

controlling the pests in which the second means exerts its effects through a different mode of action in comparison to the recombinant toxin. One means for deploying two or more toxins would be to incorporate a seed treatment containing some insecticidal composition that is effective at very low doses and which infuses into the soil or into the growing recombinant plant after sprouting. This means has been shown to be effective and economical, but has the disadvantage of subjecting the environment to a chemical pesticide that may accumulate in the food chain and that may persist in the environment. Another means would be to deploy a recombinant plant that expresses at least two different insecticidal toxins, each toxin being toxic to the same insect pest, and each toxin exerting its effects through a different mode of action. In the short term this second approach is cost effective and likely sufficient to delay the development of resistance in the pest population. However, there may be at least two disadvantages to this approach as well. One disadvantage is that any development of resistance to one of the insecticidal toxins deployed into the environment of the pest immediately increases the likelihood that resistance could develop sooner than anticipated to a second or even a third toxin. Also, there is a limited number of insecticidal crystal protein toxins that are toxic to the same insect pest that are available for use that, when combined with another insecticidal protein, would fall within the defined scope of exerting its effects through a different mode of action than toxins presently in use. Therefore, additional compositions and methods for controlling pest infestation are needed, and in particular, methods and compositions are needed for use in delaying or minimizing the development of resistance to present pest control agents.

[0008] Chemical pesticidal agents typically exert their effects by inhibiting one or more proteins within the target pest either by binding irreversibly to an active site within a particular protein, by inhibiting the protein from acting upon a naturally occurring substrate, or by poisoning a respiratory or chemical gradient pathway. Recombinant methods and compositions have typically targeted cell membrane systems by producing proteins that, upon ingestion by a pest, introduce pores that result in the loss of chemical or other gradients across disrupted cell membranes. Other than chemical compositions that directly exert their effects upon proteins involved in transcription or translation mechanisms, no method has been reported for controlling pest infestation by inhibiting in the target pest the production of essential proteins through RNA mediated interference by providing one or more double stranded RNA molecules in the diet of the pest.

[0009] Antisense methods and compositions have been reported in the art and are believed to exert their effects through the synthesis of a single-stranded RNA molecule that in theory hybridizes in vivo to a substantially complementary sense strand RNA molecule. It is believed that the antisense methods function in much the same way as double stranded RNA mediated interference methods are believed to function, except that the effectiveness of the antisense response is often substantially less than desirable, intermittent, or not evident at all. Furthermore, there has never been a report in which antisense was contemplated as a means for suppressing expression of a gene in a cell remote from the cell or biological system in which the antisense sequence was expressed. Antisense technology has only been applied as a means for achieving gene-specific interference of expression within the cell or biological system in which the antisense sequence is expressed. Antisense technology has been difficult to employ

in many systems for three principle reasons. First, the antisense sequence expressed in the transformed cell is unstable. Second, the instability of the antisense sequence expressed in the transformed cell concomitantly creates difficulty in delivery of the sequence to a host, cell type, or biological system remote from the transgenic cell. Third, the difficulties encountered with instability and delivery of the antisense sequence create difficulties in attempting to provide a dose within the recombinant cell expressing the antisense sequence that can effectively modulate the level of expression of the target sense nucleotide sequence.

[0010] The phenomenon of double-stranded RNA (dsRNA) induced silencing has been known for a number of years in plant systems. One form of dsRNA induced silencing is referred to as co-suppression and as virus-induced gene silencing (VIGS) and is reviewed in Matzke et al. (*Adv. Genet.*, 2002, 46:235-275). Co-suppression and VIGS effects in recombinant plant systems were observed but unexplained before dsRNA was identified in animal systems as the trigger for induction of the evolutionarily conserved mechanism of gene suppression. Guo et al. first observed that the use of sense RNA as a control was as effective as antisense RNA in specific silencing of a targeted gene in *C. elegans* (Guo et al., 1995, *Cell* 81:611-620). Fire et al. suspected that the single stranded RNA preparations used by Guo et al. were contaminated with dsRNA, and subsequently demonstrated that dsRNA was a much more potent trigger than single stranded RNA for achieving gene specific silencing. The observations by Fire et al. distinguished the physical attribute of double stranded RNA suppression from antisense suppression (Fire et al., 1998, *Nature* 391:806-811). It is believed that the post-transcriptional gene silencing effects observed in plants (Jorgensen, 1990, *Trends Biotechnol.* 8:340-344) and the quelling effect in fungi (Romano et al., 1992, *Mol. Microbiol.* 6:3343-3353; Bernstein et al., 2001, *RNA* 7:1509-1521) using single stranded RNA is a result of contamination of samples with double stranded RNA sequences (Dykxhoorn et al., 2003, *Nature Reviews* 4:457-467; Hannon et al., 2002, *Nature* 418:244-251). It is now clear, however, that double stranded RNA mediated inhibition of gene expression, co-suppression, and virus-induced gene silencing are triggered by dsRNA and operate by similar mechanisms (Stevenson, 2003, *Nature Reviews* 3:851-858; Bernstein et al., 2001, *RNA* 7:1509-1521).

[0011] The lack of understanding of the specific mechanisms involved in these phenomena has meant that there have been few improvements in technologies for modulating the level of gene expression within a cell, tissue, or organism, and in particular, a lack of developed technologies for delaying, repressing or otherwise reducing the expression of specific genes using recombinant DNA technology. Furthermore, as a consequence of the unpredictability of these approaches, no commercially viable means for modulating the level of expression of a specific gene in a eukaryotic or prokaryotic organism is available.

[0012] Double stranded RNA mediated inhibition of specific genes in various pests has been previously demonstrated. dsRNA mediated approaches to genetic control have been tested in the fruit fly *Drosophila melanogaster* (Tabara et al., 1998, *Science* 282:430-431). Tabara et al. describe a method for delivery of dsRNA involved generating transgenic insects that express double stranded RNA molecules or injecting dsRNA solutions into the insect body or within the egg sac prior to or during embryonic development. Research investi-