Plant Biotechnology 29, 95–101 (2012)
DOI: 10.5511/plantbiotechnology.11.1114a

Short Communication

Gentian lipid transfer protein homolog with antimicrobial properties confers resistance to *Botrytis cinerea* in transgenic tobacco

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Received August 3, 2011; accepted November 14, 2011 (Edited by Y. Watanabe)

Abstract An antifungal protein fraction against *Botrytis cinerea* was purified from the leaves of *Gentiana triflora*. Polypeptides with molecular weight ca. 8.0 K (8.0 KP) and 7.0 K (7.0 KP) were detected by Tricine-SDS PAGE under reducing conditions. The deduced amino acid sequence of 8.0 KP cDNA showed amino acid identities with lipid transfer proteins from *Apium graveolens* (32.4%), *Lycopersicon pennellii* (31.5%), *L. esculentum* (31.5%), *Nicotiana tabacum* (30.7%), non-specific lipid transfer protein from *L. esculentum* (28.0%), and an unknown protein from *Prosopis juliflora* (30.7%) (*GtLTP1*). The deduced amino acid sequence of 7.0 KP showed amino acid identities with putative lipid transfer proteins from *Arabidopsis thaliana* (26.3% and 22.9%), *Gossypium hirsutum* (21.0%), *Tamarix hispida* (19.5%), *Populus trichocarpa* (15.9%) and an unknown protein from *Vitis vinifera* (17.2%) (*GtLTP2*). Both *GtLTP1* and *GtLTP2* were present as a multigene family in the genome and were strongly expressed in the roots and stems. Overexpression of *GtLTP1* in tobacco plants improved tolerance against *B. cinerea*, demonstrating that GtLTP1 is a useful molecular tool for genetic engineering of disease-resistant plants.

Key words: Antifungal protein, *Gentiana triflora*, lipid transfer protein, transgenic tobacco.

Plants are endowed with a wide variety of defense mechanisms that protect them from attack by pathogenic microorganisms. Active defense mechanisms include hypersensitive cell death (Govrin and Levine 2000), production of phytoalexins (Kuc et al. 1972), expression of pathogenesis-related proteins (Mauch and Staehelin 1989), oxidative burst (Baker and Orlandi 1995), cross-linking of cell wall glycoproteins (Bradly et al. 1992, Brisson et al.1994) and lignification (Vance et al. 1980). Many plant proteins with antimicrobial activity have also been identified. These proteins include chitin-binding proteins (van den Bergh et al. 2004), thionines (Loeza-Angeles et al. 2008), PR-4 type protein (Friedrich et al. 1991), lipid-transfer proteins (Marison et al. 2007), plant defensines (Thomma et al. 2002), PR-1 type proteins (Kiba et al. 2007), win type proteins (Caruso et al. 1996), hevein-like protein (Kiba et al. 2003), and 7S globulin proteins (Marcus et al. 1999). Fourteen families of pathogenesis-related (PR) protein were also recognized and classified (van Loon and van Strien 1990).Some antimicrobial proteins are induced by different stress stimuli, such as infection by viruses, bacteria, and fungi or treatment with salicylic acid or jasmonic acid (Linthorst 1991). These proteins may have an important role in the protection of plants against microbial infection.

Ectopic expression of antimicrobial protein genes as a result of genetic transformation can confer enhanced disease resistance in crop plants (Evans and Greenland 1998). The overexpression of chitinase (Broglie et al. 1991), osmotin-like proteins (Zhu et al. 1996), thionin (Epple et al. 1997), non-specific lipid transfer proteins (Molina and García-Olmedo 1997), and small cysteine-rich plant defensins (Bi et al. 1999) enhance pathogenic fungi and bacterial resistance in various plant species. However, the expression of antimicrobial protein genes in transgenic plants is not always effective in conferring resistance and often only partial resistance is obtained

Abbreviations: *bar*, phosphinothricin acetyltransferase; CaMV, cauliflower mosaic virus; DET, dithioerythreitol; DIG, digoxigenin; LTP, lipid transfer protein; nsLTP, non specific lipid transfer protein; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription polymerase chain reaction; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid.
The nucleotide sequence reported in this paper has been submitted to DDBJ, EMBL and Gene Bank under accession number AB638714 (*GtLTP1*) and AB638715 (*GtLTP2*).
This article can be found at http://www.jspcmb.jp/
Published online February 20, 2012

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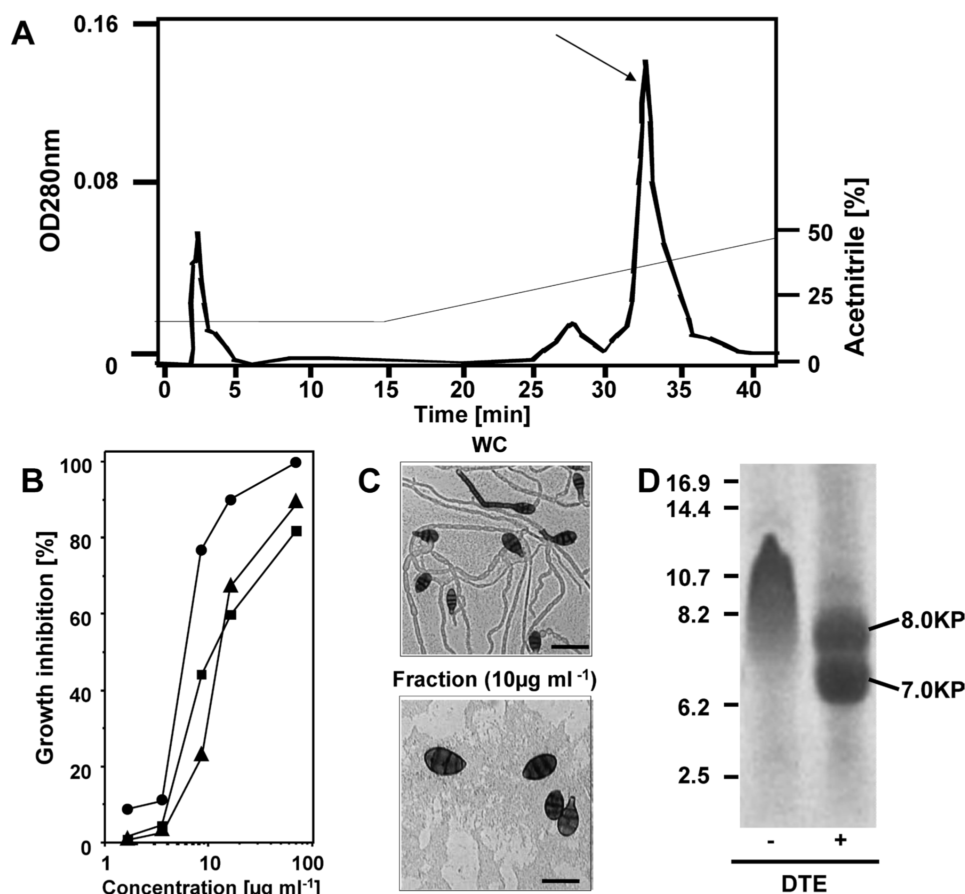

Figure 1. Purification of antimicrobial protein. (A) Protein fractions extracted from gentian leaves and separated by a reverse-phase column as described in the text. The arrow indicates the peak corresponding to antifungal fraction. (B) Growth inhibition of the fungi *Alternaria alternata* (▲), *Botrytis cinerea* (●) and *Fusarium solani* (■) at different concentration of antifungal fraction. (C) Microscopic observations of *Alternaria alternata* growth 24 h after the start of culture in the absence (WC) or presence of antimicrobial fraction at a concentration of 10 μg ml^{-1}. Bar indicates 100 μm. (D) Tricine-SDS-PAGE analysis of antimicrobial fraction in the absence (−) or presence (+) of dithioerythreitol (DET). The molecular masses (kDa) of the marker proteins are indicated on the left.

(Evans and Greenland 1998). Therefore, the development of novel proteins (substances) that show high antimicrobial activity is one of the solutions to overcome this problem.

Gentian plants are often used for medicinal purposes and are a suitable source for isolation of novel antimicrobial agents. However, we are the only group to report on antimicrobial proteins in gentian plants (Kiba et al. 2005). In this paper, we isolate an antimicrobial protein and clone its corresponding cDNA from a gentian plant. We successfully isolated 8.0 kDa and 7.0 kDa lipid transfer protein (LTP), which designated as GtLTP1 and 2, respectively. We also analyzed the antimicrobial function of GtLTP1 and create disease-resistant, stably transformed tobacco plants.

Gentiana triflora cv. Yahaba Y514-8-38 was provided by the Iwate Agricultural Research Center and maintained in a greenhouse. Antifungal protein was purified using the method described by Kiba et al. (2005), with a slight modification. One hundred grams of mature gentian leaves was homogenized in extraction buffer containing 10 mM NaH_2PO_4, 15 mM Na_2HPO_4, 100 mM KCl, 2 mM EDTA, 1.5% (w/v) polyvinylpolypyrrolidone, 2 mM thiourea and 1 mM phenylmethylsulfonylfluoride with a homogenizer (ULTRA-TURRAX T25, Janke & Kunkel, GmbH & Co., IKA-Labortechnik, Germany). The extract was then centrifuged for 30 min at 32000×g and the supernatant was extensively dialyzed against deionized water using a dialyzed membrane (Spectro-pore; cut-off 1000 Da). The dialyzed solution was adjusted to 0.1% trifluoroacetic acid (TFA) and subjected to reverse phase high performance liquid chromatography on TSKgel phenyl-5PW RP (Tosoh, Tokyo, Japan) in equilibrium with 0.1% TFA. Proteins were eluted with a linear gradient of 0–50% solvent B (0.1% TFA in 100% acetonitrile) in solvent A (0.1% TFA in deionized water) at a flow rate of 1 ml/min for 200 min and collected every 1 min. Fractions were assayed for growth inhibition against the fungal pathogen, *Botrytis cinerea*, as described below. Protein concentrations were determined by the method of Bradford (1976) with bovine serum albumin as a

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Table 1. Antifungal activity of antimicrobial fraction in the presence of different cations.

Relative inhibition of fungal germination (%)						
Medium A	Medium B	Medium A supplemented with				
		50 mM K^+	50mMNa$^+$	50 mM NH_4^+	50 mM Mg^{2+}	50 mM Ca^{2+}
66.0	72.9	63.1	60.1	84.8	86.3	64.5

Medium A: Synthetic growth medium. Medium B: medium A supplemented with 1 mM $CaCl_2$ and 50 mM KCl. Data was represented germination rate without antimicrobial fraction as 100.

standard. Tricine-SDS-PAGE was performed and stained with Coomassie brilliant blue G250 (Shagger and von Jagow 1987).

B. cinerea strain S1 and *Fusarium solani* were grown on potato dextrose agar at 25°C for two weeks under white fluorescent lighting. *Alternaria alternata* apple pathotype were grown at 25°C for two weeks under blue fluorescent lighting on potato dextrose agar or oatmeal agar (Kiba et al. 2003). Conidia of the fungal pathogen were suspended and washed with deionized water, and adjusted to 1×10^8 spores ml^{-1}. Antimicrobial activity was measured by microspectrophotometry under the conditions described by Cammue et al. (1992). Antifungal activity was measured at 25°C over a 24-h period in reaction solution consisting of 10 μl of test solution and 90 μl of fungal spore suspension (1×10^5 spores ml^{-1}). Fungal spores were suspended in a synthetic growth medium containing 2.5 mM K_2HPO_4, 50 μM $MgSO_4$, 50 μM $CaCl_2$, 5 μM $FeSO_4$, 0.1 μM $CuSO_4$, 2 μM Na_2MoO_4, 0.5 μM H_3BO_3, 0.1 μM KI, 0.5 μM $ZnSO_4$, 0.1 μM $MnSO_4$, 10 g l^{-1} glucose, 1 g l^{-1} asparagine, 20 mg ml^{-1} methionine, 2 mg l^{-1} myo-inositol, 0.2 mg l^{-1} biotin, 1 mg l^{-1} thiamine-HCl, and 0.2 mg l^{-1} pyridoxine-HCl.

Figure 1A shows the separation pattern of proteins extracted from *G. triflora* leaves. The fraction indicated by an arrow inhibited growth of the fungal pathogen *B. cinerea*. The purified antimicrobial fraction inhibited the growth of the phytopathogenic fungi, *A. alternata*, *B. cinerea* and *F. solani* in a dose-dependent manner (Figure 1B). Microscopic observations showed that the purified antimicrobial fraction inhibited both spore germination and the growth of *A. alternata* germinating hyphae (Figure 1C). The antifungal activity of the purified antimicrobial fraction was scarcely affected by the presence of cations (Table 1). Analysis of this fraction using tricine-SDS-PAGE showed a single major band corresponding approximately to 11.0 kDa under non-reducing conditions. In contrast, the fraction was divided into two polypeptides, with molecular weight approximately to 8.0 kDa (8.0 KP) and 7.0 kDa (7.0 KP), under reducing conditions (Figure 1D).

The purified proteins were separated by Tricine-SDS-PAGE and electroblotted on a polyvinylidene difluoride membrane. Proteins were visualized by staining with Coomassie brilliant blue G250. Bands of interest were cut out and sequenced using a protein sequencer (HP G1005A, TaKaRa-Bio, Shiga, Japan).

Total RNA was extracted from gentian leaves using a fast-green RNA purification kit (BIO101, CA, USA). Based on the N-terminal amino acid sequences of 8.0 KP and 7.0 KP, the following primers were designed for RT-PCR: 5′-YTIACITGYGGIGCIGTIAC-3′ and 5′-GCIGCIGTITGYAAYYTIGGIGC-3′. RT-PCR was performed with a High Fidelity RNA PCR kit with an oligodT$_{(16)}$ primer as the antisense primer (TaKaRa-Bio). 5′-RACE was performed to isolate the complete cDNA of 8.0 KP and 7.0 KP. Based on the internal nucleotide sequences of the RT-PCR product, we designed 5′-TGTTGCAACACATACTACTACATTTATAAA-3′ for 8.0 KP and 5′-GGGGTATTTCCTAAGATACCC-3′ for 7.0 KP. Using the nucleotide sequences of the RT-PCR and 5′-RACE products, the specific primers, 5′-ATATTCATAGTCATCATTCACTTACTC-3′ and 5′-GCAAAACATACATTCGTTTTC-3′ were synthesized to generate the full-length cDNA of 8.0KP and 7.0KP, respectively. The full-length cDNAs were cloned into a pCR2.1 vector (Invitrogen, California, USA). DNA sequencing was performed using a Big Dye Terminator cycle sequencing kit (Applied Biosystems, Tokyo, Japan) and an Applied Biosystems 377 Automated Sequencer. Sequence alignments and analyses were performed using DNASIS (version 3.6; Hitachi, Tokyo, Japan) and the BLAST network service from the National Center for Biotechnology Information (NCBI) (Altschul et al. 1990).

N-terminal amino acid sequences of 8.0 KP and 7.0 KP up to the 12th residue were determined as LTCGAVTSAVGP and AAVCNLGALQRP, respectively (Figure 2A). The 600 bp cDNA corresponding to 8.0 KP obtained by PCR contained an open reading frame (ORF) encoding a polypeptide 115 amino acids in length, which included the same amino acid sequence as the N-terminus, as determined by Edman digestion. A search of the protein database using NCBI's BLASTP program showed amino acid identities with LTPs from *Apium graveolens* (32.4%), *Lycopersicon pennellii* (31.5%), *L. esculentum* (31.5%), *Nicotiana tabacum* (30.7%), a non specific LTP from *L. esculentum* (28.0%), and an unknown protein from *Prosopis juliflora* (30.7%). The 660 bp long cDNA corresponding to 7.0 KP contained an ORF encoding for a polypeptide 95 amino acids in

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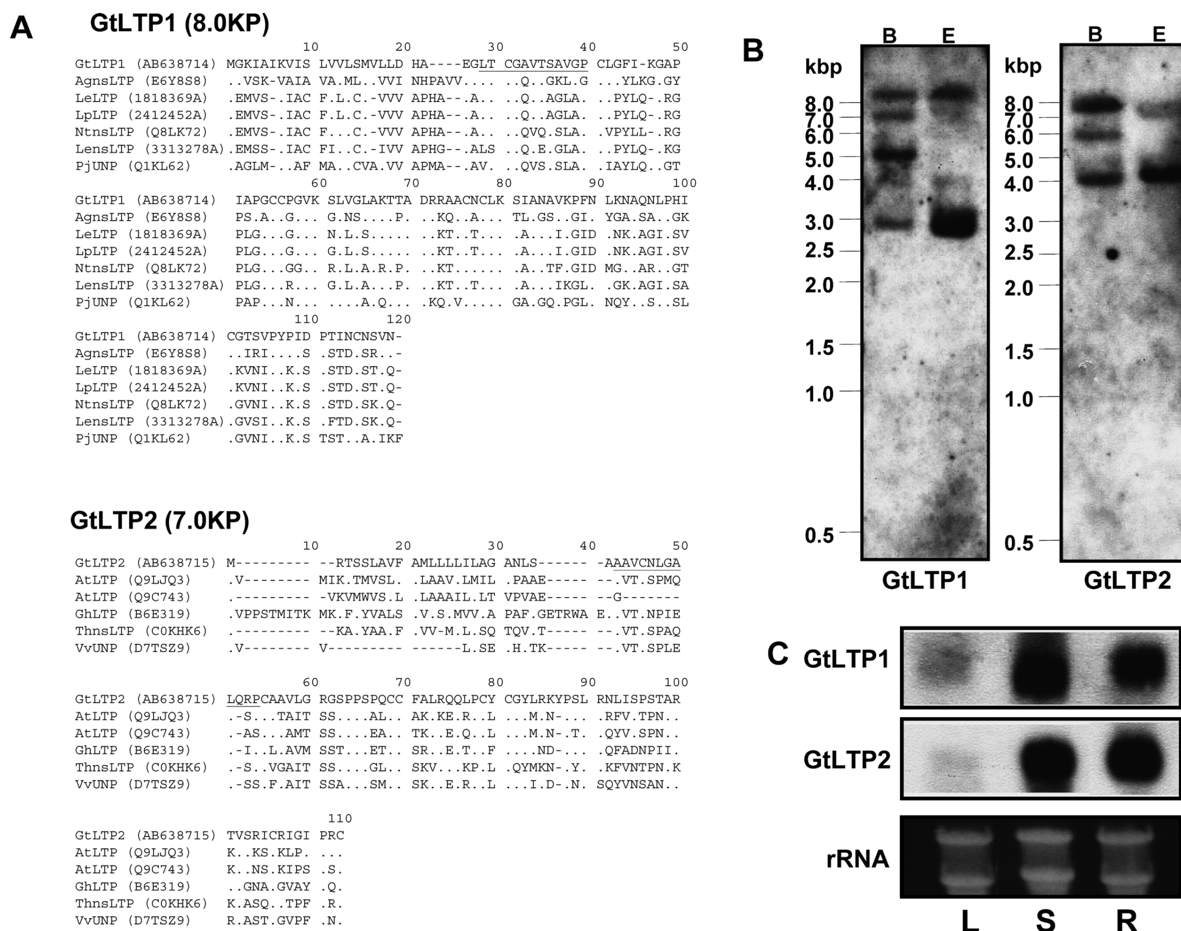

Figure 2. Characterization of antimicrobial proteins. (A) Alignment of 8.0 KP antimicrobial protein (GtLTP1; AB638714) and lipid transfer proteins from *Apium graveolens* (AdnsLTP; E6Y8S8), *Lycopersicon esculentum* (LeLTP; 1818369A), *L. pennellii* (LpLTP; 2412452A), *Nicotiana tabacum* (NtLTP; Q8LK72), non-specific lipid transfer protein from *L. esculentum* (LensLTP; 3313278A), and an unknown protein from *Prosopis juliflora* (PjUNP; Q1KL62). Alignment of 7.0 KP antimicrobial protein (GtLTP2; AB638715) and putative lipid transfer proteins from *Arabidopsis thaliana* (AtLTP; Q9LJQ3 and Q9C743), *Gossypium hirsutum* (GhLTP; B6E319), *Tamarix hispida* (ThLTP; C0KHK6), and an unknown protein from *Vitis vinifera* (VvUNP; D7TSZ9). (B) Southern blot analysis of *GtLTP1* and *GtLTP2*. Genomic DNA (10 μg) was digested with *Bam*HI and *Eco*RI. The DNA size markers are indicated on the left. (C) Expression analysis of *GtLTP1* and *GtLTP2* genes. Total RNA was isolated from leaf, stem, and root samples. Staining of rRNA with ethidium bromide was conducted as a control for equal loading of RNA.

length, which included the same amino acid sequence as the N-terminus. The sequence showed amino acid identities with putative LTPs from *Arabidopsis thaliana* (26.3% and 22.9%), *Gossypium hirsutum* (21.0%), *Tamarix hispida* (19.5%), *Populus trichocarpa* (15.9%), and an unknown protein from *Vitis vinifera* (17.2%). Plant LTPs are a homogeneous class of small (9–10 kDa), abundant, ubiquitous, and mostly basic proteins containing eight cysteine residues with four conserved disulfide bridges. LTPs are ubiquitous in the plant kingdom including rice (Lee et al. 1998), maize (Shin et al. 1995), and wheat (Simorre et al. 1991). LTPs which can enhance intermembrane transfer without lipid specificity are termed non-specific LTPs (nsLTPs), which form a multigenic family of proteins that can be subclassified as nsLTP1 and nssLTP2 (Douliez et al. 2000). Structural analysis with BLAST suggested that 8.0 KP and 7.0 KP were classified as part of the nsLTP1 family and nsLTP2 family, respectively. We therefore designated 8.0 KP and 7.0 KP as *Gentiana triflora* Lipid transfer protein 1 (GtLTP1) and *Gentiana triflora* Lipid transfer protein 2 (GtLTP2), respectively.

Total DNAs (10 μg) extracted from gentian leaves were digested with *Bam*HI and *Eco*RI restriction enzymes that did not cut the *GtLTP1*-cDNA and *GtLTP2*-cDNA. Fragments were separated on a 1% agarose gel and transferred to a Hybond N+ membrane. Hybridization was performed with a digoxigenin (DIG)-labeled cDNA probes, and the membrane was washed with 2×SSC with 0.1% SDS at 55°C and 0.1×SSC with 0.1% SDS at 65°C. Detection was performed using an alkaline phosphatase-conjugated anti-DIG antibody (Roche) and CSPD (Boehringer Mannheim) according to the supplier's instructions. Total RNA was isolated from leaves, roots, and stems from gentian plants as described above. RNA (5 μg) was separated on a 1.0% agarose gel under

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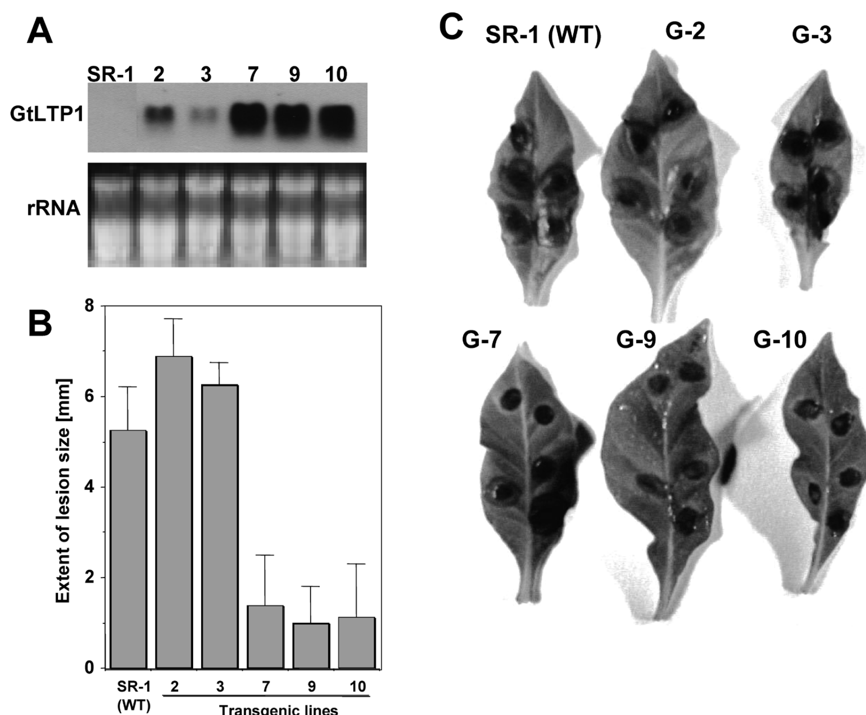

Figure 3. Expression of *GtLTP1* in transgenic tobacco plants. (A) Expression of *GtLTP1* in transgenic tobacco plants. Total RNA was isolated from fully expanded tobacco leaves of untransformed control (SR-1) or T_2 *GtLTP1*-transformed plants (nos. 2, 3, 7, 9, 10). Equal loading of RNA was verified by visualizing rRNA in gel stained with ethidium bromide (bottom). (B) Enhancement of disease resistance against the fungal pathogen, *Botrytis cinerea*, in transgenic tobacco. Lesion extension by *B. cinerea* measured 5 days after inoculation. Each value represents the mean with standard deviation (SD) of results from triplicate experiments. (C) The extent of lesion sizes caused by the fungus 5 days after inoculation in untransformed control (SR-1) and homozygous T_2 transgenic plants.

denaturing conditions and blotted onto a Hybond N+ membrane. The blot was hybridized with DIG-labeled cDNA probes as described above.

Southern blot analysis showed multiple copies of the *GtLTP1* and *GtLTP2* genes per genome (Figure 2B). To further characterize *GtLTP1* and *GtLTP2*, the organ expression pattern was determined by northern blot analysis. Both *GtLTP1* and *GtLTP2* mRNAs were abundant in the stems and roots, with less expression in the leaves (Figure 2C).

LTPs function as a carrier of the acyl monomer, which is an essential building block in cutin biosynthesis and strengthening the cell wall through the secretion or deposition of cutin (Kader 1996). LTPs show TED4/p48h-10-like functions in plant vascular development (Endo et al. 2001). In addition, LTPs are also members of the ubiquitous class of antimicrobial peptides, with some LTPs able to inhibit the growth of fungal and bacterial pathogens *in vitro* (Marison et al. 2007; Molina et al. 1997; Terras et al. 1993). Both GtLTP1 and GtLTP2 showed antifungal activity and are part of the non-specific lipid transfer protein family, so we focused on GtLTP1 to test the possibility of creating disease-resistant plants. We therefore created *GtLTP1*-overexpressing transgenic tobacco plants by cloning the ORF of *GtLTP1* between the CaMV35S promoter and the nopaline synthase terminator of pBIS221S, which was derived from pBI221 (Clontech) using *Bam*HI and *Sal*I sites. The pBIS221SGtLTP1 was cut with *Sse*I and cloned into a binary vector, pEKB, which was generated by exchanging the expression cassette of hygromycin phosphotransferase (*hph*) (pEKH) (Kiba et al. 2005) with that of phosphinothricin acetyltransferase (*bar*), resulting in pEKB-GtLTP1. The construct was transformed into *Agrobacterium tumefaciens* EHA105 by electroporation. Tobacco plants (*Nicotiana tabacum* cv. SR-1) were transformed via an *A. tumefaciens*-mediated leaf disc procedure (Horsch et al. 1985) and selected using 5 μg ml^{-1} of bialaphos (Meiji Seika, Tokyo, Japan) as the selection reagent. After rooting and acclimatization, the regenerated plants were grown in a greenhouse to set seeds. Spores of *B. cinerea* were resuspended in potato glucose liquid medium and adjusted to 1×10^8 spores ml^{-1}. Filter paper (8 mm^2) dipped in the spore suspension was then placed onto detached fully expanded leaves from 4- to 5-week-old transgenic (homozygous T_2) and wild-type tobacco plants. They were then kept in a moistened box under dim light conditions. The extent of lesion sizes was measured 5 days after inoculation (Kiba et al. 2005).

Expression of foreign genes was determined by northern blot analysis using a *GtLTP1* cDNA probe,

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and 5 transgenic lines, including expressed at high (line 7, 9, 10), intermediate (line 2), and low levels (line 3) were selected (Figure 3A). We could not observe any phenotypic changes in the transgenic plants (data not shown). Typical lesions were observed and the extent of lesion sizes reached about 5 mm in wild-type tobacco plants 5 days-after inoculation of *B. cinerea* spores. In contrast, lesion expansion was significantly reduced in the three transgenic lines (line 7, 9 and 10) that strongly expressed *GtLTP1*. Transgenic lines that showed moderate or weak *GtLTP1* expression did not show reduction of the extent of lesion sizes against *B. cinerea* (lines 2 and 3), suggesting that overexpression of *GtLTP1* suppresses disease development of *B. cinerea* and that *GtLTP1* has an antifungal activity.

The antifungal activity of cysteine-rich antimicrobial proteins, such as thionine and defensine, is reduced by cations (Cammue et al. 1992; Florack et al. 1994; Terras et al. 1993; Terras et al. 1995). These antifungal peptides might interact with fungal membrane lipids to form membrane pores and cause leakage of cytoplasmic materials (van Loon and van Strien 1990). However, the antifungal activity of purified antimicrobial fractions composed of both 8.0 KP and 7.0 KP was scarcely affected by the presence of cations (Table 1), suggesting a different mechanism of antifungal activity than defensin, thionin, and hevein-like protein. Based on the P-Sort program, the probable subcellular localization of GtLTP1 is outside the cell, including the cell wall. Certain LTPs with fungicidal activity are localized in apoplastic areas (Garcia-Olmedo et al. 1995), and GtLTP1 may show similar localization.

LTPs are involved in plant-pathogen interactions. Jung et al. (2003) reported that the transcripts of three LTPs accumulated in the leaf, stem, and fruit tissues of pepper plants following infection with *Xanthomonas campestris* pv. *vesicatoria*, *Phytophthora capsici*, and *Colletotricum gloeosporioides*, and that they were strongly induced in systemic leaves infected by pathogenic or non-pathogenic bacteria. Moreover, LTP expression increased following inoculation of the pepper plants with *Tobacco mosaic virus* (Park et al. 2002). In addition, nsLTP1s share some structural and non-specific lipid binding properties with elicitins from *Phytophthora* species, which induces a hypersensitive response. Both nsLTP1 and elicitin are commonly bound to elicitin receptors on the plasma membrane, suggesting that LTP1 can modulate intracellular signal transduction (Blein et al. 2002; Buhot et al. 2001). LTP can also play a role in long-distance systemic signaling in plants (Sarowar et al. 2009). These results indicate that plant LTPs can function both in direct plant defense as antimicrobial agents and in regulation of plant immune responses.

Transgenic plants overexpressing *GtLTP1* acquired disease resistance. We speculate that GtLTP1 shows direct toxicity to the microbial pathogen and/or regulates plant immune responses. In conclusion, the GtLTP1 gene might have potential for genetic engineering of enhanced disease resistance in plants. GtLTP2 might also contribute to antifungal activity and the regulation of plant immune responses, although further study is needed to verify this hypothesis.

Acknowledgements

We are grateful to Ms. Kaori Oomiya and Shizuka Nagasaka for technical assistance during this experiment. This work was supported by Iwate prefecture government.

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