diego, pp. 245–406

Hancock RE, Lehrer R (1998) Cationic peptides: a new source of antibiotics. Trends Biotechnol 16:82–88. doi:10.1016/S0167-7799(97)01156-6

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. Plant J 6:271–282. doi:10.1046/j.1365-313X.1994.6020271.x

Imamura T, Kusano H, Kajigaya Y, Ichikawa M, Shimada H A rice (2007) dihydrosphingosine C4 hydroxylase (*DSH1*) gene, which is abundantly expressed in the stigmas, vascular cells and apical meristem, may be involved in fertility. Plant Cell Physiol 48:1108–1120. doi:10.1093/pcp/pcm084

Imamura T, Yamamoto N, Tamura A, Murabayashi S, Hashimoto S, Shimada H, Taguchi S (2008) NMR based structure-activity relationship analysis of an antimicrobial peptide, thanatin, engineered by site-specific chemical modification: activity improvement and spectrum alteration. Biochem Biophys Res Commun 396:609–615. doi:10.1016/j.bbrc.2008.02.057

Kamakura T, Yamaguchi S, Saitoh K, Teraoka T, Yamaguchi I (2002) A novel gene, CBP1, encoding a putative extracellular chitin-binding protein, may play an important role in the hydrophobic surface sensing of Magnaporthe grisea during appressorium differentiation. Mol Plant Microbe Interact 15:437-444. doi: 10.1094/MPMI.2002.15.5.437

Kusnadi AR, Nikolov ZL, Howard JA (1997) Production of recombinant proteins in transgenic plants: practical considerations. Biotechnol Bioeng 56:473–484. doi:10.1002/(SICI)1097-0290(19971205)56:5<473::AID-BIT1>3.0.CO;2-F

Lorenzo O, Piqueras R, Sánchez-Serrano JJ, Solano R (2003) ETHYLENE RESPONSE FACTOR1 integrates signals from ethylene and jasmonate pathways in plant defense. Plant Cell 15:165–178. doi: 10.1105/tpc.007468

Marcos JF, Muñoz A, Pérez-Payá E, Misra S, López-García B (2008) Identification and rational design of novel antimicrobial peptides for plant protection. Annu Rev Phytopathol. 46:273-301. doi:10.1146/annurev.phyto.121307.094843

Mauch F, Mauch-Mani B, Gaille C, Kull B, Haas D, Reimmann C (2001) Manipulation of salicylate content in *Arabidopsis thaliana* by the expression of an engineered bacterial salicylate synthase. Plant J 25:67–77. doi:10.1111/j.1365-313X.2001.00940.x

Mitsuhara I, Matsufuru H, Ohshima M, Kaku H, Nakajima Y, Murai N, Natori S, Ohashi Y (2000) Induced expression of sarcotoxin IA enhanced host resistance against both bacterial and fungal pathogens in transgenic tobacco. Mol Plant Microbe Interact 13:860–868. doi: 10.1094/MPMI.2000.13.8.860

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 18:100–127. doi:10.1111/j.1399-3054.1962.tb08052.x

Murray MG, Tompson WF (1980) Rapid isolation of high-molecular-weight plant DNA. Nucleic Acids Res 8:4321–4325

Osusky M, Osuska L, Hancock RE, Kay WW, Misra S (2004) Transgenic potatoes expressing a novel cationic peptide are resistant to late blight and pink rot. Transgenic Res 13:181–190. doi: 10.1023/B:TRAG.0000026076.72779.60

Sharma A, Sharma R, Imamura M, Yamakawa M, Machii H (2000) Transgenic expression of cecropin B, an antibacterial peptide from *Bombyx mori*, confers enhanced resistance to bacterial leaf blight in rice. FEBS Lett 484:7–11. doi:10.1016/S0014-5793(00)02106-2

Taguchi S, Kuwasako K, Suenaga A, Okada M, Momose H (2000) Functional mapping against *Escherichia coli* for the broad-spectrum antimicrobial peptide, thanatin, based on an in vivo monitoring assay system. J Biochem 128:745–754

Twyman RM, Stoger E, Schillberg S, Christou P, Fischer R (2003) Molecular farming in plants: host systems and expression technology. Trends Biotechnol 21:570–578. doi:10.1016/j.tibtech.2003.10.002

Yevtushenko DP, Misra S (2007) Comparison of pathogen-induced expression and efficacy of two amphibian antimicrobial peptides, MsrA2 and temporin A, for engineering wide-spectrum disease resistance in tobacco. Plant Biotechnol J 5:720–734. doi:10.1093/jxb/eri165

Zasloff M (2002) Antimicrobial peptides of multicellular organisms. Nature 415:389–95.

doi:10.1038/415389a

Figure Legends

Fig. 1 *Thanatin* gene in rice transformants. (A) Schematic representation of the thanatin gene. A stop codon was introduced downstream from the open reading frame of the thanatin gene. The *thanatin* gene is followed by 727 bp of a non-coding sequence before the Nos terminator. The nucleotide sequence of the *thanatin* gene and the deduced peptide sequence were shown. RB: right border; LB: left border; HPT: hygromycin-resistance gene; 35S: CaMV 35S promoter; Thanatin: the coding region of the *thanatin* gene with its stop codon; NosT: terminator of the nopaline synthase gene; STOP: stop codon (TAG); P1 and P2: position of the PCR primers corresponding to forward and reverse primers for genomic PCR and RT-PCR, respectively. ”a” indicates the fragments detected by PCR and RT-PCR shown in the panel B and C, and ”b” indicates the fragment detected by RT-PCR shown in the panel C. (B) Detection of the introduced *thanatin* gene in transformants by genomic PCR. An arrow with ”a” indicates the 780 bp fragment corresponding to the *thanatin* gene. Numbers indicate the names of the transformant lines. PC: positive control; plasmid DNA with the introduced gene. VC: vector control transformed by pCAMBIA1302. (C) Detection by RT-PCR of transcript of the introduced *thanatin* gene in transformants. a: The 780 bp fragment for a full-length *thanatin* gene transcript. b: The 660 bp fragment indicates the *thanatin* gene transcript with unexpected splicing. Thanatin: *Thanatin* gene expression in plant; Actin I: *Actin I* gene expression in rice, used as a control. Numbers indicate the names of the transformant lines. WT: wild type plant. (D) Photographs of three weeks-old plants. WT: wild type; VC: vector control; TF: transformant expressing the introduced thanatin gene (line No. 5). (E) Fertility of the transformants harboring the thanatin gene. Fertility is estimated as the ratio of number of mature seeds in total flowers. WT, VC3 and VC8 indicate wild type plant, vector control line 3 and line 8, respectively. Each number indicates to the corresponding transformant line expressing the thanatin gene.

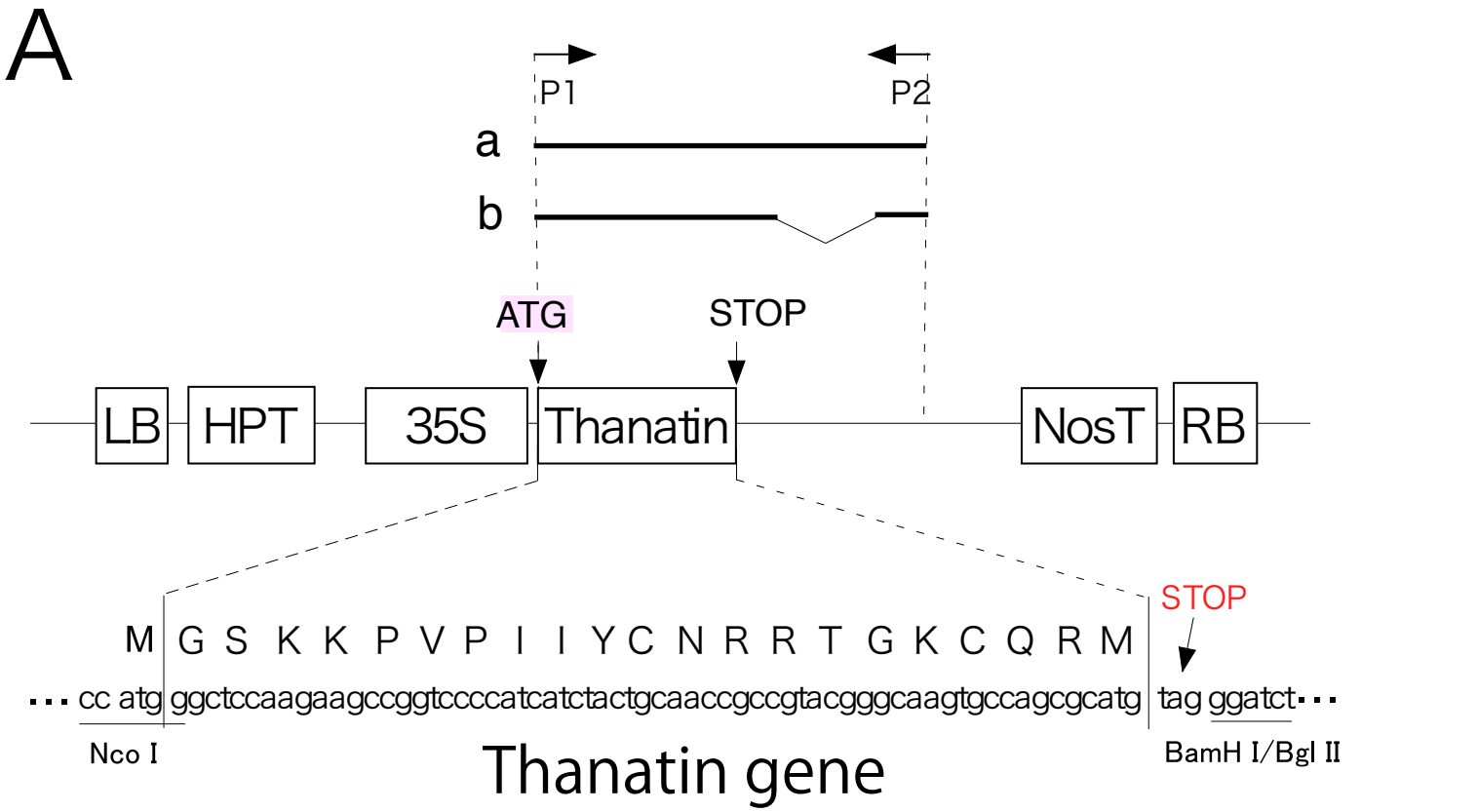
Fig. 2 Detection of antimicrobial substances in the transformants. (A) Detection of antimicrobial activity in the transformants carrying the thanatin gene. Crude extract of leaves, containing 10 μg of total protein, was used in each assay with *M. luteus.* Photographs were taken after 48 h of incubation for the assay. Activity is represented by a clear well, indicated by the red circle. Numbers shown above correspond to the numbers of the transformant line. WT: wild-type plant. (B) Chromatogram from the HPLC analysis. This analysis was carried out using the partially purified antimicrobial substances in the crude extract of transformant line 9-5, which were separated by eluted with 250 mM NaCl from a cation exchange chromatographic sepharose column before HPLC analysis. The numbers on the figure indicate the numbers of the fractions eluted between 36 min to 60 min. The eluted substances were monitored by their absorbance 225 nm. (C) Antimicrobial activity of the HPLC fractions shown in panel B. The HPLC fractions were subjected to bioassay using *M. luteus*. The Numbers correspond to the fractions shown in the panel B. PC: Assay with authentic thanatin as the positive control. NC: Assay media including *M. luteus* with no antimicrobial substance, used as a negative control.

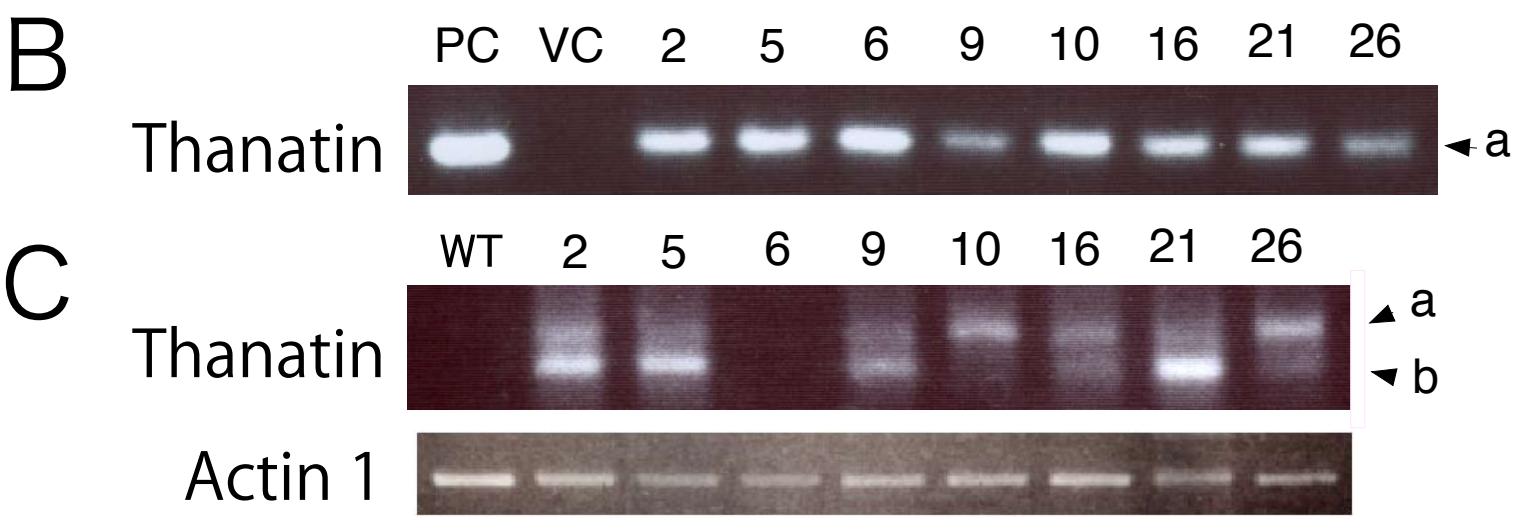
Fig. 3 ESI-MS spectra of the isolated antimicrobial substance in fraction 7 (see Fig. 2B) derived from the transformant (A), or authentic thanatin (B). Arrows indicate the representative peaks of the thanatin at *m/z* 487, 609. Vertical axis indicates the relative abundance.

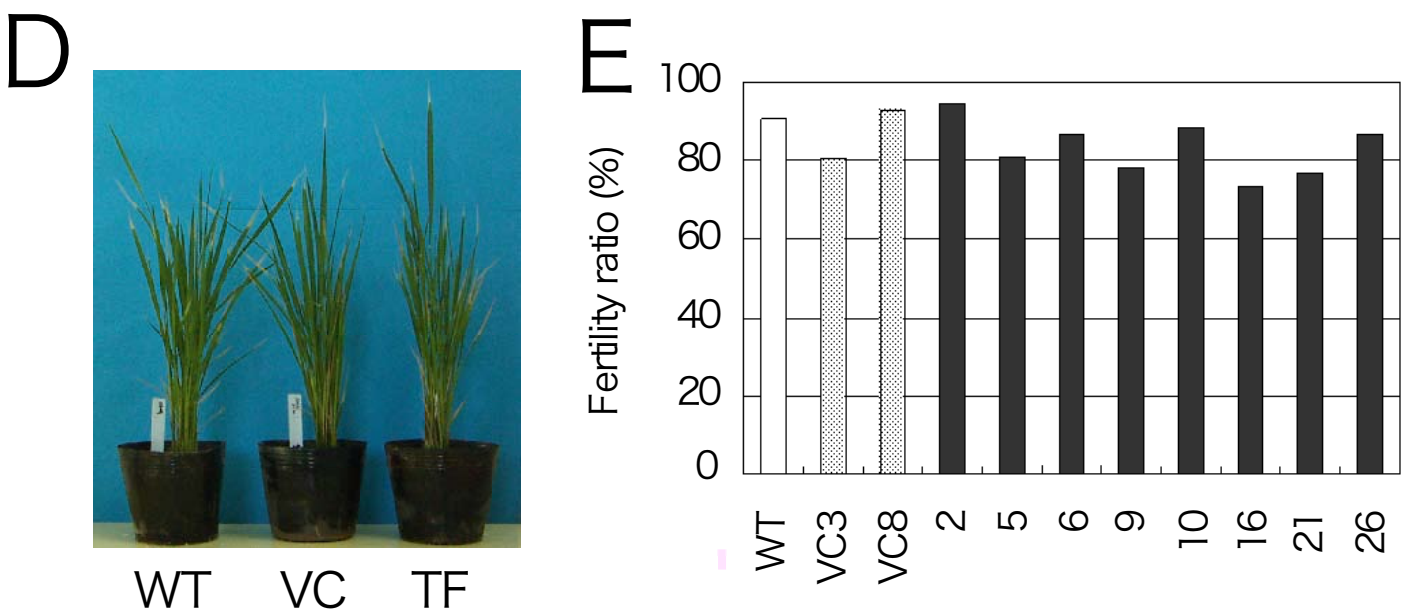
Fig. 4 *In vitro* assay for antifungal activity of thanatin to *M. oryzae*. (A) The ratio of appressorium formation of *M. oryzae* in the presence of thanatin. Formation of appressorium was observed three times under bright-field microscopy after 6 hours incubation.

Concentration of thanatin in the suspension of conidia (0 to 70 μM) is shown below. Vertical bars show standard deviations. Asterisks indicate significant differences between appressorium formation with 0 μM thanatin and others at $P < 0.01$. The chemically-synthesized thanatin was used in this assay. (B) and (C) Photographs of *M. oryzae* after 6 hours incubation in absence of thanatin (B) and in presence of 70 μM thanatin (C), respectively. (B) and (C), Bars = 20μm.

Fig. 5 Disease resistance assay to *M. oryzae*. (A) Photographs of representative disease symptoms taken five days after inoculation. Young leaves were truncated and evaluated their antifungal activities. Transformants No. 5-1 and No. 9-5 are the progenies of thanatin transformants established as homozygous lines. VC: vector control containing the empty plasmid, WT: the wild type plants. (B) The disease index represents the mean (n=20 leaves) disease severity (1= light to 5= severe). Asterisks indicate significant differences between transgenic lines and the wild type at $P < 0.01$.



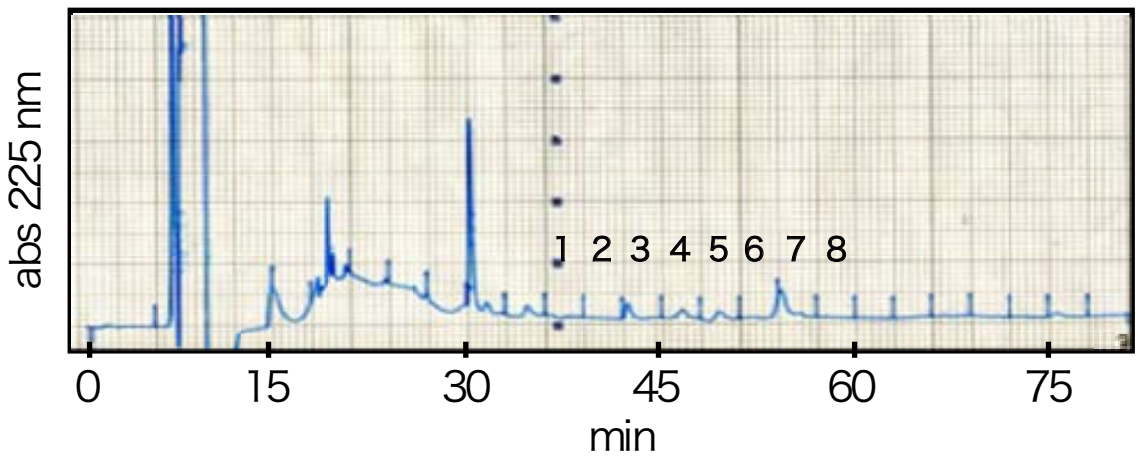




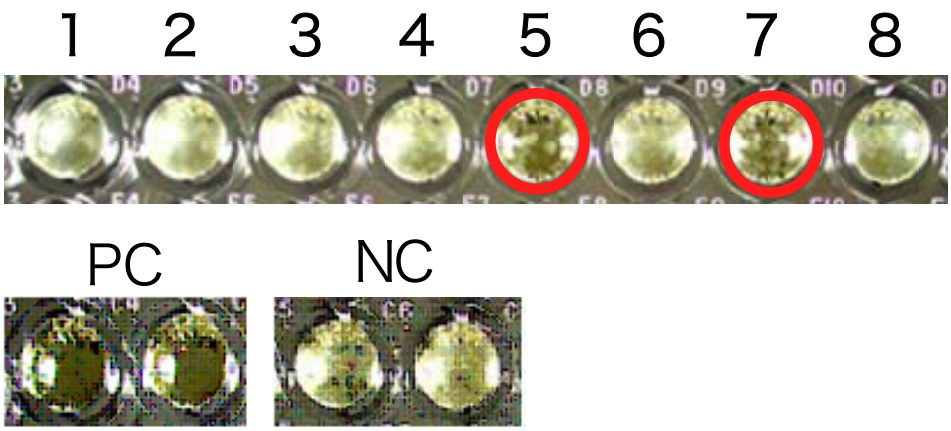
Imamura et al Fig. 1

A

B

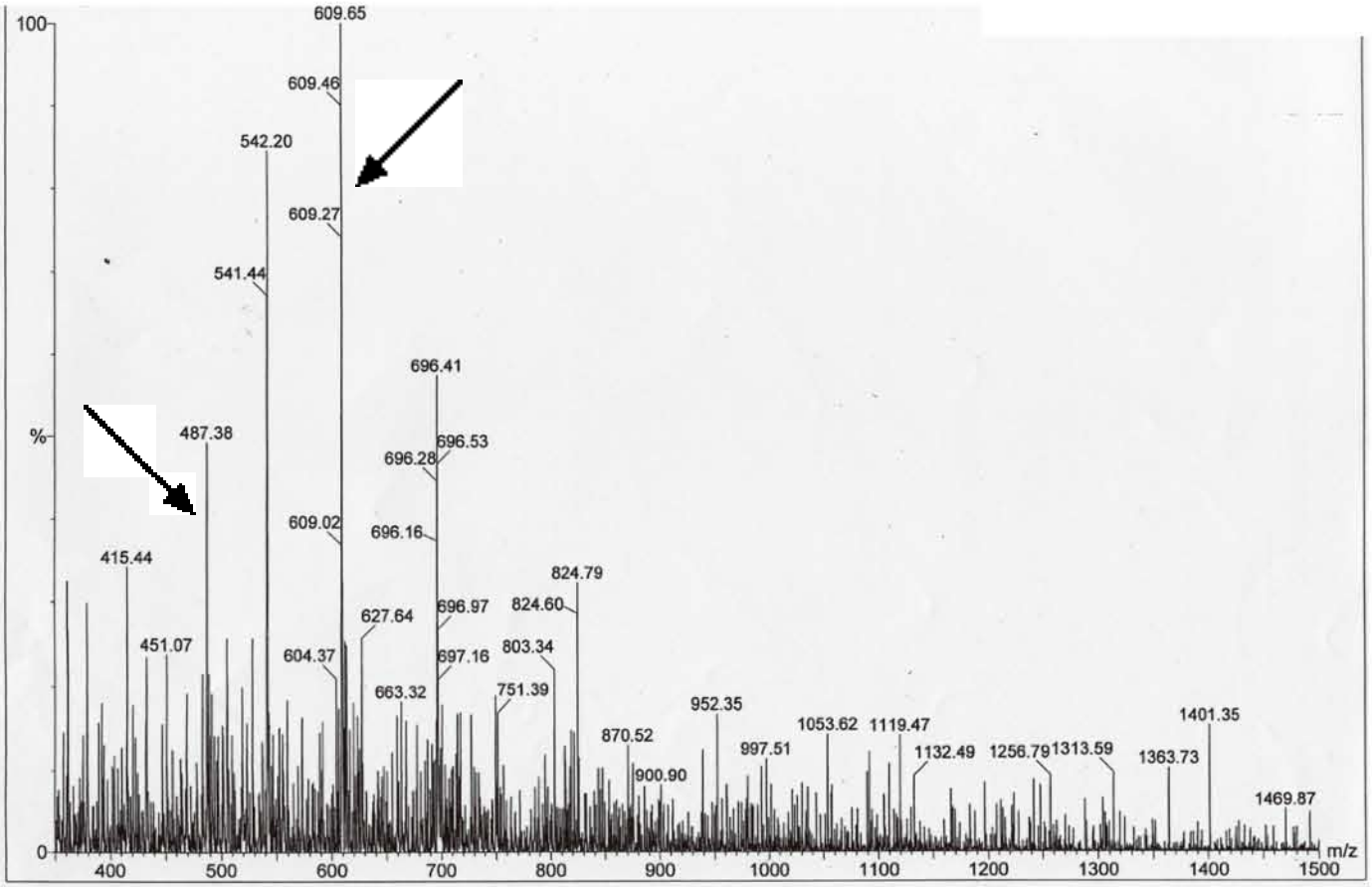


C

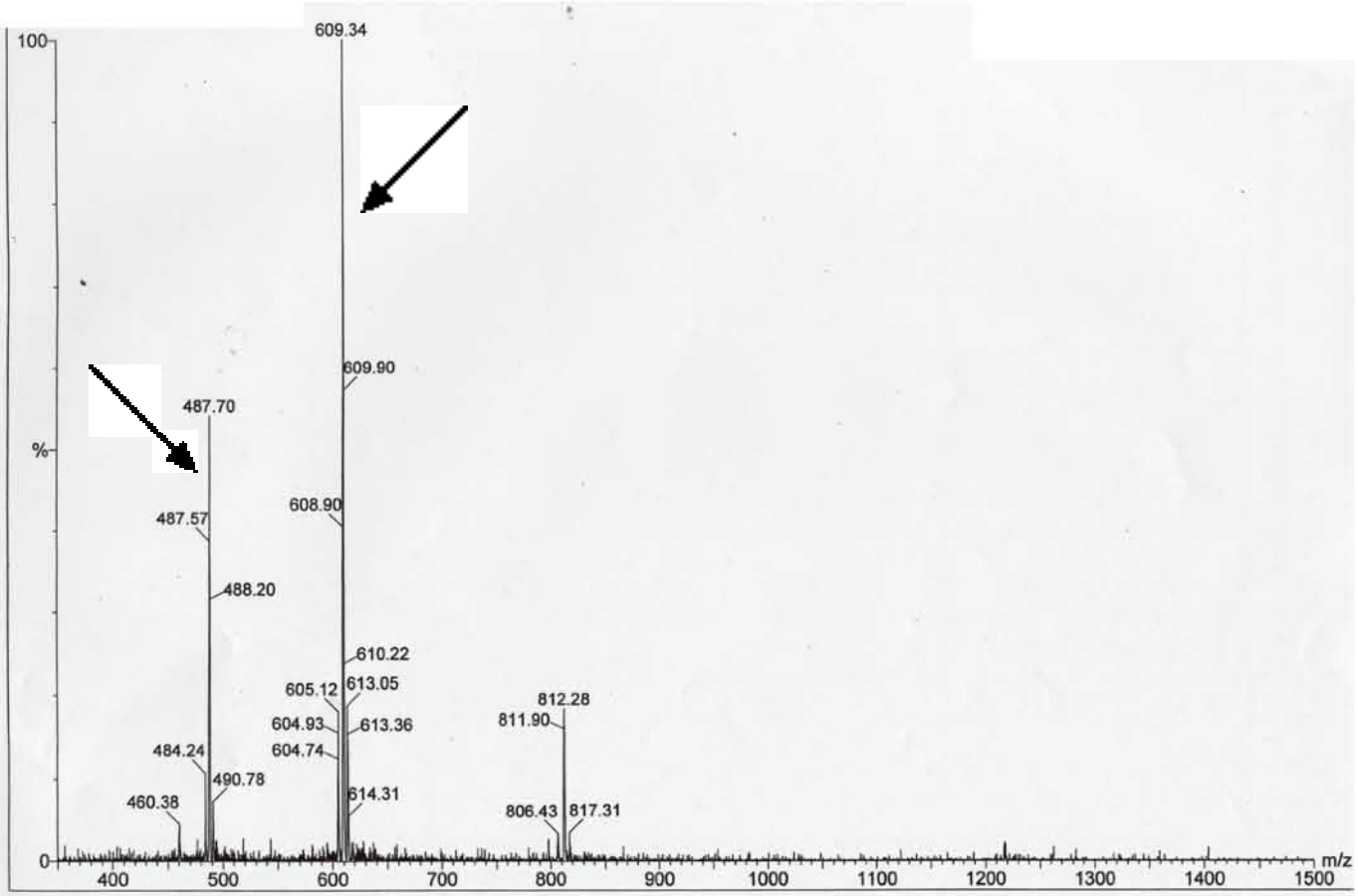


Imamura et al Fig. 2

A

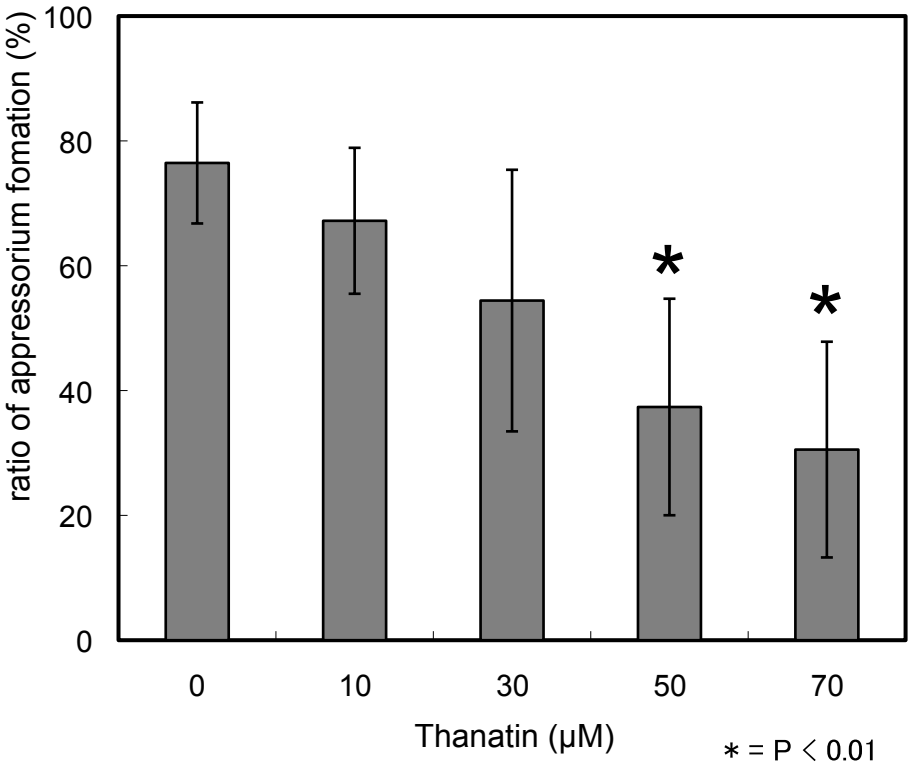


B

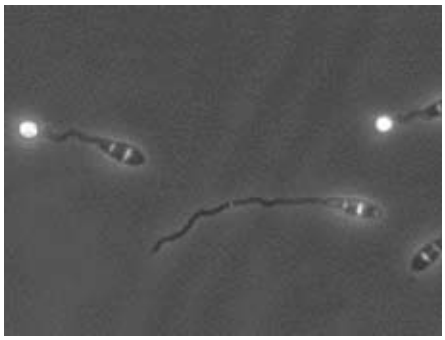


Imamura et al. Fig. 3

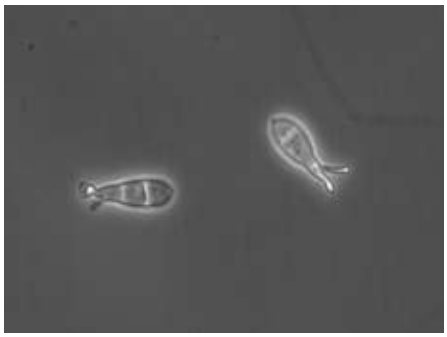
A



B

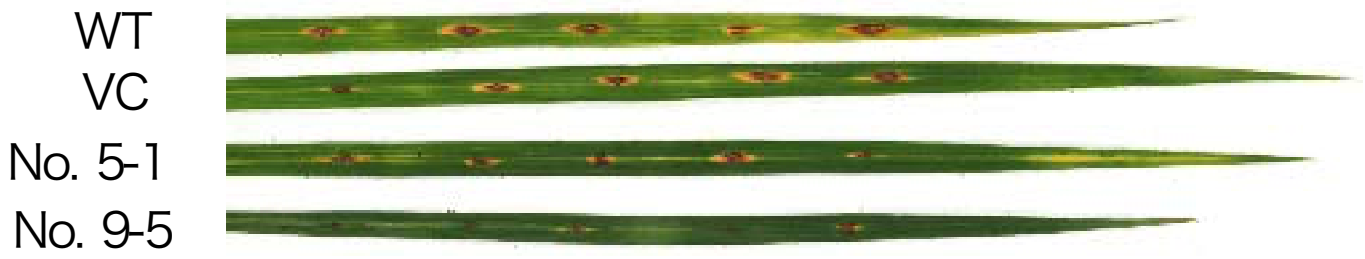


C

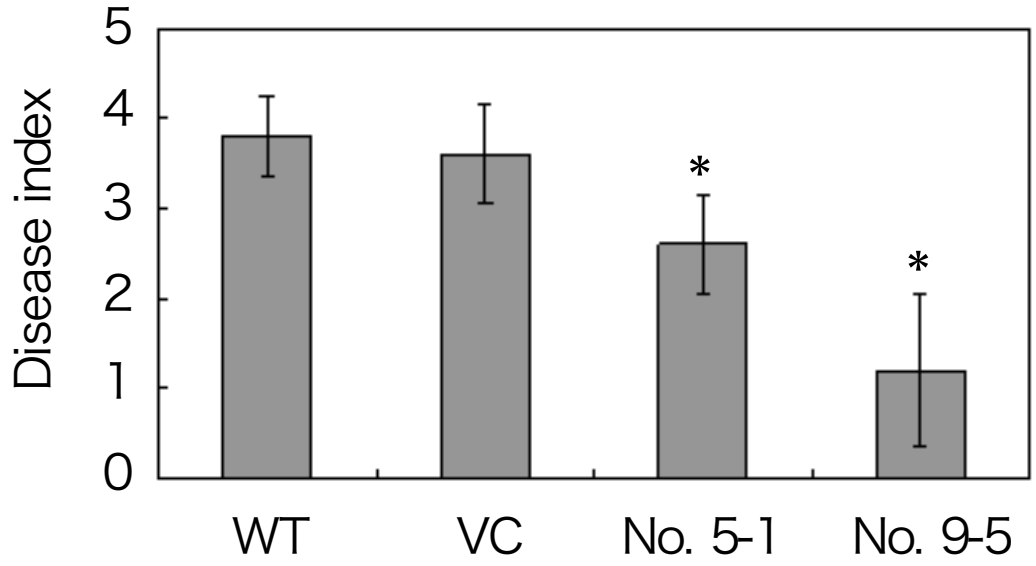


Imamura et al. Fig. 4

A



B



Imamura et al Fig. 5