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Original Paper

Transgenic tobacco plants expressing antimicrobial peptide bovine lactoferricin show enhanced resistance to phytopathogens

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Abstract Lactoferricin B, the antimicrobially active N-terminal peptide derived from acid pepsin hydrolysis of bovine lactoferrin, has broad spectrum antimicrobial activities. We expressed the lactoferricin gene in tobacco plants to evaluate its antimicrobial activity. The coding region for the chimeric peptide gene of the signal peptide from tobacco pathogen-related protein (PR-1) and bovine lactoferricin was synthesized using the recombinant polymerase chain reaction (PCR) method. Transgenic tobacco plants expressing the lactoferricin gene were developed using the *Agrobacterium*-mediated transformation method. The lactoferricin B gene was integrated into the tobacco genome and its transcription was detected by PCR, Southern blot analysis, and reverse transcription PCR (RT-PCR), respectively. The transgenic tobacco plants were challenged with the pathogenic bacteria *Pseudomonas syringae* pv. *tabaci* and *Botrytis cinerea*. At 30 or 28 days post-inoculation, the transgenic plants were still green and continued to grow, whereas the control plants were infected with bacterial or fungal pathogens from the roots to the tips, resulting in death of the plants. In conclusion, transgenic tobacco plants that overexpressed the lactoferricin gene, linked to the signal peptide of tobacco PR-1 protein under the control of a high expression constitutive promoter, showed enhanced resistance to bacterial (*P. syringae* pv. *tabaci*) and fungal (*B. cinerea*) diseases.

Key words: Lactoferricin, cationic peptide, transgenic tobacco, phytopathogens, disease resistance

Lactoferrin is a glycoprotein found in bovine whey protein fractions. Lactoferrin has multiple biological functions, mostly related to host defense, including broad-spectrum antimicrobial properties.

Lactoferricin B (Lfc) is a 25-amino acid peptide with a molecular weight of 3,124 that originates from the N-terminus of bovine lactoferrin (Bellamy et al. 1992a). Lfc has stronger antimicrobial activity against various human pathogenic bacteria (Aguilera et al. 1999; Bellamy et al. 1992b; Sánchez-Gómez et al. 2008; Shin et al. 1998; Wakabayashi et al. 2002), fungi (Bellamy et al. 1993), and viruses (Marr et al. 2009) compared to that of lactoferrin. The antimicrobial activity of Lfc against plant pathogenic bacteria (Zhang et al. 1998) and fungi (Muños and Marcos 2006) has also been studied. Lfc has characteristics of typical cationic antimicrobial peptides (Tomita et al. 2009), which are thought to be the result of actions on the cytoplasmic membrane of bacteria, such as the formation of pores, destabilization of the bilayer, thinning of the membrane, or depolarization of the membrane (Ulvatne et al. 2001).

Many studies have shown the expression of antimicrobial cationic peptides in plants, e.g., cecropin in tobacco (Hightower et al. 1994; Jaynes et al. 1993) and tomato (Jan et al. 2010), sarcotoxin in tobacco (Ohshima et al. 1999) and tomato (Radi et al. 2006), MsrA3 in potatoes (Osusky et al. 2004), and cecropin-melittin chimeric peptide in potato (Osusky et al. 2000). Transgenic plants showed considerably greater resistance to pathogens (Hightower et al. 1994; Jan et al. 2010; Jaynes et al. 1993; Ohshima et al. 1999; Osusky et al. 2000; Osusky et al. 2004) and parasitic weeds (Radi et al. 2006) than to the wild types.

Zhang et al. (1998) reported that transgenic tobacco expressing human lactoferrin protein demonstrated significant delays in developing bacterial wilt symptoms when inoculated with the bacterial pathogen *Ralstonia solanacearum*. They reported that lactoferricin, which has higher bactericidal activity and is resistant to pepsin digestion, could confer higher levels of bactericidal

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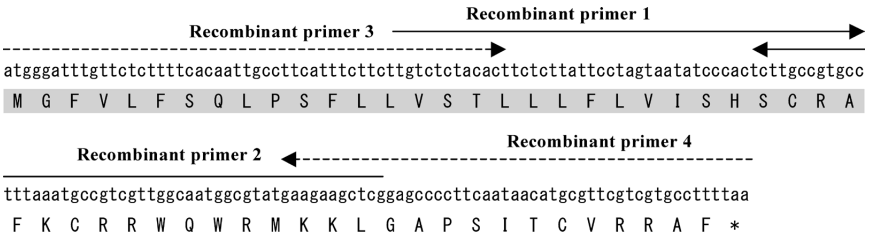

Figure 1. The sequences of the amino acids and the nucleotides of signal peptide of PR1 and lactoferricin. For improved plant expression, codon choice was made according to the codon usage in tobacco. Shadowed letters show the signal peptide of tobacco PR1 protein, and * shows the stop codon.

disease resistance to transgenic plants. In this study, we developed transgenic tobacco plants that carried the *Lfc* gene fused to the signal peptide of tobacco pathogen-related protein (PR-1) under the control of the enhanced cauliflower mosaic virus (CaMV) 35S promoter. The effects of *Lfc* gene expression in transgenic tobacco plants against the phytopathogenic bacteria *Pseudomonas syringae* pv. *tabaci* and the fungi *Botrytis cinerea* were analyzed.

Materials and methods

Assay of Lfc antibacterial activity

Synthesized bovine Lfc (*N*-FKC RRW QWR MKK LGA PSI TCV RRA F-*C*) was purchased from BioGate Co. (Gifu, Japan). The *in vitro* antibacterial activity of Lfc was determined by the method described by Zhang et al. (1998) with a slight modification. *P. syringae* pv. *maculicola* (PMC8301) and *Xanthomonas campestris* pv. *vesicatoria* (#613) were a gift from Dr. Takikawa, University of Shizuoka. *R. solanacearum* (OE1-1) was collected in Aichi Prefecture, Japan. The bacteria were grown to OD600 of 0.3 in NB liquid medium (Difco), and 1 ml aliquots were transferred to culture tubes. Synthetic Lfc was added to the culture tubes to obtain final concentrations of 100 μmol l^{-1}, 10 μmol l^{-1}, 1 μmol l^{-1} or 0 μmol l^{-1}, respectively. The bacterial suspensions were serially diluted 10-fold and plated on NB agar. The colonies were counted after an overnight culture.

Plasmid construction

The chimeric peptide gene coding region (Figure 1) of the signal peptide from tobacco PR-1 and *Lfc* (*Sig-Lfc*) was synthesized by the recombinant polymerase chain reaction (PCR) method (Fujimoto et al. 1993). The first and second recombinant PCR were performed in a total volume of 100 μl. The first reaction contained 1× AmpliTaq Gold Buffer (Applied Biosystems), 0.25 mmol l^{-1} dNTPs, 1 μmol l^{-1} of recombinant primer 1 (5′-TTG TCT CTA CAC TTC TCT TAT TCC TAG TAA TAT CCC ACT CTT GCC GTG CC-3′) and recombinant primer 2 (5′-CGA GCT TCT TCA TAC GCC ATT GCC AAC GAC GGC ATT TAA AGG CAC GGC AAG A-3′), and 2.5 units of AmpliTaq Gold (Applied Biosystems). The second reaction contained 1× AmpliTaq Gold Buffer, 0.25 mmol l^{-1} dNTPs, 1 μmol l^{-1} of recombinant primer 3 (5′-ATG GGA TTT GTT CTC TTT TCA CAA TTG CCT TCA TTT CTT CTT GTC TCT ACA C-3′) and recombinant primer 4 (5′-TTA AAA GGC ACG ACG AAC GCA TGT TAT TGA AGG GGC TCC GAG CTT CTT CA-3′), 2.5 units of AmpliTaq Gold, and 1 μl of the first PCR solution. The first and second PCR mixture were denatured for 9 min at 94°C and then amplified via five cycles of 94°C for 1 min, 45°C for 1 min, and 72°C for 2 min followed by 20 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min. This was followed by a final extension period of 10 min at 72°C. The PCR products were cloned into the pGEM T-Easy Vector System (Promega) and sequenced with the CEQ8000 System (Beckman Coulter). The vector including the *Sig-Lfc* gene was digested with *Bam*HI and *Sac*I. The DNA fragment containing the chimeric peptide was inserted into the pEC vector, which had a high-expression promoter, including the double enhancer of cauliflower mosaic virus 35S promoter and the *Kyuri green mottle mosaic virus* (KGMMV) 5′ no coding region (Fukuta and Kanbe 2002). The binary vector pEC-sigLfc is shown in Figure 2.

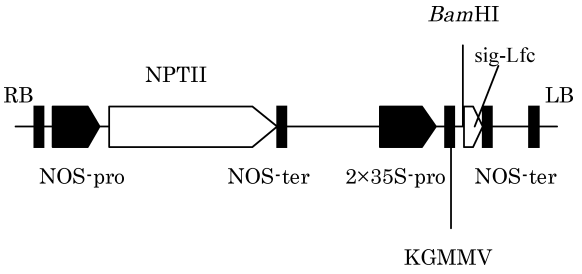

Figure 2. The expression constructs for sig-Lfc. The pEC-sigLfc plasmid expression vector for right and left border regions of the Ti plasmid; Nos-pro and Nos-ter, promoter and terminator, respectively, of the nopaline synthase gene; NPTII, neomycin phosphotransferase II; 2×35S, duplicated enhancer CaMV 35S promoter; KGMMV, 5′ no coding region of *Kyuri green mottle mosaic virus*; sig-Lfc, peptide coding sequence of sig-Lfc.

Plant material and transformation

Tobacco (*Nicotiana tabacum* cv. Samsun) seeds were treated with 1% NaOCl and 0.1% (v/v) Tween20 for 10 min and then rinsed three times with sterile deionized water. The sterilized seeds were germinated and grown on Murashige and Skoog's (MS) medium (Murashige and Skoog 1962) and maintained under a 16-h/8-h (light/dark) photoperiod at 25°C. Excised young leaves were co-cultivated with the overnight culture of

Agrobacterium tumefaciens LBA4400 containing the pEC-sigLfc plasmid for 48 h at 25°C in the dark on co-culture medium [MS medium supplemented with 30 g l^{-1} sucrose, 0.2 mg l^{-1} naphthalene acetic acid (NAA), 2 mg l^{-1} benzyladenine (BA), and 2.5 g l^{-1} gellan gum]. The explants were transferred to selection medium after two days (co-culture medium supplemented with 250 mg l^{-1} cefotaxim and 100 mg l^{-1} kanamycin) at 25°C under a 16-h/8-h (light/dark) photoperiod. When shoots appeared from explants, they were separated and transferred into root formation medium (half strength MS medium, 15 g l^{-1} sucrose, 2.5 g l^{-1} gellan gum, 250 mg l^{-1} cefotaxime, and 100 mg l^{-1} kanamycin).

DNA isolation, PCR analysis, and Southern hybridization

Genomic DNA was isolated from tobacco plant leaf tissue using the DNeasy Plant Mini Kit (Qiagen), according to the manufacturer's instruction. Integration of the *Sig-Lfc* gene into the plant genome was confirmed by PCR amplification of the CaMV 35S promoter and the *Sig-Lfc* gene using the forward primer (5′-CGC CAA GCT TGC ATG CC-3′) and the reverse primer (5′-TTA AAA GGC ACG ACG AAC -3′). PCR products were analyzed on 1% agarose gels.

Young leaves were collected to isolate DNA according to the CTAB method (Murray and Thompson 1980). About 5 μg of DNA samples were digested with *Bam*HI and used for Southern analysis using *Lfc* gene as the probe. The Gene Images Random-Prime Labeling and Detection System (GE Healthcare) was used to detect the transgene.

Analysis of Lfc gene expression

Total RNA was extracted from 100 mg of young leaves with the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. Total RNA was reverse-transcribed with an oligo-dT primer using Ready-To-Go™ You-Prime First-Strand Beads (Roche Applied Science). The resulting cDNA was used as a template for the PCR reaction to amplify the *Lfc* gene with the Lfc-F (5′-TTT AAA TGC CGT CGT TGG CAA -3′) and Lfc-B primers (5′-AAA GGC ACG ACG AAC GCA TGT -3′). PCR products were analyzed on 2% agarose gels.

Evaluation of antibacterial and antifungal activity in transgenic tobacco plants

Antibacterial and antifungal assays using the plant pathogens *P. syringae* pv. *tabaci* and *B. cinerea* were performed in a plant culture box with two leaf-stage tobacco plants grown in MS medium. Bacterial solution (100 μl) containing 10^2 *P. syringae* cells and 100 μl of spore solution containing 10^2 *B. cinerea* spores were applied to the bottom of the culture box and incubated at 25°C under a 16-h/8-h (light/dark) photoperiod. The tests were performed using six plants infected with *P. syringae* and eight plants infected with *B. cinerea*. An infection rate was calculated according to the following formula:

Infection rate
= (the number of total infected plants/
the number of total checked plants) × 100

The mean and standard deviations were calculated based on three replications.

Results

Evaluation of biological activity of synthetic lactoferricin against plant pathogenic bacteria

The biological activity of synthetic Lfc was evaluated against three strains of plant pathogenic bacteria, *P. syringae* pv. *maculicola* (PMC8301), *X. campestris* pv. *vesicatoria* (#613), and *R. solanacearum* (OE1-1). As a result of the growth inhibition experiment, even 1 μmol l^{-1} Lfc showed bactericidal activity against the three bacterial species (Table 1). Furthermore, *P. syringae* and *X. campestris* were not detected at 100 μmol l^{-1}.

Integration and expression of the Lfc gene

It is imperative that Lfc is translocated into the intercellular spaces to make it effective for controlling bacterial and fungal diseases in tobacco. This was achieved by fusing Lfc to the signal peptide from tobacco PR-1a, which directs secretion of the PR-1a protein into the intercellular spaces in tobacco. The *Sig-Lfc* gene was constructed using the recombinant PCR method.

Tobacco (*Nicotiana tabacum* cv. Samsun) was transformed with pEC-sigLfc by *A. tumefaciens*-mediated transformation method. Integration of the *Lfc* gene into the genomic DNA of tobacco plants was confirmed by PCR amplification of the CaMV 35S promoter and *Lfc* gene sequences. A suitable 970 bp DNA fragment was detected in T_0 transgenic tobacco lines (No. 33 and 74), whereas no bands were detected in the untransformed control tobacco plant (data not shown).

Table 1. Antibacterial activity of Lfc against three phytopathogenic bacteria

Bacterial species	μM Lfc concentration	Log10 CFU*/ml
P. syringae	0	9.47±0.12
	1	5.51±0.45
	10	4.19±0.04
	100	<1
X. campestris	0	9.06±0.23
	1	7.67±0.12
	10	5.51±0.17
	100	<1
R. solanacearum	0	10.67±0.09
	1	7.80±0.11
	10	6.57±0.06
	100	5.69±0.29

* Colony-formation unit.

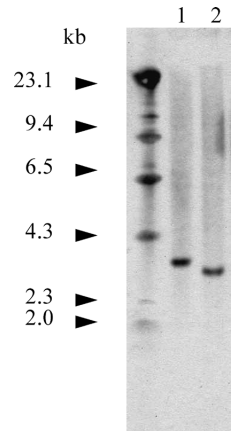

Figure 3. Southern blot analysis of transgenic tobacco plants carrying the Lfc gene. DNA from transgenic tobacco plants No. 33 (lane 1) and No. 74 (lane 2) was digested with *Bam*HI. The Southern blot was probed with Lfc gene. The molecular weight marker was lambda/*Hin*dIII.

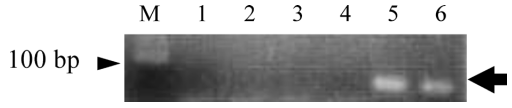

Figure 4. Analysis of the Lfc gene expression in the transgenic tobacco plants by RT-PCR. PCR products from RNA extracted from control (lane 1) and transgenic plants No. 33 (lane 2) and No. 74 (lane 3) without reverse transcription, and RT-PCR products from RNA extracted from control (lane 4) and transgenic plants No. 33 (lane 5) and No. 74 (lane 6) were analyzed. Allow indicates Lfc gene amplified by RT-PCR. Lane M, 100 bp ladder.

Furthermore, the transgenic tobacco lines were confirmed to have the transgene using Southern blot analysis. As a result, a signal was detected in No. 33 and 74 (Figure 3), which revealed that the *Lfc* gene was transformed in these two lines.

Lfc expression was tested at the RNA level using RT-PCR. The appropriate 78 bp DNA fragments were detected in two transgenic lines (Figure 4, lanes 5 and 6), whereas no bands appeared in the untransformed control tobacco plants (Figure 4, lane 4).

Evaluation of antimicrobial activity in transgenic tobacco

To evaluate the resistance to bacterial disease, control tobacco plants and two kanamycin resistant T_1 transgenic lines (No. 33 and 74) were challenged with *P. syringae* pv. *tabaci*, an endemic pathogen of wildfire disease.

Figure 5A shows the progression of *P. syringae* infection rate. The first symptoms were observed on the lower leaves in control and transgenic tobacco plants nine days post-infection. The infection rate of control tobacco increased immediately and all control plants were more or less damaged by bacterial infection, 16 days post-infection, resulting in the yellowing of leaves and softening of stems. In contrast, symptoms in the transgenic tobacco plants were delayed. The average infection rates were much lower than those in the control plants. At 30 days post inoculation, the control plants were infected with the disease from the roots to the tips, resulting in death of the plants. However, the transgenic plants were still green and continued to grow (Figure 5B).

To investigate the ability of transgenic tobacco plants to resist fungal infection, control tobacco plants and two kanamycin resistant T_1 transgenic lines (No. 33 and 74) were challenged with a pathogenic fungus (*B. cinerea*), and symptoms were recorded. The results are shown in Figure 6A. The fungus had grown all over the surface of the MS medium, and the roots and stems of the control plants were damaged 6 days post-inoculation. The control plants were infected from the roots to the tips 28 days post-inoculation, and five control plants were dead. In contrast, symptoms in the transgenic tobacco plants were delayed until 11 days post-inoculation. The average infection rates were much lower in the transgenic plants than those in control plants. The control plants were damaged 28 days post-inoculation, resulting in yellowing of leaves. However, the transgenic plants were still green and continued to grow normally (Figure 6B).

Discussion

Lactoferrin is an iron-binding glycoprotein in the transferrin family. A high concentration of lactoferrin is found in human milk and has been suggested to have several biological activities, including protection from pathogens, regulation of iron absorption, modulation of the immune system, and cellular growth promoting activity (Lönnerdal and Iyer 1995). Recombinant lactoferrin has been produced in plants to protect against plant pathogenic bacteria (Mitra and Zhang 1994; Zhang et al. 1998) and to increase nutritive value (Nandi et al. 2002; Nandi et al 2005). Lfc is the antimicrobial fragment derived from the full length lactoferrin protein upon pepsin cleavage. Lfc has characteristics of typical cationic antimicrobial peptides and higher antimicrobial activity than that of lactoferrin. Moreover, Lfc is not toxic to plant or animal cells (Muños and Marcos 2006). In the present study, transgenic tobacco plants expressing the *Lfc* gene were developed to enhance resistance against a broad spectrum of plant pathogens. As a result, Lfc exhibited bactericidal activity as high as bactericidal peptides, such as sarcotoxin IA derived from fresh fly *Sarcophaga peregrina* (Ohshima et al. 1999) and cecropin derived from giant silk moth *Hylophora cecropiaie* (Hightower et al. 1994; Jan et al. 2010; Jaynes et al. 1993).

Trials for the overexpression of bactericidal peptides under the control of the CaMV35S promoter have been conducted without positive results, possibly due to the instability of the expressed peptides (Hightower et al. 1994; Sharma et al. 2000; Zhang et al. 1998). Ohshima

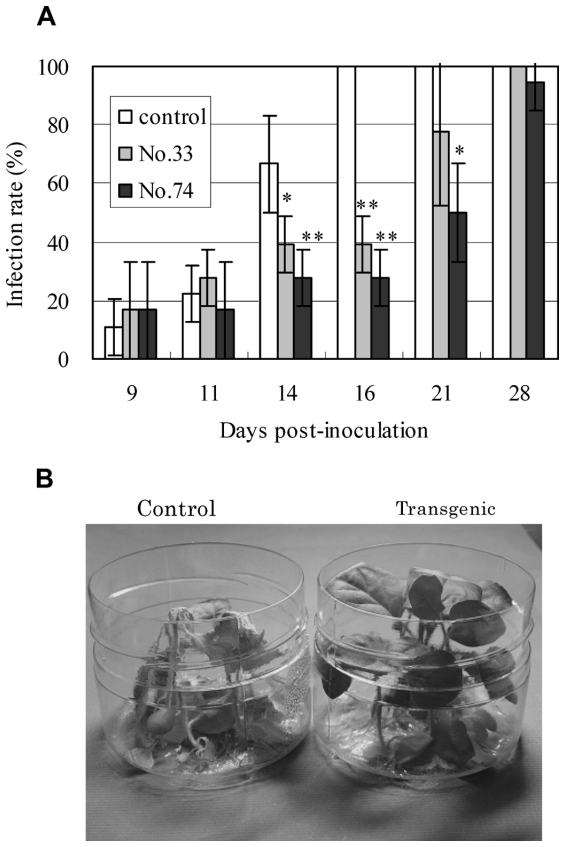

Figure 5. Transgenic tobacco challenged with the bacterial pathogen *P. syringae* pv. *tabaci*. Progression of the infection rate in transgenic tobacco plants (No. 33 and No. 74) and control tobacco plants (A). Points represent the mean of three replicates inoculations using 6 plants. Vertical bars represent standard deviations of three replications. Differences between values compared with control plants were tested for significance with Student's *t*-test (* $p<0.05$, ** $p<0.01$). Transgenic (No. 74) and control tobacco plants on 30 days post-inoculation (B).

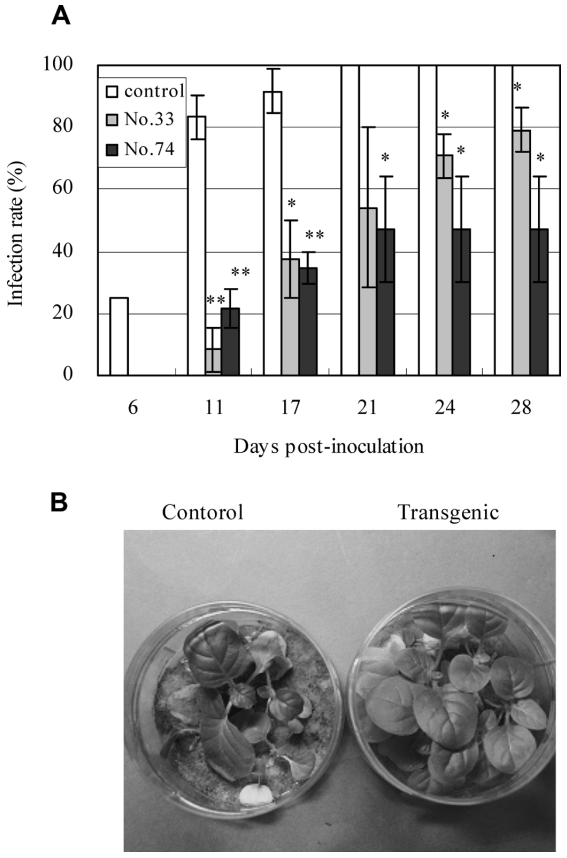

Figure 6. Transgenic tobacco plants challenged with the fungal pathogen *B. cinerea*. Progression of the infection rate in transgenic tobacco plants (No. 33 and No. 74) and control tobacco plants (A). Points represent the mean of three replicates inoculations using 8 plants. Vertical bars represent standard deviations of three replications. Differences between values compared with control plants were tested for significance with Student's *t*-test (* $p<0.05$, ** $p<0.01$). Transgenic (No. 74) and control tobacco plants on 28 days post-inoculation (B).

et al. (1999) and Osusky et al. (2000) reported that antimicrobial peptides overproduced by a strong promoter have antimicrobial activity in transgenic plants. They succeeded in improving the resistance against bacterial and fungal disease. High expression promoter, which is an artificial constitutive promoter including tandem repeats of the 5′ enhancer sequence of the CaMV35S promoter and the 5′ no coding region of KGMMV, was generated in this study. This artificial promoter could express the GUS protein at a 10 times higher level in comparison with that of the original 35S promoter (Fukuta and Kanbe 2002).

Moreover, Lfc must be secreted outside the cells for effective protection against pathogenic bacteria and fungi (Ohshima et al. 1999). The PR-1a signal peptide has been successfully used to translocate expressed proteins or peptides to the apoplast (van Esse et al. 2006). Ohshima et al. (1999) and Sharma et al. (2000) reported that transgenic plants expressing the cationic bactericidal peptide as a form of fusion protein with the signal peptide are highly resistant to bacterial disease. Therefore, we used the signal peptide of the tobacco pathogenesis-related protein PR-1a to transfer Lfc peptides to the apoplast. As a result, the transgenic tobacco plants were highly resistant to *P. syringae* pv. *tabaci* and *B. cinerea* inoculation owing to the distinct delay in development of symptoms. Lfc peptides should be expected to secret into apoplast. Accumulation of the antimicrobial peptide in the apoplast should effectively enhance host resistance to pathogens that invade through the intercellular space.

Lfc has been reported to have high antimicrobial activity *in vitro* (Bellamy et al. 1993; Shin et al. 1998; Wakabayashi et al. 2002). Zhang et al. (1998) found that Lfc antibacterial activity is higher than that of shiva-1, which is one of the most widely reported bactericidal peptides. In the present study, Lfc was also demonstrated to have extremely high antibacterial activity against *P. syringae*, *X. campestris*, and *R. solanacearum*, which represent plant pathogenic bacteria (Table 1). In addition,

Lfc has antiviral (Andersen et al. 2001; Marr et al. 2009), antitumour (Iigo et al. 1999; Yoo et al. 1997), and anti-inflammatory activities (Levay and Viljoen 1995). Introducing the *Lfc* gene into crops and fruit plants is one method to develop plant resistance to bacterial and fungal diseases and to produce functional food for human health.

Acknowledgments

We would like to thank Dr. Takikawa for generous gift *P. syringae* pv. *maculicola* (PMC8301) and *X. campestris* pv. *vesicatoria* (#613), and Enago (http://www.enago.jp) for the English language review.

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