DEVELOPMENT OF A TRANSFORMATION CONSTRUCT FOR ENHANCED DISEASE RESISTANCE

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Abstract

Magainins are 23-residue antibiotic peptides isolated from the skin of the African clawed frog (*Xenopus laevis*). They inhibit the growth of numerous species of bacteria and fungi by making pores in the membranes thus disrupting the ionic balance across membranes. The objective of this research is to study the cytotoxicity of magainin to isolated chloroplasts, and to incorporate the gene encoding an analog of magainin into tobacco and eventually cotton to enhance the resistance to various disease causing pathogens. Polymerase chain reaction (PCR) was utilized to synthesize the magainin gene with different signal peptides and the PCR products were cloned into pGEM vector. The gene was cloned into a binary vector and mobilized into *Agrobacterium*. Tobacco leaf discs were infected and transgenic plants were regenerated. Future research involves molecular analysis of transgenic plants and the assessment of resistance of transformed plants to various fungal pathogens.

Introduction

Plants are constantly exposed to pathogens trying to tap into the food reservoir, which ensures their continuation of species. Several disease management strategies are employed to control the proliferation of these microbial pathogens. Plants by their inherent nature produce antimicrobial compounds, most of which are peptides that possess a broad-spectrum antimicrobial activity. Several of these peptides have been characterized from a wide range of organisms. Genes encoding these peptides have been isolated and utilized in transforming plants in hope to enhance the resistance of various crop species. One of the bioactive peptides that has received much attention is magainin.

Magainins are peptide antibiotics with a broad antiparasitic and antibiotic activities, are derived from the skin (Zasloff, 1987) and gastric mucosa (Moore et al., 1991) of the African clawed frog (*Xenopus laevis)*. Magainin 1 and 2 are 23-residue peptides which inhibit the growth of fungi and both Gram-positive and Gram-negative bacteria and also brings about lysis of protozoa (Zasloff, 1987). Magainin interacts directly with the lipid bilayer and ruptures the cellular membrane of target cells. Magainin disrupts the membrane integrity by forming ion channels across lipid bilayers (Duclohier et al., 1989; Cruciani et al., 1992), causes vessels to be leaky (Matsuzaki et al., 1989, 1991), and depolarizes the membrane (Westerhoff et al., 1989), leading to cell death. However, each peptide exhibits membrane selectivity (Maloy and Kari, 1995; Tytler et al., 1995). Kristyanne et al. (1996) reported the antifungal activity of magainin on several species of fungus such as *Thielaviopsis basicola*, *Rhizoctonia solani*, *Fusarium oxysporum*, *Verticillium dahliae*, and *Pythium ultimum*. Magainin 2 at 0.05 µg/µl completely inhibited all hyphal growth. Electronmicroscopy revealed degradation of the mitochondrial and cytoplasmic matrices, a reduction in number of ribosomes, and vacuolization of the cytoplasm. Zasloff (1987) et al., contend that magainin acts as an antimicrobial at relatively low concentration, but it does not affect normal eukaryotic cells unless at high concentration (Cruciani et al., 1992; Zasloff, 1987). The antibiotic activity of magainin and its potential phytotoxicity may prove useful in genetic engineering of crop plants to increase their resistance to pathogens.

We hypothesize that the antimicrobial activity and the selectivity of magainins to pathogenic microbes can be used to our advantage as sources of disease resistance genes for transgenic plants.

Materials and Methods

Dose Effect Relationship of Magainin to Isolated Chloroplasts

Chloroplasts were isolated from pea seedlings by percoll gradient as outlined by Cline et al., 1981. Pure chloroplast preparation was exposed to magainin at different concentrations ranging from 0.1 to 0.5µg/µl and at 25ºC for 10 minutes. The treated chloroplasts were layered on top of a percoll gradient and centrifuged to separate any ruptured chloroplasts. The rupture of chloroplasts was measured as a function of loss in chlorophyll concentration, which was determined by spectrophotometry. No decrease in chlorophyll concentration was evident. However there is a possibility that magainin could have inserted in the chloroplastic membranes and may have inactivated the chloroplasts without any loss of chlorophyll.

Synthesis of Magainin and PCR amplification

Two gene constructs differing in their signal peptides were made utilizing the Polymerase Chain Reaction (PCR) Oligonucleotides were synthesized and restriction sites were added to the 5' and 3' ends of the fragment to facilitate unidirectional cloning into the binary vector. Primers (25 mers) for the 5' and the 3' ends were used to synthesize and amplify the 150 bp and 90 bp fragments. The reaction conditions were: preheat at 95 ºC for 15 min, 94ºC for 30 sec., 65ºC for 1 min., followed by a 5 min final extension time at 72ºC.

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Following synthesis of the chimaeric gene, it was cloned into pGEM-T Easy vector (Promega) which possess T overhangs to facilitate ligation of PCR products. White colonies were picked and verified by PCR and restriction digestion for inserts. The fragment was also sequenced to confirm the sequence.

Cloning into Binary Vector and Tobacco Transformation

The fragments were digested with *BamHI* and *SacI* or *EcoR1* and *Sac1* and purified and cloned into the binary vector pBIN-GFP. The presence of the gene fragment was confirmed by PCR and restriction digestion. The plasmid was then mobilized into *Agrobacterium* strain EHA 105. The presence of the gene was again confirmed at this stage by PCR.

Tobacco leaf disks were sterilized and cocultivated with *Agrobacterium*. The disks were transferred to shoot initiation media and transformed tissues were selected on Kanamycin. Plantlets were then transferred to the root initiation media containing kanamycin. Regenerated Plants were then moved to soil and hardened. Preliminary confirmation of the gene was done by PCR on genomic DNA of transformed plants.

Future Research

Southern, Northern, and Western blots will be performed to obtain a molecular confirmation of the gene integration event and also its activity in the transgenic plants. Transformed plants will be tested for resistance to various pathogens in the greenhouse. Resistance will be tested against *T. basicola, R. solani, F. oxysporum, and V. dahliae* which are infamous for seedling diseases.

Discussion and Conclusion

Preliminary research work done on magainin reveals magainin as a potential candidate for plant transformation. All research data indicate strongly that, not only magainin, but also its homologues, exhibit biocidal activity. But, at the same time we have to be aware of the cytotoxic activity of magainin on plant cells, especially on the mitochondrial membranes. A balance has to be sought between the protective activity of magainin and potential cytotoxicity in plants. Such transgenic adjustment in plants will cut down the use of pesticides and will provide an efficient and alternative method to controlling diseases.

References

Cline, K., J. Andrews, B. Mersey, EH Newcomb, and K. Keegstra. 1981. Separation and characterization of inner and outer envelope membranes of pea chloroplasts. *Proc. Natl. Acad. Sci.* USA. 78: 3595-3599.

Cruciani, R. C., J. L. Barker, S. R. Durell, G. Raghunathan, H. R. Guy, M. Zasloff, and E. F. Stanley. 1992. Magainin 2, a natural antibiotic from frog skin forms ion channels in lipid bilayer membranes. *Eur. J. Pharmacol. Mol. Pharmacol.* Sect. 226: 287-296.

Duclohier, H., G. Molle, and G. Spach. 1989. Antimicrobial peptide magainin 1 from Xenopus skin forms anion-permeable channels in planar lipid bilayers. *Biophys. J*. 56: 1017-1021

Kristyanne, E.S., J. McD. Stewart, and K.S. Kim. 1996. Magainin 2 effects on the ultrastructure of five plant pathogens. *Mycologia*. 89 (3). 353-360.

Maloy, W. L., and U. P. Kari. 1995. Structure-Activity studies on host magainins and other host defense peptides. *Biopolymers(Peptide science).* 37: 105-122.

Matsuzaki, K., S. Nakai, T. Handa, Y. Takaishi, T. Fujita, and K. Miyajima. 1989. Magainin 1-induced leakage of entrapped calcein out of negatively-charged lipid vesicles. *Biochem.* 28: 9392-9398.

Matsuzaki, K., M. Harada, S. Funakoshi, N. Fujii, and K. Miyajima. 1991. Interactions of an antimicrobial peptide, tachylepsin I, with lipid membranes. *Biochem. Biophys. Acta*. 1063: 162-170.

Moore, K. S., C. L. Bevins, M. M. Brasseur, N. Tomassini, K. Turner, H. Eck, and M. Zasloff. 1991. Antimicrobial peptides in the stomach of Xenopus laevis. *J. Biol. Chem*. 226: 19851-19857.

Tytler, E. M., G. M. Anantharamaiah, D. E. Walker, V. K. Mishra, M. N. Palgunachari, and J. R. Segrest. 1995. Molecular basis for prokaryotic specificity of magainin-induced lysis. *Biochem*. 34: 4349-4401

Westerhoff, H. V., D. Juretic, R. W. Hendler, and M. Zasloff. 1989. Magainins and the disruption of membrane linked free-energy transduction. *Proc. Natl. Acad. Sci*. 86: 6597-6601.

Zasloff, M. 1987. Magainins, a class of antimicrobial peptides from Xenopus skin: Isolation, characterization of two forms, and partial cDNA sequence of a precursor. *Proc. Natl. Acad. Sci*. 84: 5449-5453.