Expression of a Human Lactoferrin cDNA in Tobacco Cells Produces Antibacterial Protein(s)*

Amitava Mitra* and Zhanyuan Zhang

Department of Plant Pathology and the Center for Biotechnology (A.M.) and Department of Horticulture (Z.Z.), University of Nebraska, Lincoln, Nebraska 68583-0722

A suspension tobacco (Nicotiana tabacum L.) cell line was transformed to express human lactoferrin, an iron-binding glycoprotein. The transgenic calli produced a protein that was significantly smaller than the full-length lactoferrin protein. Total protein extracts made from transgenic tobacco callus exhibited much higher antibacterial activity than commercially available purified lactoferrin as determined by the decrease of colony-forming units when tested with four phytopathogenic species of bacteria. Introduction of the lactoferrin gene in crop plants may provide resistance against phytopathogenic bacteria.

Lactoferrin, originally found in milk, is a member of a family of iron-binding glycoproteins that includes transferrin and melanotransferrin found in polymorphonuclear leukocytes and in mucosal and epithelial secretions. It is the major iron-binding protein of human mammary secretions. Lactoferrin genes from various sources show significant homology and code for a single polypeptide chain of about 700 amino acids (M, approximately 80,000), with an iron-binding site in each of the similar amino- and carboxy-terminal regions. High levels of lactoferrin expression have been shown in neutrophils (Masson et al., 1969), in lactating mammary glands (Green and Pastewka, 1978), and in the uterus under different types of regulation, whereas a low level of constitutive expression is found in many biological fluids (Liu and Teng, 1991). Lactoferrin has been associated with the promotion of cell growth, regulation of hematopoiesis, and protection against microbial infection and immune modulating properties in vitro. Little, however, is known about the function of lactoferrin in vivo. Extracellular lactoferrin has been reported to have both antibacterial and antiviral activities (Arnold et al., 1977; Lu et al., 1987).

Diseases caused by phytopathogenic bacteria are of major economic importance and result in losses of more than $100 million annually (Kennedy and Alcorn, 1980; Perombelon and Kelman, 1980). Advancement in plant genetic engineering methodologies has allowed introduction of foreign genes in plants, creating the potential for development of disease-resistant plants. Expression of an antibacterial gene in a plant, in theory, could produce resistance or tolerance in transgenic crop plants. Lactoferrin has been known as an antibacterial agent for a long time. Since the initial inoculum required for development of plant bacterial disease is usually small, we thought that high-level, constitutive expression of an antibacterial agent such as lactoferrin might be able to reduce or control plant bacterial diseases. As a first step in achieving this objective, an attempt was made to express a human lactoferrin cDNA in tobacco (Nicotiana tabacum L.) suspension cells. In this study we demonstrate the expression of the lactoferrin protein in plant cells. Furthermore, it appears that the gene product undergoes posttranslational processing, because only a truncated protein is detected in plant cells. The protein extract from transgenic calli exhibited a strong bactericidal activity.

MATERIALS AND METHODS

Construction of Plasmids

The original plasmid pLACMODC/18.1 containing a modified human lactoferrin cDNA was kindly provided by Dr. M. Powell (Delta Biotechnology Limited, Nottingham, UK) (Powell and Ogden, 1990). The plasmid contained the 2082-bp mature lactoferrin protein sequence preceded by a synthetic ATG but without the sequence encoding the signal peptide. The lactoferrin cDNA was subsequently subcloned into the binary plant expression vector pAM1400 using the BamHI or HindIII sites downstream from the cauliflower mosaic virus 35S promoter to achieve a transcriptional fusion between the cauliflower mosaic virus 35S promoter and the lactoferrin cDNA. The resultant binary plasmid vector was named pAM1401 (Fig. 1, top). The plant expression vector pAM1400 was constructed by replacing the neomycin phosphotransferase-selectable marker in the plasmid pGA643 (An et al., 1988) with the bar-selectable marker (Fromm et al., 1990). This allowed selection of transgenic calli using the herbicide bialaphos and its derivatives. The binary plasmid also contained a transcriptional termination signal from the T-DNA gene nopaline synthase immediately following the lactoferrin stop codon.

Transformation of Tobacco Cells

A suspension tobacco cell line NT-1 (Nicotiana tabacum L. cv Bright Yellow) (Nagata et al., 1981) was transformed by the Agrobacterium co-cultivation method (Horsch et al., 1985; Mitra and An, 1989). Transformed tobacco cells were selected on Murashige and Skoog (1962) plates containing 40 mg/L...
Ignite. Ignite, a commercial formulation of glufosinate-ammonium, was a gift from the Hoechst-Roussel Agri-Vet Co (Loland, MS). Individual transformants were pooled to form 10 groups, each group consisting of 10 independently transformed calli.

**Verification of Insert**

Integration of the plasmid DNA into callus DNA was determined by Southern blot analysis (Southern, 1975). Five grams of fresh callus tissue from each of the 10 groups were used to prepare DNA as described by Guillemaut and Maréchal-Drouard (1992). Briefly, callus tissue was ground in liquid nitrogen with extraction buffer (100 mM sodium acetate, pH 4.8, 50 mM EDTA, pH 8.0, 500 mM NaCl, 2% PVP-40, mixture adjusted to pH 5.5, then SDS to 1.4%, and Cys to 10 mM), incubated at 65°C for 20 min and centrifuged. One-half volume of 3 M potassium acetate (pH 4.8) was added to the supernatant and incubated at 0°C for 1 h. The DNA was recovered by ethanol precipitation, dissolved in Tris-EDTA (10 mM Tris-Cl, 1 mM EDTA, pH 8.0), and centrifuged overnight in a cesium gradient. DNA was digested with HindIII, separated on a 1% (w/v) agarose gel, and transferred to a Zeta-probe membrane (Bio-Rad) according to manufacturer's recommendations. The membrane was hybridized with a randomly labeled DNA probe using 2082 bp of human lactoferrin cDNA as the template.

To determine expression levels of the lactoferrin cDNA in the transformed NT-1 calli, total RNA was prepared from each of the 10 groups of transformants as described by Logemann et al. (1987). RNA extraction was done in a buffer containing 8 mM guanidinium chloride, 20 mM Mes, 20 mM EDTA, and 50 mM β-mercaptoethanol, pH 7.0. RNA samples were separated on formaldehyde gel, transferred to the Zeta-probe membranes, and probed as above. As an internal control, a labeled β-tubulin fragment was used to probe total RNA from transgenic and control tobacco calli.

**Extraction of Total Protein from the NT-1 Calli**

A total cellular protein extract was prepared by homogenization of the transformed callus in protein extraction buffer (0.5 M Suc, 0.1% ascorbic acid, 0.1% Cys-HCl, 0.01 M Tris-HCl, pH 7.5) (Mitra et al., 1989) in the presence of 15 μg of PMSF in liquid nitrogen. The homogenate was centrifuged at 14,000g for 30 min at 4°C, and the supernatant containing the soluble proteins was recovered. Protein extracts containing 100 μg of total protein were separated on a 12.5% (w/v) acrylamide gel (Laemmli, 1970) along with 200 ng of commercially available lactoferrin (Sigma) as a standard. The resolved proteins were transferred to a nitrocellulose membrane using a Bio-Rad transblot apparatus at 250 mA for 1 h. The membrane was immersed in a blocking solution (5% nonfat dry milk in Tris-buffered saline) and incubated at

---

**Figure 1.** Transformation of tobacco suspension culture cells with the gene encoding human lactoferrin. Top, Schematic diagram of the plant expression cassette from the plasmid pAM1401. RB and LB, T-DNA right and left border sequences, respectively; bar, herbicide resistance gene; P35S, cauliflower mosaic virus 35S promoter; Tnos, T-DNA nopaline synthase gene terminator. Bottom left, Southern blot of transgenic calli. Total genomic DNA was digested with HindIII, transferred to a membrane, and hybridized with 32P-labeled randomly primed fragments generated from 2082 bp of human lactoferrin cDNA (hLF) probe as marked in the diagram. Numbers at right are size markers in kb. Bottom right, Northern blot analysis of the transgenic calli. Total cellular RNA was isolated and electrophoresed on a 1% agarose-formaldehyde gel, transferred to a membrane, and hybridized with the same probe as in bottom left panel. The bottom panel shows β-tubulin (tub) transcripts.
Expression of an Antibacterial Gene in Tobacco

28°C for 1 h. The membrane was washed and incubated with 1:1000 dilution of a commercially available (Sigma) polyclonal antibody prepared against human lactoferrin antigen. Following incubation in the lactoferrin antibody, the membrane was placed in the biotinylated goat anti-rabbit antibody solution for 1 h at 28°C with gentle agitation. The membrane was washed and incubated in the streptavidin-biotinylated alkaline phosphatase complex for 1 h, followed by immersion in the color development solution until the purple bands became visible, usually within 10 min.

Assay of Antibacterial Activity

For determinations of antibacterial activity, total protein extract was made from the transgenic callus in protein extraction buffer as stated above except that no PMSF was used and the protein extracts were concentrated using a concentrator. Four different phytopathogenic bacterial strains, Xanthomonas campestris pv phaseoli, Pseudomonas syringae pv phaseolicola, Pseudomonas syringae pv syringae, and Clavibacter flaccumfaciens pv flaccumfaciens, were grown to an A600 of 0.4, and aliquots of 2 mL were transferred to culture tubes. Various amounts of callus extract, ranging from 0 to 500 µg of total protein, were added to each culture tube and incubated at 27°C until the A600 of the control cell suspension reached to 1.00. The final volume of suspensions in each sample of all treatments was adjusted to 2.5 mL by adding appropriate amounts of culture medium. Commercially available lactoferrin solution and extract from tobacco calli transformed with the plasmid pAM1400 were used as positive and negative controls, respectively. After the incubation, the total number of colony-forming units was determined by serial dilution on nutrient agar plates and counting the number of colonies.

RESULTS AND DISCUSSION

Tobacco Cell Transformation with Lactoferrin cDNA

Transformed tobacco calli were selected on 40-mg/L Ignite and subcultured three times on herbicide-supplemented Murashige-Skoog plates. The transformed transgenic calli had a normal appearance and had similar growth characteristics as the control calli. Southern blot analysis of HindIII-digested genomic DNA from several independently transformed transgenic calli showed the expected 2.4-kb bands (Fig. 1, bottom left). All herbicide-resistant calli tested had this band. Northern blot analysis of the same calli indicated the presence of 2.3-kb transcripts (Fig. 1, bottom right).

Expression of Lactoferrin Protein in Transgenic Tobacco Calli

The transgenic tobacco calli were tested for the expression of the lactoferrin protein by western blot analysis. The amount of total protein in the extract was quantified by the Bradford (1976) method. A known amount of protein from transformed calli was loaded on a gel and transferred to a nitrocellulose membrane for immunoblotting. All transgenic calli expressed lactoferrin protein, but the control calli extract did not react with the anti-lactoferrin antibody (Fig. 2B). The immunoreactive lactoferrin levels did not differ significantly among all the transgenic calli tested. The plant-produced lactoferrin was compared to the full-length human lactoferrin (Fig. 2B, lane 2 versus lane 3). The lactoferrin cDNA that was expressed in the tobacco calli had a 2082-bp coding sequence, indicating that the mature protein should be approximately 80 kD. The full-length protein was never detected in transgenic tobacco callus, although a total of 50 independently transformed calli were screened. Furthermore, all of the calli produced a truncated lactoferrin protein of the same size. This implies a posttranslational processing of the protein, since the northern blot analysis indicated generation of a single 2.3-kb transcript. We do not know at present whether truncation is at the amino- or carboxy-terminal end of the protein. The immunoblot showed a major protein band of 48 kD in extracts from transgenic calli. A faint but slightly larger
protein band was consistently detected in all transgenic calli tested. Since a human lactoferrin gene was successfully expressed in *Aspergillus nidulans* with the production of large amounts of full-length lactoferrin (Ward et al., 1992), it is not known why full-length lactoferrin is not produced in tobacco cells. It is possible that the plant-produced lactoferrin protein does not undergo proper folding and the unfolded part is degraded. It is also not known whether the protein is glycosylated in tobacco.

**Antibacterial Properties of the Transgenic Calli Extract**

To test the antibacterial activities of the transformed tobacco calli, total protein extracts from these calli were made in a protein extraction buffer as mentioned earlier. The protein extracts were further concentrated using a filtration apparatus. Antibacterial activity was determined by adding a known amount of protein to a 2-mL culture of bacteria grown to an *A*<sub>600</sub> of 0.4. Control extracts were prepared from tobacco calli and used at the same concentration as the protein extracts from the transgenic calli. Protein extracts from the transformed tobacco calli clearly had significant antibacterial activity (Table I). Furthermore, the protein extracts were bactericidal for all of the bacterial species tested. Extracts from control calli had no antibacterial activity. Since lactoferrin produced in the tobacco cells was much more effective than the full-length lactoferrin, it is possible that the truncation of the lactoferrin in plant cells made it more toxic. A similar observation was made with the *Bacillus thuringiensis* crystal protein toxin, in which the strongest insecticidal activity was found in the truncated protein. Actual cytosolic concentration of lactoferrin in individual transformed calli varied from 0.6 to 2.5% of the total cellular protein as determined by ELISA (data not shown). Extract prepared from pooled calli had an average of 1.8% lactoferrin protein. Since protein extracts were concentrated for the antibacterial assay, the effectiveness of cytosolic lactoferrin levels is not known. Arnold et al. (1982) reported that the antibacterial activity of lactoferrin was dependent on its iron-free state and is mediated by iron chelation. Commercially available lactoferrin was only marginally antibacterial. A fairly high concentration (500 µg) of commercially available lactoferrin was required to achieve only 10% of the antibacterial activity conferred by the transgenic tobacco cell extract. The antibacterial activity of purified lactoferrin was totally abolished when the culture medium was supplemented with 0.1 mM FeSO<sub>4</sub>. However, FeSO<sub>4</sub> had no effect on the plant cell extract-induced antibacterial activity. This indicates that there must be some other mechanism for the bactericidal activity of the transgenic tobacco extract. Because transformed calli were grown in the presence of 40 mg/L herbicide, the bacterial species were also tested in medium supplemented with the herbicide, but no effect on the growth of bacteria was observed (data not shown).

Tomita et al. (1991) reported that the low mol wt peptides generated by enzymatic hydrolyzation of bovine lactoferrin showed strong broad-spectrum antibacterial activity against a number of Gram-negative and Gram-positive bacterial species. The antibacterial activities of the enzymatic hydrolysates were at least 8-fold greater than that of undigested lactoferrin. They hypothesized that the smaller peptides had better access to the target sites on the bacterial membrane and, hence, were more effective. In transgenic tobacco cells we detected only a single peptide of 48 kD, although generation of smaller antibacterial peptides that either ran off the gel or remained undetected by immunoblot cannot be ruled out. Yamauchi et al. (1993) showed that a pepsin-derived bovine lactoferrin peptide fragment had a strong bactericidal effect, whereas bovine lactoferrin was at most bacteriostatic. The protein extracts from the transformed tobacco calli were effective against both Gram-negative and Gram-positive phytopathogenic bacteria. Among the species tested, the extract was almost equally effective against *P. syringae pv phaseolicola*, *X. campestris*, and *C. flaccumfaciens pv flaccumfaciens*, whereas *P. syringae pv syringae* was slightly less sensitive.

In conclusion, it is evident from our data that the expression of a human lactoferrin gene produces a potent antibacterial activity. The transgenic tobacco cell extracts were at least 8-fold greater than that of undigested lactoferrin. They hypothesized that the smaller peptides had better access to the target sites on the bacterial membrane and, hence, were more effective. In transgenic tobacco cells we detected only a single peptide of 48 kD, although generation of smaller antibacterial peptides that either ran off the gel or remained undetected by immunoblot cannot be ruled out. Yamauchi et al. (1993) showed that a pepsin-derived bovine lactoferrin peptide fragment had a strong bactericidal effect, whereas bovine lactoferrin was at most bacteriostatic. The protein extracts from the transformed tobacco calli were effective against both Gram-negative and Gram-positive phytopathogenic bacteria. Among the species tested, the extract was almost equally effective against *P. syringae pv phaseolicola*, *X. campestris*, and *C. flaccumfaciens pv flaccumfaciens*, whereas *P. syringae pv syringae* was slightly less sensitive.

**Table I. Antibacterial activity of total protein extract from transgenic tobacco calli expressing a human lactoferrin cDNA**

<table>
<thead>
<tr>
<th>Bacterial Species</th>
<th>Protein Concentration µg/ml</th>
<th>Control Callus Extract log&lt;sub&gt;10&lt;/sub&gt; colony-forming units/ml</th>
<th>Lactoferrin (commercial) Extract log&lt;sub&gt;10&lt;/sub&gt; colony-forming units/ml</th>
<th>Transgenic Callus Extract log&lt;sub&gt;10&lt;/sub&gt; colony-forming units/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>X. campestris pv phaseoli</em></td>
<td>0</td>
<td>7.43&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.44&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.34&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>7.48&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.94&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>6.43&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>7.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.70&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.67&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>P. syringae pv phaseolicola</em></td>
<td>0</td>
<td>6.80&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.81&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.72&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>6.75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.04&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>6.64&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.52&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>P. syringae pv syringae</em></td>
<td>0</td>
<td>7.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.32&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>7.32&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.96&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.18&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>7.31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.71&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.78&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>C. flaccumfaciens pv flaccumfaciens</em></td>
<td>0</td>
<td>7.30&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.05&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>7.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.84&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.06&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>7.28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.72&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.73&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
protein(s) in transgenic tobacco calli. Expression of the lactoferrin gene in transgenic crop plants may introduce a broad-spectrum resistance against phytopathogenic bacteria.

ACKNOWLEDGMENTS

We would like to thank Dr. Mike Powell for making the lactoferrin cDNA available to us, Dr. Anne K. Vidaver and Pat Lambrecht for providing the bacterial strains and their generous help with the antibacterial activity assay, and Hoechst Roussell Agri-Vet for the gift of Ignite. We are grateful to Drs. M.B. Dickman and A.E. Zipf for comments concerning the manuscript. The technical assistance of Dan Higgins and the assistance of S. Mitra with plant tissue culture are sincerely appreciated.

Received May 4, 1994; accepted July 11, 1994.

LITERATURE CITED