

nidae (e.g. *Leptinotarsa* spp., *Phaedon* spp., *Epilachna* spp., *Anthonomus* spp., *Tribolium* spp., *Myzus* spp., *Nilaparvata* spp., *Chilo* spp., *Plutella* spp., or *Acheta* spp. Additionally, the nucleotide sequences for use as a target sequence in the present invention may also be derived from viral, bacterial, fungal, insect or fungal genes whose functions have been established from literature and the nucleotide sequences of which share substantial similarity with the target genes in the genome of an insect.

[0132] For many of the insects that are potential targets for control by the present invention, there may be limited information regarding the sequences of most genes or the phenotype resulting from mutation of particular genes. Therefore, genes may be selected based on available information available concerning corresponding genes in a model organism, such as *Caenorhabditis* or *Drosophila*, or in some other insect species. Genes may also be selected based on available sequence information for other species, such as nematode or fungal species, in which the genes have been characterized. In some cases it will be possible to obtain the sequence of a corresponding gene from a target insect by searching databases, such as GenBank, using either the name of the gene or the gene sequence. Once the sequence is obtained, PCR may be used to amplify an appropriately selected segment of the gene in the insect for use in the present invention.

[0133] In order to obtain a DNA segment from the corresponding gene in an insect species, for example, PCR primers may be designed based on the sequence as found in *C. elegans* or *Drosophila*, or an insect from which the gene has already been cloned. The primers are designed to amplify a DNA segment of sufficient length for use in the present invention. Amplification conditions are selected so that amplification will occur even if the primers do not exactly match the target sequence. Alternately, the gene, or a portion thereof, may be cloned from a genomic DNA or cDNA library prepared from the insect pest species, using a known insect gene as a probe. Techniques for performing PCR and cloning from libraries are known. Further details of the process by which DNA segments from target insect pest species may be isolated based on the sequence of genes previously cloned from an insect species are provided in the Examples. One of ordinary skill in the art will recognize that a variety of techniques may be used to isolate gene segments from insect pest species that correspond to genes previously isolated from other species.

[0134] III. Methods for Inhibiting or Suppressing a Target Gene

[0135] The present invention provides methods for inhibiting gene expression of one or multiple target genes in a target pest using stabilized dsRNA methods. The invention is particularly useful for modulating eukaryotic gene expression, in particular modulating the expression of genes present in pests that exhibit a digestive system pH level that is from about 4.5 to about 9.5, more preferably from about 5.0 to about 8.0, and even more preferably from about 6.5 to about 7.5. For pests with a digestive system that exhibits pH levels outside of these ranges, delivery methods may be desired for use that do not require ingestion of dsRNA molecules.

[0136] The methods of the invention encompass the simultaneous or sequential provision of two or more different double-stranded RNAs or RNA constructs to the same insect, so as to achieve down-regulation or inhibition of multiple target genes or to achieve a more potent inhibition of a single target gene.

[0137] Alternatively, multiple targets are hit by the provision of one double-stranded RNA that hits multiple target sequences, and a single target is more efficiently inhibited by the presence of more than one copy of the double stranded RNA fragment corresponding to the target gene. Thus, in one embodiment of the invention, the double-stranded RNA construct comprises multiple dsRNA regions, at least one strand of each dsRNA region comprising a nucleotide sequence that is complementary to at least part of a target nucleotide sequence of an insect target gene. According to the invention, the dsRNA regions in the RNA construct may be complementary to the same or to different target genes and/or the dsRNA regions may be complementary to targets from the same or from different insect species. Use of such dsRNA constructs in a plant host cell, thus establishes a more potent resistance to a single or to multiple insect species in the plant. In one embodiment, the double stranded RNA region comprises multiple copies of the nucleotide sequence that is complementary to the target gene. Alternatively, the dsRNA hits more than one target sequence of the same target gene. The invention thus encompasses isolated double stranded RNA constructs comprising at least two copies of said nucleotide sequence complementary to at least part of a nucleotide sequence of an insect target. DsRNA that hits more than one of the above-mentioned targets, or a combination of different dsRNA against different of the above mentioned targets are developed and used in the methods of the present invention. Suitable dsRNA nucleotides and dsRNA constructs are described in WO2006/046148 by applicant, which is incorporated herein in its entirety.

[0138] The terms “hit”, “hits”, and “hitting” are alternative wordings to indicate that at least one of the strands of the dsRNA is complementary to, and as such may bind to, the target gene or nucleotide sequence.

[0139] The modulatory effect of dsRNA is applicable to a variety of genes expressed in the pests including, for example, endogenous genes responsible for cellular metabolism or cellular transformation, including house keeping genes, transcription factors, and other genes which encode polypeptides involved in cellular metabolism.

[0140] As used herein, the phrase “inhibition of gene expression” or “inhibiting expression of a target gene in the cell of an pest” refers to the absence (or observable decrease) in the level of protein and/or mRNA product from the target gene. Specificity refers to the ability to inhibit the target gene without manifest effects on other genes of the cell and without any effects on any gene within the cell that is producing the dsRNA molecule. The inhibition of gene expression of the target gene in the pest may result in novel phenotypic traits in the pest.

[0141] “Gene suppression” refers to any of the well-known methods for reducing the levels of gene transcription to mRNA and/or subsequent translation of the mRNA. Gene suppression is also intended to mean the reduction of protein expression from a gene or a coding sequence including post-transcriptional gene suppression and transcriptional suppression. Posttranscriptional gene suppression is mediated by the homology between of all or a part of a mRNA transcribed from a gene or coding sequence targeted for suppression and the corresponding double stranded RNA used for suppression, and refers to the substantial and measurable reduction of the amount of available mRNA available in the cell for binding by ribosomes. The transcribed RNA can be in the sense orientation to effect what is called co-suppression, in the