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RNA interference in Colorado potato beetle: steps toward development of dsRNA as a commercial insecticide

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Abstract

Colorado potato beetle (CPB) is a notorious pest on potatoes and has a remarkable ability to detoxify plant chemicals and develop resistance against insecticides. dsRNA targeting CPB genes could be expressed in potato plants to control this pest. However, previous attempts at introducing transgenic potato plants to control CPB were not highly successful. Recent studies showed that feeding dsRNA expressed in bacteria works very well to kill CPB. To realize the potential of RNAi to control this and other economically important pests, more efficient methods for production and delivery of dsRNA need to be developed. Extensive research to determine off-target and non-target effects, environmental fate and potential for resistance development is also essential.

Colorado potato beetle, a notorious pest that is difficult to control

The Colorado potato beetle (CPB) is a major insect pest on potatoes, tomatoes and eggplants. This beetle is a voracious feeder and could consume 40 cm² of potato leaves during the larval stage and 10 cm² of foliage per day during the adult stage [1]. Very high fecundity (300–800 eggs/adult [2]), a flexible life cycle that includes adult diapause [3] and remarkable detoxification ability make this insect a challenging pest to control. These attributes may have contributed to the development of resistance against most of the insecticides introduced to control this pest [4]. Target site mutations, increase in metabolism by detoxifying enzymes (e.g. P450), reduction in insecticide penetration and increase in insecticide excretion are among the resistance mechanisms employed by this insect [5–19]. P450 mediated detoxification is the most dominant mechanism of resistance in this insect. The P450 inhibitor, piperonyl butoxide was shown to increase efficacy of azinphosmethyl [20], carbofuran and carbaryl [33], fenvalerate [21], permethrin [22], imidacloprid [23] and abamectin [24] in resistant strains of CPB. Increase in carboxylesterase activity in permethrin [25] resistant CPB and higher levels of glutathione-S-transferase activity in beetles resistant to azinphosmethyl, permethrin and carbofuran [20] have also been reported. In laboratory selection experiments, CPB was able to develop resistance to *Bacillus thuringiensis* toxin [26]. Thus, CPB has developed resistance to almost all synthetic insecticides introduced for its control [23,27–30,31**]. Because of difficulties faced by farmers in controlling this pest, novel approaches need to be developed to manage CBP

populations. RNA interference-based insecticide for controlling this pest might help in this respect.

RNA interference (RNAi) to the rescue

The ability of double-stranded RNA to silence genes was discovered in the nematode, *Caenorhabditis elegans* [32••]. Within a few years of its discovery, RNAi was shown to function in nematodes, insects and plants [33–41]. Over the course of the next decade, numerous improvements to the design, synthesis and delivery of dsRNA or siRNA led to the development of RNAi applications in both human and plant health. DsRNA or siRNA mediated RNAi is achieved after these RNA molecules bind to complimentary DNA/RNA interfering with translation, transcription or replication of the target gene. For efficient recognition and subsequent interference, the RNAs and the target DNA/RNA need to have almost 100% identity. Therefore, unlike other methods of pest control, RNAi is highly specific to target species.

RNAi works efficiently in coleopteran insects

RNAi works well in some insects, especially those that belong to order Coleoptera [42•] and RNAi does not seem to work well in other insects especially those that belong to order Lepidoptera [43•]. Extensive studies in coleopteran insects such as in the red flour beetle, *Tribolium castaneum*, the western corn rootworm (WCR, *Diabrotica virgifera virgifera* LeConte) and the Colorado potato beetle showed the utility of RNAi in both basic and applied science [44]. Although *in vivo* amplification of dsRNA/ siRNA has not been shown in beetles, small quantities of dsRNA appear to be sufficient to initiate RNAi response in these insects. RNAi does not seem to work uniformly in all beetles. While systemic RNAi works well in most beetle species studied, significant differences in the efficacy of orally delivered dsRNA have been reported. For example, orally delivered dsRNA works much better in WCR and CPB than in the red flour beetle [45••,46•,47••]. Tomoyasu *et al.* [42•] identified the genes coding for proteins involved RNAi pathway in *T. castaneum*. These studies showed that *T. castaneum* genome contains a larger inventory of RNAi core component genes than in *D. melanogaster* where feeding or injection of dsRNA does not initiate RNAi response efficiently. On the basis of the data on knockdown in expression of each of the three sid-like genes individually these studies suggested that *T. castaneum* sid-like genes are not required for systemic RNAi. A study in WCR showed that dsRNA of a minimum length of 60 base pairs (bp) is required for the initiation of RNAi response in artificial diet bioassays [48•]. These authors also reported that 21 bp siRNAs are not taken up by the midgut cells and therefore are not effective in silencing *snf7* gene. Recently, an *in vivo* assay system has been established to identify important proteins involved in RNAi response in WCR [49]. Using this system, Dicer-2 and Argonaute-2 were shown to be important for RNAi response. Knockdown of two WCR sid-like transporter genes caused a reduction in knockdown of a marker gene, *ebony* but the phenotype of this gene was not severe suggesting that there may be other players in the transport of dsRNA.

Various degrees of success in silencing target genes by feeding or injecting dsRNA has been reported in a number of insects belonging to almost all insect orders. Interestingly, in locust,

injecting dsRNA efficiently knocks down target genes but feeding dsRNA does not cause knockdown of target genes [50]. Degradation of dsRNA by dsRNases secreted by the midgut cells is thought to be the major contributor to the lack of knockdown by oral application of dsRNA. Tissue-specific RNAi response to injected dsRNA has been observed in both desert and migratory locusts [51,52]. RNAi works very well in the fat body and other tissues but ovaries are insensitive. Differences in the stability and uptake of dsRNA among these tissues are probably responsible for differential effects of dsRNA among tissues tested [51]. RNases responsible for degradation of dsRNA and proteins involved in uptake of dsRNA have been identified in desert and migratory locusts [50,53–55]. Recent studies in the pea aphid, *Acyrtosiphon pisum* showed that dsRNA degradation is one of the factors contributing to the lack of response to feeding or injecting dsRNA [56]. Understanding the mechanisms that contribute to the differences in RNAi efficiency among insect species is the crucial step for successful commercialization of dsRNA-based insecticides. Recent studies on this subject point to differences in the expression levels of genes coding for proteins involved in dsRNA uptake, spread among cells and tissues, dsRNA processing and formation of Risk Complex may play key roles in defining the success of RNAi. Genes coding for these proteins may be induced by dsRNA in target cells and the differences in the sensitivity of this induction by dsRNA also could contribute to RNAi success. These initial results on dsRNA stability, uptake, spread, processing and Risk Complex formation began to uncover mechanisms of dsRNA action and its differential efficacy among insects. Future studies along these lines will help in further understanding the mechanisms of dsRNA action.

Overview of methods for delivery of dsRNA

Expression of dsRNA in plants

When RNAi technology was employed to silence a gene critical for survival of pest insect by expressing dsRNA targeting this gene in plants, a reduction in the survival of target insect pests feeding on these transgenic plants was observed [45,57]. The bollworm larvae reared on transgenic plants expressing dsRNA targeting its P450 gene showed retarded growth and caused less damage [58]. Expression of dsRNA targeting genes coding for hexose transporter, carboxypeptidase and trypsin-like serine protease gene in rice plants caused a decrease in mRNA levels of these genes in nymphs of brown plant hopper feeding on transgenic the rice plants, but the survival of these nymphs was not affected [59]. The aphid, *Myzus persicae* fed on *Arabidopsis thaliana* plants producing *MpC002* and *Rack-1* dsRNAs showed a reduction in mRNA levels of the target genes and the aphids feeding on these plants produced less progeny [60]. *Helicoverpa armigera* larvae fed on transgenic tobacco plants expressing 482 bp ecdysone receptor (EcR) dsRNA showed molting defects and larval lethality. Interestingly, these transgenic tobacco plants expressing *H. armigera* EcR dsRNA also affected the beet armyworm, *Spodoptera exigua*. This may be due to the longer and conserved region of the EcR gene fragment used to prepare dsRNA. The dsRNA expressed in the transgenic tobacco plants would have produced enough siRNAs that matched 100% with the regions of *S. exigua* EcR mRNAs [61]. *H. armigera* hormone receptor 3 dsRNA expressed in transgenic tobacco plants also disrupted development of larvae feeding on the transgenic tobacco plants [62]. Whitefly *Bemisia tabaci* feeding on

transgenic tobacco plants expressing *v-ATPaseA* dsRNA showed reduced levels of *v-ATPaseA* mRNA and increased mortality [63]. These studies showed that the expression of dsRNA in plants to target critical insect genes in insect pests is an excellent approach. The application of RNAi technology for pest management by expressing dsRNA in plants to control coleopteran pests such as WCR is at the forefront of development. This will probably be the first commercialized RNAi-based insect control product. However, public hesitance in accepting food produced from genetically modified plants and lack of reliable methods for production of transgenic plants for many minor crops make it necessary to explore alternative methods of production and delivery of dsRNA.

Feeding *in vitro* synthesized dsRNA

Successful silencing of target genes after feeding *in vitro* synthesized dsRNA to insects has been reported by many groups suggesting that it is possible to feed dsRNA and achieve silencing of target genes [46•]. However, the major challenge in achieving insect control by feeding dsRNA is the availability of cost effective methods for producing large quantities of dsRNA. Large quantities of dsRNA could be produced by employing enzymatic (*in vitro* synthesis using RNA polymerases) and chemical synthesis (based on polymerization of nucleotides) methods. dsRNA synthesized by these methods was successfully field tested under natural beekeeping conditions to prevent Israeli Acute Paralysis Virus in honey bees [64]. *In vitro* synthesized and topically applied dsRNA targeting an inhibitor of apoptosis protein I gene in *Aedes aegypti* was able to kill these mosquitoes [65]. Chemical synthesis of dsRNA could be a cost effective method to produce large quantities of dsRNA required for pest management applications. For example, AgroRNA sells 100 grams of dsRNA for \$4500 (http://www.agroRNA.com/sub_05.html).

Production of dsRNA in bacteria

Synthesis in bacteria could be an alternative method for production of large quantities of dsRNA. *Escherichia coli* strain that is deficient in RNAase III (the enzyme that degrades dsRNAs in the normal bacterial cell) has been used to produce dsRNA. Feeding heat-killed bacteria that produced dsRNA targeting five housekeeping genes efficiently knocked down all five target genes tested in CPB. Loss of function of these target genes caused larval mortality, decrease in feeding and insect growth [47••]. Similar results have been reported for tephritid fruit fly [66] and beet armyworm [67].

Successful applications of dsRNA feeding in CPB

The CPB gut transcriptome was generated and most of the genes implicated in RNAi pathway have been identified in this transcriptome suggesting that CPB has all the necessary machinery to transport and process dsRNAs [68]. Several recent studies demonstrated successful knockdown of target genes in dsRNA fed CPB (Table 1). Feeding bacterially expressed AdoHcy hydro-lase (SAHase) dsRNA to CPB decreased SAHase and Kr-h1 mRNA levels, reduced JH titer, and caused the death of larvae, and pupae and blocked adult emergence [69]. Another study in CPB showed that feeding ryanodine receptor (RyR) dsRNA reduced RyR mRNA levels in the larvae and adults and caused a decrease in chlorantrani-liprole-induced mortality confirming that RyR is the target site for this

insecticide [70]. Feeding dsRNA method has been used to knockdown expression of the gene coding for P450 enzyme, Shade. A reduction in the hydroxylation of ecdysone, delay in development and death of CPB larvae and pupae were observed in RNAi insects [71].

A synergism between dsRNA and *Bacillus thuringiensis* toxin

In an interesting study, prohibitin, an essential protein for CPB viability, has been identified as Cry3Aa binding protein [72]. Prohibitin has been shown as an important protein involved in important cellular processes including mitochondrial function, cell proliferation, and development [73]. A combination of feeding prohibitin dsRNA and treatment with Cry3Aa enhanced Cry3Aa toxin induced mortality by threefold and the time to kill was reduced and as a result 100% mortality was achieved in five days. Although the molecular mechanisms of synergism between prohibitin RNAi and Cry3Aa toxin application are not known yet, this study proposes an interesting method of combining RNAi with toxins derived from microbes and other sources to improve the efficacy of RNAi in pest control.

Improvements needed to develop dsRNA as a commercial insecticide

RNAi played a key role in advancing insect science and helped in identifying functions of many genes involved in physiological, developmental, behavioral and reproductive processes of insects. The contribution of RNAi toward a basic understanding of insect development and physiology is irrefutable. However, the contribution of RNAi for controlling insect pests is still at the developmental stage. RNAi holds a great promise to help in development of target-specific environmentally friendly pest management methods. The initial results on the use of RNAi for controlling insect pests are encouraging. However, the development and commercialization of RNAi-based products for controlling insects is rather slow and requires progress in several fronts (Figure 1).

Development of methods for economical production and formulation of dsRNA

To compete well with chemical insecticides, methods for economical production and formulation of dsRNA need to be developed. Expression in plants, chemical synthesis and production in bacteria and other microorganisms are the three major methods that are being developed (Figure 2). Each of these three methods has its own pros and cons and may have to be chosen depending on the target pest and the crop being protected. Expression in transgenic plants might work well for commercial crops such as Corn. On the other hand, dsRNA synthesized in bacteria or by *in vitro* synthesis may be a better choice for pests attacking minor crops and those that produce food that is consumed directly (e.g. Potato). However, methods for synthesis, scale-up as well as formulation need further development to make dsRNA a cheaper alternative to chemical insecticides.

Studies on off-target and non-target effects and environmental fate of dsRNAs

The unintended effects caused by dsRNA include off-target silencing of genes in the target as well as in non-target insects, silencing of target gene homologs in non-target organisms, stimulation of immune response and saturation of RNAi machinery. All these effects could influence performance of non-target organisms including, parasites, predators and pollinators resulting in adverse effects on crop performance. Therefore, the persistence of

dsRNA in the field as well as the effect of dsRNA on organisms present in the pest and crop ecosystem need to be investigated thoroughly before using dsRNA in the field. In a recent study, the off-target and non-target effects of a 240 bp dsRNA targeting the *Snf7* gene in WCR were evaluated in many insects belonging to 10 families and 4 orders. These studies concluded that the spectrum of activity for *Snf7* dsRNA is narrow and the activity is limited to a subset of beetles within the *Galerucinae* subfamily of *Chrysomelidae* [74••]. The *Snf7* homolog sequence in the beetles that were affected by *Snf7* dsRNA showed more than 90% identity with 240 bp WCR *Snf7* sequence used to prepare dsRNA. Interestingly, all the active *Snf7* orthologs contained at least three 21 nucleotide fragments that showed 100% match with WCR *Snf7* suggesting that in order to interfere with gene expression, the dsRNA needs to contain 100% match with at least three 21-nt-long fragments in the target sequence. One could use this information to develop bioinformatics approaches to select dsRNA that precisely targets only gene of choice in the pest species as long as the genome sequence of the organisms in the pest–crop ecosystem are known. With the recent advances in genome sequencing methods and a rapid reduction in sequencing cost, there is a real possibility that we will obtain genome sequences of most of the insects and other organisms present in crop–pest ecosystem.

Thorough analysis of the environmental fate of dsRNA expressed in the plant and sprayed on the plant need to be conducted prior to commercialization of dsRNA-based insecticides. Recent studies showed that 90% of *Snf7* dsRNA was degraded in less than 35 hours in three different soils tested. Also, the biological activity of this dsRNA was undetectable within two days after application to soils [75]. On the basis of these data the authors concluded that accumulation of the *Snf7* dsRNA in the environment is highly unlikely.

Studies on potential for resistance development

Insects such as diamondback moth and CPB developed resistance to almost all insecticides introduced for their control. Therefore, there is no reason to believe that dsRNA is immune to resistance development by these and other insects. Mutations to genes coding for proteins involved in dsRNA transport, processing, Risk Complex formation and other processes involved in RNAi pathway as well as mutations to dsRNA target genes are potential mechanisms of resistance development. Recent studies on WCR suggest that resistance to *Snf7* dsRNA due to single nucleotide polymorphisms in the 240 nt target sequence is highly improbable because potentially 221 SiRNAs could target *Snf7* gene; non-target studies on a number of insect species showed that Dv*Snf7* dsRNA showing a minimum of three 21-nt matches with the target gene is sufficient to silence target gene [76]. Therefore, loss of some of the 221 SiRNAs due to mutation may not lead to significant levels of resistance. Preemptive studies on the possible mechanisms of resistance development by insects against dsRNA would be valuable in developing dsRNA as a pesticide. Differences in efficacy of dsRNA on target pest could also aid in the evolution of resistance. In a recent study, evaluation of effects of RNAi treatments against immune gene *att1* on adults of three western corn rootworm populations exhibiting different levels of gut cysteine protease activity showed differences in RNAi efficiency among these three populations [76]. The authors concluded that the effectiveness of dsRNA could vary among field populations depending on their physiological state and genetic background.

Conclusions

RNAi contributed enormously toward advances in functional genomics in insects. RNAi has great potential to contribute toward development of modern pest management methods. The major road blocks in successful commercialization of dsRNA-based insecticides are: a) lower efficiency in key pest species; b) higher production and formulation costs; c) lack of much information on off-target and non-target effects and environmental fate; d) unknown potential for resistance development; and e) lack of thorough knowledge on mechanisms of action. Papers published during the past two years made significant contributions toward eliminating some of these road blocks and the studies during the next few years will probably increase our knowledge in these areas to eliminate fear of the unknown resulting in successful commercialization of dsRNA-based insecticides.

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Figure 1.

Steps toward commercialization of dsRNA as an insecticide. Research on various aspects required prior to commercialization of dsRNA as an insecticide are shown.

Figure 2.

Three possible methods for mass-production of dsRNA for pest control. Expression of dsRNA in plants using transgenic technologies, chemical synthesis of dsRNA in factory and production of dsRNA in microorganisms in a bioreactor are three possible methods that could be used for production of large quantities of dsRNA.

Table 1
Examples of successful RNAi in CPB fed on dsRNA

Target gene	Application method	Observed effects	Reference
Vacuolar ATPase subunits A and E	Feeding by contaminating artificial diet	Neonates tested showed mortality	[45]
Actin, Sec23, vATPase E and B and COP β	Bacteria and <i>in vitro</i> synthesized dsRNA on potato leaves	Reduction in mRNA levels of target gene, reduced growth and increased mortality	[47]
S-adenosyl-L-homocysteine	Bacteria synthesized dsRNA on potato leaves	Reduction in mRNA levels of target gene, developmental arrest and mortality	[69]
Prohibitin	<i>In vitro</i> synthesized dsRNA on potato leaves	Reduction in prohibitin mRNA levels and enhanced Cry3Aa toxicity	[72]
Ryanodine receptor	Bacteria synthesized dsRNA on potato leaves	Decrease in susceptibility to chlorantraniliprole	[70]
Shade	Bacteria synthesized dsRNA on potato leaves	Decrease in ecdysone hydroxylation, developmental arrest and mortality	[71]