

Heat Treatment of Bacteria

[0329] Bacteria are killed by heat treatment in order to minimize the risk of contamination of the artificial diet in the test plates. However, heat treatment of bacteria expressing double-stranded RNA is not a prerequisite for inducing toxicity towards the insects due to RNA interference. The induced bacterial culture is centrifuged at 3000 g at room temperature for 10 minutes, the supernatant discarded and the pellet subjected to 80° C. for 20 minutes in a water bath. After heat treatment, the bacterial pellet is resuspended in 1.5 ml MilliQ water and the suspension transferred to a microfuge tube. Several tubes are prepared and used in the bioassays for each refreshment. The tubes are stored at -20° C. until further use.

G. Laboratory Trials to Test *Escherichia coli*
Expressing dsRNA Targets Against *Epilachna*
varivetis

Plant-Based Bioassays

[0330] Whole plants are sprayed with suspensions of chemically induced bacteria expressing dsRNA prior to feeding the plants to MBB. The are grown from in a plant growth room chamber. The plants are caged by placing a 500 ml plastic bottle upside down over the plant with the neck of the bottle firmly placed in the soil in a pot and the base cut open and covered with a fine nylon mesh to permit aeration, reduce condensation inside and prevent insect escape. MMB are placed on each treated plant in the cage. Plants are treated with a suspension of *E. coli* AB309-105 harbouring the pGBNJ001 plasmids or pGN29 plasmid. Different quantities of bacteria are applied to the plants: for instance 66, 22, and 7 units, where one unit is defined as 10⁹ bacterial cells in 1 ml of a bacterial suspension at optical density value of 1 at 600 nm wavelength. In each case, a total volume of between 1 and 10 ml sprayed on the plant with the aid of a vaporizer. One plant is used per treatment in this trial. The number of survivors are counted and the weight of each survivor recorded.

[0331] Spraying plants with a suspension of *E. coli* bacterial strain AB309-105 expressing target dsRNA from pGBNJ003 lead to a dramatic increase in insect mortality when compared to pGN29 control. These experiments show that double-stranded RNA corresponding to an insect gene target sequence produced in either wild-type or RNaseIII-deficient bacterial expression systems is toxic towards the 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 6

Anthonomus grandis (Cotton Boll Weevil)

A. Cloning *Anthonomus grandis* Partial Sequences

[0332] High quality, intact RNA was isolated from the 3 instars of *Anthonomus grandis* (cotton boll weevil; source: Dr. Gary Benzon, Benzon Research Inc., 7 Kuhn Drive, Carlisle, Pa. 17013, 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.

[0333] To isolate cDNA sequences comprising a portion of the AG001, AG005, AG010, AG014 and AG016 genes, a series of PCR reactions with degenerate primers were performed using Amplitaq Gold (Cat. Nr. N8080240, Applied Biosystems) following the manufacturer's instructions.

[0334] The sequences of the degenerate primers used for amplification of each of the genes are given in Table 2-AG. These primers were used in respective PCR reactions with the following conditions: for AG001, AG005 and AG016, 10 minutes at 95° C., followed by 40 cycles of 30 seconds at 95° C., 1 minute at 50° C. and 1 minute and 30 seconds at 72° C., followed by 7 minutes at 72° C.; for AG010, 10 minutes at 95° C., followed by 40 cycles of 30 seconds at 95° C., 1 minute at 54° C. and 2 minutes and 30 seconds at 72° C., followed by 7 minutes at 72° C.; for AG014, 10 minutes at 95° C., followed by 40 cycles of 30 seconds at 95° C., 1 minute at 55° C. and 1 minute 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 pCR8/GW/TOPO vector (Cat. Nr. K2500-20, Invitrogen) and sequenced. The sequences of the resulting PCR products are represented by the respective SEQ ID NO:s as given in Table 2-AG and are referred to as the partial sequences. The corresponding partial amino acid sequence are represented by the respective SEQ ID NO:s as given in Table 3-AG.

B. dsRNA Production of the *Anthonomus grandis*
(Cotton Boll Weevil) Genes

[0335] 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.

[0336] 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-AG. A touchdown PCR was performed as follows: 1 minute at 95° C., followed by 20 cycles of 30 seconds at 95° C., 30 seconds at 60° C. with a decrease in temperature of 0.5° C. per cycle 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-AG. 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