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Expression of Human Lactoferrin cDNA Confers Resistance to *Ralstonia solanacearum* in Transgenic Tobacco Plants

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ABSTRACT

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A construct containing a human lactoferrin cDNA was used to transform tobacco (*Nicotiana tabacum*) using an *Agrobacterium*-mediated DNA-transfer system to express this human protein in transgenic plants. Transformants were analyzed by Southern, Northern, and Western blots to determine integration of the cDNA into the plant genome and lactoferrin gene expression levels. Most transgenic plants demonstrated significant delays of bacterial wilt symptoms when inoculated with the bacterial pathogen *Ralstonia solanacearum*. Quantification of the expressed lactoferrin protein by enzyme-linked immunosorbent assay in transgenic plants indicated a significant positive relationship between lactoferrin gene expression levels and levels of disease resistance. Incorporation of the lactoferrin gene into crop plants may enhance resistance to other phytopathogenic bacteria as well.

Additional keywords: antibacterial proteins, *Burkholderia*, lactoferricin, *Pseudomonas*, shiva-1.

Expression of nonplant antibacterial protein genes in engineered plants has great potential for combating bacterial diseases (12,17, 24). Transgenic potato or tobacco plants have been developed using constructs encoding shiva-1 (14), SB37 (10,19), or T4 lysozyme (7) for enhancing resistance of these plants to either bacterial wilt caused by *Ralstonia solanacearum* or to soft rot incited by *Erwinia carotovora* pv. *atroseptica*. Both shiva-1 and SB37 are well-characterized bactericidal peptides (about 4 kDa) that are synthetic derivatives of cecropin found in insect guts (6,12–14). T4 lysozyme, on the other hand, is an antibacterial bacteriophage protein (7) that disrupts the bacterial membrane (17).

Among numerous nonplant antibacterial proteins isolated to date, mammalian lactoferrin (Lf), an iron-binding glycoprotein, is of special interest to us. This protein has long been reported as a major component of infant defense systems. Its mode of action against bacteria is more bactericidal than bacteriostatic (5,15). A study on human and bovine Lf (both sharing a high degree of identity in their amino acid sequences) revealed that a potent bactericidal domain exists in a short peptide sequence termed lactoferricin near the N-terminal region of the Lf molecule (2). While Lf by itself is at most bacteriostatic, its N-terminal peptide (about 25 to 47 amino acids), which can be released by proteolytic cleavage, is highly bactericidal (23). This peptide, lactoferricin, is the shortest active amino acid sequence that is resistant to further enzymatic cleavage (2). Another prominent property of Lf or lactoferricin is its potent activity against a wide range of microorganisms including both gram-negative and gram-positive bacteria, as well as fungi and viruses. Lethal concentrations of bovine lactoferricin, for instance, ranged from 3 to 150 µg/ml against bacteria (1), 18 to 150 µg/ml against yeast (3,21), and 3 to 60 µg/ml against filamentous fungi (4). Inhibition of a human herpes virus by human and bovine Lf occurs at concentrations ranging from 0.5 to 1 mg/ml (9). Our previous research (18) demonstrated high levels of in vitro inhibition of several phytopathogenic bacteria using extracts from Lf-transformed tobacco calli. These reports suggest that the human Lf gene, once expressed in plants, may enhance plant resistance to some bacterial pathogens. Since the tobacco calli used previously (18) were nonregenerable, the current work was then undertaken to genetically engineer tobacco with this gene and determine the degree of resistance of transgenic plants to *R. solanacearum*. An in vitro assay was further conducted using synthetic bovine lactoferricin and shiva-1 to compare their antibacterial activity.

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MATERIALS AND METHODS

Binary vectors and *Agrobacterium* strain. Plasmids employing an *Agrobacterium* Ti-plasmid binary vector system that contained the human Lf gene (*hlf*) (pAM1400/*hlf*) were constructed as previously described (18), except that the neomycin phosphotransferase II selectable marker was retained (Fig. 1). The vector plasmid pAM1400 without an insert was used as a negative control. *A. tumefaciens* strain LBA 4404 was then transformed with either of these two plasmids as before (18).

Plant material and transformation. Leaf disks of *Nicotiana tabacum* cv. Xanthi nc were transformed as described earlier (11). Kanamycin-resistant primary transformed plants (designated as T_0) were obtained as reported previously (24). Stable integration of the Lf gene in the genomes of T_0 tobacco plants was confirmed by Southern blots (20) using an *hlf* cDNA fragment as a probe. Kanamycin-resistant, *hlf*-cDNA–containing T_0 plants were self-fertilized to obtain T_1 plants.

Analysis of Lf gene expression. Young seedlings of T_0 progeny were screened on modified Murashige and Skoog medium (24) containing 200 µg of kanamycin per ml, and only kanamycin-resistant seedlings were later challenged with *R. solanacearum*. For Northern blots, frozen leaf samples were ground in liquid nitrogen, and total RNA was extracted using Trizol reagent (Life Technologies, Inc., Gaithersburg, MD) following the manufacturer's instructions. Twenty micrograms of total RNA from each sample was electrophoresed on 1% agarose gels in 1× Tris-borate-EDTA buffer (20). The gel was then soaked in 7% formaldehyde and 2× SSPE

(1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]) (20) for 15 min each. RNA was blotted onto Zeta-Probe membranes (BioRad Laboratories, Richmond, CA) in 20× SSPE transfer buffer overnight by capillary transfer. Both prehybridization and hybridization were performed at 65°C in the same solution containing 1 mM EDTA, 0.5 M Na_2HPO_4, and 7% sodium dodecyl sulfate (SDS), for 30 min and 16 h, respectively. The membrane was washed for 30 min using a washing solution (1 mM EDTA, 40 mM $NaHPO_4$, and 5% SDS) twice at 65°C and further washed using another washing solution (1 mM EDTA, 40 mM $NaHPO_4$, and 1% SDS). The Lf probe was generated by excising a *hlf*-containing *Hin*dIII fragment from the original plasmid and labeling it with a ^{32}P random labeling system (Gibco BRL; Gaithersburg, MD). Western blots (BioRad Laboratories) and enzyme-linked immunosorbent assay (ELISA) (BioRad Laboratories) were conducted following manufacturer's instructions. Rabbit polyclonal antisera (Sigma Chemical Co., St. Louis) generated using human LF protein was used as the primary antibody. For sampling, two young, healthy, and fully expanded leaves from the upper portion of each plant were collected and stored frozen. These frozen leaf samples were from T_1 transgenic controls (transformed with vector alone) and from different T_1 Lf transformants representing different resistance responses to *R. solanacearum*. These T_1 transgenic plants were the progeny of respective primary (T_0) transgenic plant lines.

Culturing, storage of *R. solanacearum*, and in planta tests. *R. solanacearum* strain NC251 was provided by T. Denny, Department of Plant Pathology, University of Georgia. *R. solanacearum* was maintained in 80% glycerol at –20°C. To prepare *R. solanacearum* inoculum, 50 µl of glycerol suspension was evenly spread on nutrient broth yeast (NBY) (22) solid medium and grown at 28°C until separate colonies formed. A single colony was inoculated into tryptic soy broth (Difco Laboratories, Detroit) liquid medium and allowed to grow overnight at 28°C with shaking at 200 rpm. The cell suspension, when grown to exponential phase (A_{650} = 1), was serially diluted in 12.5 mM phosphate buffer and cooled on ice for at least 10 min before use. The culturing of bacteria and all steps of in vivo tests followed previously described procedures (16). When transformed plants developed 7 to 10 leaves, they were inoculated with *R. solanacearum*.

For in planta tests, 5 µl of two different inocula (4.8×10^4 CFU and 4.8×10^6 CFU) of the *R. solanacearum* strain were manually stabbed into the vascular system from a nodal region of the secondary leaf of the bottom stem (16) using a calibrated #20 sewing needle. In vivo resistance or susceptibility was recorded as the number of days needed for all the leaves to become totally wilted. Disease progress was recorded at 3-day intervals. Besides inoculated transgenic vector-only controls and Lf transgenic plants, additional wild-type plants were inoculated with only 12.5 mM phosphate buffer. The plants were grown under a 16-h photoperiod at an average temperature of 28 ± 2°C in growth chambers.

Reisolation of bacteria from inoculated plants. The populations of bacteria in infected tissues of both the most resistant transformants and susceptible controls were determined at the time when wilted control plants began to die. The reisolation was conducted as follows: 60 mg of a basal portion of the leaf petiole of a fully expanded young leaf from the top of each inoculated plant was ground separately in 2 ml of 12.5 mM phosphate buffer using a mortar and pestle. The bacterial populations were determined as CFU on NBY plates (22), based on three replicate plate counts for each dilution.

Synthesis of bovine lactoferricin and shiva-1 and assay of their antibacterial activity. The bovine lactoferricin containing the antibacterial domain (25 amino acids) and shiva-1 (45 amino acids) were synthesized following essentially the method reported elsewhere (13) through the Protein Core Facility Center at the University of Nebraska-Lincoln. The resultant high-performance liquid chromatography profiles showed a purity of more than 98%. The synthesized peptides were lyophilized and stored at –20°C before use. The in vitro antibacterial activity of these two peptides was determined by the method described earlier (18). All three bacterial strains were grown to A_{600} of 0.4 in NBY liquid medium (22), and 2-ml aliquots were transferred to culture tubes. Various amounts of synthetic peptides were added to the culture tubes to obtain final concentrations of 0, 1, 10, and 100 µM. Ten microliters of bacterial suspension at different dilution series was spotted onto the NBY plate. Viable cell counts were then made on NBY agar as described earlier (18).

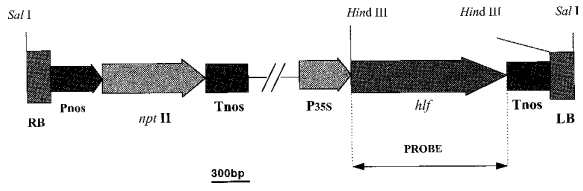

Fig. 1. Schematic diagram of the T-DNA region from the binary vector pAM1400/*hlf*. RB and LB, T-DNA right and left border sequences, respectively; P_{nos} and T_{nos}, nopaline synthase gene promoter and terminator, respectively; *npt* II, neomycin phosphotransferase II conferring kanamycin resistance; P_{35S}, cauliflower mosaic virus 35S promoter; *hlf*, human lactoferrin cDNA; and Probe, segment of DNA used to hybridize blots in Figure 3.

RESULTS

Reactions of transgenic tobacco plants to *R. solanacearum*. Primary tobacco (*N. tabacum* cv. Xanthi nc) transformants (T_0) were transformed with a cDNA encoding human Lf (Fig. 1). Individual transformants were evaluated for bacterial resistance under growth chamber conditions by manually inoculating them with *R. solanacearum* at two inoculum levels (Table 1). A total of 12 T_0 transformed plants including eight Lf transgenic and four transgenic control plants (transformed with vector alone) as well as three *N. tabacum* cv. Xanthi nc 1 (XNC-1) wild-type plants were tested. The first symptoms of wilt were observed in inoculated control plants 1 week after inoculation. *R. solanacearum*-inoculated T_0 transgenic tobacco plants expressing Lf protein were significantly delayed in wilting of both the first leaf and 100% of leaves compared with either transgenic vector-only controls (XNC-T_0-1 and XNC-T_0-2) or nontransgenic wild type (XNC-1) (Table 1). Plants started to show wilting from the very bottom leaf; hence, 100% leaf wilting

TABLE 1. Responses of T_0 human lactoferrin gene (*hlf*) transgenic tobacco plants inoculated with *Ralstonia solanacearum* (9.5×10^6 and 9.5×10^8 CFU/ml)

Inoculum	Tobacco line	100% leaf wilt DAI[a]	Delayed wilt (days)[b]
9.5×10^6	XNC-1	23	0
	XNC-T_0-1	23	0
	XNC-T_0-2	22	0
	XNC/*hlf*-T_0-1	54	31
	XNC/*hlf*-T_0-2	44	21
	XNC/*hlf*-T_0-3	54	31
	XNC/*hlf*-T_0-4	29	6
9.5×10^8	XNC-1	12	0
	XNC-T_0-3	11	0
	XNC-T_0-4	12	0
	XNC/*hlf*-T_0-5	29	17
	XNC/*hlf*-T_0-6	23	11
	XNC/*hlf*-T_0-7	23	11
	XNC/*hlf*-T_0-8	44	32
Buffer only	XNC-1	NA[c]	NA

[a] Days after inoculation.

[b] Delayed wilt is relative to transgenic control plants that wilted 23 days and 12 days after inoculation with 9.5×10^6 and 9.5×10^8, respectively. Delay of wilting was determined as the number of days needed for the transgenic plants to show 100% wilt in all the leaves compared with control plants. Disease progress was recorded at 3-day intervals. The average number of days in delayed wilting shown by lactoferrin T_0 transgenic lines were significantly different from those of control lines across two inoculum levels ($P \leq 0.05$) as determined by protected least significant difference.

[c] NA = not applicable because plants inoculated with buffer did not wilt.

meant all the leaves were wilted. Although partial wilting in a single leaf was observed, a leaf was scored as a wilted leaf only when the whole leaf became wilted in order to make unbiased scoring. About 3 weeks after inoculation with the 9.5×10^6-CFU/ml inoculum, all the leaves of the control plants wilted, and then they died 1 week later. At a higher inoculum (9.5×10^8 CFU/ml) concentration, all the leaves of the control plants wilted within 11 to 12 days after inoculation. In contrast, wilting was delayed in Lf transgenic plants from 6 days (XNC/*hlf*-T_0-4) to 32 days (XNC/*hlf*-T_0-8) compared with controls, even at the higher inoculum concentration. Transgenic plants with a delay in wilting of more than 21 days eventually flowered and produced mature seeds. The plants with the higher resistance levels produced normal seeds similar to those of wild-type control plants inoculated only with buffer. However, micropropagation of seedling nodes had to be used in order to rescue the Lf transgenic lines (XNC/*hlf*-T_0-2, -4, -5, -6, and -7) and transgenic control plants that showed early wilting. To confirm the disease reaction levels and characterize the transgenic plants further, seeds from these rescued T_0 plants were planted, and young seedlings were screened on kanamycin-selection growth medium. T_1 kanamycin-resistant seedlings (Table 2), representing four different T_0 lines, were selected. A total of 48 T_1 plants were then challenged with the 2.5×10^6-CFU/ml inoculum. About 14 days after inoculation, all leaves of the control plants were wilted, whereas wilting of most Lf transgenic plants was significantly delayed (Fig. 2). However, two progenies of line XNC/*hlf*-T_1-4 did not show any resistance compared with the controls (Table 2). The maximum delay in wilting (3 weeks) was observed for transgenic lines XNC/*hlf*-T_1-2 and XNC/*hlf*-T_1-8 (Table 2 and Fig. 2). Most of the T_1 plants exhibiting a 2-week delay in wilting grew to maturity and produced seeds; however, a few plants with a 15- to 21-day delay in wilting did not produce fully mature seeds. Plants with a delay in wilting of more than 21 days always produced normal seeds.

TABLE 2. Responses of T_1 human lactoferrin gene (*hlf*) transgenic tobacco plants inoculated with *Ralstonia solanacearum* (2.5×10^6 CFU/ml inoculum)[a]

Plant line[b]	Total number of plants	Delayed wilt (days)[c]					
		0	3	6	9	15	21
XNC-1-1*	1	NA[d]					
XNC-T_1-1**	6	6					
XNC-T_1-3**	6	6					
XNC/*hlf*-T_1-2***	12			6		4	2
XNC/*hlf*-T_1-4***	12	2	2	6		2	
XNC/*hlf*-T_1-5***	12		2	2	2	6	
XNC/*hlf*-T_1-8***	12			2		4	6

[a] Data represent the number of plants that have a given delay
[b] The plant lines were derived from the seeds of corresponding T_0 lines.
[c] Delayed wilting of over 3 days is significantly different ($P \leq 0.05$) from controls as detected by protected least significant difference. Delay of wilting was determined as the number of days needed for the transgenic plants to show 100% wilt in all the leaves compared with control plants. Disease progress was recorded at 3-day intervals. *, wild-type control plant; **, transgenic vector-only control plants; and ***, transgenic plants containing the lactoferrin gene.
[d] NA = not applicable because plants inoculated with buffer did not wilt.

Fig. 2. Representative picture of phenotypic symptoms observed in three different tobacco lines 12 days after inoculation. Left, lactoferrin transgenic tobacco line (XNC/*hlf*-T_1-8) showing a significant delay in wilting; middle rear, transgenic control (vector only) tobacco line (XNC-T_1-2) showing severe wilt symptoms and stunted growth; and right, wild-type tobacco line (XNC-1) that was inoculated with only buffer solution, showing no wilt symptoms.

Analysis of Lf gene expression. RNA extracted from transformants representing different resistance levels (0, 3, 6, 9, 15, and 21 days of delayed wilting) was analyzed by Northern blotting (Fig. 3). Transcripts of about 2.3 kb, which corresponded to the expected full-length Lf mRNA, were detected in all transgenic plants with various resistance levels to *R. solanacearum*, except for controls. Western blot results (Fig. 4) demonstrated differences in Lf translational levels as indicated by different intensities of Lf protein bands. The size of these bands (about 80 kDa) corresponded to full-length human lactoferrin protein (HLF). A low-molecular-weight protein band (48 kDa) was also seen in plants with high levels of resistance, especially in line XNC/*hlf*-T_1-8, in addition to the full-length Lf bands (Fig. 4). This 48-kDa band, previously detected in *hlf* transgenic calli (18), was only seen in line XNC/*hlf*-T_1-8, which had the highest bacterial disease resistance level. It appears that a small portion of Lf protein was truncated or degraded within this transformant. Further quantification of total Lf proteins by ELISA assay (Fig. 5) revealed a positive correlation (+0.964) of enhanced disease resistance with Lf levels. The estimated HLF protein concentrations ranged from 0.1 to 0.8% of total soluble proteins, depending on the HLF expression levels in different plants as determined by ELISA.

Reisolation of *R. solanacearum* from resistant and susceptible plants. To establish the relationship of disease severity with various levels of *R. solanacearum* inoculum, *R. solanacearum* was isolated from both resistant and vector-only transgenic control plants when the wilted wild-type control plants started to die. Viable cells counts (data not shown) could not detect *R. solanacearum* in the highly resistant XNC/*hlf*-T_1-8 transgenic plants even at high

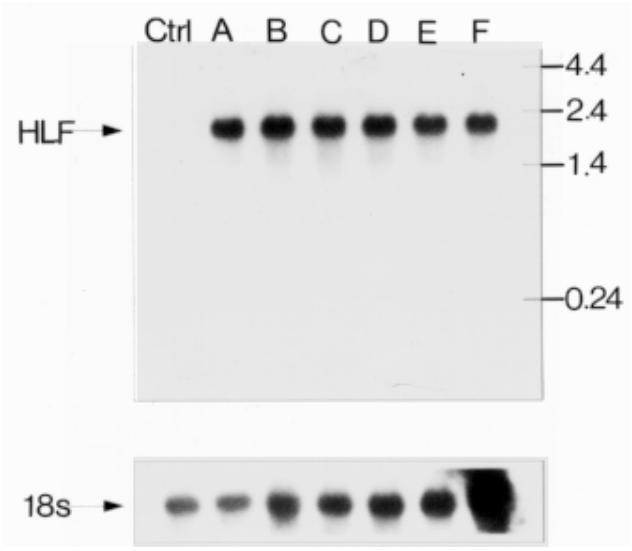

Fig. 3. Northern blot analysis of the tobacco plants representing different resistance levels. Ctrl, mRNA from transgenic control; lanes A, C, and E, mRNA from lactoferrin transgenic plants (XNC/*hlf*-T_1-5, XNC/*hlf*-T_1-4, and XNC/*hlf*-T_1-8, respectively) in which wilting was delayed 9, 15, and 21 days, respectively; lanes B, D, and F, mRNA from lactoferrin transgenic plants (XNC/*hlf*-T_1-4*, XNC/*hlf*-T_1-5*, and XNC/*hlf*-T_1-2, respectively) in which wilting was delayed 0, 3, and 6 days, respectively; and HLF, lactoferrin mRNA. The bands in the bottom panel show 18s transcripts as an internal control. The numbers at left are size markers in kb. * = Different progeny from the same T_0 lines.

inoculum levels and the lowest dilution (<300 CFU/g), while approximately 8.3×10^8 CFU/g was obtained from the susceptible controls at 2 weeks after inoculation.

Assay of antibacterial activity of bovine lactoferricin and shiva-1. A previous study demonstrated enhanced bacterial disease resistance to *R. solanacearum* in transgenic potato and tobacco plants expressing shiva-1 (12,14). Therefore, we further wanted to determine if the bovine lactoferricin, as both a key antibacterial component of Lf and a representative of lactoferricin, was more bactericidal than shiva-1 against phytopathogenic bacteria. We synthesized both bovine lactoferricin and shiva-1 according to their published sequences (2,6). Three gram-negative pathogens of crop plants were selected for the experiment: *Xanthomonas campestris* pv. *phaseoli* (common blight of common beans), *R. solanacearum* (bacterial wilt), and *Pseudomonas syringae* pv. *phaseolicola* (bean halo blight). These bacteria represent major phytopathogens of either common beans (*Phaseolus vulgaris*) or other crop species. At concentrations of 10 μM or above, lactoferricin showed more bactericidal activity in vitro than did shiva-1 against all three bacterial strains (Table 3) as indicated by a decreased number of CFU. In particular, strain *R. solanacearum* was more sensitive to lactoferricin than to shiva-1. At the 100-μM peptide concentration, bovine lactoferricin completely inhibited colony formation of three bacterial species at the lowest dilution (10^{-2}) series (≤100 CFU was designated in this dilution series when no colonies were found) (Table 3).

DISCUSSION

Transferring a human Lf cDNA into tobacco genome enhanced bacterial disease resistance. Previous results indicated that Lf was expressed in transgenic calli, and the extracted Lf in total protein preparations inhibited bacterial multiplication of four bacterial pathogens in vitro (18). Our current results indicate that inhibition of bacterial growth leading to increased resistance occurs in planta as well. These results are encouraging, because wilting was delayed over 3 weeks in most resistant transgenic plant lines. There are no previous reports on the use of the Lf gene to confer resistance to plant pathogens.

It is interesting that intact full-length instead of truncated Lf is the major protein product (about 80 kDa molecular weight) in transgenic tobacco plants, in contrast with our earlier findings (18) that consistently showed major Lf-derived bands at 48 kDa in all transgenic tobacco calli. Post-translational processing in transgenic plants may differ from that in transgenic calli. Cells in the callus stage, especially after undergoing several passages under tissue culture conditions, undergo different physiological changes and are known to produce reduced or modified proteins. The effectiveness of both truncated HLF in our in vitro tests (18) and the full-length HLF expressed here in in planta tests suggested that their mechanisms against bacteria may be correlated. In transgenic plants, however, it is not clear how Lf affects *R. solanacearum* in planta. Perhaps, the full-length HLF may first act as an iron-chelating agent that reduces the biological availability of iron for the invading bacteria, causing decreased multiplication of the bacterial population. It is also likely that lactoferricin could be released from the full-length HLF molecule by plant proteases and become bactericidal, because smaller-sized proteins cross-reacting with HLF antiserum were detected in plants with high expression levels of HLF by using Western blots (Fig. 4). This postulation is deduced from the fact that the full-length Lf was at most bacteriostatic, whereas lactoferricin was bactericidal (2). We further speculate that this truncated, 48-kDa protein could contain the lactoferricin active domain, although more molecular evidence confirming this hypothesis

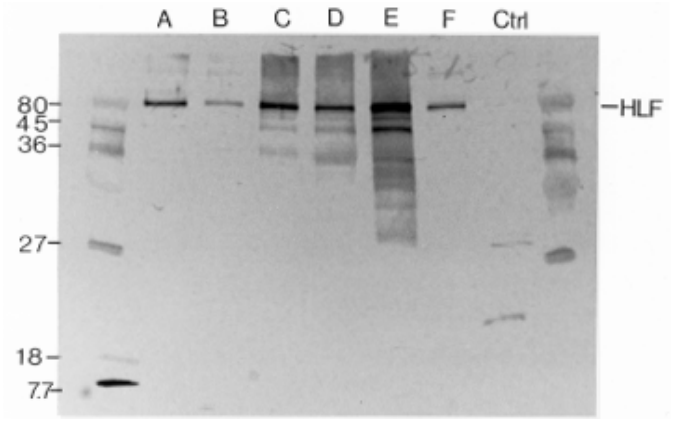

Fig. 4. Western blot analysis of the tobacco plants representing different resistance levels. Ctrl, transgenic (vector only) control; lanes A, C, and E, lactoferrin transgenic plants (XNC/*hlf*-T_1-5, XNC/*hlf*-T_1-4, and XNC/*hlf*-T_1-8, respectively) in which wilting was delayed 9, 15, and 21 days, respectively; lanes B, D, and F, lactoferrin transgenic plants (XNC/*hlf*-T_1-4*, XNC/*hlf*-T_1-5*, and XNC/*hlf*-T_1-2, respectively) in which wilting was delayed 0, 3, and 6 days, respectively; and HLF, intact human lactoferrin protein. The numbers at left are protein standard markers in kDa. * = Different progeny from the same T_0 lines.

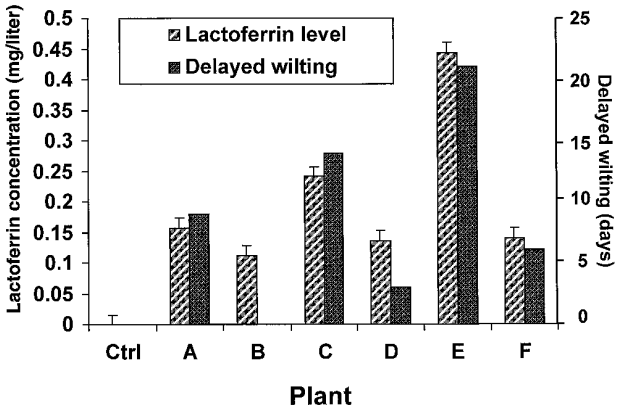

Fig. 5. Relationship between lactoferrin protein levels and different disease resistance responses of transgenic tobacco and control plants. Ctrl, transgenic (vector only) control; columns A, C, and E, lactoferrin transgenic plants (XNC/*hlf*-T_1-5, XNC/*hlf*-T_1-4, and XNC/*hlf*-T_1-8, respectively) in which wilting was delayed 9, 15, and 21 days, respectively; columns B, D, and F, lactoferrin transgenic plants (XNC/*hlf*-T_1-4*, XNC/*hlf*-T_1-5*, and XNC/*hlf*-T_1-2, respectively) in which wilting was delayed 0, 3, and 6 days, respectively. Enzyme-linked immunosorbent assay data were obtained from 10 replications. Bars represent standard error of a mean. * = Different progeny from the same T_0 lines.

TABLE 3. Antibacterial activity of synthetic shiva-1 and bovine lactoferricin peptides against three phytopathogenic bacteria

Bacterial species	μM Peptide concentration	$\log_{10}$ CFU/ml[a] shiva-1	BLFcn[b]
Xanthomonas campestris pv. *phaseoli*	0	9.68 ± 0.08	9.51 ± 0.35
	1	9.65 ± 0.11	9.20 ± 0.23
	10	9.47 ± 0.07	8.84 ± 0.14
	100	2.33 ± 0.58	≤2.00 ± 0.00
Ralstonia solanacearum	0	9.55 ± 0.18	9.55 ± 0.10
	1	9.42 ± 0.09	6.74 ± 0.06
	10	9.31 ± 0.12	4.00 ± 0.00
	100	8.77 ± 0.02	≤2.00 ± 0.00
Pseudomonas syringae pv. *phaseolicola*	0	9.32 ± 0.10	9.62 ± 0.02
	1	9.29 ± 0.20	8.59 ± 0.08
	10	8.92 ± 0.38	3.13 ± 0.15
	100	5.44 ± 0.02	≤2.00 ± 0.00

[a] Means are from two independent experiments, each of which contained three replications. A lower limit of 100 CFU/ml ($\log_{10}$ CFU/ml ≤ 2) was designated for plate counts when no colony was found at the lowest dilution series (no dilution). Numbers immediately following the mean indicate the standard deviation of the mean, i.e., standard deviation of log CFU.

[b] BLFcn = bovine lactoferricin.

is needed. Degradation of HLF proteins appeared to be less pronounced in our Lf transgenic plants than in transformed calli (18). Degradation of Lf may, however, be beneficial because of the release of lactoferricin, which would be more antibacterial.

Variations in resistance levels to *R. solanacearum* were observed among and within different transgenic plant lines. These may be due to location of the transgene insertion, different copy numbers, or homozygosity and heterozygosity of the *hlf* gene among the T_0 progenies. Differential modulation of HLF by Ca^{2+} and Mg^{2+}, possibly due to variations in levels of these ions caused by different physiological conditions, may also be responsible for the nonuniform resistant responses observed in different T_1 transgenic lines (Fig. 4, lanes B and D, in which Lf levels appear to be the same based on band intensities in Western blots). This mechanism of modulation has been shown previously under in vitro conditions (1,8). The average height of Lf transformed plants was about 10% shorter than those of either transgenic or wild-type controls (data not shown). This effect might be due to the perturbations and imbalance of iron availability caused by Lf, since Lf has strong iron-binding ability.

Bovine lactoferricin is a close relative of human lactoferricin (2). Both peptides are the only antibacterial fragment derived from their corresponding full-length Lf proteins upon pepsin cleavage, but are resistant to further pepsin digestion. Furthermore, both peptides contain an 18-amino acid loop that is critical for their antibacterial activity (2). However, bovine lactoferricin (25 amino acids and about 3.1 kDa) is more bactericidal than human lactoferricin (47 amino acids and about 5.6 kDa) (2). Our previous study (18) and data obtained here also strongly suggest that lactoferricin, rather than full-length Lf, has stronger in planta antibacterial activity in transgenic plants. Therefore, we chose bovine lactoferricin as a first step to evaluate its in planta antibacterial potential and compared its activity with that of shiva-1, one of the most widely reported nonplant bactericidal peptides based on an in vitro assay system. Our data here clearly showed that bovine lactoferricin was even more potent than shiva-1 against all three representative phytopathogenic bacteria. In fact, bovine lactoferricin and shiva-1 are both basic peptides and serve as membrane-disruptive agents upon interaction with bacterial membranes (1,2,6,12,14,23,24). However, bovine lactoferricin is much smaller and more cationic than shiva-1 and contains an 18-amino acid loop (2). These chemical and structural features may help lactoferricin penetrate the bacterial membrane more efficiently and protect it from further degradation by plant proteases. This may, in part, explain why lactoferricin showed a more potent effect than shiva-1 in our assay. These collective results suggest that lactoferricin, once properly translated but not severely degraded in engineered plants expressing the lactoferricin gene, could confer higher levels of bacterial disease resistance to transgenic plants than could shiva-1.

The expression of human Lf cDNA conferred resistance to *R. solanacearum* in transgenic tobacco plants. It would be useful to determine if the enhanced bacterial disease resistance may be achieved by incorporating this mammalian gene or a short synthetic DNA sequence encoding lactoferricin into other economically important crop plants using plant transformation.

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