

down to 0.01 ng/ $\mu$ l was applied topically onto the solid artificial diet in the wells of a 24-well plate (Nunc). The diet was dried in a laminar flow cabin. Per treatment, twenty-four Colorado potato beetle larvae (2<sup>nd</sup> stage), with two insects per well, were tested. The plates were stored in the insect rearing chamber at 25 $\pm$ 2 $^{\circ}$  C., 60% relative humidity, with a 16:8 hours light:dark photoperiod. The beetles were assessed as live or dead at regular intervals up to day 14. After seven days, the diet was replaced with fresh diet with topically applied dsRNA at the same concentrations. The dsRNA targets were compared to diet only.

**[0259]** Feeding artificial diet containing intact naked dsRNAs of different targets to *L. decemlineata* larvae resulted in high larval mortalities at concentrations as low as between 0.1 and 10 ng dsRNA/ $\mu$ l as shown in FIG. 5-LD.

#### H. Cloning of a CPB Gene Fragment in a Vector Suitable for Bacterial Production of Insect-Active Double-Stranded RNA

**[0260]** While any efficient bacterial promoter may be used, a DNA fragment corresponding to an MLB gene target was cloned in a vector for the expression of double-stranded RNA in a bacterial host (See WO 00/01846).

**[0261]** The sequences of the specific primers used for the amplification of target genes are provided in Table 8. The template used is the pCR8/GW/topo vector containing any of target sequences. The primers are used in a PCR reaction with the following conditions: 5 minutes at 98 $^{\circ}$  C., followed by 30 cycles of 10 seconds at 98 $^{\circ}$  C., 30 seconds at 55 $^{\circ}$  C. and 2 minutes at 72 $^{\circ}$  C., followed by 10 minutes at 72 $^{\circ}$  C. The resulting PCR fragment is analyzed on agarose gel, purified (QIAquick Gel Extraction kit, Cat. Nr. 28706, Qiagen), blunt-end cloned into Srf I-linearized pGNA49A vector (reference to WO0188121A1), and sequenced. The sequence of the resulting PCR product corresponds to the respective sequence as given in Table 8. The recombinant vector harboring this sequence is named pGBNJ003.

**[0262]** The sequences of the specific primers used for the amplification of target gene fragment LD010 are provided in Table 8 (forward primer SEQ ID NO: 191 and reverse primer SEQ ID NO: 190). The template used was the pCR8/GW/topo vector containing the LD010 sequence (SEQ ID NO: 11). The primers were used in a PCR reaction with the following conditions: 5 minutes at 98 $^{\circ}$  C., followed by 30 cycles of 10 seconds at 98 $^{\circ}$  C., 30 seconds at 55 $^{\circ}$  C. and 2 minutes at 72 $^{\circ}$  C., followed by 10 minutes at 72 $^{\circ}$  C. The resulting PCR fragment was analyzed on agarose gel, purified (QIAquick Gel Extraction kit, Cat. Nr. 28706, Qiagen), blunt-end cloned into Srf I-linearized pGNA49A vector (reference to WO 00/188121A1), and sequenced. The sequence of the resulting PCR product corresponds to SEQ ID NO: 188 as given in Table 8. The recombinant vector harboring this sequence was named pGBNJ003.

#### I. Expression and Production of a Double-Stranded RNA Target in Two Strains of *Escherichia coli*: (1) AB309-105, and, (2) BL21(DE3)

**[0263]** The procedures described below were followed in order to express suitable levels of insect-active double-stranded RNA of target LD010 in bacteria. An RNaseIII-deficient strain, AB309-105, was used in comparison to wild-type RNaseIII-containing bacteria, BL21(DE3).

**[0264]** Transformation of AB309-105 and BL21(DE3)

**[0265]** Three hundred ng of the plasmid was added to and gently mixed in a 50  $\mu$ l aliquot of ice-chilled chemically competent *E. coli* strain AB309-105 or BL21(DE3). The cells were incubated on ice for 20 minutes before subjecting them to a heat shock treatment of 37 $^{\circ}$  C. for 5 minutes, after which the cells were placed back on ice for a further 5 minutes. Four hundred and fifty  $\mu$ l of room temperature SOC medium was added to the cells and the suspension incubated on a shaker (250 rpm) at 37 $^{\circ}$  C. for 1 hour. One hundred  $\mu$ l of the bacterial cell suspension was transferred to a 500 ml conical flask containing 150 ml of liquid Luria-Bertani (LB) broth supplemented with 100  $\mu$ g/ml carbenicillin antibiotic. The culture was incubated on an Innova 4430 shaker (250 rpm) at 37 $^{\circ}$  C. overnight (16 to 18 hours).

**[0266]** Chemical Induction of Double-Stranded RNA Expression in AB309-105 and BL21(DE3)

**[0267]** Expression of double-stranded RNA from the recombinant vector, pGBNJ003, in the bacterial strain AB309-105 or BL21(DE3) was made possible since all the genetic components for controlled expression are present. In the presence of the chemical inducer isopropylthiogalactoside, or IPTG, the T7 polymerase will drive the transcription of the target sequence in both antisense and sense directions since these are flanked by oppositely oriented T7 promoters.

**[0268]** The optical density at 600 nm of the overnight bacterial culture was measured using an appropriate spectrophotometer and adjusted to a value of 1 by the addition of fresh LB broth. Fifty ml of this culture was transferred to a 50 ml Falcon tube and the culture then centrifuged at 3000 g at 15 $^{\circ}$  C. for 10 minutes. The supernatant was removed and the bacterial pellet resuspended in 50 ml of fresh S complete medium (SNC medium plus 5  $\mu$ g/ml cholesterol) supplemented with 100  $\mu$ g/ml carbenicillin and 1 mM IPTG. The bacteria were induced for 2 to 4 hours at room temperature.

**[0269]** Heat Treatment of Bacteria

**[0270]** Bacteria were 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 was centrifuged at 3000 g at room temperature for 10 minutes, the supernatant discarded and the pellet subjected to 80 $^{\circ}$  C. for 20 minutes in a water bath. After heat treatment, the bacterial pellet was resuspended in 1.5 ml MilliQ water and the suspension transferred to a microfuge tube. Several tubes were prepared and used in the bioassays for each refreshment. The tubes were stored at -20 $^{\circ}$  C. until further use.

#### J. Laboratory Trials to Test *Escherichia coli* Expressing dsRNA Target LD010 Against *Leptinotarsa decemlineata*

**[0271]** Two bioassay methods were employed to test double-stranded RNA produced in *Escherichia coli* against larvae of the Colorado potato beetle: (1) artificial diet-based bioassay, and, (2) plant-based bioassay.

**[0272]** Artificial Diet-Based Bioassays

**[0273]** Artificial diet for the Colorado potato beetle was prepared as described previously in Example 4. A half milliliter of diet was dispensed into each of the wells of a 48-well multiwell test plate (Nunc). For every treatment, fifty  $\mu$ l of an OD 1 suspension of heat-treated bacteria (which is equivalent to approximately 5 $\times$ 10<sup>7</sup> bacterial cells) expressing dsRNA was applied topically onto the solid diet in the wells and the