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PRESENT AND FUTURE STRATEGIES FOR IMPROVEMENT OF *BACILLUS THURINGIENSIS* THROUGH GENE MANIPULATION

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ABSTRACT

The information gained from studies on the molecular biology and genetics *of Bacillus thuringiensis* over the past 10-15 years has led to attempts, and undoubtedly will lead to future attempts, at providing more effective insect control agents based on *B. thuringiensis.* The use of either natural gene transfer among the strains to yield broader spectrum isolates (Battisti etal., 1985; Gonzalez et al., 1982; Klier et al., 1983) or through genetic engineering of the insecticidal protein gene itself are available techniques to achieve such a goal (Andrews et al., 1987). Reports of transferring truncated or full-length sequences of the toxin gene into crops such as tobacco, tomatoes, cabbage, cotton (Adang et al., 1987; Barton et al., 1987; Fischkoff et al., 1987) and into other bacteria associated with crop plants (e.g., *Pseudomonas fluorescens* and *Clavibacter xyli)* to give unique delivery systems that reportedly will be available longer for insect ingestion have begun to appear.

A number of recent reports (Anonymous, 1987; Graf, 1985; Obukowicz et al., 1986a,b, 1987; Watrud et al., 1985; Hofte and Whiteley, 1989) of genetic engineering of the *B. thuringiensis* toxin gene have been indicative of future strategies in directing the modification of the activity of the insecticidal crystal proteins. Such modifications should result in the production of new forms of toxin protein, a broadened insect host range, creation of biocides possessing activities against two or more different target insects, increased persistence, ability to recycle, increased plant tolerance and resistance to insect pests, elimination of processing, manufacturing, transportation, and farmer application of toxin in some instances, and perhaps increased toxin yield. Present and future strategies include the use of transforming/transducing/mating systems, recombinant DNA manipulation/mutagenesis, molecular cloning of toxin genes (e.g., transfer of two or more genes for distinct insecticide activity in the same host cell or transfer of the insecticidal toxin gene into other prokaryotes). Transfer of the toxin gene into insect viral genomes, and/or transfer of the toxin gene into the insect's food source (e**.g.,** algae, tobacco, potato, soybean, com, cabbage, cotton, tomato, etc.) are additional strategies. The improvement of bio-insecticides to control insect pests should further decrease the dependence on chemical insecticides.

Application of intra- and interspecies transconjugation should be useful in attempts at broadening the host-range specificity by introducing several crystal gene containing plasmids of different origins into the same recipient strain, This achievement could lead to the development of a multipurpose biocide possessing activities against lepidopteran, dipteran, and/or coleopteran insects. Strains of *B. thuringiensis* having broader spectra have already been obtained using a transconjugation system (Battisti et al., 1985; Gonzalez et al., 1982; Klier et al., 1983). Klier et al. (1983) transferred the toxin gene of *B. thuringiensis* subsp. *thuringiensis* (Berliner 1715) that had been cloned in *B. subtilis* to an acrystalliferous *B. thuringiensis* mutant of subspecies *kurstaki* and also to *B. thuringiensis*

subsp. *israelensis*. In the latter transfer, the resulting transcipient strain produced both types of insecticidal protein toxins and was active against both lepidopteran and dipteran larvae. Gonzalez et al. (1982) have also demonstrated the production of more than one protein toxin in the same cell from two different subspecies of *B. thuringiensis* (*thuringiensis* and *kurstaki*) that are each toxic to specific lepidopteran larvae.

If expression problems can be overcome, and in some cases they have been solved, the genes for the *B. thuringiensis* protein toxin might be introduced into a variety of atypical systems to improve the total efficiency of this biocide. A few such efforts have recently been publicized (Anonymous, 1987; Fischkoff et al., 1987; Graf, 1985; Kronstad and Whiteley, 1986; Obukowicz et al., 1986a,b, 1987; Oeda et al., 1987; Prefontaine, 1987; Watrud, 1985).

For example, the gene might be introduced into bacteria commensurable with plants. Seeds could be watered with these modified bacteria, and the seeds and resultant seedlings might be afforded some measure of protection against susceptible insect pests as well as the growing plant. Both the Monsanto Company and Mycogen have cloned the protein toxin gene of *B. thuringiensis* subsp. *kurstaki* into *Pseudomonas* species. The Monsanto Company's biocide, a recombinant *P. fluorescens*, which in the natural state is presumably found on the roots of corn plants, is intended to colonize corn roots for control of the black cutworm, *Agrotis ipsilon*. Corn seeds are to be coated with the preparation. To ensure that the toxin gene is not transferred to another microbial species, the company's scientists inactivated the transposase enzyme to prevent the movement of the transposon, which is associated with the toxin gene. Mycogen's approach involved creating a "biological package", or microcapsule, for the toxin to protect it from environmental stresses. The company "fixed" or killed the *Pseudomonas* organism for use in the field. The killed vegetative cells will presumably protect the toxin, thus preventing premature deactivation. The Company suggests that using killed cells should make the product easier to formulate and handle. More recently, Crop Genetics International Corp. has inserted the insecticidal toxin gene from *B. thuringiensis* subsp. *kurstaki* into the chromosome of the endophytic bacterium, *C. xyli* subsp. *cynodontis* for control of the European corn borer, one of the largest uncontrolled insect pests of corn (Anonymous, 1987). The endophytic microorganism is capable of colonizing corn internally.

Other bacteria, such as natural pond microflora, might be suitable hosts for insertion of mosquitocidal crystal protein genes. The suitability of the cyanobacteria for molecular cloning purposes has improved with the identification and construction of useful shuttle-cloning vectors. These cloning systems in which the mosquitocidal protein gene from *B. thuringiensis* could be introduced into a blue-green algae and maintained on an autonomous replicon should be helpful in developing algae-based mosquito controls having persistance and ability to recycle. A report from Plant Genetic Systems indicates that Company scientists have succeeded in inserting the mosquitocidal gene of *B. thuringiensis* subsp. *israelensis* into a blue-green algae (unpublished data). Additionally, de Marsac et al. (1987) have reported transferring a 3.6kb Hind III DNA fragment containing the mosquitocide gene of *B. sphaericus* 1593M into the cyanobacterium *Anacystis nidulans* R2. The fragment was initially cloned and expressed in *E. coli* and *B. subtilis* using pHV33 as a shuttle vector, and then cloned into the blue-green algae with a pUC303 shuttle vector. The cyanobacteria grow on the upper layers of aquatic habitats, persist relatively long in the environment, and presumably should be more effective in controlling mosquito larvae. These reports are good indicators of potential development of biocides with amplified persistance in mosquito habitats.

Other recent efforts at developing novel biocides involve incorporating the toxin genes directly into the genetic background of plants thereby allowing the plant to synthesize its own insecticidal material. The success of such efforts should result in bypassing the need for repeated applications of the agent, and eliminate the processing, manufacturing, and transportation of insecticidal products. Such modifications have been made possible since both efficient DNA vector constructs are now available for use in transporting foreign genes into higher plant cells. In vitro systems for genetic engineering of plants have also been developed for a number of crop plants including corn, cotton,

soybean, cabbage, tobacco, tomato, potato, lettuce, carrot, sugarbeet, etc. However, this strategy could result in hastened development of resistance to the toxin.

The vectors for transferring foreign genes into higher plant cells or tissues currently available are mainly based on *Agrobacterium tumefaciens* or the cauliflower mosaic virus, although other sources of vector materials being studied include *A. rhizogenes*, other DNA caulimoviruses, geminiviruses (e.g. bean golden mosaic virus), potato leafroll virus, and liposomes (Andrews et al., 1987). The Rohm and Haas Company (Plant Genetic Systems), Agrigenetics Advanced Science Co., Agricetus, and the Monsanto Company have reported that the *B. thuringiensis* toxin gene of subsp. *kurstaki* has been inserted into tobacco where it is expressed (Adang et al., 1987; Andrews et al., 1987; Freedman, 1985). Both leaves and the tobacco plant tissues synthesize the insecticidal toxin. Barton et al. (1987) have also reported successful transfer and expression of the *B. thuringiensis* protein toxin gene in tobacco where it provides the plants with resistance to lepidopteran pests. Tobacco plants carrying the toxin gene should be resistant to tobacco budworm, cabbage looper, and the tobacco hornworm. Other important agricultural insects susceptible to the *B. thuringiensis* subsp. *kurstaki* toxin are the corn earworm, cottom bollworm, and the beet armyworm. A few other publicized reports indicate that work is underway on transferring the toxin gene to cabbage, cotton, and potato.

A significant step in developing insect tolerant transgenic plants other than tobacco was recently reported by Fischkoff et al. (1987). Two truncated genes from *B. thuringiensis* subsp. *kurstaki* HD–1 were incorporated into a plant expression vector for *Agrobacterium*-mediated transformation. The genes were transferred into tomato plants where they were expressed. Expression of the insecticidal protein genes conferred insect tolerance on the transgenic tomato plants and their progeny. Larvae (*Manduca sexta, Heliothis zea,* and *H. virescens*) were killed within 48 hours with very little evidence of feeding damage to the leaf. Some of the transgenic plants were able to kill 100% of the *M. sexta* and *H. virescens* larvae. The investigators also obtained preliminary evidence that some toxin activity was detectable in tomato fruit.

The future development of such novel microbial insecticide products by genetic manipulation, will require the availability of efficient and appropriate cloning/transfer systems that incorporate the technical advantage of high-level expression of a variety of *B. thuringiensis* insecticide crystal protein genes. Transfer of important genes to select procaryotes and to higher plants appears to be a facet of intense research that is now beginning to yield significant results.

Much basic and applied research is required to develop a long lasting and viable biocide product for field application. The advances will have increasing importance in efforts at the genetic engineering of the *B. thuringiensis* toxin in order to optimize its use as a biocide insect control agent. It is highly likely that we can expect the application of molecular biology and biotechnology to provide greater efficacy for products based on *B. thuringiensis*. The feasibility of such approaches have been demonstrated and are now on the forefront of a number of research efforts. The location of the insecticidal crystal protein genes on transmissible plasmids permits construction of novel insecticidal combinations by transconjugation between diverse isolates. Through molecular cloning, gene transfer, recombinant DNA manipulation, and site directed mutagenesis more sophisticated biocides will undoubtedly be developed and progress has already been made using some of these strategies. The nucleotide and deduced amino sequences and protoxin subdomains are now known for a wide-variety of the subspecies that affect at least 3 orders of insects. Their comparisons have given some insight into the differences between their activities. The use of gene splicing methods and reconstruction of the various toxin domains should allow for greater insight and perhaps the creation of novel biocides based on *B. thuringiensis* that may exceed the present day naturally-occurring isolates in efficacy.

Although genetically engineered crop biocides and control agents for insect vectors of disease will likely have a real impact, some very important concerns and questions must be addressed as advances are made and the genetically engineered biocides are put to use: What, if any, adverse

effects might occur to the general ecology of the biosphere? Will a substantial increase of resistant insects from constant and total exposure to the insecticidal protein in transgenic plants occur? Will artificially inserted DNA and the expressed protein be innocuous and stable? Will the efforts be cost effective? Already, the opposition to genetic engineering by parts of society have been deleterious to progress and increase secrecy due to proprietary concerns may result in decreasing exchange of scientific information. Since the lethality of the insecticidal crystal protein likely involves a complex multistep process which is not well understood, the cloning and genetic analysis of the toxin gene only gives us a partial picture of the overall mechanisms important to the activity. These areas have to be more intensely studied and considered as we move forward in our efforts at testing strategies discussed here. Conventional strategies are still of great importance and their use together with the recent pioneering research thrusts should compliment each other. We expect both efforts to be increasingly important to the biopesticide industry, the industrialized countries, and the lesser-developed countries.

REFERENCES

Adang, M.J., Firoozabady, E., Klein, J., DeBoer, D., Sekar, V., Kemp, J.D., Murray, E., Rocheleau, T.A., Rashka, K., Staffeld, G., Stock, C., Sutton, D. and Merlo, D.J. 1987. Expression of s *Bacillus thuringiensis* insecticidal crystal protein gene in tobacco plants. In Molecular Strategies for Crop Protection. (Edit. C.J. Arntzen and C. Ryan) pp 345–353. Alan R. Liss, Inc., NY.

Andrews, R.E., Faust, R.M., Wabiko, H., Raymond, K.C. and Bulla, L.A., Jr. 1987. The biotechnology of *Bacillus thuringiensis*. In Critical Reviews in Biotechnology, Vol. 6, Issue 2 (Edit G. Stewart and I. Russell) pp. 163–232. CRC Press, Inc., Fl.

Anonymous. 1987. EPA Application for an Experimental Use Permit to Ship and Use a Pesticide for Experimental Purposes Only (Transmittal document), Crop Genetics International Corporation, Hanover, MD.

Barton, K.A., Whiteley, H.R. and Yang, N.S. 1987. *Bacillus thuringiensis* delta-endotoxin expressed in transgenic *Nicotiana tabacum* provides resistance to lepidopteran insects. *Plant Physiol.* 885:1103–1109.

Battisti, L., Green, B.D. and Thorne, C.B. 1985. Mating system for transfer of plasmids among *Bacillus anthracis, Bacillus cereus,* and *Bacillus thuringiensis*. *J. Bacteriol.* 162:543–550.

Fischkoff, D.A., Bowdish, K.S., Perlak, F.J., Marrone, P.G., McCormick, S.M., Niedermeyer, J.G., Dean, D.A., Kusano-Kretzmer, K., Mayer, E.J., Rochester, E.E., Rogers, S.G. and Fraley, R.T. 1987. Insect tolerant transgenic tomato plants. *Bio/Technology* 5:807–813.

Freedman, A.M. 1985. Rohm and Haas puts bug-battling gene into tobacco plants. *Wall Street J.* (January 24) p. 18.

Gonzalez, J.M., Brown, B.J. and Carlton, B.C. 1982. Transfer of *Bacillus thuringiensis* plasmids coding for delta-endotoxin among strains of *B. thuringiensis* and *B. cereus*. *Proc. Natl. Acad. Sci. USA.* 79:6951–6955.

Graf, J.S. 1985. Mycogen gets EPA go ahead to test recombinant pesticide. *Gen. Eng. News.* 5:1.

Hofte, H. and Whiteley, H.R. 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol. Rev.* 2:242–255.

Klier, A., Bourgouin, C. and Rapoport, G. 1983. Mating between *Bacillus subtilis* and *Bacillus thuringiensis* and transfer of cloned crystal genes. *Mol. Gen. Genet.* 191:257–262.

Kronstad, J.W. and Whiteley, H.R. 1986. Three classes of homologous *Bacillus thuringiensis* crystal protein genes. *Gene* 43:29–40.

de Marsac, N.T., de la Torre, F. and Szulmajster, J. 1987. Expression of the larvicidal gene of *Bacillus sphaericus* 1593M in the cyanobacterium *Anacystis nidulans* R2. *Mol. Gen. Genet.* 209:396–398.

Obukowicz, M.G., Perlak, F.J., Kusano-Kretzmer, K., Mayer, E.J., Bolten, S.L. and Watrud, L.S. 1986a. Tn5-mediated integration of the delta-endotoxin gene from *Bacillus thuringiensis* into the chromosome of root-colonizing pseudomonads. *J. Bacteriol.* 168:982–989.

Obukowicz, M.G., Perlak, F.J., Kusano-Kretzmer, K., Mayer, E.J. and Watrud, L.S. 1986b. Integration of the delta-endotoxin gene of *Bacillus thuringiensis* into the chromosome of root-colonizing strains of pseudomonads using Tn5. *Gene* 45:327–331.

Obukowicz, M.G., Perlak, F.J., Bolten, S.L., Kusano-Kretzmer, K., Mayer, E.J. and Watrud, L.S. 1987. IS50L as a non-self transposable vector used to integrate the *Bacillus thuringiensis* delta-endotoxin gene into the chromosome of root-colonizing pseudomonads. *Gene* 51:91–96.

Oeda, K., Oshie, K., Shimizu, M., Nakamura, K., Yamomoto, H., Nakayama, I. and Ohkawa, H. 1987. Nucleotide sequence of the insecticidal protein gene of *Bacillus thuringiensis* strain *aizawi* IPL7 and its high-level expression in *Escherichia coli*. *Gene* 53:113–119.

Prefontaine, G., Fast, P., Lau, P.C.K., Hefford, M.A., Hanna, Z. and Brousseau, R. 1987. Use of oligonucleotide probes to study the relatedness of delta-endotoxin genes among *Bacillus thuringiensis* subspecies and strains. *Applied Environ. Microbiol.* 53:2808–2814.

Watrud, L.S., Perlak, F.J., Tran, M.-T., Kusano, K., Mayer, E.J., Miller-Wideman, M.A., Obukowicz, M.G., Nelson, D.R., Kreitinger, J.P. and Kaufman, R.J. 1985. Cloning of the *Bacillus thuringiensis* subsp. *kurstaki* delta-endotoxin gene into *Pseudomonas fluorescens*: Molecular biology and ecology of an engineered microbial pesticide. In Engineered Organisms in the Environment. (Edit H.O. Halvorson, D. Pramer and M. Rogul) pp. 40–46. American Society for Microbiology, Washington, DC.