

insect in terms of substantial increases in insect mortality and growth/development delay for larval survivors. It is also clear from these experiments that an exemplification is provided for the effective protection of plants/crops from insect damage by the use of a spray of a formulation consisting of bacteria expressing double-stranded RNA corresponding to an insect gene target.

Example 5

Epilachna varivettis (Mexican Bean Beetle)

A. Cloning *Epilachna varivettis* Partial Gene Sequences

[0311] High quality, intact RNA was isolated from 4 different larval stages of *Epilachna varivettis* (Mexican bean beetle; source: Thomas Dorsey, Supervising Entomologist, New Jersey Department of Agriculture, Division of Plant Industry, Bureau of Biological Pest Control, Phillip Alampi Beneficial Insect Laboratory, PO Box 330, Trenton, N.J. 08625-0330, USA) using TRIzol Reagent (Cat. Nr. 15596-026/15596-018, Invitrogen, Rockville, Md., USA) following the manufacturer's instructions. Genomic DNA present in the RNA preparation was removed by DNase treatment following the manufacturer's instructions (Cat. Nr. 1700, Promega). cDNA was generated using a commercially available kit (SuperScript™ III Reverse Transcriptase, Cat. Nr. 18080044, Invitrogen, Rockville, Md., USA) following the manufacturer's instructions.

[0312] To isolate cDNA sequences comprising a portion of the EV005, EV009, EV010, EV015 and EV016 genes, a series of PCR reactions with degenerate primers were performed using Amplitaq Gold (Cat. Nr. N8080240, Applied Biosystems) following the manufacturer's instructions.

[0313] The sequences of the degenerate primers used for amplification of each of the genes are given in Table 2-EV, which displays *Epilachna varivettis* target genes including primer sequences and cDNA sequences obtained. These primers were used in respective PCR reactions with the following conditions: for EV005 and EV009, 10 minutes at 95° C., followed by 40 cycles of 30 seconds at 95° C., 1 minute at 50° C. and 1 minute 30 seconds at 72° C., followed by 7 minutes at 72° C.; for EV014, 10 minutes at 95° C., followed by 40 cycles of 30 seconds at 95° C., 1 minute at 53° C. and 1 minute at 72° C., followed by 7 minutes at 72° C.; for EV010 and EV016, 10 minutes at 95° C., followed by 40 cycles of 30 seconds at 95° C., 1 minute at 54° C. and 1 minute 40 seconds at 72° C., followed by 7 minutes at 72° C. The resulting PCR fragments were analyzed on agarose gel, purified (QIAquick Gel Extraction kit, Cat. Nr. 28706, Qiagen), cloned into the pCR4/TOPO vector (Cat. Nr. K₄₅₃₀-20, Invitrogen), and sequenced. The sequences of the resulting PCR products are represented by the respective SEQ ID NO:s as given in Table 2-EV and are referred to as the partial sequences. The corresponding partial amino acid sequences are represented by the respective SEQ ID NO:s as given in Table 3-EV, where the start of the reading frame is indicated in brackets.

B. dsRNA Production of the *Epilachna varivettis* Genes

[0314] dsRNA was synthesized in milligram amounts using the commercially available kit T7 Ribomax™ Express RNAi System (Cat. Nr. P1700, Promega). First two separate

single 5' T7 RNA polymerase promoter templates were generated in two separate PCR reactions, each reaction containing the target sequence in a different orientation relative to the T7 promoter.

[0315] For each of the target genes, the sense T7 template was generated using specific T7 forward and specific reverse primers. The sequences of the respective primers for amplifying the sense template for each of the target genes are given in Table 8-EV.

[0316] The conditions in the PCR reactions were as follows: 1 minute at 95° C., followed by 20 cycles of 30 seconds at 95° C., 30 seconds at 60° C. and 1 minute at 72° C., followed by 15 cycles of 30 seconds at 95° C., 30 seconds at 50° C. and 1 minute at 72° C. followed by 10 minutes at 72° C. The anti-sense T7 template was generated using specific forward and specific T7 reverse primers in a PCR reaction with the same conditions as described above. The sequences of the respective primers for amplifying the anti-sense template for each of the target genes are given in Table 8-EV. The resulting PCR products were analyzed on agarose gel and purified by PCR purification kit (Qiaquick PCR Purification Kit, Cat. Nr. 28106, Qiagen) and NaClO₄ precipitation. The generated T7 forward and reverse templates were mixed to be transcribed and the resulting RNA strands were annealed, DNase and RNase treated, and purified by sodium acetate, following the manufacturer's instructions. The sense strand of the resulting dsRNA for each of the target genes is given in Table 8-EV.

C. Laboratory Trials to Test dsRNA Targets Using Bean Leaf Discs for Activity Against *Epilachna varivettis* Larvae

[0317] The example provided below is an exemplification of the finding that the Mexican bean beetle (MBB) larvae are susceptible to orally ingested dsRNA corresponding to own target genes.

[0318] To test the different double-stranded RNA samples against MBB larvae, a leaf disc assay was employed using snap bean (*Phaseolus vulgaris* variety Montano; source: Aveve NV, Belgium) leaf material as food source. The same variety of beans was used to maintain insect cultures in the insect chamber at 25±2° C. and 60±5% relative humidity with a photoperiod of 16 h light/8 h dark. Discs of approximately 1.1 cm in diameter (or 0.95 cm²) were cut out off leaves of 1- to 2-week old bean plants using a suitably-sized cork borer. Double-stranded RNA samples were diluted to 1 µg/µl in Milli-Q water containing 0.05% Triton X-100. Treated leaf discs were prepared by applying 25 µl of the diluted solution of target Ev005, Ev010, Ev015, Ev016 dsRNA and control gfp dsRNA or 0.05% Triton X-100 on the adaxial leaf surface. The leaf discs were left to dry and placed individually in each of the 24 wells of a 24-well multiplate containing 1 ml of gellified 2% agar which helps to prevent the leaf disc from drying out. A single neonate MBB larva was placed into each well of a plate, which was then covered with a multiwell plastic lid. The plate was divided into 3 replicates of 8 insects per replicate (row). The plate containing the insects and leaf discs were kept in an insect chamber at 25±2° C. and 60±5% relative humidity with a photoperiod of 16 h light/8 h dark. The insects were fed on the leaf discs for 2 days after which the insects were transferred to a new plate containing freshly treated leaf discs. Thereafter, 4 days after the start of the bioassay, the insects were transferred to a petriplate contain-