Use of Vip3A-like Insecticidal Toxin Gene from *Bacillus thuringiensis* to develop Designer Rice Cultivars Resistant to Lepidopteran Pests

Srimonta Gayen[1], Munshi Azad Hossain[2], Pradip Kumar Biswas[3] and Soumitra Kumar Sen

Advanced Laboratory for Plant Genetic Engineering, Indian Institute of Technology, Kharagpur- 721302, India. Presently: [1]Department of Human Genetics, University of Michigan, Ann Arbor, USA; [2]Molecular Biology Section, Division of Biology, University of California, San Diego, USA; [3]DuPont Knowledge Center, E.I. DuPont (India) Pvt. Ltd., Hyderabad 500078, India. Email: soumitrakumar.sen@gmail

Abstract

The Vip3BR toxin, like the well known Vip3A toxin protein produced by Bacillus thuringiensis, contains a broad spectrum lepidopteran specific insecticidal property against most of the major lepidoteran insect pests that invade farmer's field in the Indian subcontinent. A deletion mutant of the Vip3BR toxin protein, Ndv200 showed increased insecticidal potency than that of the native form of the toxin against feeding larvae of yellow stem borer. Ligand blot analysis revealed that Ndv200 toxin recognized the same larval midgut target site of rice yellow stem borers as that of the native Vip3BR toxin, but different from those recognized by Cry toxins. Broad spectrum toxicity towards lepidopteran insects makes Ndv200 toxin to be a strong candidate for the potential insecticidal gene of interest for development of insect resistant GM rice plants that would be especially suitable for small to medium holding farming community in developing countries. Ectopically expressing the Ndv200 toxin gene in transgenic rice plants has confirmed on their practical utility.

Keywords: *Bacillus thuringiensis*, Vip3BR toxin, Ndv200 toxin, transgenic rice, pest resistance, Lepidopteran specific cry toxin, rice stem borers

Introduction

Agricultural biotechnology holds much potential to contribute towards productivity gains for small-farmers' holdings in India and other developing countries. There is currently the need to grow more food while taking additional measures to prevent yield losses from biotic and/or abiotic stresses, and post-harvest storage, and waste in the supply chain. The necessary growth in production will come about in large measure from small farms, which represent a substantial part of the food agriculture industry in India. Molecular breeding can accelerate crop improvement timescales and enable greater use of diverse gene sources. However, constraints to the development and adoption of technology-based solutions to reduce yield gaps need to be overcome.

For example, the development of such technology has focused perhaps more on the potential economic returns rather than the potential crop yield. Furthermore, because of the regulatory systems controlling the release of transgenic crops, the development of insect-resistant transgenic crops has become difficult and expensive, so that large companies can only afford to carry products forward when the projected returns are commercially viable. As a result, the economic consequences to the public of the failure to adopt insect-resistant transgenic plants for the developing world and to apply the associated technologies have become serious (Huang et al 2005). Therefore, new developments in strategic research to meet the practical challenges to controlling insect-inflicted yield losses are rarely made available for public use.

Insect-resistant *Bacillus thuringiensis* (Bt) toxins have been the second most widely used trait in transgenic crops, after herbicide tolerance, and have resulted in many agricultural and biotechnological successes. The production of transgenic insect-resistant plants, and their continuing development, has been a major scientific achievement that has translated into practical success of a limited number of pest-resistant genetically modified crops. However, these successes must be set against the failure to make this technology more widely available. It is important for plant scientists to generate products that are more suitable to meet the challenges

that the farmers in small farm holdings, in particular, currently face.

Progress in molecular genetics has made it possible to use the Bt *cry* gene encoding δ-endotoxin as a genetic resource for transgenesis and for the development of transgenic plants that are resistant to insects. The value of this strategic use of *cry* gene-based technology has significantly decreased in cases where secondary pests are not killed by the Bt toxins produced by the current transgenic varieties of corn and cotton, as recently experienced in India and China. Thus, future advancement of transgenic technology for the generation of insect-resistant rice cultivars will depend on whether it is possible to improve the performance of the Bt lines in terms of their insecticidal activity cultivars that will be suitable for growth by farmers with small-holdings.

Lepidopteran pests of rice that cause major damages to the crop are borers, such as the yellow stem-borer (YSB; *Scirpophaga incertulus*), white stem borer (*Sesamia innotata*), pink stem borer (*Se. inferens*), striped stem borer (*Chilo suppressalis*), gold fringed stem borer (*C. auricilius*) and the dark-headed stem borer (*C. polychrysis*). The YSB in particular causes massive yield loss. The transgenic approaches used until now have been primarily aimed at the generation of Bt rice lines to combat yield loss resulting from YSB damage alone. However, most rice fields are also attacked by a variety of other lepidopterans. Additionally, there exists the need to avoid the emergence of insect that are resistant to the toxin. From our experience working with Bt cotton and lepidopteran pest control management in cotton fields, we believe that, on a long-term basis, there is a need to refocus the current transgenic strategy on the development of transgenic rice that is resistant to a variety of insect pests, and to manage that resistance by using a new generation of insect-resistant transgenic rice lines that are significantly different from those carrying the *CryIAb* and/or *CryIAc* toxin genes. The *Vip3A* gene, or its analogs, from *B. thuringiensis* appears to be a suitable additional genetic resource for the design of insect-resistant rice cultivars. This approach is in no way original, as Syngenta has adopted the same line of approach in the form of VipCot™ in cotton and Agrisure Viptera™ in corn. However, the novelty of our approach lies with the use of an engineered Vip3Aa-like *Vip3BR* toxin gene in the development of insect-resistant transgenic rice cultivars for a wide adoption by farmers including those with small farms.

Research initiatives underway in the laboratory

The identification and characterization of a variant *Vip3A*-like toxin gene from a local isolate of *B. thuringiensis* has already been carried out. The full nucleotide sequence of the toxin gene has been determined and submitted to GenBank (Acc#AY739647). The gene contained several changes in its amino acid sequences and was named Vip3BR (Biswas 2004; Hossain 2005). The insecticidal property of the native Vip3BR toxin to the first-instar larvae of rice YSB was found to be lower than that of the Cry1Ab and Cry1Ac toxins, but higher than that of the Cry2Aa toxin. In vitro proteolysis of the native toxin protein by midgut juice extracted from YSB larvae showed that a dominant and stable, approximately 62-kDa protein is generated by proteolysis of the native protein. To determine the active component of the native toxin, several deletion mutants were generated using polymerase chain reactions (PCR). Each of these genes was expressed in a suitable bacterial expression system. The recombinant toxin proteins generated were then tested using an insect feeding assay. A deletion mutant of the Vip3BR strain expressed an approximately 66-kDa toxin protein. The modified toxin protein was found to be almost three times as toxic to first-instar YSB larvae compared with the native toxin peptide. This enhanced potency of the engineered Vip3BR toxin peptide was considered to be significant in its use in the generation of insect-resistant transgenic rice cultivars. The engineered Vip3BR toxin peptide has also been found to have enhanced potency as an insecticidal agent against the first-instar larvae of cotton boll worm (*Helicoverpa armigera*), black cut worm (*Agrotis ipsilon*) and cotton leaf worm (*Spodoptera littoralis*) (Gayen 2011). It is believed that the modified structural form of the Vip3BR toxin peptide could be developed to be toxic to any sensitive target insect, rather than to only one target species.

Ligand blot analysis for the identification of the insect-specific receptor binding properties of the modified Vip3BR toxin protein showed that, similar to the native Vip3BR toxin, it recognized an approximately 180-kDa-like receptor protein of the midgut epithelial cell surface of YSB. This receptor protein differs from the receptor proteins that recognize the Cry1Ab and/or Cry1Ac toxins.

The native as well as the engineered *Vip3BR* toxin genes have been reconstructed with plant preferred codon usage. The reconstructed *Vip3BR* genes have been transferred to several rice elite cultivars and transgenic lines are currently being developed. However, their ability to protect rice crops against lepidopteran pests is yet to be tested.

The current state of research

The vegetative insecticidal protein (Vip3) is produced as a secretory protein by certain Bacillus species during

their vegetative growth stage. The protein has insecticidal properties against many lepidopteran species. The gene encoding the Vip3A toxin contains no sequence homology with known δ-endotoxins. The best-known vegetative insecticidal proteins include the Vip3Aa protein from *B. thuringiensis* strain AB88 and the Vip3Ab protein from Bt strain AB424 (Estruch et al 1996). Interaction of the Vip3A protein with ribosomal protein S2 seems to be related to the insecticidal activity of the Vip3a protein (Singh et al 2010). Additional Vip toxins have recently been identified, including Vip3BR. The molecular mass of each of these proteins is approximately 90 kDa and they are toxic to a broad spectrum of lepidopteran species, including many of the major crop pests in India. Compared with the 300- plus Bt cry toxin genes currently identified, approximately 15 Vip3 toxin genes have been cloned and characterized, many of which have insecticidal activities similar to that of the Vip3Aa1 protein.

It was observed that Vip3 kills insects by lysing their midgut cells (Yu et al 1997) via cell membrane pore formation (Lee et al 2003). Therefore, Vip proteins have an insecticidal mode of action similar to that of δ-endotoxins. As with Cry proteins, Vip proteins also have an active proteolytic core, which is activated by insect gut proteases, and binds to specific sites localized on the midgut lining of susceptible insect species.

Differences were found in the mode of action of Vip3A compared with that of Cry proteins. The onset time of the symptoms provoked by ingestion of Vip3A was delayed (36–48 h) compared to 16-24 h in case of δ-endotoxins (Yu et al 1997). However, the Vip3A protein targets different receptors in the midgut lining, and the binding results in the formation of ion channels distinct from those formed by δ-endotoxins (Lee et al 2003). Thus, Vip proteins are not expected to affect other invertebrates and vertebrates, including beneficial arthropods, birds and mammals, including livestock and humans. Only lepidopteran species that have Vip-binding sites on the surface of their gut epithelia will be affected.

Little is known about the structural and functional relations of this insecticidal toxin. However, strong evidence from bioinformatics indicates that Vip proteins are subject to high rates of positive selection and 16 possible sites have been identified to be under positive selection. These sites are located from site 705 to site 809 in the C terminus of the protein. It has been also postulated that the high divergence in the C terminus might not result in functional constraints, but instead help to adapt the protein to its target insect (Wu et al 2007).

Given that the Vip toxin has a different mortality mechanism compared with Cry toxins, it could be used strategically in the generation of transgenic crop plants that are resistant to insect damage. For example, significant progress has been made by Syngenta in the development of Agrisure Viptera™ in corn and VipCot™ in cotton. Both transgenic lines contain the *Vip3Aa* toxin gene as the major component for protection against insect pests, and have both been subject to crucial tests for environmental biosafety considerations.

Transgenic rice plants expressing a fused protein of Cry1Ab/Vip3H have shown resistance to rice YSB damage. Domain swapping between Vip3Aa1 and Vip3Ac1 resulted in the generation of a chimeric Vip3A toxin protein that demonstrated a differential insect sensitivity range not shown by any single toxin. Cotton plants carrying both the Cry1Ab and Vip3A toxins showed enhanced efficacy in the control of insect pests.

Prospects of achieving the goal

Research activities currently center on the development of insect-resistant transgenic rice lines as a resource for plant breeders. Over the past two decades, attempts in different laboratories have been aimed at generating transgenic rice lines that are resistant to the YSB, with some success. However, the potential of the genetically engineered rice lines could not be realized in the field because of regulatory restrictions. Only two transgenic insect resistant rice lines harboring Bt *cry* genes has earned the required permission (Lu, 2010) for commercialization in China (in August 2009). Despite the 2010 report of the genetically modified organism (GMO) panel of the European Food Safety Authority that stated that the products of biotechnology are no riskier *per se* than conventional plant-breeding technologies (Fagerström et al 2012), the embargo imposed on genetically engineered Bt rice remains. However, we suggest that it is unethical to withhold solutions to problems that currently result in hunger and malnutrition in thousands of people around the world. Thus, the failure to expand agricultural research in and for developing countries will make food security goals elusive. Under the current state of waiting for field trials, we further fine tuned our strategy to protect rice crops against insect pests in the field. In such a context, we believe that the potential of the insecticidal *Vip3A* toxin gene as a resource for the development of insect-resistant transgenic crop plants has not yet been fully realized. This is made even more obvious when one considers that agriculture in Asian countries offers different challenges in insect management-related issues compared with other areas of the world. Farmers owning small-farms cannot devote their land to refuges. In such circumstances, some of the advantages of using

the insecticidal *Vip* toxin gene might be realized; for example; (i) the approach of insect-resistance management of Bt crops with two or more genes expressing insecticidal proteins at high doses, such that a single insect mutation is unlikely to confer resistance, might not be realistic. The Vip3A-like toxins in this situation are a broad spectrum of lepidopteran pests that invade rice crops as secondary pests, has special significance; (iii) the diverse forms of native Vip3A-like toxin and their modified versions have been shown to carry altered insecticidal potential, similar to that found in Cry proteins. This would add additional possibilities to their use that have yet to be explored; (iv) the transgenic expression of the *Vip3A* gene in cotton is consistent throughout the life time of the annual crop, unlike that of the Cry protein in cotton; (v) Vip3A-expressing transgenic cotton plants do not require supplementary insecticidal applications to prevent yield loss; (vi) crops producing Vip3A do not adversely affect biological control organisms; and (vii) the combination of *Vip3A* and *cry* genes would result in crop plants that would be resistant to a range of lepidopteran species, in addition to enabling the Bt products to be more durable.

Bearing the above points in mind, the prospective utility of the modified *Vip3BR* toxin gene in the development of insect-resistant rice cultivars appears to be well founded. GM plants with Vip3A toxin have already been commercialized and are considered to be safe for human consumption.

REFERENCES

Biswas, PK. 2004. *Isolation and characterization of an entomocidal secretory protein from vegetatively gwowing Bacillus thurngiensis*. Ph.D. thesis, Indian Institute of Technology, Kharagpur, India, pp 24-56.

Estruch JJ, Warren GW Mullins MA, Nye GJ, Craig JA and Koziel MG. 1996. Vip3A, a novel *Bacillus thuringiensis* vegetative insecticidal protein with a wide spectrum of activities against lepidopteran insects. *Proc Natl Acad Sci USA* 93: 5389-5394.

Estruch JJ, Koziel MG, Yu CG, Desai NM, Nye GJ and Warren GW. 1998. *Plant pest control.* Patent WO. 9844137.

Fagerström T, Dixelius C, Magnusson U and Sundström F. 2012. Stop worrying; start growing. *EMBO Sci Soc* doi:10.1038/embor.2012.59.

Gayen S. 2011. *Development of molecular strategies and their experimental execution in the making of second generation of genetically modified plants, resistant against lepidopteran insects.* Ph.D. thesis, University of Calcutta, India, pp 33-51.

Hossain MH. 2005. *Development of transgenic crop plant expressing entomocidal gene.* Ph.D. thesis, University of Calcutta, India, pp.23-37.

Huang J, Hu R, Rozelle S and Pray C. 2005. Insect-resistant GM rice in farmer's fields: Assessing productivity and health effects in China. *Science* 3084 (5722): 688-690.

Lee MK, Walters FS, Hart N, Palekar N and Chen JS. 2003. The mode of action of the *Bacillus thuringiensis* vegetative insecticidal protein Vip3A differs from that of cry1Ab δ-endotoxin. *Appl Environ Microbiol* 69: 4648-4657.

Lu C. 2010. The first approved transgenic rice in China, www. landesbioscience.com/journala/gmcrops/article/12377.

Singh G, Sachdev B, Sharma N, Seth R and Bhatnagar RK. 2010. Interaction of *Bacillus thuringiensis* vegetative insecticidal protein with ribosomal S2 protein triggers larvicidal activity in *Spodoptera frugipertein*. *Appl Environ Microbiol* 76 (21): 7202-7209.

Wu J, Zhao F, Bai J, Deng G, Qin S and Bao Q. 2007. Evidence for positive Darwinian selection of *Vip* gene in *Bacillus thuringiensis*. *J Genet Genomics* 34(7): 649-660.

Yu CG, Mullins MA, Warren GW, Koziel MG and Estruch JJ. 1997. The *Bacillus thuringiensis* vegetative insecticidal protein Vip3A lyses midgut epithelium cells of susceptible insects. *Appl Environ Microbiol* 63:532-536.

Citation: Gayen S, Hossain MA, Biswas PK and Sen SK. 2013. Use of Vip3A-like insecticidal toxin gene from *Bacillus thuringiensis* to develop designer rice cultivars resistant to lepidopteran pests. In: Muralidharan K and Siddiq EA, eds. 2013. *International Dialogue on Perception and Prospects of Designer Rice*. Society for Advancement of Rice Research, Directorate of Rice Research, Hyderabad 500030, India, pp 188-191.