PROCEEDINGS

OF THE

26th ANNUAL MEETING

July 29 to August 4, 1990

Mayaguez, Puerto Rico

Published by:

Caribbean Food Crops Society

with the cooperation of the USDA-ARS-TARS

Mayaguez, Puerto Rico

POTATO TRANSFORMATION FOR CONFERRING DISEASE RESISTANCE

J.H. Dodds[1], L. Destefano-Beltran[2], F. Buitron[1], R. Salinas[1], C. Sigueñas[1], P. Toyar[2], V. Zambrano[1], and J. Jaynes[2]

[1] Genetic Resources Department, International Potato Center, P.O. Box 5969, Lima-Peru.
[2] Department of Biochemistry, Louisiana State University, Baton Rouge, LA 70803 U.S.A.

ABSTRACT

Over the last 5 years rapid advantages have been made both in terms of transformation technology, using both Agrobacterium rhizogenes and Agrobacterium tumefaciens. Also new methods have been found for foreign gene introduction. Coupled to this rapid advance has been the availability of a wide range of genes that can be introduced. Among the genes that are currently available are genes to confer resistance to viruses, bacteria, fungi and insects. These genes are of various degrees of effectiveness and much depends on the type of promotors used in conjunction with the gene. CIP (The International Potato Center) has developed a network of collaborating institutions that work on varying aspects of these pest and disease resistances. In this presentation data is given on the analysis of various transformations for bacterial disease resistance. The genes used to confer this bacterial resistance code for lytic proteins and are isolated from silk moths. Discussions will be oriented towards the advantages and disadvantages of this type of approach over conventional breeding.

INTRODUCTION

Genetic engineering of crop plants is a subject that has received a great deal of attention in recent years. Care must be exercised however in proposing the use of this type of technique to resolve agricultural problems. Many scientists have talked about the potential of genetic engineering for crop plant improvement, while few have shown concrete results leading to that objective.

In the case of potato, concrete successes have been obtained to insert and express foreign genes, as will be described later. It is not therefore unrealistic to believe that this type of approach may be fruitful to prevent the problems of bacterial diseases.

As new heat adapted potato clones are produced, allowing the potato to be grown in lowland tropical conditions, control of bacterial diseases becomes a high priority. The cultivation of genetically resistant material coupled to good field management

has so far been the only effective method for the control of bacterial wilt. However experience has shown that the available genetic resistance has a tendency to break down if the material is moved into climatic zones for which it is ill adapted. The genetic basis of resistance traditionally used in breeding is extremely small, two Colombian clones of Solanum phureja. The reliance on this small genepool has been a concern to breeders and pathologists for a long time.

In the genetic engineering approach that we will go on to describe, we have moved away from looking for new sources of resistance genes within potato germplasm. The logic we have taken is that we understand little about the structure and location of genes within the potato controlling bacterial disease resistance, however, we know a great deal about the bacterial pathogens. By attacking the pathogens from our knowledge of their structure we may have a chance of success.

ADVANTAGES OF A GENETIC ENGINEERING APPROACH

There are a number of advantages to the use of a genetic engineering approach to inserting genes for bacterial disease resistance. Firstly, by this method it is possible to insert a single gene or small group of genes into an adapted genotype or variety without major perturbations of other genetic characters. Secondly, it should be possible to obtain resistance to both Pseudomonas and Erwinia in the same clone, this is something which would be extremely hard to achieve by conventional methods.

The probability of success in the short or medium term by this type of approach is low, however, the problem of bacterial disease is of such high priority and there are so many limitations to the conventional approaches that an attempt by genetic engineering technology is justified.

BACTERIAL AND FUNGAL DISEASES OF POTATO

The Peruvian potato (Solanum tuberosum L.) is susceptible to a great number of diseases, some of which are of worldwide importance, whereas others are of more localized significance, The causal agents include bacteria, fungi, viruses, viroids, mycoplasms, and nematodes (Rich, 1983). There are more numerous diseases in potato grown in the tropics than those cultivated in temperate areas. Indeed, Wellman (1972) reported that "Solanum andigenum-tuberosum is afflicted with at least 175 different diseases in the tropics compared to 91 found in the temperate areas". Of those in the United States, ten have been reported to be caused by bacteria and thirty from fungal infections (Anonymous, 1960). Total crop losses, in the potato resulting from bacterial and fungal diseases, can amount to hundreds of millions of dollars (US$) annually in developing countries. During particularly bad years, damages can approach several billion dollars worldwide. The extent of loss varies greatly from

country to country and is influenced by climate and conditions of growth and storage.

In the last 15 years there has been a tremendous effort expended to introduce new varieties appropriate for the tropical areas (Sawyer, 1984). One primary component in restricting potato production in those regions is the presence of bacterial and fungal diseases. Among the most important are bacterial soft rot, caused by Erwinia carotovora and bacterial wilt (Brown Rot), caused by Pseudomonas solanacearum. Total losses from these two diseases can be between 30-100% during cultivation and the 2-6 months storage period where temperature can be between 27-32^{o}C. The most important fungal diseases are caused by Rhizoctonia solani, Alternaria solani, Verticillium dahliae, and Phytophtora infestans (Annual Report, 1988, International Potato Center).

HUMORAL IMMUNITY IN INSECTS

Humoral immunity can be induced in insects by an injection of either live, nonpathogenic bacteria or heat-killed pathogens. This phenomenon has been studied by many investigators in a number of different insects, especially those of the orders Lepidoptera and Diptera. However, diapausing pupae of the giant silk moth, Hyalophora cecropia, have proven to be particularly useful (mainly because of their large size) to study the humoral defense mechanisms of insects. When H. cecropia pupae are immunized they produce a set of 15 proteins which are normally not present in the hemolymph of the animals. Boman and his co-workers have exploited this fact, both in the purification of these immune proteins and in the isolation of mRNA which was used for the preparation of a cDNA bank (Boman and Steiner, 1981). After a short period of RNA synthesis, the insects respond to the exposure of bacteria by the production of a potent antibacterial activity which is due to the synthesis of three (of which two are novel), classes of bactericidal proteins: cecropins, attacins, and a lysozyme (Hultmark et al., 1980). The three principal cecropins, A, B, and D, are small basic proteins with a comparatively long hydrophobic region. The attacins are larger and there are two main forms: basic and acidic The lysozyme protein is very similar to that found in chicken egg white.

Cecropins

The cecropins are the most potent group of the antibacterial factors found in the cecropia hemolymph. These peptides possess a broad spectrum of antibacterial activity against both gram negative and gram positive forms. They are small, 35-37 amino acid residues, strongly basic, and comprise three major forms: A, B, and D.

Comparison of the amino acid sequences of the different forms has revealed a high degree of homology 60-80%. The peptides

all have a basic N-terminal region and a hydrophobic stretch in the C-terminal part of the molecule. It seems that the cecropins are products of three related genes which, as in the case of the atacin genes (see below), have originated by gene duplication. Recombinant cDNA clones, corresponding to the cecropin B form, have been isolated which when analyzed together revealed that it is processed from a 62 amino acid precursor chain including a 26-amino acid leader peptide and a C-terminal Gly residue which is decarboxylated to render an amidated Leu (Van Hofsten et al., 1985). Recently, several genomic clones have been characterized and cecropin B was shown to exist in at least 3-5 copies in the cecropia genome (Xanthopouloss et al., 1988).

Additional cecropin-like lytic peptides have been described in Antheraea pernyi (Qu et al., 1982), Bombyx mori (Shiba et al., 1983), and Drosophila melanogaster (Flyg et al., 1987). The sarcotoxins: IA, IB, & IC, were isolated from the hemolymph of Sarcophaga peregrina larvae (Okada and Natori, 1983). These peptides, although small and basic, possess a somewhat different structural motif from that exhibited by the cecropins (Jaynes et al., in preparation). However, all appear to act by causing generalized membrane disruption.

Attacins

Attacins are the largest antibacterial molecules found in the hemolymph of immunized H. cecropia pupae, with a molecular weight of about 20,000 daltons. They are comprised of six different isoforms (A through F) which can be fractionated according to their isoelectric point (Hultmark et al., 1983). The results from amino acid sequence analysis of the N-terminus of five of the attacins indicate the presence of three basic and two acidic species. The basic type has similar sequences while the acidic pair are identical. It has been suggested that they are the products of two related genes. Comparison of cDNA clones has revealed 76% homology in the coding region and is, thus, evidence for the origination of the genes from a common ancestral form. The six attacin isoforms found in the hemolymph are thought to be products of secondary modification of the two precursor molecules, however, a purification artifact cannot be ruled out (Boman et al., 1985). Attacin like factors have been described in Manduca secta, Drosophila melanogaster, and S. peregrina (Spies et al., 1986, Flyg et al., 1987, and Ando et al., 1987).

Lysozyme

H. cecropia lysozyme was purified from immune hemolymph and was identified as a chicken-type lysozyme. The primary structure of this enzyme was elucidated by determination of the complete amino acid sequence. It is composed of 120 amino acids with considerable similarity with vertebrate lysozymes. The amino acid regions responsible for the catalytic activity and for the binding of substrate are highly conserved (Engstrom et al., 1985).

INTRODUCTION OF THE 'DISEASE RESISTANCE' GENES INTO TOBACCO AND POTATO PLANTS

Tobacco was selected as a model system to test the efficacy of our constructs in enhancing plant disease resistance. Also, established tobacco transformation and regeneration protocols were available (Horsh et al., 1985). Prior to selfing, all mother plants were screened for kanamycin resistance and GUS activity as appropriate and Southern analysis demonstrated the integration of single-copy genes. Northern and western analyses of some of the lines have shown the proper expression of the respective genes.

We have adopted a two-pronged approach to incorporate these genes into potato. Because of our previous experience with A. rhizogenes (Yang et al., 1989). we decided to start with our binary constructs, i.e. pBI 121-derivatives, in A. rhizogenes strain R1000 and infected stems of in vitro grown potato plants (Desiree, LT-9 and a yet to be named new cultivar from the Philippines, tentatively designated 86007). As expected, GUS-positive and kanamycin resistant hairy roots were obtained after two weeks of infection and plantlets were regenerated 3-4 weeks later. These plants have been evaluated and Southern analysis has confirmed the stable incorporation of these genes. The other approach utilized A. tumefaciens strain LBA 4404 containing the pBI 121-derivative constructs to infect leaf-disks of in vitro grown Desiree plants. GUS-positive and kanamycin resistant plants have been obtained after a few weeks. At present, most of the transformed lines are being propagated in order to conduct the pathogen challenge tests with P. solanecearum and E. carotovora.

BACTERIAL CHALLENGE

A. tumefaciens strain LBA 4404, containing the binary vector pWIShiva-1, was used to infect tobacco leaf disks utilizing established protocols. Eventually, a number of transgenic plants were obtained and shown to be kanamycin resistant GUS positive. Southern blots have confirmed the presence of single copies of the Shiva-1 gene integrated into the genomes of these plants, Northern analysis has verified that the expression of the gene is triggered by mechanical and pathogen induced wounding. F1 plants demonstrate a 3:1 segregation pattern for kanamycin resistance and in preliminary disease-challenge experiments, these plants exhibit delayed symptoms, reduced disease severity, and lower mortality after infection by P. solanacearum when compared to transgenic control plants.

CONCLUSIONS

In 1987, we first proposed that lytic peptide-encoding genes could be used to enhance overall resistance against bacterial and fungal diseases (Jaynes et al., 1987). Recently, it has been suggested that the transfer and expression of a new class of

peptides found in honeybee, the apidaecins, could generate similar effects in plants (Casteels et al., 1989). However, these peptides lack lytic activity and are merely bacteriostatic, the significance of these differences still must be determined.

Our promising results, so far obtained, are merely the beginning. The extreme malleability of peptide design portends many new avenues of research, not only in the arena of plant disease control, but also in human and veterinary medicine (Jaynes, 1989).

Even though the lytic peptides are the most active components in hand, we must not disregard the potential of the other antibacterial proteins in our overall strategy. The giant silk moth's humoral immune system has been forged for millions of years through the evolutionary process and has demonstrated the wisdom of having a multiple defensive system in which the different elements can work in synergy with one another. This multilevel defense system would present a formidable challenge to the invading microbe, one which would be very difficult to evolve a means to circumvent. The probability that a pathogen would become naturally resistant to all three components at once is rather remote (about 1 in 10^{18}).

REFERENCES

Ando, K., Okada, M., and Natori, S. 1987. Purification of Sarcotoxin II, Antibacterial proteins of Sarcophaga peregrina (flesh fly) larvae. Biochemistry 26:226-230.

Annual Report 1988, International Potato Center, P.O. Box 5969, Lima, Peru.

Anonymous. 1960. Index of plant diseases in the United States. Agricultural Handbook 165, U.S. Dept. of Agriculture, Washington, D.C.

Boman, H.G. and Steiner, H. 1981. Humoral immunity in Cecropia pupae. In: Curr. Top. Microbiol. Immunol. 9495:75-91.

Engstrom, A., Xanthopoulos, K., Boman, H.G., and Bennich, H. 1985. Amino acid and cDNA sequences of lysozyme from Hyalophora cecropia. EMBO J. 4:2119-2122.

Flyg, C., Dalhammar, G., Rasmuson, B., Boman, H.G. 1987. Insect immunity. Inducible antibacterial activity in Drosophila. Insect Biochem. 17:153-160.

Horsh, R., Fry, N., Hoffmann, D. Eichholtz. 1985. A simple and general method for transferring genes into plants. Science 223:1229-1231.

Hultmark, D., Steiner, H., Rasmuson, T., and Boman, H.G. 1980. Purification and properties of three inducible bacterial proteins from the hemolymph of immunized pupae of Hyalophora cecropia. Eur. J. Biochem. 106:7-16.

Hultmark, D., Engstrom, A., Anderson, K., Steiner, H., Bennich, H., and Boman, H.G. 1983. Insect immunity: attacins, a family of antibacterial proteins from Hyalophora cecropia. EMBO J. 2:571-576.

Okada, K.A., and Clausen, A.M. Collecting potatoes in Northwest Argentina. American Potato Jour. (USA) ISSR 0003-0589, 61(5):301-305.

Qu, X., Steiner, H., Engstrom, A., Bennich, H., and Boman, H.G. 1982. Insect immunity: Isolation and structure of cecropins B and D from Pupae of the Chinese Oak Silk Moth, Antheraea pernyi. Eur. J. Biochem. 127:219-224.

Rich, A.E. 1983. Potato diseases. New York (USA). Academic Press, 238 pp.

Sawyer, R.L. 1984. Potatoes for the developing world. International Potato Center, Lima, Peru.

Shiba, T., Ueki, Y., Kubota, I., Teshima, T., Sugiyama, Y., Oba, Y., and Kikuchi, M. 1983. Structure of lepidopteran, a self-defense substance produced by silkwork. In: Peptide Chemistry 1983, E. Munekata, (ed.), Protein Res. Foundation, Osaka (1984), 209-214.

Spies, A.G., Karlinsey, J.E., and Spence, K.D. 1986. Antibacterial hemolymph proteins of Manduca sexta. Comp. Biochem. Physiol. B-83:125-133.

Van Holsten, P., Faye, I., Kockum, K., Lee, J.-Y., Xanthopoulos, K.G., Boman, I.A., Boman, H.G., Engstrom, A., Andreu, D., and Merrifield, R.B. 1985. Molecular cloning, cDNA sequencing, and chemical synthesis of Cecropin B from Hyalophora cecropia. Proc. Natl. Acad. Sci. 82:2240-2243.

Wellman, F.L. 1972. Tropical American plant disease (Neotropical) phytopathology problems. Scarcerou Press, 989 pp. New Jersey (USA).

Xanthopoulos, K., Lee, J.Y., Gan, R., Kockum, K., Faye, I. and Boman, H.G. 1988. The structure of the gene for Cecropin B, and antibacterial immune protein from Hyalophora cecropia. Eur. J. Biochem. 1:371-376.

Yang, Z., Crawer, C.L., and Lacy, G.R. Bacterial and plant gene expression in potato soft rot. 1989. Annual Meeting of the Amer. Phytopathol. Soc., Richmond (USA), pp. 20-24.