

[0355] Red flour beetles, *Tribolium castaneum*, were maintained at Insect Investigations Ltd. (origin: Imperial College of Science, Technology and Medicine, Silwood Park, Berkshire, UK). Insects were cultured according to company SOP/251/01. Briefly, the beetles were housed in plastic jars or tanks. These have an open top to allow ventilation. A piece of netting was fitted over the top and secured with an elastic band to prevent escape. The larval rearing medium (flour) was placed in the container where the beetles can breed. The stored product beetle colonies were maintained in a controlled temperature room at 25±3° C. with a 16:8 hour light:dark cycle.

[0356] Double-stranded RNA from target TC014 (with sequence corresponding to SEQ ID NO: -799) was incorporated into a mixture of flour and milk powder (wholemeal flour: powdered milk in the ratio 4:1) and left to dry overnight. Each replicate was prepared separately: 100 µA of a 10 µg/µl dsRNA solution (1 mg dsRNA) was added to 0.1 g flour/milk mixture. The dried mixture was ground to a fine powder. Insects were maintained within Petri dishes (55 mm diameter), lined with a double layer of filter paper. The treated diet was placed between the two filter paper layers. Ten first instar, mixed sex larvae were placed in each dish (replicate). Four replicates were performed for each treatment. Control was Milli-Q water. Assessments (number of survivors) were made on a regular basis. During the trial, the test conditions were 25-33° C. and 20-25% relative humidity, with a 12:12 hour light:dark photoperiod.

[0357] Survival of larvae of *T. castaneum* over time on artificial diet treated with target TC014 dsRNA was significantly reduced when compared to diet only control, as shown in FIG. 1.

D. Cloning of a RFB Gene Fragment in a Vector Suitable for Bacterial Production of Insect-Active Double-Stranded RNA

[0358] What follows is an example of cloning a DNA fragment corresponding to an RFB 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).

[0359] The sequences of the specific primers used for the amplification of target genes are provided in Table 8-TC. 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 WO0188121A1), and sequenced. The sequence of the resulting PCR product corresponds to the respective sequence as given in Table 8-TC. The recombinant vector harbouring this sequence is named pGBNJ00 XX.

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

[0360] 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-defi-

cient strain, AB309-105, is used in comparison to wild-type RNaseIII-containing bacteria, BL21(DE3).

Transformation of AB309-105 and BL21(DE3)

[0361] 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)

[0362] 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.

[0363] 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.

Heat Treatment of Bacteria

[0364] Bacteria are killed by heat treatment in order to minimise 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.

F. Laboratory Trials to Test *Escherichia coli* Expressing dsRNA Targets Against *Tribolium castaneum*

Plant-Based Bioassays

[0365] Whole plants are sprayed with suspensions of chemically induced bacteria expressing dsRNA prior to feeding the plants to RFB. The are grown from in a plant growth