

formed using Amplitaq Gold (Cat. Nr. N8080240, Applied Biosystems) following the manufacturer's instructions.

**[0446]** The sequences of the degenerate primers used for amplification of each of the genes are given in Table 2-AD. These primers were used in respective PCR reactions with the following conditions: 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. 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-AD 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-AD.

#### B. dsRNA Production of the *Acheta domesticus* Genes

**[0447]** 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.

**[0448]** 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-AD. The conditions in the PCR reactions were as follows: 1 minute at 95° C., followed by 20 cycles of 30 seconds at 95° C., 30 seconds at 60° C. (-0.5° C./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-AD. The resulting PCR products were analyzed on agarose gel and purified by PCR purification kit (Qiaquick PCR Purification Kit, Cat. Nr. 28106, Qiagen) and NaClO<sub>4</sub> 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 manufacturer's instructions. The sense strand of the resulting dsRNA for each of the target genes is given in Table 8-AD.

#### C. Laboratory Trials to Test dsRNA Targets, Using Artificial Diet for Activity Against *Acheta domesticus* Larvae

**[0449]** House crickets, *Acheta domesticus*, were maintained at Insect Investigations Ltd. (origin: Blades Biological Ltd., Kent, UK). The insects were reared on bran pellets and cabbage leaves. Mixed sex nymphs of equal size and no more than 5 days old were selected for use in the trial. Double-stranded RNA is mixed with a wheat-based pelleted rodent diet (rat and mouse standard diet, B & K Universal Ltd., Grimston, Aldbrough, Hull, UK). The diet, BK001P, contains the following ingredients in descending order by weight:

wheat, soya, wheatfeed, barley, pellet binder, rodent 5 vit min, fat blend, dicalcium phosphate, mould carb. The pelleted rodent diet is finely ground and heat-treated in a microwave oven prior to mixing, in order to inactivate any enzyme components. All rodent diet is taken from the same batch in order to ensure consistency. The ground diet and dsRNA are mixed thoroughly and formed into small pellets of equal weight, which are allowed to dry overnight at room temperature.

**[0450]** Double-stranded RNA samples from targets and gfp control at concentrations 10 µg/µl were applied in the ratio 1 g ground diet plus 1 ml dsRNA solution, thereby resulting in an application rate of 10 mg dsRNA per g pellet. Pellets are replaced weekly. The insects are provided with treated pellets for the first three weeks of the trial. Thereafter untreated pellets are provided. Insects are maintained within lidded plastic containers (9 cm diameter, 4.5 cm deep), ten per container. Each arena contains one treated bait pellet and one water source (damp cotton wool ball), each placed in a separate small weigh boat. The water is replenished ad lib throughout the experiment.

**[0451]** Assessments are made at twice weekly intervals, with no more than four days between assessments, until all the control insects had either died or moulted to the adult stage (84 days). At each assessment the insects are assessed as live or dead, and examined for abnormalities. From day 46 onwards, once moulting to adult has commenced, all insects (live and dead) are assessed as nymph or adult. Surviving insects are weighed on day 55 of the trial. Four replicates are performed for each of the treatments. During the trial the test conditions are 25 to 33° C. and 20 to 25% relative humidity, with a 12:12 hour light:dark photoperiod.

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

**[0452]** What follows is an example of cloning a DNA fragment corresponding to a HC 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).

**[0453]** 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-AD. The recombinant vector harbouring this sequence is named pGBNJ00XX.

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

**[0454]** 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-