Lactoferrin-Derived Resistance against Plant Pathogens in Transgenic Plants

Dilip K. Lakshman,*[,†] Savithiry Natarajan,[‡] Sudhamoy Mandal,[§] and Amitava Mitra*[,§]

[†]Floral and Nursery Plants Research Unit and Sustainable Agricultural Systems Laboratory, [‡]Soybean Genomics and Improvement Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705, United States
[§]Department of Plant Pathology, University of Nebraska, Lincoln, Nebraska 68583, United States

ABSTRACT: Lactoferrin (LF) is a ubiquitous cationic iron-binding milk glycoprotein that contributes to nutrition and exerts a broad-spectrum primary defense against bacteria, fungi, protozoa, and viruses in mammals. These qualities make lactoferrin protein and its antimicrobial motifs highly desirable candidates to be incorporated in plants to impart broad-based resistance against plant pathogens or to economically produce them in bulk quantities for pharmaceutical and nutritional purposes. This study introduced bovine LF (BLF) gene into tobacco (*Nicotiana tabacum* var. Xanthi), *Arabidopsis* (*A. thaliana*) and wheat (*Triticum aestivum*) via *Agrobacterium*-mediated plant transformation. Transgenic plants or detached leaves exhibited high levels of resistance against the damping-off causing fungal pathogen *Rhizoctonia solani* and the head blight causing fungal pathogen *Fusarium graminearum*. LF also imparted resistance to tomato plants against a bacterial pathogen, *Ralstonia solanacearum*. Similarly, other researchers demonstrated expression of LF and LF-mediated high-quality resistance to several other aggressive fungal and bacterial plant pathogens in transgenic plants and against viral pathogens by foliar applications of LF or its derivatives. Taken together, these studies demonstrated the effectiveness of LF for improving crop quality and its biopharming potentials for pharmaceautical and nutritional applications.

KEYWORDS: *antimicrobial peptide, broad-spectrum disease resistance, biopharming, iron-binding glycoprotein, lactoferricin, lactoferrampin, LF1-11, transferrin*

■ INTRODUCTION

Cultivated soil is rich with a multitude of microflora, some of which are beneficial to soil and plant health; others are apparently neutral, and a few are pathogenic to plants. According to Sullivan[1] a typical teaspoon of native grassland soil would contain at least 10000 species of microbes, of which around 5000 species are fungi. Soilborne plant pathogenic fungi and nematodes are the major players of soilborne plant pathogens.[2] Considering the diversity of soil microflora, it is not surprising that about 90% of the 2000 major diseases of principal crops in the United States are caused by soilborne plant pathogens,[3] resulting in losses in excess of $4 billion/year.[4] Major soilborne plant pathogenic fungi and fungus-like pathogens (oomycetes) are species of *Rhizoctonia, Fusarium, Sclerotium, Sclerotinia, Thielaviopsis, Phytophthora, Pythium*, etc. In contrast, only a few groups of plant pathogenic bacteria are considered to be soilborne such as *Ralstonia solanacearum*, causal agent of bacterial wilt of tomato,[5] and *Agrobacterium tumefaciens*, the well-studied causal agent of crown gall.[6] In addition, some filamentous bacteria (i.e., *Streptomyces*) and a few viruses (i.e., *Nepoviruses*) are soil inhabitants.[2]

Unlike aerial plant pathogens, soilborne pathogens are difficult to control without aggravating the beneficial rhizo-spheric microflora, and control using fungicides alone can be unreliable at times.[7,8] Some pesticides used in commercial vegetable and fruit production to control soilborne diseases can be highly toxic and deleterious to the environment, and pathogens often develop resistance to pesticides.[9,10] There are only a very limited fungicides registered for horticultural use, and no suitable alternatives exist to control pathogens in

organic production systems. Resistant germplasm against *Rhizoctonia solani* is unavailable, and only a very few commercial agronomic cultivars are partially resistant to the pathogen. In the case of the *Fusarium* head blight (FHB, caused by *Fusarium graminearum*) of wheat, even the optimal fungicide applications may only provide a 50−60% reduction in FHB incidence.[11] Use of biological control has often proved inconsistent or did not work at all under field conditions.[12]

An alternative to natural resistance against plant pathogens is the development of genetically engineered resistance by incorporation of disease-altering or pathogen-suppressing genes into plants. Currently transgenic crops are commercially grown in at least 25 countries,[13] and transgenic disease-resistant plants represent approximately 10% of the total number of approved field trials in North America.[14] There are three important strategies employed for transgenic resistance: (a) direct interference with pathogenicity or inhibition of pathogen physiology; (b) manipulation of the natural induced host defense; and (c) pathogen mimicry or pathogen-derived resistance (PDR), where the plant is designed to express important, recognizable features of the pathogen.[13,15]

The majority of transgenic trials against bacteria and fungi involve crops expressing antimicrobial proteins, which impart

resistance against a broad range of pathogens.[16] One such antimicrobial peptide function is derived from lactoferrin (LF), a globular milk protein known to nourish and defend newborns against fungal, bacterial, and viral pathogens. In addition to its use for transgenic resistance, recombinant LF (RLF) has been produced in filamentous fungi, economic plants, and animal systems for pharmaceutical and nutritional (nutraceutical) purposes.[17−26] The focus of this paper is to review the current status of LF-mediated transgenic resistance against plant pathogens. Although a major emphasis has been placed on research on plant disease resistance conducted by our group, an attempt has also been made to summarize all relevant and current literature on the subject. Moreover, the readers may check recent reviews on milk protein-derived antimicrobial peptides[27−29] as well as transgenic production of RLF in plants, cell cultures, fungi, and mammals for nutraceutical purposes.[30,31]

■ LACTOFERRIN: A MILK PROTEIN WITH MULTIPLE ANTIMICROBIAL PEPTIDES

Lactoferrin is a protein present in milk, tears, saliva, and mucus membrane of most mammals, and it plays a major role in the immune system of newborns.[30] LF is a cationic iron-binding glycoprotein of 80 kDa belonging to the transferrin family. LF may have a role in iron absorption and/or excretion and in gastric health of newborns.[32] It has anti-inflammatory and wound-healing properties and detoxicant, antioxidant, and anticancer activities.[33,34] Another prominent property of LF is its potent activity against a wide range of microorganisms including both Gram-negative and Gram-positive bacteria, as well as fungi and viruses.[35] LF consists of two globular domains, namely, the N- and C-lobes, and each lobe has two subdomains (i.e., N1, N2 and C1, C2, respectively).[36] The respective domains create one iron binding site on each lobe. The two lobes are connected by an α-helix hinge. Although several peptides with antimicrobial activities have been identified in LF, the three most characterized are the LF1-11, lactoferricin, and lactoferrampin; all of them are present in close proximity to each other in the N-terminal lobe of bovine LF (BLF) (Figure 1) or human LF (HLF). Lactoferricin and lactoferrampin can
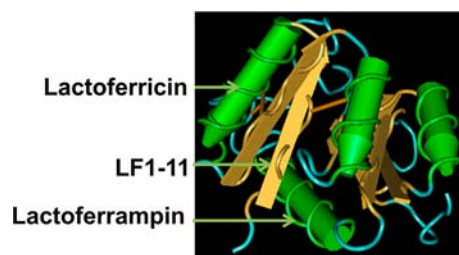
Figure 1. N-lobe of bovine lactoferrin. The three motifs LF1-11, lactoferrampin, and lactoferricin are shown. The 3-D structure was constructed from GenBank sequence L19981.1, using the Cn3D software from the National Center for Biotechnology Information.

be released by proteolytic cleavage in the gastrointestinal tracts of mammals.[37] All three peptides have higher pI values, are hydrophobic, and likely interact with negatively charged cellular elements such as lipopolysaccharides (LPS), DNA, lysozyme, and proteoglycans. Lactoferricin has an N-terminal amphipathic helix connected to a β-strand with a loop and is held together with a disulfide bond. It is exposed to the outer surface of the N-lobe of LF and acts by disrupting the permeability of

bacterial cytoplasmic membrane.[38] A comparison of antimicrobial activities of lactoferricin motifs derived from human, cow, goat, and mouse indicated that bovine lactoferricin has the highest antimicrobial activity.[39] Similarly, lactoferrampin has an amphipathic α-helix structure with a C-terminal tail. It binds to bacterial membrane and causes its disruption.[40,41]

■ LACTOFERRIN INDUCES PLANT RESISTANCE AGAINST *RHIZOCTONIA SOLANI*

The basidiomycetous soilborne fungus *Rhizoctonia solani*, sensu lato (Tele: *Thanatephorus cucumeris*, *T. praticola*, etc.) is known to attack 188 species of higher plants in 32 families, including various staple crops, ornamentals, and turfgrasses.[42,43] Some *R. solani* isolates infect distinct tissues on the same host plant, causing multiple diseases. The most economically important diseases caused by *R. solani* are pre- and postemergence damping-off, root and crown necrosis of seedlings, root rots of carrot and beet, aerial blights on foliage, flower, and fruits, head blight of cabbage and lettuce, soil rots of vegetables, patch diseases of turfgrasses, and sheath blight of rice, etc.[44]

In an agar-gel diffusion assay, transgenically expressed LF from tobacco was found to inhibit *R. solani*.[45] In the detached tobacco leaf bioassay, necrotic areas developed around the mycelial plug in control leaves, which rapidly increased in size each day until the entire leaf surface became necrotic 9 days after inoculation. On the contrary, transgenic leaves did not show any visible necrosis for 5−6 days. A small necrotic area was visible in transgenic leaves on day 7 and only increased slightly on day 9 (Figure 2). However, the transgenic resistance
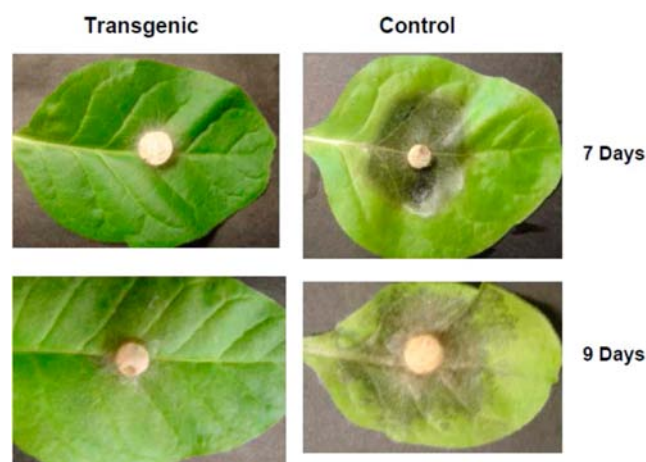
Figure 2. Detached leaf assay of the fourth leaf of transgenic tobacco seedlings inoculated with *R. solani*. A 5 mm mycelial disk was placed in the center of each leaf. The leaves were incubated in a humid chamber under fluorescent light at room temperature. The diameters of necrotic symptom developed around the inoculation disks were recorded 7 and 9 days after inoculation.

seemed to break down after 10 days as the necrotic area covered the entire leaf during the next 2 days, possibly due to rapid senescence and associated protein degradation in the detached leaves.[45] In a damping-off bioassay of *Arabidopsis* seedlings expressing LF, the seeds from two T2 transgenic *Arabidopsis* lines were sown in pots containing *Rhizoctonia* inoculum. Most seedlings in the control pots died soon after germination, although germination of seeds was comparable for both transgenic and control *Arabidopsis* plants. On the other hand, most transgenic seedlings grew normally, indicating

resistance against *Rhizoctonia* damping-off (Figure 3). Thus, our experiment demonstrated nearly complete protection of
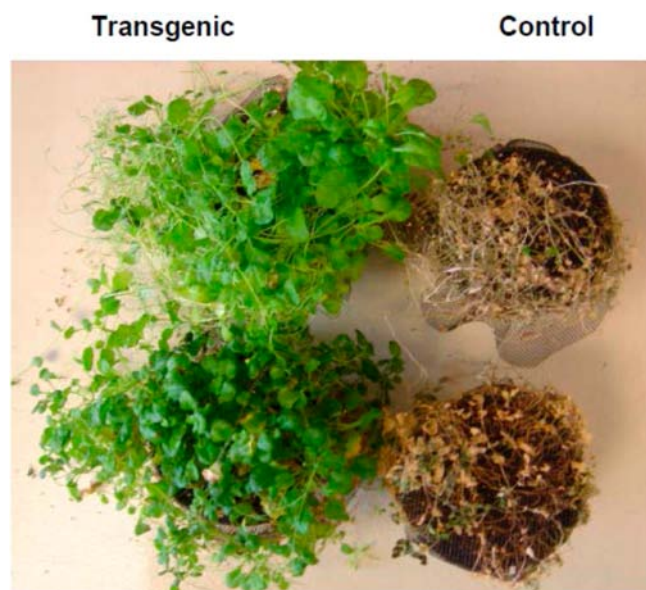
Figure 3. Seedling damping-off bioassay. Twelve-day-old seedlings of two BLF−*Arabidopsis* transgenic lines (top and bottom left) and two vector-only control *Arabidopsis* lines (top and bottom right) were inoculated with *R. solani* inoculum mixed with (0.75% w/w) autoclaved potting mix.

Arabidopsis seedlings against pre- and postemergence damping-off caused by *R. solani* within the duration of experiment.[45] Because *Rhizoctonia* normally attacks the juvenile tissues, bedding plants grown from seeds are especially vulnerable to pre-emergence damping-off.[46,47] On the other hand, seedlings tend to develop resistance or tolerance to the pathogen with age.[48,49] Thus, LF-imparted protection against *Rhizoctonia* during the most vulnerable early stage of seedling developmental seems to be significant. It could be envisaged that slowing the onset and progress of *Rhizoctonia* by transgenic expression of LF along with reduced amount or frequency of pesticide application has an advantage to the host plant in terms of epidemiology and management of the disease.

■ LACTOFERRIN INDUCES RESISTANCE AGAINST *FUSARIUM GRAMINEARUM* IN TRANSGENIC WHEAT

FHB caused by *F. graminearum* has emerged as a major threat to wheat and barley crops around the world. The disease can occur on all small grain crops when the spore of the fungus germinates and infects developing kernels on the wheat head. FHB reduces grain yield and quality and is frequently associated with fungal toxins that are hazardous to the health of both humans and animals.[50] The U.S. Department of Agriculture ranks FHB as the worst plant disease to hit the United States since the stem rust (*Puccinia graminis*) epidemic over 50 years ago.[51,52] Improving FHB resistance is a high priority in wheat and barley breeding programs.

We demonstrated that LF inhibits the growth of *F. graminearum* both in vitro and in vivo.[53] We also demonstrated that the level of resistance in the highly susceptible wheat cultivar 'Bobwhite' was significantly higher in transgenic plants expressing LF compared to the control 'Bobwhite' and in two

nontransformed commercial wheat cultivars, 'Wheaton' and 'ND 2710', which are susceptible or tolerant to *F. graminearum*, respectively (Figure 4). The mean percent of infection in LF

Figure 4. Fungal resistance in transgenic wheat: (A) transgenic and (B−D) empty vector control wheat lines. The transgenic line is healthy, whereas control lines show various levels of scab infection following inoculation with *F. graminearum* in the greenhouse.

expressing 'Bobwhite' wheat varied from 14 to 46%, whereas the mean percent infections in nontransformed 'Bobwhite', 'Wheaton', and 'ND 2710' were 82, 61, and 39%, respectively (Table 1). Quantification of the expressed LF protein by ELISA

Table 1. Disease Severity in Seven Transgenic Wheat Lines[a]

wheat line[b]	disease severity (%)
BLFW 119 (L+)	15
BLFW 351 (L+)	46
BLFW 378 (L+)	15
BLFW 424 (L+)	19
BLFW 685 (L+)	32
BLFW 892 (L+)	13
BLFW 1102 (L+)	15
BW (L−)	82
Wheaton (NTC)	61
ND 2710 (NTC)	39

[a]Transgenic lines and three control wheat varieties were spray-inoculated with a conidial suspension of *F. graminearum*. Disease severity was calculated as percent of infection in the sprayed heads. Six of the seven transgenic lines showed significant Fusarium head blight resistance compared to two commercial wheat varieties, 'Wheaton' and 'ND 2710', and a transgenic 'Bobwhite' control carrying an empty vector. BLFW, transgenic 'Bobwhite' wheat lines; BW, wheat cultivar 'Bobwhite' carrying an empty vector; 'Wheaton' and 'ND2710', susceptible and tolerant wheat breeding lines, respectively. All BLFW lines are significantly different from BW at $P < 0.01$ by Student's t test.[53] [b]L+, transgenic expressing lactoferrin; L−, vector-only transgenic control; NTC, nontransgenic cultivar.

in transgenic wheat indicated a positive correlation between the LF gene expression levels and the levels of disease resistance. More importantly, deoxynivalenol (DON) levels in five transgenic lines, BLFW-119, -378, -424, -892, and -1102, were below the 1 ppm limit established by the U.S. Food and Drug Administration for finished wheat grain products for human consumption. Under greenhouse conditions, artificially spray-inoculated control 'Bobwhite' lines had an average of 28.5 ppm DON. Although natural infection in Nebraska wheat fields varies widely, we routinely detect over 5 ppm DON in mildly

11732

dx.doi.org/10.1021/jf400756t | *J. Agric. Food Chem.* 2013, 61, 11730−11735

infected wheat fields and over 50 ppm in moderately infected wheat fields.[53]

■ LACTOFERRIN INDUCES PLANT RESISTANCE AGAINST OTHER FUNGAL PATHOGENS

Thus far, only limited studies have been conducted on LF-imparted transgenic resistance against other fungal plant pathogens. Takase et al.[54] expressed the full-length LF and the N-terminal half of the molecule in rice plants under the control of the cauliflower mosaic virus 35S promoter. Transgenic plants failed to show resistance against *Pyricularia oryzae* (causal agent of rice blast fungus). However, Fukuta et al.[55] recently expressed bovine lactoferricin attached to the signal peptide of pathogenesis-related protein (PR-1) in tobacco. The signal peptide is supposed to secrete lactoferricin to apoplast. The transgenic tobacco demonstrated high resistance against *Botrytis cinerea* (causal agent of fruit rot and other plant disease). In nontransgenic experiments, the milk product whey, of which LF is a component, was found to control the powdery mildew of grapevine, zucchini, and cucumber.[56−58]

■ LACTOFERRIN INDUCES PLANT RESISTANCE AGAINST BACTERIAL PATHOGENS

Mitra and Zhang[19] showed that tobacco (*Nicotiana tabacum*) calli transformed with human LF exhibited much higher antibacterial activity than commercially available purified LF against four phytopathogenic species, namely, *Xanthomonas campestris* pv *phaseoli*, *Pseudomonas syringae* pv *phaseolicola*, *P. syringae* pv *syringae*, and *Clavibacter flaccumfaciens* pv *flaccum faciens*. In addition, transgenic tobacco plants expressing LF demonstrated significant delays of bacterial wilt symptoms when inoculated with the bacterial pathogen *Ralstonia solanacearum*.[59] Quantification of the expressed LF protein by enzyme-linked immunosorbent assay in transgenic plants indicated a significant positive relationship between LF gene expression levels and the levels of disease resistance. Later, the same group[60] demonstrated that transgenic tomato plants expressing BLF exhibited early resistance and subsequent susceptibility to *R. solanacearum*, whereas 44−55% of infected plants survived until fruit ripening. The transgene was inherited in tomato in a stable Mendelian pattern, suggesting a potential new approach for controlling bacterial wilt of tomato.[60] Similarly, other researchers have demonstrated that transgenically expressed LF imparts increased resistance against *Erwinia amylovora* (causal agent of fire blight) in pear, *Burkholderia plantarii* (causal agent of rice bacterial seedling blight) in rice, *P. syringae* pv *tabaci* (causal agent of wildfire disease of tobacco, angular leaf spot) in tobacco, and *P. syringae* pv *syringae* and *C. michiganensis* in alfalfa.[24,25,54,61]

■ LACTOFERRIN-INDUCED PLANT RESISTANCE AGAINST VIRAL PATHOGENS

In a study on transgenic experiment with LF to test effectiveness against a plant virus, Takase et al.[54] have shown that rice seedlings expressing BLF or the N-terminal half of BLF do not impart demonstrable resistance against rice dwarf virus. On the contrary, Wang et al.[62] has recently reported that LF and esterified LF (ELF) impart resistance against tobacco mosaic virus (TMV) in tobacco seedlings using a half-leaf method. The virus inhibitory effect of ELF was higher than that of LF, and both proteins worked in a dose- and time-dependent

manner. Several pathogenesis-related protein (PR) genes and defense-related enzymes and chemicals were also induced both locally and systemically as a result of LF and ELF applications. Abdelbacki et al. have demonstrated that foliar spray with bovine lactoferricin enhances resistance to tomato leaf curl virus (TYLCV) on infected tomato plants.[56]

■ COMMERCIAL PRODUCTION OF LACTOFERRIN AND ITS DERIVATIVES

The biopharming potentials and applications of LF and its motifs for nutraceutical uses have been reviewed recently.[30,31] LF has been expressed in fungi, insects, poultry eggs, various mammalian cell culture systems, and transgenic animals, including goats, rabbits, and cows. However, to an extent those systems have disadvantages such as long development time, expensive purification processes, logistics issues, and potential zoonotic contaminations. On the other hand, plants are often suitable for cost-effective production of transgenic proteins. In this context, LF has been expressed in rice, potato, tobacco, maize, barley, ginseng, alfalfa, etc. Organ-specific expressions of LF in leaves, fruits, cereal grains, and tubers were obtained by utilizing gene expression with organ-specific promoters and protein transport signal sequences. The concerns of cell binding, immunogenicity, and nutritional values arising from plant glycosylation patterns, retention of terminal amino acid sequences and antimicrobial properties, structure and physiochemical stability, and agricultural issues as well as economics of various plant-expressed LFs have been addressed in many primary papers and discussed in recent reviews.[30,31]

■ LACTOFERRIN IS A MULTIFUNCTIONAL ANTIMICROBIAL PROTEIN

Agriculturally important crop plants need to be protected from devastating diseases to ensure food security in a changing world climate. As crop plants are routinely infected by serious fungal, bacterial, and viral pathogens, simultaneous control of multiple pathogen groups is invaluable. One recent approach to controlling plant diseases has been to express antimicrobial genes in transgenic plants. LF appears to be one of the promising broad-spectrum nonplant antimicrobial genes with the potential to control aggressive plant pathogens. LF in milk is a component of nutrition and organ development of newborns. Copious amounts of LF can be readily detected in milk and other routinely consumed dairy products.[63] It is also released in bodily secretions, including saliva, tears, bile, and pancreatic fluids, etc.[30] Transgenic expression of LF also imparts a broad-based resistance against plant bacterial and fungal pathogens.[19,45,53,59,60] Moreover, LF and its motifs and derivatives have been demonstrated to inhibit plant viruses.[62,64] This, together with the fact that LF occurs naturally in the diet of humans, makes it a novel candidate to introduce plant resistance against diseases. Thus, LF may be considered alone or in combination with other transgenes and practices to manage soilborne plant pathogenic fungi and bacteria. However, to exploit the full potentials of LF, it will be important to demonstrate that the observed resistance against plant pathogens by LF will hold up under field conditions. Also, the risk assessments of LF in biopharming should be adequately addressed.[65,66] Moreover, the effect of LF-expressing transgenic plants on beneficial rhizospheric, phyllospheric, and endosym-

11733

dx.doi.org/10.1021/jf400756t | *J. Agric. Food Chem.* 2013, 61, 11730−11735

biotic microflora and the acceptability of LF-transgenic cultivars by the end-users need to be evaluated.

■ AUTHOR INFORMATION

Corresponding Author
*(D.K.L.) E-mail: Dilip.Lakshman@ars.usda.gov. Phone: 1 (301) 504-6413. (A.M.) E-mail: amitra1@unl.edu. Phone: 1 (402) 472-7054.

■ ABBREVIATIONS USED

DON, deoxynivalenol; FHB, *Fusarium* head blight; LF, lactoferrin; BLF, bovine lactoferrin; ELF, esterified lactoferrin; HLF, human lactoferrin; RLF, recombinant lactoferrin; PR, pathogenesis related protein; TYLCV, tomato leaf curl virus

■ REFERENCES

(1) Sullivan, P. Sustainable management of soil-borne plant diseases − SlideShare, 2004; http://www.slideshare.net/ElisaMendelsohn/sustainable-management-of-soilborne-plant-diseases.
(2) Agrios, G. N. *Plant Pathology*, 5th ed.; Elsevier-Academic Press: San Diego, CA, 2005; 922 pp.
(3) Lewis, J. A.; Papavizas, G. C. Biocontrol of plant diseases: the approach for tomorrow. *Crop Prot.* 1991, 10, 95−102.
(4) Lumsden, R. D.; Lewis, J. A.; Fravel, D. R. Formulation and delivery of biocontrol agents for use against soilborne plant pathogens. In *Biorational Pest Control Agents: Formulation and Delivery*; Hall, F. R., Barry, J. W., Eds.; American Chemical Society: Washington, DC, 1995; pp 166−182.
(5) Genin, S.; Boucher, C. Lessons learned from the genome analysis of *Ralstonia solanacearum*. *Annu. Rev. Phytopathol.* 2004, 42, 107−134.
(6) Nester, E. *Agrobacterium: The Natural Genetic Engineer 100 Years Later*; 2008; online, APSnet features; DOI: 10.1094/APSnetFeatures-2008-0608.
(7) Kataria, H. R.; Gisi, U. Chemical control of *Rhizoctonia* species. In *Rhizoctonia Species: Taxonomy, Molecular Biology, Ecology, Pathology and Disease Control*; Sneh, B., Jabaji-Hare, S., Neate, S., Dijst, G., Eds.; Kluwer Academic Publishers: London, UK, 1996; Chapter VI.D, pp 537−547.
(8) Cotterill, P. J. Evaluation of in-furrow fungicide treatments to control *Rhizoctonia* root rot of wheat. *Crop Prot.* 1991, 10, 473−478.
(9) *Crop Protection Handbook*; Meister Publishing: Willoughby, OH, 2003; Vol. 89, p C 274.
(10) Walters, D. *Disease Control in Crops: Biological and Environmentally Friendly Approaches*; Walters, D., Ed.; Wiley-Blackwell: Chichester, UK, 2009.
(11) Leonard, K. J.; Bushnell, W. *Fusarium Head Blight of Wheat and Barley*; APS Press: St. Paul, MN, 2003.
(12) Xu, X.; Nicholson, P. Community ecology of fungal pathogens causing wheat head blight. *Annu. Rev. Phytpathol.* 2009, 47, 83−103.
(13) Collinge, D. B.; Jørgensen, H. J. L.; Lund, O. S.; Lyngkjær, M. F. Engineering pathogen resistance in crop plants: current trends and future prospects. *Annu. Rev. Phytopathol.* 2010, 48, 269−291.
(14) Raymond Park, J.; McFarlane, I.; Hartley Phipps, R.; Ceddia, G. The role of transgenic crops in sustainable development. *Plant Biotechnol. J.* 2011, 9, 2−21.

(15) Lomonossoff, G. P. Pathogen-derived resistance to plant-viruses. *Annu. Rev. Phytopathol.* 1995, 33, 323−343.
(16) Marcos, J. F.; Perez-Paya, E.; Misra, S.; Lopez-Barcia, B. Identification and rational design of novel antimicrobial peptides for plant protection. *Annu. Rev. Phytopathol.* 2008, 46, 273−301.
(17) Salmon, V.; Legrand, D.; Slomianny, M. C.; Yazidi, I. E.; Spik, G.; Gruber, V. Production of human lactoferrin in transgenic tobacco plants. *Protein Express. Purif.* 1998, 13, 127−135.
(18) Ward, P. P.; Piddington, C. S.; Cunningham, G. A.; Zhou, X.; Wyatt, R. D.; Connelly, O. M. A. System for production of commercial quantities of human lactoferrin: a broad spectrum natural antibiotic. *Bio/Technology* 1995, 13, 498−503.
(19) Mitra, A.; Zhang, Z. Expression of a human lactoferrin cDNA in tobacco cells produces antibacterial protein(s). *Plant Physiol.* 1994, 106, 977−981.
(20) Kamenarova, K.; Gecheff, K.; Stoyanova, M.; Muhovski, Y.; Anzai, H.; Atanassov, A. Production of recombinant human lactoferrin in transgenic barley. *Biotechnol. Biotechnol. Equip.* 2007, 21, 18−27.
(21) Nandi, S.; Suzuki, Y. A.; Huang, J.; Yalda, D.; Pham, P.; Wu, L.; et al. Expression of human lactoferrin in transgenic rice grains for the application in infant formula. *Plant Sci.* 2002, 163, 713−722.
(22) Rachmawati, D.; Mori, T.; Hosaka, T.; Takaiwa, F.; Inoue, E.; Anzai, H. Production and characterization of recombinant human lactoferrin in transgenic Javanica rice. *Breed. Sci.* 2005, 55 (2), 213−222.
(23) Samyn-Petit, B.; Gruber, V.; Flahaut, C.; Wajda-Dubos, J. P.; Farrer, S.; Pons, A.; et al. N-glycosylation potential of maize: the human lactoferrin used as a model. *Glycoconjugate J.* 2001, 18, 519−27.
(24) Stefanova, G.; Slavov, S.; Gecheff, K.; Vlahova, M.; Atanassov, A. Expression of recombinant human lactoferrin in transgenic alfalfa plants. *Biol. Plant.* 2013, DOI: 10.1007/s10535-013-0305-5.
(25) Chong, D. K.; Langridge, W. H. Expression of full-length bioactive antimicrobial human lactoferrin in potato plants. *Transgenic Res.* 2000, 9, 71−78.
(26) Min, S. R.; Woo, J. W.; Jeong, W. J.; Han, S. K.; Lee, Y. B.; Liu, J. R. Production of human lactoferrin in transgenic cell suspension cultures of sweet potato. *Biol. Plant.* 2006, 50, 131−134.
(27) Fadaei, V. Milk proteins-derived antibacterial peptides as novel functional food ingredients. *Ann. Biol. Res.* 2012, 3 (5), 2520−2526.
(28) Sinha, M.; Kaushik, S.; Kaur, P.; Sharma, S.; Singh, T. P. Antimicrobial lactoferrin peptides: the hidden players in the protective function of a multifunctional protein. *Int. J. Pept.* 2013, article ID 390230, 12, DOI:10.1155/2013/390230.
(29) Adlerova, L.; Bartoskova, A.; Faldyna, M. Lactoferrin: a review. *Vet. Med. Czech* 2008, 53, 457−468.
(30) Conesa, C.; Calvo, M.; Sánchez, L. Recombinant human lactoferrin: a valuable protein for pharmaceutical products and functional foods. *Biotechnol. Adv.* 2010, 28 (6), 831−838, DOI: 10.1016/j.biotechadv.2010.07.002.
(31) Stefanova, G.; Vlahova, M.; Atanassov, A. Production of recombinant human lactoferrin from transgenic plants. *Biol. Plant.* 2008, 52 (3), 423−428.
(32) van Berkel, P. H. C.; Welling, M. M.; Geerts, M.; van Veen, H. A.; Ravensbergen, B.; Salaheddine, M.; Pauwels, E. K. J.; Pieper, F.; Nuijens, J. H.; Nibbering, P. H. Large scale production of recombinant human lactoferrin in the milk of transgenic cows. *Nat. Biotechnol.* 2002, 20, 484−487.
(33) Goldman, I.; Deikin, A.; Sadchikova, E. Human lactoferrin can be alternative to antibiotics. *Proceedings of the World Medical Conference*; WSEAS Press, 2010; pp 27−38, ISBN 978-960-474-224-0.
(34) Takayama, Y. *Lactoferrin and Its Role in Wound Healing*; Springer: New York, 2012; 107 pp.
(35) Farnaud, S.; Evans, R. W. Lactoferrin − a multifunctional protein with antimicrobial properties. *Mol. Immunol.* 2003, 40, 395−405.
(36) Shashank, R.; Singh, R. *In-Silico Analysis of Lactoferrin*; Lambert Academic Publishing: Saarbrücken, Germany, 2012; 196 pp.
(37) Haney, E. F.; Nazmi, K.; Bolscher, J. G.; Vogel, H. J. Structural and biophysical characterization of an antimicrobial peptide chimera

comprised of lactoferricin and lactoferrampin. *Biochim. Biophys. Acta* **2012**, *1818*, 762−775.

(38) Andrés, M. T.; Fierro, J. F. Antimicrobial mechanism of action of transferrins: selective inhibition of H+-ATPase. *Antimicrob. Agents Chemother.* **2010**, *54*, 4335−42.

(39) Kalra, E. K. Nutraceutical − definition and introduction. *AAPS pharmSci* **2003**, *5* (3), E25.

(40) Ward, P. P.; Paz, E.; Conneely, O. M. Multifunctional roles of lactoferrin: a critical overview. *Cell. Mol. Life Sci.* **2005**, *62*, 2540−2548.

(41) Valenti, P.; Antonini, G. Lactoferrin: an important host defense against microbial and viral attack. *Cell. Mol. Life Sci.* **2005**, *62*, 2576−2587.

(42) Manibhushanrao, K.; Zuber, M.; Manian, S. Phytotoxic metabolites of *Rhizoctonia solani. J. Sci. Ind. Res.* **1981**, *40*, 602.

(43) Anderson, N. A. The genetics and pathology of *Rhizoctonia solani. Annu. Rev. Phytopathol.* **1982**, *20*, 329−347.

(44) Sneh, B.; Jabaji-Hare, S.; Neate, S.; Dijst, G. *Rhizoctonia Species: Taxonomy, Molecular Biology, Ecology, Pathology and Disease Control*; Kluwer Academic Publishers: New York, 1996.

(45) Nguyen, T. C.; Lakshman, D. K.; Han, J.; Galvez, L. C.; Mitra, A. Transgenic plants expressing antimicrobial lactoferrin protein are resistant to a fungal pathogen. *J. Plant Mol. Biol. Biotechnol.* **2011**, *2*, 1−8.

(46) Benson, D. M.; Cartwright, D. K. Ornamental diseases insighted by *Rhizoctonia* spp. In *Rhizoctonia Species: Taxonomy, Molecular Biology, Ecology, Pathology and Disease Control*; Sneh, B.; S. Jabaji-Hare, Neate, S.; , and Dijst, G., Eds.; Kluwer Academic Publishers: London, UK, 1996; pp 303−314.

(47) Lewis, J. A.; Lumsden, R. D. Biocontrol of damping-off of greenhouse-grown crops caused by *Rhizoctonia solani* with a formulation of *Trichoderma* spp. *Crop Prot.* **2001**, *20*, 49−56.

(48) Bateman, D. F.; Lumsden, R. D. Relationship of calcium content and the nature of the pectic substances in bean hypocotyls of different ages and susceptibility to an isolate of *Rhizoctonia solani.* *Phytopathology* **1965**, *55*, 734−738.

(49) Kus, J. V.; Zaton, K.; Sarkar, R.; Cameron, R. K. Age-related resistance in Arabidopsis is a developmentally regulated defense response to *Pseudomonas syringae. Plant Cell* **2002**, *14*, 479−490.

(50) Foroud, N. A; Eudes, F. Tricothecenes in cereal grains. *Int. J. Mol. Sci.* **2009**, *10*, 147−173.

(51) McMullen, M.; Halley, S.; Schatz, B.; Meyer, S.; Jordahl, J.; Ransom, J. Integrated strategies for *Fusarium* head blight management in the United States. *Cereal Res. Commun.* **2008**, *36*, 563−568.

(52) The U.S. Wheat and Barley Scab Initiative (USWBSI), 2013; http://scabusa.org.

(53) Han, J.; Lakshman, D. K.; Galvez, L. C.; Mitra, S.; Baenziger, P. S.; Mitra, A. Transgenic expression of lactoferrin imparts enhanced resistance to head blight of wheat caused by *Fusarium graminearum. BMC Plant Biol.* **2012**, *12*, 33−41.

(54) Takase, K.; Hagiwara, K.; Onodera, H.; Nishizawa, Y.; Ugaki, M.; Omura, T.; Numata, S.; Akutsu, K.; Kumura, H.; Shimazaki, K. Constitutive expression of human lactoferrin and its N-lobe in rice plants to confer disease resistance. *Biochem. Cell Biol.* **2005**, *83*, 239−249.

(55) Fukuta, S.; Kawamoto, K.; Mizukami, Y.; Yoshimura, Y.; Ueda, J.; Kanbe, M. Transgenic tobacco plants expressing antimicrobial peptide bovine lactoferricin show enhanced resistance to phytopathogens. *Plant Biotechnol.* **2012**, *29* (4), 383−389.

(56) Bettiol, W.; Harllen, S. A.; Reis, R. C. Effectiveness of whey against zucchini squash and cucumber powdery mildew. *Sci. Hortic.* **2008**, *117*, 82−84.

(57) Crisp, P.; Scott, E. S.; Wicks, T. J. Evaluation of biological and novel control of grapevine powdery mildew. *Proceedings of the 15th Australasian Plant Pathology Society Conference Handbook*, Geelong, Australia, 2005; p 85.

(58) Crisp, P.; Wicks, T. J.; Troup, G.; Scott, E. S. Mode of action of milk and whey in the control of grapevine powdery mildew. *Aust. Plant Pathol.* **2006**, *35*, 487−493.

(59) Zhang, Z.; Coyne, D. P.; Vidaver, A. K; Mitra, A. Expression of human lactoferrin cDNA confers resistance to *Ralstonia solanacearum* in transgenic tobacco plants. *Phytopathology* **1998**, *88*, 730−734.

(60) Lee, T. J.; Coyne, D. P.; Clemente, T. E.; Mitra, A. Partial resistance to bacterial wilt in transgenic tomato plants expressing antibacterial Lactoferrin gene. *J. Am. Soc. Hortic. Sci.* **2002**, *127*, 158−164.

(61) Malnoy, M.; Venisse, J. S.; Brisset, M. N.; Chevreau, E. Expression of bovine lactoferrin cDNA confers resistance to *Erwinia amylovora* in transgenic pear. *Mol. Breed.* **2003**, *12*, 231−244.

(62) Wang, J.; Wang, H. Y.; Xia, X. M.; Li, P. P.; Wang, K. Y. Inhibitory effect of esterified lactoferin and lactoferin against tobacco mosaic virus (TMV) in tobacco seedlings. *Pestic. Biochem. Physiol.* **2013**, *105*, 62−68.

(63) de Ferrer, P. A. R.; Baroni, A.; Sambucetti, M. E.; López, N. E.; Ceriani Cernadas, J. M. Lactoferrin levels in term and preterm milk, American College of Nutrition, 2010; http://www.am-coll-nutr.org/nutrition/lactoferrin-levels-in-milk/.

(64) Abdelbacki, A. M.; Taha, S. H.; Sitohy, M. Z.; Dawood, A. I. A.; Hamid, M. M.; Rezk, A. A. *Inhibition of Tomato Yellow Leaf Curl* Virus (TYLCV) using whey proteins. *Virol. J.* **2010**, *7*, 26 DOI: 10.1186/1743-422X-7-26.

(65) Heinemann, J. A. Human lactoferrin biopharming in New Zealand − scientific risk assessment. *Constructive Conversations/Kōrero Whakaaetanga Rpt 15* **2008**, 31.

(66) Broz, A.; Huang, N.; Unruh, G. Plant-based protein biomanufacturing. *Genetic Eng. Biotechnol. News* **2013**, *33* (4).