

following the manufacturer's instructions. The sense strand of the resulting dsRNA for each of the target genes is given in Table 8-PC.

C. Laboratory Trials to Test dsRNA Targets, Using Oilseed Rape Leaf Discs for Activity Against *Phaedon cochleariae* Larvae

[0294] The example provided below is an exemplification of the finding that the mustard leaf beetle (MLB) larvae are susceptible to orally ingested dsRNA corresponding to own target genes.

[0295] To test the different double-stranded RNA samples against MLB larvae, a leaf disc assay was employed using oilseed rape (*Brassica napus* variety SW Oban; source: Nick Balaam, Sw Seed Ltd., 49 North Road, Abington, Cambridge, CB1 6AS, UK) leaf material as food source. The insect cultures were maintained on the same variety of oilseed rape in the insect chamber at $25\pm 2^\circ\text{C}$. and $60\pm 5\%$ relative humidity with a photoperiod of 16 h light/8 h dark. Discs of approximately 1.1 cm in diameter (or 0.95 cm^2) were cut out off leaves of 4- to 6-week old rape plants using a suitably-sized cork borer. Double-stranded RNA samples were diluted to $0.1\ \mu\text{g}/\mu\text{l}$ 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 PC001, PC003, PC005, PC010, PC014, PC016, PC027 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. Two neonate MLB larvae were placed into each well of the plate, which was then covered with a multiwell plastic lid. The plate (one treatment containing 48 insects) was divided into 4 replicates of 12 insects per replicate (each row). The plate containing the insects and leaf discs were kept in an insect chamber at $25\pm 2^\circ\text{C}$. and $60\pm 5\%$ relative humidity with a photoperiod of 16 h light/8 h dark. The insects were fed leaf discs for 2 days after which they were transferred to a new plate containing freshly treated leaf discs. Thereafter, 4 days after the start of the bioassay, the insects from each replicate were collected and transferred to a Petri dish containing untreated fresh oilseed rape leaves. Larval mortality and average weight were recorded at days 2, 4, 7, 9 and 11.

[0296] *P. cochleariae* larvae fed on intact naked target dsRNA-treated oilseed rape leaves resulted in significant increases in larval mortalities for all targets tested, as indicated in FIG. 1(a). Tested double-stranded RNA for target PC010 led to 100% larval mortality at day 9 and for target PC027 at day 11. For all other targets, significantly high mortality values were reached at day 11 when compared to control gfp dsRNA, 0.05% Triton X-100 alone or untreated leaf only: (average value in percentage \pm confidence interval with alpha 0.05) PC001 (94.4 ± 8.2); PC003 (86.1 ± 4.1); PC005 (83.3 ± 7.8); PC014 (63.9 ± 20.6); PC016 (75.0 ± 16.8); gfp dsRNA (11.1 ± 8.2); 0.05% Triton X-100 (19.4 ± 10.5); leaf only (8.3 ± 10.5).

[0297] Larval survivors were assessed based on their average weight. For all targets tested, the mustard leaf beetle larvae had significantly reduced average weights after day 4 of the bioassay; insects fed control gfp dsRNA or 0.05% Triton X-100 alone developed normally, as for the larvae on leaf only (FIG. 1(b)—PC).

D. Laboratory Trials to Screen dsRNAs at Different Concentrations Using Oilseed Rape Leaf Discs for Activity Against *Phaedon cochleariae* Larvae

[0298] Twenty-five μl of a solution of dsRNA from target PC010 or PC027 at serial ten-fold concentrations from $0.1\ \mu\text{g}/\mu\text{l}$ down to $0.1\ \text{ng}/\mu\text{l}$ was applied topically onto the oilseed rape leaf disc, as described in Example 4D above. As a negative control, 0.05% Triton X-100 only was administered to the leaf disc. Per treatment, twenty-four mustard leaf beetle neonate larvae, with two insects per well, were tested. The plates were stored in the insect rearing chamber at $25\pm 2^\circ\text{C}$., $60\pm 5\%$ relative humidity, with a 16:8 hours light:dark photoperiod. At day 2, the larvae were transferred on to a new plate containing fresh dsRNA-treated leaf discs. At day 4 for target PC010 and day 5 for target PC027, insects from each replicate were transferred to a Petri dish containing abundant untreated leaf material. The beetles were assessed as live or dead on days 2, 4, 7, 8, 9, and 11 for target PC010, and 2, 5, 8, 9 and 12 for target PC027.

[0299] Feeding oilseed rape leaf discs containing intact naked dsRNAs of the two different targets, PC010 and PC027, to *P. cochleariae* larvae resulted in high mortalities at concentrations down to as low as $1\ \text{ng dsRNA}/\mu\text{l}$ solution, as shown in FIGS. 2 (a) and (b). Average mortality values in percentage \pm confidence interval with alpha 0.05 for different concentrations of dsRNA for target PC010 at day 11, $0\ \mu\text{g}/\mu\text{l}$: 8.3 ± 9.4 ; $0.1\ \mu\text{g}/\mu\text{l}$: 100; $0.01\ \mu\text{g}/\mu\text{l}$: 79.2 ± 20.6 ; $0.001\ \mu\text{g}/\mu\text{l}$: 58.3 ± 9.4 ; $0.0001\ \mu\text{g}/\mu\text{l}$: 12.5 ± 15.6 ; and for target PC027 at day 12, $0\ \mu\text{g}/\mu\text{l}$: 8.3 ± 9.4 ; $0.1\ \mu\text{g}/\mu\text{l}$: 95.8 ± 8.2 ; $0.01\ \mu\text{g}/\mu\text{l}$: 95.8 ± 8.2 ; $0.001\ \mu\text{g}/\mu\text{l}$: 83.3 ± 13.3 ; $0.0001\ \mu\text{g}/\mu\text{l}$: 12.5 ± 8.2 .

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

[0300] 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).

[0301] 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°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. The recombinant vector harbouring this sequence is named pGBNJ00 (to be completed).

[0302] The sequences of the specific primers used for the amplification of target gene fragment PC010 are provided in Table 8-PC. The template used was the pCR8/GW/topo vector containing the PC010 sequence (SEQ ID NO: 253). The primers were used in a touch-down PCR reaction with the following conditions: 1 minute at 95°C ., followed by 20 cycles of 30 seconds at 95°C ., 30 seconds at 60°C . with temperature decrease of -0.5°C . per cycle and 1 minute at 72°C ., followed by 15 cycles of 30 seconds at 95°C ., 30