Mining versus *in vitro* evolution for the selection of novel microbial insecticidal proteins

Alejandra Bravo | Mario Soberón

Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca, Mexico

**Correspondence**
Mario Soberón, Instituto de Biotecnología, Universidad Nacional Autónoma de México, Cuernavaca 62250, Morelos, México.
Email: mario.soberon@ibt.unam.mx

Microbial insecticidal proteins, mainly identified from the insect pathogen *Bacillus thuringiensis*, are an effective strategy for insect control. These proteins have been extensively used worldwide, first as sprayable formulations and later in transgenic plants like maize, cotton or soja, lowering the use of chemical insecticides. However, some insect pests show low susceptibility to the insecticidal proteins that are used in transgenic plants, or some insect pests have already evolved resistance to these insecticidal proteins. Thus, novel insecticidal genes that codify for proteins with different modes of action highly active against resistant insects are needed in order to have a continuous and sustained use of this technology. Here, we discuss ways to select novel powerful insecticidal variants, involving mining and *in vitro* evolution of insecticidal genes. Furthermore, we hypothesized that the larger number of insecticidal genes described, the biggest possibility to tailor novel insecticidal genes highly active against specific crop pests.

Transgenic crops expressing microbial insecticidal proteins have been proven to be a successful technology for the control of insect pests worldwide, lowering the use of chemical insecticides (Sanahuja et al., 2011). The main source of the insecticidal protein genes expressed in transgenic crops is the soil bacterium *Bacillus thuringiensis* (Bt), an insect pathogen that produces diverse types of insecticidal proteins such as the Cry and Vip toxins. These proteins show high specificity against particular-insect-pests, including lepidopteran, coleopteran and dipteran species (Pardo-López et al., 2012). Different Bt strains produce multiple insecticidal proteins that are classified in at least 14 groups, based on their three-dimensional structure (Crickmore et al., 2021). To date >100 different classes and >700 different genes, belonging to the 14 structural groups of insecticidal proteins, have been described (Crickmore et al., 2021). The large number of Bt toxin genes described has broadened the possibilities for their continuous application in insect control.

However, despite the large number of insecticidal proteins already identified, just a few of them have been commercially used in transgenic crops for pest control. Different reasons account for this, such as the requirement of their efficient expression in the plant tissues; also, that few insecticidal genes display high toxicity against the most important crop-pests; and that many insecticidal proteins share the same mode of action.

The major threat on the use of Bt-plants is the evolution of resistance by insect pests which has posed pressure for the identification of novel insecticidal genes codifying for new insecticidal proteins that could counter resistance (Jurat-Fuentes et al., 2021). These novel insecticidal genes should be highly efficacious against the resistant insects, implying that the novel genes should codify for proteins that have a different mode of action from the toxin showing no cross-resistance with the Bt insecticidal toxin that evolved resistance in the first place. High levels of resistance to Cry toxins from Bt have been linked to mutations affecting expression of larval gut proteins known as 'toxin-receptors' such as GPI-anchored proteins (aminopeptidase and alkaline phosphatase) or some transmembrane proteins (cadherin and ABC transporters) that participate in the insect gut function. Thus, the novel insecticidal proteins should preferably bind to different larval gut proteins to overcome resistance. This is the case, for example, of Bt Vip3Aa or Cry2Ab proteins that show high toxicity against different lepidopteran pests that have evolve resistance to Cry1 toxins, since these two proteins bind to...
different midgut receptors than Cry1 toxins (Sanahuja et al., 2011). Also, it is important to mention that there are still some insect pests which are not effectively controlled by the known Bt insecticidal proteins.

Whole genome sequencing of multiple insect pathogens has speed up the mining and discovery of insecticidal genes. Since new insecticidal toxins with novel modes of action are needed to deal with the evolution of resistance to Bt-crops, mining new insecticidal proteins from other bacterial sources is an interesting alternative. Recently, new insecticidal proteins active against *Diabrotica virgifera virgifera*, a corn root pest, resistant to Cry3 proteins used in transgenic plants were identified in other bacteria such as *Pseudomonas chlororaphis* and *Pseudomonas mosselii* (Schellenberger et al., 2016; Wei et al., 2018).

What is the best strategy for the identification of these novel genes? Mining or evolving known insecticidal genes for different modes of action?

Regarding evolving known insecticidal genes, a novel and efficient *in vitro* evolution system was described, where Cry1Ac mutants were selected to bind to a different gut protein, resulting in the selection of an insecticidal Cry1Ac protein that was highly effective against Cry1Ac-resistant insects (Badran et al., 2016). Besides this example, several efficient display systems for selecting Bt toxins that bind to different gut proteins and counter resistance have also been developed (Pacheco et al., 2015).

Describing new gene sequences provides clues and means for their *in vitro* improvement of insecticidal activities. For example, the description of many insecticidal genes has provided clues for the *in vitro* evolution of the Cry gene family of Bt insecticidal proteins. Early phylogenetic analysis of Bt Cry toxins, commonly used in transgenic crops and composed of three structural domains, showed that domain III swapping contributed to the natural evolution of this family of proteins, creating insecticidal proteins with novel specificities (Bravo et al., 2013). Domain III, as well as domain II, have been shown to be involved in binding to toxin receptors found in the insect gut. In the case of domain II, exposed loop regions have been shown to be important for receptor binding and insect specificity. In fact, some Cry proteins with higher toxicity against certain insect pests were created by domain III swapping and are currently used in transgenic crops. The Cry1A105, that is, composed of domains I and II from Cry1Ac and domain III from Cry1Fa, shows high toxicity against different *Spodoptera* species. Another interesting case is the eCry3.1Ab, that is, composed of domains I and II from Cry3Aa and domain III from Cry1Ab showing high toxicity against *Diabrotica virgifera virgifera* (Bravo et al., 2013).

Figure 1 shows how gene mining and *in vitro* evolution could be interconnected for the discovery of new insecticidal genes with novel modes of action. Domain or loop swapping among different toxins could yield

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**Figure 1** Mining versus evolution of microbial insecticidal genes. Squares represent different insecticidal genes identified in different insect pathogens. Different colours represent different structural domains. Some of the known insecticidal genes are subject for *in vitro* evolution of insecticidal activity by different experimental approaches, domain swapping, loop swapping, gene shuffling and site directed or random mutagenesis. Evolved insecticidal genes could be screened by binding to different insect gut proteins using efficient display systems. Finally, selection of novel insecticidal genes with high activity and no cross-resistance to insecticidal genes already used in transgenic crops.
proteins with novel modes of action that could overcome resistance to Bt-crops. In addition, gene shuffling among different toxin genes could create proteins with novel specificities or increased toxicity. The larger the number of insecticidal genes described, the biggest possibility to tailor novel insecticidal genes against specific crop pests that are not naturally susceptible to the known insecticidal toxins or that have become resistant to the known insecticidal genes. Thus, mining of novel insecticidal genes is likely to provide novel insecticidal genes with novel modes of action but also provide novel protein scaffolds for in vitro evolution to create insecticidal proteins for the sustained use of insect-resistant transgenic crops.

CONFLICT OF INTEREST
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ORCID
Alejandra Bravo © https://orcid.org/0000-0002-7573-7475
Mario Soberón © https://orcid.org/0000-0002-1593-9227

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