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Enhanced Resistance to Bacterial Diseases of Transgenic Tobacco Plants Overexpressing Sarcotoxin IA, a Bactericidal Peptide of Insect[1]

Masahiro Ohshima,* Ichiro Mitsuhara,* Masaji Okamoto,† Sadanori Sawano,‡ Koji Nishiyama,§ Hisatoshi Kaku,* Shunji Natori,‖ and Yuko Ohashi*,[2]

**National Institute of Agrobiological Resources, Tsukuba, Ibaraki 305-8602; †Bioscience Research Dept., Toa Gosei Co., Ltd., 2 Ohkubo, Tsukuba, Ibaraki 300-2611; ‡Gifu Prefecture Agricultural Research Center, 3481-2 Kamihachiya, Hachiya, Minokamo, Gifu 505-0004; §National Institute of Agro-environmental Science, Tsukuba, Ibaraki 305-0856; and ‖Faculty of Pharmaceutical Science, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8654*

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Sarcotoxin IA is a bactericidal peptide of 39 amino acids found in the common flesh fly, *Sarcophaga peregrina*. Many agronomically important bacteria in Japan are killed by this peptide at sub-micro molar levels, and the growth of tobacco and rice suspension cultured cells is not inhibited with less than 25 μM. Transgenic tobacco plants which overexpress the peptide, *i.e.* over 250 pmol per gram of fresh leaf, under the control of a high expression constitutive promoter showed enhanced resistance to the pathogens for wild fire disease (*Pseudomonas syringae* pv. *tabaci*) and bacterial soft rot disease (*Erwinia carotovora* subsp. *carotovora*).

Key words: artificial strong promoter, bacterial disease resistant plant, bactericidal peptide, sarcotoxin IA, transgenic plant.

Bacterial diseases of crop and fruit plants are a serious problem which needs to be solved in agronomy. To protect plants from phytobacterial-attack, antibiotics such as streptomycin and copper-containing agriculture chemicals have been used in fields, posing environmental problems such as the occurrence of antibiotic-resistant bacteria. When such antibiotics are not available, infected plants become diseased and die. Conversely, the cross breeding of resistant cultivars is difficult because suitable genetical sources containing specific genes for resistance to these bacterial diseases are limited or in some cases not yet known. Thus, for the generation of resistant plants, molecular breeding, involving such as the introduction of genes for bactericidal proteins, is a more promising strategy compared to classical breeding.

Antibacterial proteins have been identified in various organisms including vertebrates (*1*), insects (*2*), and plants (*3*). These proteins are thought to act as the major defense molecules for self-defense against bacterial infection especially in insects (see review, *2*). Among these proteins, sarcotoxin IA, which was found in the larvae of the common flesh fly, *Sarcophaga peregrina*, is one of most well characterized ones. It is a short peptide that is induced by wounding of larvae and then secreted into the haemolymph (*4*). It is synthesized in the fat bodies as a precursor consisting of 63 amino acids. The mature form consisting of 39 amino acids is produced after processing of the signal peptide and release of the C-terminal glycine followed by amidation of the next arginine moiety as the C-terminal amino acid. The mature peptide consists of two amphiphilic alpha helical regions, helix I (Leu3-Gln23) and helix II (Ala28-Ala38), with a hinge region (Gln24-Ile27). The lengths of these amphiphilic helixes are thought to be identical to the thickness of the hydrophobic layer of the bacterial membrane, and they are considered to act as ionophores (*5*). The minimum bactericidal concentration (MBC) for *Escherichia coli* is 200-300 nM (*6*). This peptide is effective against a wide variety of important causal pathogens of human diseases, such as *Staphylococcus aureus, Shigaella* spp., and *Diplococcus pneumoniae* (*7*). In plants, although other types of antibacterial peptides such as thionine, plant defensin and lipid transfer proteins have been found (*3*), a bactericidal peptide with simple two α-helices has not been found. We previously constructed fusion genes consisting of the sarcotoxin IA and GUS (β-glucuronidase) genes, and introduced them into tobacco plants. Although these fusion proteins were produced at high levels in transgenic plants, they were not suitable for plant improvement (*8*). Here, we report the generation of transgenic plants overexpressing free sarcotoxin IA at considerable levels in the intercellular spaces.

The anti-bacterial activity of synthetic sarcotoxin IA against agronomically important phytopathological bacteria in Japan listed in Table I was analyzed by means of an *in vitro* growth inhibitory assay. All bacteria used were sensitive to the synthetic sarcotoxin IA, particularly *Xanthomonas campestris* pv. *citri*, which is one of the most destructive pathogens for citrus plants in Japan. The size of the clearance zone was dependent on the concentration of the peptide and almost identical or larger than that in the case of *E. coli* with both concentrations tested. The MBC of

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[2] To whom correspondence should be addressed. Tel: +81-298-38-7440, Fax: +81-298-38-7044, E-mail: yohashi@abr.affrc.go.jp

TABLE I. **The inhibitory effect of sarcotoxin IA against phytopathogenic bacteria in Japan.**

Phytopathogenic bacterium (strain)	Disease	Diameter of inhibited area (mm)	
		Sarcotoxin IA	
		5 μg	10 μg
Xanthomonas campestris pv. *citri* (MAFF03-01079)	Bacterial canker	11.0	13.0
Xanthomonas campestris pv. *vitians* (MAFF03-01352)	Bacterial spot	9.5	10.6
Xanthomonas campestris pv. *pruni* (MAFF03-01420)	Bacterial spot of stone fruits	9.3	10.5
Xanthomonas campestris pv. *oryzae* (MAFF03-01230)	Bacterial leaf blight	8.8	9.5
Erwinia carotovora subsp. *carotovora* (MAFF03-01393)	Bacterial soft rot	10.3	11.5
Pseudomonas syringae pv. *tabaci* (MAFF03-01075)	Wild fire	9.5	10.8
Pseudomonas cichorii (MAFF03-01369)	Bacterial rot	10.0	10.8
Escherichia coli (K-12)		9.0	10.0

The diameter of inhibited area was measured after 2 days incubation with 5 or 10 μg of synthesized sarcotoxin IA. The mature sarcotoxin IA peptide was synthesized, using an Applied Biosystem 430A peptide synthesizer, as a mature form with an amidated C-terminal arginine, which is the naturally occurring form found in insects, and purified by HPLC to 95% purify. The phytopathogenic bacteria were supplied by the Gene Bank of the Ministry of Agriculture, Fisheries and Forestry of Japan (MAFF). The bactericidal activity of synthetic sarcotoxin IA was determined by means of an *in vitro* growth inhibitory assay. Ten or 5 μg of the synthetic peptide in 15 μl of water was placed in apertures (5 mm in diameter) in a plate (90 mm in diameter) containing 30 ml of 1.8% agar with bacteria at a final concentration of 10^6/ml, followed by incubation for 8 h at 4°C for diffusion of the peptide into agar and then transfer to 25°C. The diameters of the clear circles around the apertures were measured as the inhibitory zone 2 days after incubation at 25°C.

sarcotoxin IA prepared from *S. peregrina* and the synthetic peptide, 200–300 nM (*6*), was almost the same for *E. coli* in liquid culture, suggesting that these phytopathogenic bacteria were susceptible to Sarcotoxin IA at sub-micro molar levels.

The primary target site of sarcotoxin IA was reported to be the cell membrane of bacteria (*5*). To study the toxicity of the peptide toward plants, we used tobacco BY-2 (*9*) and rice Oc (*10*) suspension cultured cells, which are most frequently used as model cell lines exhibiting vigorous proliferation. These cell lines were maintained, at 25°C with shaking at 60 rpm, by transferring them every 7 days. One milliliter of the cell suspension on the 7th day was harvested and transferred to a 30 ml Erlenmeyer flask containing 10 ml of fresh medium containing 0, 5, or 25 μM sarcotoxin IA. Before or after the 7 days' culture, the packed volume of the cell suspension was measured after centrifugation at $100 \times g$ for 10 min. The packed volume of BY-2 cells at 0 time was 0.12 ml, and after 7 days' culture it had increased to 2.1 ± 0.1, 2.1 ± 0.4, and 2.4 ± 0.2 ml with 0, 5, and 25 μM sarcotoxin IA, respectively. Similarly, no inhibitory effect was found on the growth of the rice Oc cell lines (data not shown). In contrast, antibiotics such as kanamycin and tetracycline at 10 μM inhibited the growth of these cell lines by about 40%. These results indicated that sarcotoxin IA is a natural antibiotic which is not toxic to these dicot and monocot plant cells at less than 25 μM, when it is present outside the cells.

As sarcotoxin IA was shown to have the desired characteristics for practical use for plant breeding, we initially introduced its gene into tobacco plants under the control of the Cauliflower mosaic virus 35S gene (CaMV35S) promoter. However, in transgenic lines in which the introduction of the transgene was confirmed by PCR, no immunologically positive signals for the gene products and no improved resistance were found. To improve the expression level of the transgene, we used a high expression promoter, El2Ω, which is an artificial constitutive promoter, including tandem repeats of the 5′ enhancer sequence of the CaMV35S promoter and the omega sequence from the tobacco mosaic virus (TMV). This artificial promoter could drive the *GUS* gene to a 10 times higher level in comparison with in the case of the CaMV35S promoter in transgenic tobacco plants (*11*). The DNA fragment containing the coding sequence of mature sarcotoxin IA was isolated from pTO19 (*12*) as a template by PCR. In *S. peregrina*, sarcotoxin IA is secreted outside cells and accumulates in the haemolymph for self-defense against harmful bacteria. In plants, secretion of the peptide outside cells would be necessary for effective protection against harmful bacterial attacks, which preferentially start in the intercellular spaces of plant tissues. Therefore, we added the DNA sequence of the signal peptide of the tobacco pathogenesis-related (PR) 1a protein, which is an antifungal protein found in the intercellular space and accounts for a high percentage of the total soluble protein that accumulates in TMV-infected tobacco plants (*13*). The signal sequence of PR1a protein gene, which consists of 120 nucleotides, was also isolated by PCR from pPR-γ (*14*). Both fragments were directly joined by recombinant PCR (*15*) in frame. *Bam*HI and *Sac*I recognition sequences were added to the 5′ and 3′ ends at the same time, respectively. The resultant recombinant PCR product was digested with *Bam*HI and *Sac*I, and then subcloned into pBE2113-GUS (*11*) by replacing the *GUS* gene. The resultant construct, PST10, was introduced into tobacco (*Nicotiana tabacum* cv. Samsun NN) plants *via Agrobacterium*-mediated gene transfer with LBA4404 (*16*), about 30 independent transgenic lines being generated. All of transformants showed the normal phenotype and fertility. The presence of the introduced gene was confirmed by PCR for the mature sequence of the sarcotoxin IA peptide.

We at first evaluated transgenic lines as to the resistance to bacterial soft rot disease caused by *Erwinia carotovora* subsp. *carotovora* using discs of upper full-developed leaves. Several selected resistant lines (first generation) were self-pollinated. Leaves of the progeny grown in a greenhouse (second generation) were inoculated with *Pseudomonas syringae* pv. *tabaci*. This bacterium is known to only infect parenchyma tissues and not to spread to the vessel system (*17*). Among transgenic lines of the second generation, PST10-5-1 and PST10-6-1 showed reduced symptoms in comparison with wild-type plants. Self-pollinated progeny of the lines, PST10-5-1-24 and PST10-6-1-15 (third generation), that are homozygotes as to the transgene were used for further analysis. These plants

showed constant resistance to repeated infection with *P. syringae* pv. *tabaci*. In wild-type plants, the inoculated area was initially detected as a yellow-colored halo around the needle wounds (Fig. 1A, left). For example, the mean size of halos in 16 trials (8 plants×2 spots) at 10 days after inoculation for greenhouse grown plants was 17.4 mm. The size of the halo increased thereafter, the halo developing into a necrotic lesion. In contrast, on leaves of the PST10-5-1-24 and PST10-6-1-15 lines grown under the same conditions, no halo was found (Fig. 1A, right). Detached leaves of these plants were further subjected to infection with *E. carotovoraa* subsp. *carotovora*. In wild-type plants and 35S-GUS plants, 35S-GUS, which harbor the *GUS* gene driven by the *CaMV 35S* gene promoter, water-soaked regions appeared around the inoculated regions and then increased daily (Fig. 1B, left and middle, respectively), the decayed area eventually covering to the entire leaf. In contrast, in the transgenic lines, the decayed region was restricted to around the inoculated area and it did not extend thereafter (Fig. 1B, right). Furthermore, the levels of resistance were quantitatively determined. Leaf discs were floated on a bacterial suspension (10^6 cell/ml) and then incubated for 3 days with gentle shaking, and then the ratio of the water-soaked area on the leaf discs was determined. As shown in Fig. 1C, 60% of the total leaf area of wild-type tobacco leaves was consumed by rot 40 h after inoculation and the entire leaf had decayed at 84 h. In contrast, the rot symptoms on the leaf discs from transgenic plants were delayed, and almost the entire area remained healthy at 40 h and 40% remained intact at 84 h after inoculation.

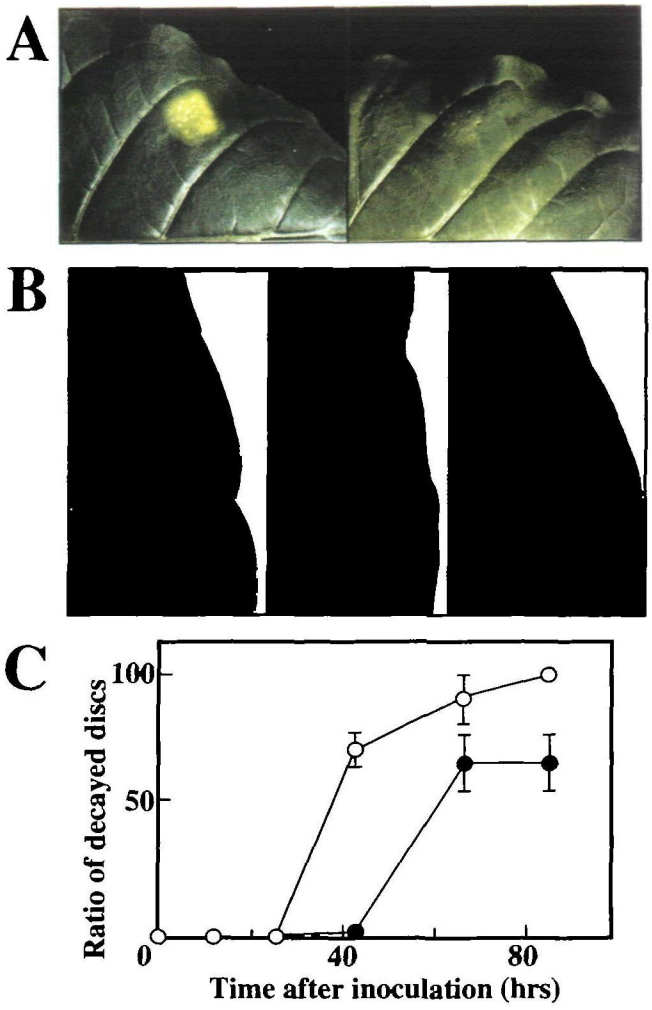

Fig. 1. **Resistance against bacterial pathogen attack in transgenic plants.** A: Fully expanded upper leaves of 4- to 6-week-old tobacco plants grown in a greenhouse at 28°C were wounded with ten fine needles at sites 3 mm apart within a 10 mm circle, and inoculated with a suspension (10^8 cell/ml) of *Pseudomonas syringae* pv. *tabaci*, the pathogen for wild fire disease, using a cotton pad. After inoculation, the plants were maintained in the greenhouse. Leaves of wild-type (left) and PST10-5-1-24 plants (right) 10 days after inoculation are shown. B: Detached tobacco leaves were similarly inoculated with a suspension of *Erwinia carotovora* subsp. *carotovora*, the pathogen for bacterial soft rot disease, and incubated in moist chambers under continuous light. The sizes and degrees of symptoms induced by bacterial multiplication were measured. Leaves of wild-type (left), 35S-GUS (middle), and PST10-6-1-15 (right) plants 3 days after inoculation are shown. C: A set of 4 leaf discs (7 mm in diameter) was uniformly cut out from detached, fully expanded upper leaves. After washing with distilled water, the discs were floated on 5 ml of the suspension of *Erwinia carotovora* subsp. *carotovora* (10^6 cell/ml) in a Petri dish (6 cm in diameter). The water-soaked leaf area was estimated after gentle shaking at 28°C for an appropriate period. The ratio of the decayed leaf area of wild-type (open circles) and PST10-6-1-15 (closed circles) plants was monitored with time. The values are averages of at least triplicate assays with SD.

The expression level of the sarcotoxin IA peptide in transgenic plants was examined by protein blot analysis using a specific antibody to synthetic sarcotoxin IA. The Tricine-SDS PAGE system is advantageous for the separation of lower molecular weight proteins, and we detected immunoreactive signals in the extracts of resistant transgenic lines (Fig. 2). The signal strength was proportional to the degree of resistance. Unexpectedly, the signal was not detected as a single band at 4 kDa, but appeared as a doublet corresponding to a molecular size of 8 kDa. The signal was observed for the extracts of transformants which showed resistance against bacterial infection, but not for wild-type plants. We estimated the quantity of the expressed peptide in PST10-5-1-24 to be about 1 μg/g fresh leaf, which corresponds to about 250 pmoles sarcotoxin IA per one gram of fresh leaf (Fig. 2, lane 5). Furthermore, we observed that at least two-fold sarcotoxin IA can be extracted under high salt conditions such as 0.5 M NaCl (lanes 8–10), suggesting that at least half of the expressed peptide in tobacco plants was ionically bound to the cell wall fraction. We also detected this peptide in ICF of healthy leaves after vacuum-infiltration, suggesting it was secreted outside cells, and accumulated in the intercellular spaces and cell wall.

We report here the generation of transformants resistant against bacterial pathogens by introduction of chimeric gene in combination with a strong artificial promoter (*11*) and the gene for the sarcotoxin IA peptide, which kills bacteria at sub-micro molar levels. We found at least 1 μg/g of fresh leaf of the expressed peptide in healthy transgenic lines, and when NaCl was added to 0.5 M, twice the amount of peptide was recovered from the leaves. Thus, the actual concentration of the peptide in transgenic plants is over 2 μg per gram fresh leaf, which corresponds to an average of 500 nM. This amount is much higher than the MBC for *E. coli* in liquid culture (200–300 nM). As shown in Table I, this peptide exhibits similar or higher bacte-

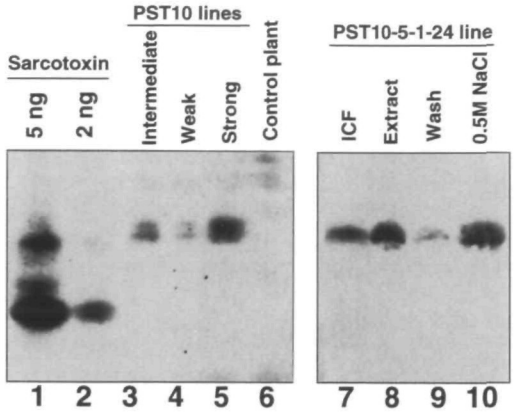

Fig. 2. **Immunological detection of the sarcotoxin IA peptide in the leaf extracts of transgenic plants.** Discs from fully expanded upper leaves were homogenized with an equal volume of an extraction buffer (50 mM phosphate buffer, pH 7.0, 1 mM EDTA, 10 μM A-PMSF, 0.5 μg/ml leupeptin, 2 mM DTT). After centrifugation for 10 min at 10,000$\times g$, an aliquot of the supernatant was denatured with SDS (*24*), and the subjected to tricine-SDS-PAGE as described by Schaegger and Jagow (*25*). After semi-dry electroblotting (*26*) onto a polyvinylidene difluoride membrane (Millipore), the immobilized sarcotoxin IA peptide was immunologically detected using anti-sarcotoxin IA rabbit polyclonal antibodies as described by Okamoto *et al.* (*8*). Alkaline phosphatase-conjugated anti-rabbit IgG was used as the second antibody. Intercellular fluid (ICF) of tobacco leaves was collected as follows. Leaf discs (7 mm in diameter) were subjected to vacuum infiltration in the extraction buffer, and then the liquid portion recovered from leaf discs after centrifugation at 4,500$\times g$ for 5 min was collected as ICF and freeze-dried. Extract equivalent to 1.25 mg fresh leaf was loaded on each lane except lane 7. Lanes 1 and 2: synthesized sarcotoxin IA, 5 ng and 2 ng, respectively. Lanes 3-5: leaf extracts of transgenic plants with intermediate resistance (PST-10-5-1-14, lane 3), weak resistance (PST10-5-1-25, lane 4), and strong resistance (PST10-5-1-24, lane 5). Lane 6: leaf extract of wild-type tobacco. Lanes 7-10: leaf extracts of PST10-5-1-24. Lane 7: ICF recovered from the equivalent of 6.25 mg fresh leaf. Lane 8: supernatant of leaf extract after centrifugation at 10,000$\times g$ for 10 min. Lane 9: the washings of the cell debris containing cell wall with the extraction buffer. Lane 10: extract of the washed cell debris with the extraction buffer containing 0.5 M NaCl. The arrowhead and arrow indicate the monomer and dimer of sarcotoxin IA, respectively.

ricidal activity toward some phytopathogenic bacteria compared to *E. coli*, and exhibits a wide inhibitory spectrum as to phytopathogenic bacteria with no toxicity toward plant cells at less than 25 μM. These findings indicated that the resistance to bacterial-attack found in transgenic plants is reasonable. Unexpectedly, the transgene product detected in transgenic tobacco plants was similar to the doublet of sarcotoxin IA. The peptide was considered to function as a dimer in the cell membrane of bacteria. Furthermore, an immunologically active signal at 8 kDa was observed when the authentic sample was used (Fig. 2, lanes 1 and 2). Therefore, this peptide possibly formed a dimer retaining the bactericidal activity, although it is plausible that the doublet was an artifact of the extraction procedure.

Sarcotoxin IA exhibits higher specific bactericidal activity than similar bactericidal peptides, such as cecropin derived from the giant silk moth, *Hyalophora cecropiaie*. The MBC of sarcotoxin IA toward *E. coli* in liquid culture is 200 to 300 nM, while that of cecropin is about 1 μM (*18*). Various trials for the overexpression of cecropin under the control of the CaMV35S promoter were conducted with negative results to the production of resistant plants, possibly due to the instability of the expressed cecropin (*18-20*). Mills *et al.* found high levels of proteolytic activity in the intercellular fluid of peach leaves and very rapid degradation of authentic cecropin (*21*). This problem was also found in the case of sarcotoxin IA. However, as shown above, the higher bactericidal activity of sarcotoxin IA and the application of a strong promoter may overcome this problem.

Norelli *et al.* (*22*) and Jaynes *et al.* (*23*) used wound inducible promoter for protease inhibitor II (PI-II) instead of the CaMV35S promoter to overproduce attacin E, an antibacterial peptide derived from the cecropia moth, and Shiva-1, which is an analog of cecropin B altered by protein engineering with higher specific activity toward phytopathogenic bacteria, respectively. They succeeded in improving the resistance against fire blight disease (*22*) and bacterial wilt disease (*23*). However, constitutive expression of a bactericidal peptide would be advantageous for inhibiting the early step of pathogen infection *via* stomata, such as in the case of *P. syringae* pv. *tabaci*. This is an important step for improving the resistance of plants grown in fields. As pathogenic bacteria easily penetrate plants from wounded sites, the combination of constitutive and inducible production systems will be ideal for plant improvement and growth.

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