

were prepared by applying 20 μ A of a 10 ng/ μ l solution of target LD002 dsRNA or control gfp dsRNA on the adaxial leaf surface. The leaf discs were allowed to dry and placed individually in 24 wells of a 24-well multiplate (Nunc). A single second-larval stage CPB was placed into each well, which was then covered with tissue paper and a multiwell plastic lid. The plate containing the insects and leaf discs were kept in an insect chamber at 28° C. with a photoperiod of 16 h light/8 h dark. The insects were allowed to feed on the leaf discs for 2 days after which the insects were transferred to a new plate containing fresh treated leaf discs. Thereafter, the insects were transferred to a plate containing untreated leaf discs every day until day 7. Insect mortality and weight scores were recorded.

[0246] Feeding potato leaf discs with surface-applied intact naked dsRNA of target LD002 to *L. decemlineata* larvae resulted in a significant increase in larval mortalities (i.e. at day 7 all insects were dead; 100% mortality) whereas control gfp dsRNA had no effect on CPB survival. Target LD002 dsRNA severely affected the growth of the larvae after 2 to 3 days whereas the larvae fed with gfp dsRNA at the same concentration developed as normal (FIG. 3-LD).

D. Screening Shorter Versions of dsRNAs Using Artificial Diet for Activity Against *Leptinotarsa decemlineata*

[0247] This example exemplifies the finding that shorter (60 or 100 bp) dsRNA fragments on their own or as concatemer constructs are sufficient in causing toxicity towards the Colorado potato beetle.

[0248] LD014, a target known to induce lethality in Colorado potato beetle, was selected for this example. This gene encodes a V-ATPase subunit E (SEQ ID NO: 15).

[0249] A 100 base pair fragment, LD014_F1, at position 195-294 on SEQ ID NO: 15 (SEQ ID NO: 159) and a 60 base pair fragment, LD014_F2, at position 235-294 on SEQ ID NO: 15 (SEQ ID NO: 160) were further selected. See also Table 7-LD.

[0250] Two concatemers of 300 base pairs, LD014_C1 and LD014_C2, were designed (SEQ ID NO: 161 and SEQ ID NO: 162). LD014_C1 contained 3 repeats of the 100 base pair fragment described above (SEQ ID NO: 159) and LD014_C2 contained 5 repeats of the 60 base pair fragment described above (SEQ ID NO: 160). See also Table 7-LD.

[0251] The fragments LD014_F1 and LD014_F2 were synthesized as sense and antisense primers. These primers were annealed to create the double strands DNA molecules prior to cloning. XbaI and XmaI restrictions sites were included at the 5' and 3' ends of the primers, respectively, to facilitate the cloning.

[0252] The concatemers were made as 300 base pairs synthetic genes. XbaI and XmaI restrictions sites were included at the 5' and 3' ends of the synthetic DNA fragments, respectively, to facilitate the cloning.

[0253] The 4 DNA molecules, i.e. the 2 single units (LD014_F1 & LD014_F2) and the 2 concatemers (LD014_C1 & LD014_C2), were digested with XbaI and XmaI and subcloned in pBluescriptII SK+ linearised by XbaI and XmaI digests, resulting in recombinant plasmids p1, p2, p3, & p4, respectively.

[0254] Double-stranded RNA production: dsRNA was synthesized 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. For LD014_F1, the sense T7 template was generated using the specific T7 forward primer oGBM159 and the specific reverse primer oGBM164 (represented herein as SEQ ID NO: 204 and SEQ ID NO: 205, respectively) in a PCR reaction with the following conditions: 4 minutes at 95° C., followed by 35 cycles of 30 seconds at 95° C., 30 seconds at 55° C. and 1 minute at 72° C., followed by 10 minutes at 72° C. The anti-sense T7 template was generated using the specific forward primer oGBM163 and the specific T7 reverse primer oGBM160 (represented herein as SEQ ID NO: 206 and SEQ ID NO: 207, respectively) in a PCR reaction with the same conditions as described above. The resulting PCR products were analyzed on agarose gel and purified by PCR purification kit (Qiaquick PCR Purification Kit, Cat. Nr. 28106, Qiagen) and NaClO₄ 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 is herein represented by SEQ ID NO: 203.

[0255] For LD014_F2, the sense T7 template was generated using the specific T7 forward primer oGBM161 and the specific reverse primer oGBM166 (represented herein as SEQ ID NO: 209 and SEQ ID NO: 210, respectively) in a PCR reaction with the following conditions: 4 minutes at 95° C., followed by 35 cycles of 30 seconds at 95° C., 30 seconds at 55° C. and 1 minute at 72° C., followed by 10 minutes at 72° C. The anti-sense T7 template was generated using the specific forward primer oGBM165 and the specific T7 reverse primer oGBM162 (represented herein as SEQ ID NO: 211 and SEQ ID NO: 212, respectively) in a PCR reaction with the same conditions as described above. The resulting PCR products were analyzed on agarose gel and purified by PCR purification kit (Qiaquick PCR Purification Kit, Cat. Nr. 28106, Qiagen) and NaClO₄ 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 is herein represented by SEQ ID NO: 208.

[0256] Also for the concatemers, 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. The recombinant plasmids p3 and p4 containing LD014_C1 & LD014_C2 were linearised with XbaI or XmaI, the two linear fragments for each construct purified and used as template for the in vitro transcription assay, using the T7 promoters flanking the cloning sites. Double-stranded RNA was prepared by in vitro transcription using the T7 Ribomax™ Express RNAi System (Promega). The sense strands of the resulting dsRNA for LD014_C1 and LD014_C2 are herein represented by SEQ ID NO: 213 and 2114, respectively.

[0257] Shorter sequences of target LD014 and concatemers were able to induce lethality in *Leptinotarsa decemlineata*, as shown in FIG. 4-LD.

G. Screening dsRNAs at Different Concentrations Using Artificial Diet for Activity Against *Leptinotarsa decemlineata*

[0258] Fifty μ l of a solution of dsRNA at serial ten-fold concentrations from 1 μ g/ μ l (for target LD027 from 0.1 μ g/ μ l)