

ing untreated fresh bean leaves every day until day 10. Insect mortality was recorded at day 2 and every other day thereafter.

**[0319]** Feeding snap bean leaves containing surface-applied intact naked target dsRNAs to *E. varivestis* larvae resulted in significant increases in larval mortalities, as indicated in FIG. 1. Tested double-stranded RNAs of targets Ev010, Ev015, & Ev016 led to 100% mortality after 8 days, whereas dsRNA of target Ev005 took 10 days to kill all larvae. The majority of the insects fed on treated leaf discs containing control *gfp* dsRNA or only the surfactant Triton X-100 were sustained throughout the bioassay (FIG. 1-EV).

#### D. Laboratory Trials to Test dsRNA Targets Using Bean Leaf Discs for Activity Against *Epilachna varivestis* Adults

**[0320]** The example provided below is an exemplification of the finding that the Mexican bean beetle adults are susceptible to orally ingested dsRNA corresponding to own target genes.

**[0321]** In a similar bioassay set-up as for Mexican bean beetle larvae, adult MBBs were tested against double-stranded RNAs topically-applied to bean leaf discs. Test dsRNA from each target Ev010, Ev015 and Ev016 was diluted in 0.05% Triton X-100 to a final concentration of 0.1 µg/µl. Bean leaf discs were treated by topical application of 30 µl of the test solution onto each disc. The discs were allowed to dry completely before placing each on a slice of gellified 2% agar in each well of a 24-well multiwell plate. Three-day-old adults were collected from the culture cages and fed nothing for 7-8 hours prior to placing one adult to each well of the bioassay plate (thus 24 adults per treatment). The plates were kept in the insect rearing chamber (under the same conditions as for MBB larvae for 24 hours) after which the adults were transferred to a new plate containing fresh dsRNA-treated leaf discs. After a further 24 hours, the adults from each treatment were collected and placed in a plastic box with dimensions 30 cm×15 cm×10 cm containing two potted and untreated 3-week-old bean plants. Insect mortality was assessed from day 4 until day 11.

**[0322]** All three target dsRNAs (Ev010, Ev015 and Ev016) ingested by adults of *Epilachna varivestis* resulted in significant increases in mortality from day 4 (4 days post bioassay start), as shown in FIG. 2(a)-EV. From day 5, dramatic changes in feeding patterns were observed between insects fed initially with target-dsRNA-treated bean leaf discs and those that were fed discs containing control *gfp* dsRNA or surfactant Triton X-100. Reductions in foliar damage by MBB adults of untreated bean plants were clearly visible for all three targets when compared to *gfp* dsRNA and surfactant only controls, albeit at varying levels; insects fed target 15 caused the least damage to bean foliage (FIG. 2(b)-EV).

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

**[0323]** What follows is an example of cloning a DNA fragment corresponding to an MLB gene target in a vector for the expression of double-stranded RNA in a bacterial host, although any vector comprising a T7 promoter or any other promoter for efficient transcription in bacteria, may be used (reference to WO0001846).

**[0324]** The sequences of the specific primers used for the amplification of target genes are provided in Table 8-EV. 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° C., followed by 30 cycles of 10 seconds at 98° C., 30 seconds at 55° C. and 2 minutes at 72° C., followed by 10 minutes at 72° 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 WO00188121A1), and sequenced. The sequence of the resulting PCR product corresponds to the respective sequence as given in Table 8-EV. The recombinant vector harbouring this sequence is named pGBNJ00XX.

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

**[0325]** The procedures described below are followed in order to express suitable levels of insect-active double-stranded RNA of insect target in bacteria. An RNaseIII-deficient strain, AB309-105, is used in comparison to wild-type RNaseIII-containing bacteria, BL21(DE3).

#### Transformation of AB309-105 and BL21(DE3)

**[0326]** Three hundred ng of the plasmid are added to and gently mixed in a 50 µl aliquot of ice-chilled chemically competent *E. coli* strain AB309-105 or BL21(DE3). The cells are incubated on ice for 20 minutes before subjecting them to a heat shock treatment of 37° C. for 5 minutes, after which the cells are placed back on ice for a further 5 minutes. Four hundred and fifty µl of room temperature SOC medium is added to the cells and the suspension incubated on a shaker (250 rpm) at 37° C. for 1 hour. One hundred µl of the bacterial cell suspension is transferred to a 500 ml conical flask containing 150 ml of liquid Luria-Bertani (LB) broth supplemented with 100 µg/ml carbenicillin antibiotic. The culture is incubated on an Innova 4430 shaker (250 rpm) at 37° C. overnight (16 to 18 hours).

#### Chemical Induction of Double-Stranded RNA Expression in AB309-105 and BL21(DE3)

**[0327]** Expression of double-stranded RNA from the recombinant vector, pGBNJ003, in the bacterial strain AB309-105 or BL21(DE3) is 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.

**[0328]** The optical density at 600 nm of the overnight bacterial culture is measured using an appropriate spectrophotometer and adjusted to a value of 1 by the addition of fresh LB broth. Fifty ml of this culture is transferred to a 50 ml Falcon tube and the culture then centrifuged at 3000 g at 15° C. for 10 minutes. The supernatant is removed and the bacterial pellet resuspended in 50 ml of fresh S complete medium (SNC medium plus 5 µg/ml cholesterol) supplemented with 100 µg/ml carbenicillin and 1 mM IPTG. The bacteria are induced for 2 to 4 hours at room temperature.