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RNAi as a management tool for the western corn rootworm, *Diabrotica virgifera virgifera*

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Abstract

The western corn rootworm (WCR), *Diabrotica virgifera virgifera*, is the most important pest of corn in the US Corn Belt. Economic estimates indicate that costs of control and yield loss associated with WCR damage exceed $US 1 billion annually. Historically, corn rootworm management has been extremely difficult because of its ability to evolve resistance to both chemical insecticides and cultural control practices. Since 2003, the only novel commercialized developments in rootworm management have been transgenic plants expressing *Bt* insecticidal proteins. Four transgenic insecticidal proteins are currently registered for rootworm management, and field resistance to proteins from the Cry3 family highlights the importance of developing traits with new modes of action. One of the newest approaches for controlling rootworm pests involves RNA interference (RNAi). This review describes the current understanding of the RNAi mechanisms in WCR and the use of this technology for WCR management. Further, the review addresses ecological risk assessment of RNAi and insect resistance management of RNAi for corn rootworm.

Keywords: RNAi, western corn rootworm, *Diabrotica,* RNAi risk assessment, insect resistance management, mode of action

1 Introduction

The western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae) is a remarkably adaptable and invasive pest,[1] and arguably the most important pest of corn, *Zea mays* (L.), throughout the US Corn Belt.[1–3] Current economic analysis estimates that costs of control and yield loss associated with WCR damage exceed $US 1 billion annually.[2] WCR is a univoltine pest that overwinters in the soil as eggs, with larvae hatching in late spring to early summer, depending on soil temperatures.[4] WCR larvae feed on roots and have an obligatory relationship with grasses (Graminae), especially corn. The majority of crop damage is thus caused by larval injury to roots, resulting in reduced yields and plant lodging. Historically, crop rotation to a non-host crop [e.g. *Glycine max* (L.)] and insecticide applications have been the primary methods to control rootworm. Corn rootworm management has been challenging due to its exceptional capacity to evolve resistance to both chemical insecticides[1,5–8] and cultural control practices such as crop rotation.[1,9] In instances where resistance has been documented, it has always been associated with uniform adoption of a given technology over large geographic areas. This has been true of chemical insecticides, crop rotation and, most recently, with transgenic corn lines that express insecticidal proteins from *Bacillus thuringiensis* (*Bt*). As a consequence, increasing rootworm management options that include multiple modes of action that encourage growers to utilize a diversity of cost-effective methods to protect their corn yield is critical to future sustainability of *Bt* technologies. It is clear that reliance on a single management approach is not sustainable, and increasing the diversity of control options, including transgenic plants, chemical insecticides, biological control, and cultural practices, is paramount. Inherent to this diversity of control options is the identification of novel control methods that are effective and safe to the environment.

Since 2003, when the first *Bt* plants for corn rootworm management became available, novel commercialized events have been limited to new *Bt* proteins, and only four insecticidal proteins are currently registered.[10] Cross-resistance has been documented between at least two of these proteins (Cry3Bb1 and mCry3A), where field-evolved resistance to one confers resistance to the other.[11,12] Characteristics of at least one documented field-evolved Cry3Bb1 resistant strain include non-recessive inheritance and a lack of fitness costs, which are expected to favor the development and maintenance of resistance in the field.[13] The Cry34Ab1/Cry35Ab1 (Cry34/35) binary insecticidal protein does not exhibit cross-resistance to Cry3 proteins and is used as a single trait or as a partner to Cry3 proteins in pyramids. Consequently, it is likely to be subject to increasing selective pressures, especially in fields where Cry3 resistance is established. The current state of the rootworm-active *Bt* traits in the field highlights the importance of developing new modes of action to control this economically important insect pest.

One of the newest approaches for managing rootworm pests involves RNA interference or RNAi. First described in the nematode *Caenorhabditis elegans*, RNAi refers to a process in which small double-stranded RNAs direct sequence-specific repression of gene expression.[14–16] The RNAi pathway has been implicated as a mechanism that evolved for defense against viruses or integration of mobile genetic elements;[15] RNAi is also effective in regulating gene expression in virtually all eukaryotic organisms, including plants and insects.[14,17–20] RNAi has become a popular functional genomics and genetics tool that is widely used to study gene functions through 'knockdown' of cognate gene targets. Both academia and the agricultural industry use RNAi in their research and development, and recognize its potential as a product for pest management. In insects, effective RNAi has been described in several species, yet the responses vary greatly across taxa.[19,21] The initial examples of root protection against western corn rootworm by transgenic RNAi plants expressing double-stranded RNA[22] foreshadow the likelihood that new commercial corn events based on RNAi will soon be available to complement *Bt* corn technology for WCR management.[23] This review describes the current understanding of the RNAi mechanisms in WCR and the use of this technology for WCR management.

2 RNAi Traits

2.1 Lethal RNAi

Unlike other agronomically important pests, such as leaf-chewing Lepidoptera,[21] both larvae and adult WCR exhibit a robust RNAi response upon ingestion of environmental dsRNA. This oral response enables the use of high-throughput artificial diet-based feeding assays as a method for testing dsRNA molecules targeting essential genes.[22,23] In 2007, Baum *et al.*[22] interrogated a set of 290 genes and identified numerous gene targets that exhibited lethality and stunting in WCR larvae. In that study, one of the most effective RNAi gene targets was *vacuolar ATPase subunit A* (*V-ATPase*); exposure of larvae to *V-ATPase-A* dsRNA resulted in a rapid suppression of corresponding endogenous mRNA, mortality and/or growth inhibition. Importantly, oral exposure of WCR larvae to corn plants expressing dsRNA directed against the *V-ATPase* gene protected the plants from root damage, documenting for the first time the potential for *in planta* RNAi as a possible pest management tool.[22] Further studies demonstrated that WCR *Snf7*, a vacuolar protein sorting gene of the (ESCRT-III) Endosomal Sorting Complex Required for Transport-III (*Vps32* or *shrub* in *Drosophila*), dsRNA also protected corn roots from WCR feeding damage,[23–26] emphasizing the value of the RNAi for the control of WCR.

A successful lethal RNAi response in WCR greatly depends on the selection of the target gene. As RNAi is systemic in WCR,[27] target gene selection does not have to be limited to midgut epithelial cells as is the case for gut-active insecticidal proteins such as *Bt* Cry proteins. The selection of RNAi targets should take into consideration factors relating to target sensitivity and dsRNA design. It is also important that the dsRNA target sequence is highly conserved both within and across target species, but is not conserved across broad taxonomic groups.[28] Almost by definition, a lethal RNAi target should be an essential gene (e.g. housekeeping gene).[24] An essential function incorporates the idea that the gene is necessary during the life stage and timeframe in which the oral exposure occurs. However, it is important to consider that, even for essential biological processes, parallel pathways or homologous genes may substitute for the function of a targeted gene. Other relevant factors may include transcript expression level, the dose sensitivity of a gene and the turnover rate of the protein. A short half-life of a protein will likely allow more rapid protein depletion and a faster appearance of the corresponding phenotype.[29] Unfortunately, *a priori* knowledge of protein half-life is generally lacking. Other parameters, such as the annotation of all homologs or transcript splice isoforms, are difficult to determine in WCR, which lacks a published genome. Consequently, experimental screening of the RNAi candidate genes is still the best approach for the identification of lethal RNAi targets in WCR.

Potential RNAi targets in WCR can also leverage genome-wide testing approaches in other insects and insect cell lines.[30,31] For example, Ulrich *et al.*[31] identified dsRNA for 100 targets in the red flour beetle *Tribolium castaneum* causing more than 90% mortality through larval and pupal injection bioassays. In that dsRNA injection study, the authors also tested the *Tribolium* orthologs of the five most active WCR targets described by Baum *et al.*[22] Those RNAi targets were also active in *Tribolium*, although the activity did not reach levels of the most active *Tribolium* RNAi targets. That result suggests that, while leveraging RNAi targets from other insects increases the probability of success, the overall efficacy of an RNAi target may vary among insects.

Independent of the target gene, the outcomes of the RNAi bioassays in WCR largely depend on the design of the bioassay itself. Conditions such as the length of the dsRNA fragment, the dose sensitivity of the target gene and the duration of the bioassay itself influence the result of RNAi bioassays. Bolognesi *et al.*[24] described bioassays that were carried out for 12 days, and Baum *et al.*[22] noted that a seven-day bioassay resulted in little if any effect. For *Snf7*, Bolognesi *et al.*[24] noted a considerable growth inhibition (GI) within 5 days. It is likely that shortening the observation period to less than 12 days may produce a lower number of RNAi targets but lead to the identification of more efficacious or faster-acting dsRNAs. Measurements other than the overall lethality, such as LC_{50} (concentration that leads to 50% lethality), LT_{50} (time to reach 50% mortality in the tested population) or GI_{50} (concentration that leads to 50% growth inhibition), can be useful to identify potential target sequences and discriminate among multiple efficacious dsRNA targets.

2.2 Adult and parental RNAi

Rangasamy and Siegfried[32] was the initial study reporting lethal RNAi effects in adult WCR. They observed that feeding dsRNA for *V-ATPase A* to WCR adults via artificial diet led to a reduction in transcript levels and protein expression, and eventual mortality within 14 days of exposure. Adult bioassays may be useful as an alternative screening method for identification of lethal RNAi targets.[24,33] The susceptibility of WCR adult beetles to dsRNA[32] offered the possibility of exploring transgenerational control of WCR. This effect, also called parental RNAi (pRNAi), has been observed in multiple insects.[34,35] The premise of pRNAi is the application of dsRNA to adult insects while the effect is observed in the progeny. The primary applications of pRNAi in insects thus far have been for the purpose of developmental studies.[34,36–43] Therefore, pRNAi in WCR may provide an additional population management strategy for this important insect pest.

The first description of pRNAi in WCR identified the developmental genes *hunchback* and *brahma* as robust RNAi targets that reduced the fecundity of WCR under laboratory conditions.[27] Although gene targets such as *hunchback* and *brahma* do not cause short-term mortality in WCR adults, the pRNAi

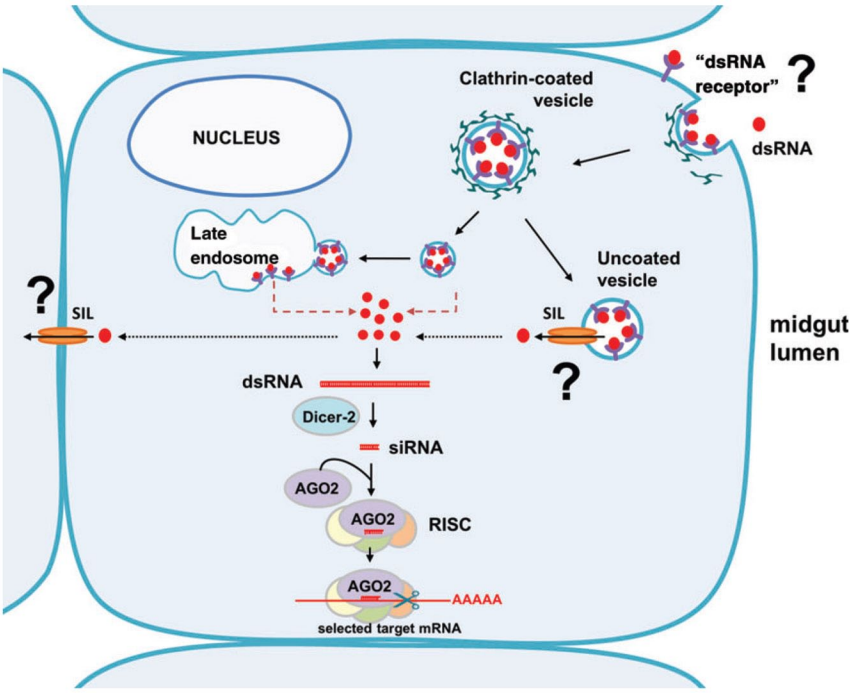

Figure 1. Cell machinery used for uptake to process dsRNA. **(1)** dsRNA enters the cell via clathrin-mediated endocytosis and possibly SID-like proteins. In *C. elegans,* SID-1 is believed to function as a dsRNA channel, and it is also necessary for the cell-to-cell transport. In insects, the specific functions of SID-like (SIL) proteins are unknown. The presence of dsRNA receptors (Eater and SR-CI) in *Drosophila* suggests that WCR may also have dsRNA receptors. **(2)** Inside the cell, dsRNA is processed into siRNAs (21–24 bp) by Dicer-2. **(3)** One strand of the siRNA is loaded onto AGO2,within the RNA-induced silencing complex (RISC). **(4)** Target mRNA is cleaved by the AGO2 component of the RISC.

gene target is not mutually exclusive to lethal RNAi effects, and one could envision a trait that would cause lethality in some individuals, and effective sterility in the insects that survive. Alternatively, pRNAi technology could be deployed in a multigene stack with lethal RNAi for larvae, or other control methods (i.e. *Bt* insecticidal proteins). Furthermore, pRNAi could potentially be used as a tool to remediate resistance to chemical insecticides or *Bt* insecticidal proteins. Detailed characteristics of pRNAi response, such as effective plant dose, the minimum duration of exposure, the onset of the response, and the duration of pRNAi response after feeding, will determine the utility of each individual gene target for parental control.

3 RNAi Mechanisms

3.1 Potential uptake mechanisms

The robust oral activity of dsRNA in WCR already assumes that the dsRNA is not degraded in the insect's digestive system. Successful oral response to environmental RNAi in WCR can be viewed in terms of two key mechanistic components: (1) the initial uptake of dsRNA in the midgut, and (2) the systemic spread of the RNAi signal. The systemic RNAi signal may be the intact dsRNA, dsRNA processed into small interfering RNAs (siRNAs) or the above RNAs being chaperoned by specific proteins or other cellular components. A third component, which may be distinct from the midgut uptake, is the cellular uptake that is initiated when RNAi in WCR is induced by injection of dsRNA. Theoretically, these three components of RNAi response in WCR can be mediated by the same or different mechanisms.

In *C. elegans,* a combination of systemic RNA interference deficient proteins 1 (SID-1) and 2 (SID-2) has been ascribed the function of dsRNA uptake from the environment (Fig. 1).[44,45] In contrast, only SID-1 is implicated in the spread of RNAi from cell to cell.[46] Expression of the *C. elegans* SID-1 in *Drosophila* S2 cells enables passive size-independent dsRNA uptake, implying that SID-1 is a dsRNA-gated channel.[47–49] SID-2 is primarily localized in the gut and is needed for the initial uptake of dsRNA of 50 bp or more from the gut lumen by intestinal cells.[45] Interestingly, SID-2-dependent dsRNA transport takes place under acidic conditions and is likely dependent on endocytosis.[45] The function of another SID protein of *C. elegans*, SID-5, in RNAi by releasing dsRNA from the endosomes further corroborates the involvement of endocytosis in dsRNA uptake.[50]

In insects, SID or SID-like (SIL) proteins have been identified,[51,52] yet it is not clear whether the SID/SIL homologs contribute to dsRNA uptake in all insects. The *Tribolium* and WCR SIL genes have similarity to SID-1[52]; however, insect SID-2 homologs have not been identified. A recent study that included the SID-like *silA* and *silC* genes of WCR showed a moderate but 'not robust' effect on oral RNAi response after *sil* gene knockdown.[53] The above results suggested that SIL proteins are not the sole mediators of dsRNA uptake. A study in *Tribolium* postulates that the best candidates for SID-like genes may be more closely related to the *C. elegans* Tag-130, which is not necessarily associated with the systemic RNAi response in *C. elegans*.[52] The authors concluded that the *Tribolium sil* genes are more likely to be Tag-130 orthologs rather than SID orthologs. Other reports also suggest that the RNAi response is not dependent on SID or SIL proteins in Orthoptera and Lepidoptera species.[54,55] SID/SIL proteins seem to be completely absent in Diptera.[52,56] Those results, however, do not imply that the function of SID proteins for the uptake and systemic spread of RNAi can be dismissed in insects altogether. In addition to clarifying

the degree to which the SIL proteins participate in the systemic RNAi response in insects, it would be interesting to find out whether they are functional in the initial dsRNA uptake in the midgut, release from the endosome and/or cell-to-cell spread of the RNAi effect (Fig. 1).

Assuming that SID-like proteins are either not involved in the systemic spread of the RNAi effect in WCR or not solely responsible for dsRNA uptake, other uptake and spread mechanisms may be involved. Recently, Cappelle *et al.*[57] compared relative contributions of the SIL genes (*silA* and *silC*) and endocytic components [clathrin heavy chain and the vacuolar H^+ ATPase 16 kD subunit (*Vha16*)] in Colorado potato beetle (CPB), *Leptinotarsa decemlineata*. A side-by-side comparison indicated that clathrin-mediated endocytosis-related genes played a more pronounced role in dsRNA uptake in CPB, yet both *silA* and *silC* showed weak but significant effects on CPB's oral response to dsRNA.[57] Earlier, Xiao *et al.*[58] demonstrated that clathrin-dependent endocytosis is needed for RNAi response in *Tribolium*. Their study showed that injection-based RNAi response in *Tribolium* could be blocked by inhibitors of clathrin-dependent endocytosis (bafilomycin-A1 and chlorpromazine) but not by the inhibitors of other types of endocytosis. Further, Xiao *et al.*[58] demonstrated that knockdown of several *Tribolium* genes directly involved in clathrin-dependent endocytosis (clathrin heavy chain, clathrin coat assembly protein AP50, vacuolar H^+ ATPase subunit H, and small GTPase Rab7) inhibited the RNAi response (see Fig. 1 for the clathrin-mediated endocytosis of dsRNA). Earlier studies in *Drosophila* S2 cells also pointed to the components of the endocytic pathway, including clathrin heavy chain, AP50, Rab7, Arf72A, vacuolar sorting protein Vsp41 and the subunits of vacuolar H^+ ATPase (*VhaSFD* and *Vha16-1*), along with scavenger receptors Sr-CI and Eater.[59,60] Although identified in different species, this set of genes may provide a basis for investigating the involvement of clathrin-dependent endocytosis in dsRNA uptake and the systemic spread of RNAi in WCR. The relative contribution of endocytosis versus SIL genes in dsRNA uptake and their genetic interactions may provide other interesting areas for exploration in WCR.

3.2 Pathway genes

The RNAi phenomenon takes advantage of the endogenous cellular machinery that has evolved as defense against viruses[36,61] and to process endogenous regulatory non-protein-coding RNAs [e.g. microRNAs (miRNAs) or endogenous siRNAs (endo-siRNAs) that can be produced from endogenous hairpin sequences in insects].[62] The core components of the RNAi response in insects are similar to those described in other eukaryotes. One of the key differences between other animals and insects in the biogenesis of active RNAi molecules (siRNAs and miRNAs) is that in nematodes and vertebrates a single type III RNase, Dicer, produces functional 21–23 nt RNAs from both long dsRNAs and miRNA precursors, while in insects Dicer-1 exclusively recognizes miRNA precursors[63,64] and Dicer-2 recognizes dsRNA (Fig. 1).[65] These conclusions were originally based on *Drosophila*; subsequently, Dicer-2 was also identified in other insect species.[66–68] An early description of Dicers 1 and 2 in *Tribolium* has speculated that the function of *Tribolium* Dicer-2 may not be as specialized as in *Drosophila,* based on its similarity to *C. elegans* Dicer.[52] Experimental results, however, confirmed that Dicer-2 in *Tribolium* guides the processing of dsRNA into siRNA.[52] Another key protein in dsRNA-mediated RNAi response is a type III RNase Argonaute 2 (AGO2), the 'slicer' component of the RNA-induced silencing complex (RISC).[69,70] One strand of the siRNA is loaded onto the RISC, and this siRNA guides the RISC complex to a target mRNA in a sequence-dependent manner, which is then cleaved by the AGO2 protein within the RISC (Fig. 1).

Both Dicer-2 and AGO2 have been identified in the WCR transcriptome.[53,71] Knockdown of these genes in both WCR larvae[53] and adults,[71] followed by an attempted knockdown of a reporter gene, showed an inhibited RNAi response. These observations argue for critical roles of Dicer-2 and AGO2 in the dsRNA-mediated RNAi pathway in WCR. These reports also highlight a potential resistance mechanism in WCR to RNAi (i.e. downregulation or mutations in Dicer-2 or AGO2 may lead to lower sensitivity to dsRNA), but there may be fitness costs to mutations in Dicer-2, AGO2 or other RNAi pathway genes. As there is no described resistance to RNAi in insects, targeted mutations in Dicer-2, AGO2 and other pathway genes provide a means to assess the risk of resistance to RNAi and the associated fitness costs.

Proteins other than Dicer-2 and AGO2 that are important in the biogenesis of siRNA and the RNAi response include the dsRNA-binding proteins R2D2 and Loquacious (Loqs-PB, Loqs-PD). R2D2 interacts with Dicer-2 and helps load exo-siRNAs into the AGO2-containing RISC complexes.[72–74] Loqs has been primarily associated with endo-siRNAs,[75] but has also been proposed to function in the processing of exogenous dsRNA.[73] In a recent study in the mosquito *Aedes aegypti*, researchers reported that *A. aegypti* lacks a Loqs-PD isoform,[76] which in *Drosophila* is specific to dsRNA processing.[73,75] To compensate for this deficiency, the *A. aegypti* Loqs-PA isoform seems to interact with both dsRNA and miRNA processing centers.[76] R2D2 and Loqs have not been studied in WCR, and their functions and involvement in exo-siRNA, endo-siRNA and miRNA may be distinct as well. The overlap of function of these and other components of the core non-coding RNA-processing machinery between exo-siRNA and other RNA types may determine the fitness costs of resistance to dsRNA and thus influence the likelihood of such resistance.

3.3 Effectiveness of small RNA species in initiating RNAi

Upon entering an insect cell, dsRNA is processed by Dicer-2 into siRNAs that are ~21–23 bp in length.[29,65] siRNAs of foreign origin are also called exogenous siRNAs (exo-siRNAs) to distinguish them from endogenous siRNAs (endo-siRNAs).[62] Although the siRNA is the functional unit of RNAi response, dsRNA length is a determining factor of the environmental RNAi response in insects. Studies in WCR showed that an approximate minimum length of 60 bp is needed to achieve a lethal RNAi effect.[24,53,77,78] This is true both in the larvae and adults of WCR, via feeding and injection.[78] Conversely, 21 nt siRNAs, Dicer-substrate 27 bp dsRNAs and dsRNAs shorter than 60 bp did not initiate RNAi.[24,53,77] Interestingly, 27-mer dsRNA sequences that were extended to over 60 bp with a neutral double-stranded carrier sequence produced high mortality in WCR.[24] Bolognesi *et al.*[24] postulated that the lack of an RNAi response to short dsRNA or siRNA in WCR was due to absence of uptake by larval midgut cells. That conclusion is supported by an investigation in *Tribolium*, where RNAi response was achieved by injection of ~30 bp dsRNA fragments into syncytial (uncellularized) embryos but not larvae.[79] However, the ineffectiveness of short dsRNAs and siRNAs may also be compounded by the inability of siRNAs to be loaded onto the RISC or ineffective Dicer processing of short dsRNAs.[24,49,72]

3.4 Systemic spread

Western corn rootworm is remarkably efficient at eliciting a strong systemic RNAi response to orally delivered dsRNA. This raises the question of whether or not transitive RNAi is a mechanism that functions in WCR to amplify the RNAi response via production of secondary siRNA. In other organisms, secondary siRNA production is achieved through RNA-dependent RNA polymerase (RdRP) activity, with the primary dsRNA acting as guides in either the primed or unprimed complementary RNA (cRNA) synthesis pathway.[80] The resulting secondary siRNAs trigger a secondary gene silencing that is termed transitive RNAi.[81] The amplification of secondary dsRNA likely reuses the RNA-loaded RISC complexes, dramatically magnifying the RNAi response. Transitive RNAi has been found in nematodes,[81–83] plants[84–86] and fungi,[87] but not in insects.[56] In *C. elegans*, except for a small proportion of primary siRNA molecules derived from the Dicer products of the externally applied dsRNA, most of the siRNA molecules are generated through RdRP activity, following cRNA synthesis initiated by the antisense strand of the primary siRNAs. The distribution of these secondary siRNAs exhibits a clear 5′→3′ antisense polarity and exceeds the 5′, but not the 3′ sequence border of the original trigger region along the target mRNA.[81–83] In plants, secondary siRNAs are also produced, mainly by an unprimed-cRNA synthesis pathway, and they may spread in both directions, surpassing both the 5′ and 3′ sequence boundaries of the initial target region, and cleave both the upstream and downstream sequences.[84,88] In fungi, the spreading of transitive RNAi is similar to that in *C. elegans*, spreading only in the 3′ to 5′ direction along the target mRNA.[87]

Early BLAST searches of the WCR transcriptome indicated that, as in other insects, RdRP is absent in WCR.[22] This is in agreement with the absence of RdRP and transitive RNAi in*Drosophila*.[56,89] The fact that insect genomes do not have a homolog of RdRP, which is considered essential for secondary siRNA amplification, suggests that insects lack the transitive RNAi pathway. This argues that the potent RNAi response in WCR might not involve transitive RNAi guided by secondary siRNA. However, it is also possible that transitive RNAi exists in WCR, but is dependent on an enzyme other than RdRP. RNAi transitivity in WCR may be demonstrated by the spread of silencing beyond the dsRNA trigger sequence. Our unpublished work to sequence small RNAs in WCR fed dsRNA revealed no siRNA sequencing reads in either 3′ or 5′ directions distal to the target sequence regions homologous to the dsRNA. These results indicate a lack of transitive RNAi in WCR. It is therefore remarkable that WCR mounts such a strong systemic RNAi response to environmental RNA without production of secondary siRNA. There is also a possibility that the secondary siRNAs generated by WCR are modified in such way that they are not detectable by the standard sequencing methods. It is therefore necessary to explore further other potential secondary dsRNA production pathways, mediated by as yet unknown mechanisms, to explain the potency and self-sustaining nature of RNAi observed in insects such as WCR.

In addition to the initial uptake in the digestive system of WCR, the dsRNA or siRNA must spread from cell to cell. Detection of diet-applied long dsRNA in tissues other than the gut in *L. decemlineata* and WCR described by Ivashuta *et al.*[77] suggested that long dsRNA species can travel to distal tissues within insects. As described above, it is believed that in insects the spread is not dependent on dsRNA amplification or an RNA replicative mechanism, and thus intact ingested dsRNA or processed dsRNA must mediate the spread of the RNAi response. Although it was demonstrated that long dsRNA sequences are necessary for oral RNAi response in WCR, it is still not clear whether the minimal dsRNA length is critical for the initial uptake or includes the spread of RNAi from cell to cell. An *ex vivo* approach showed that, while the WCR midgut cells take up only long dsRNA, the fat body can take up both dsRNA and siRNA.[77] On the other hand, injection of siRNA that targets *vacuolar-ATPase C* (a lethal dsRNA target) into the hemocoel of WCR did not cause lethality.[78] These observations indicate that, while siRNA uptake into cells is possible, the lack of lethal phenotype upon siRNA injections suggests that the cellular uptake pathway for siRNA may not be robust enough to trigger a strong RNAi response.

As discussed in Section 3.1, mechanisms that are involved in dsRNA uptake in the insect's midgut may also be responsible for the systemic spread of the RNAi effect throughout the organism. While comparisons of oral delivery with injections and direct *ex vivo* dsRNA uptake by WCR tissues can differentiate dsRNA uptake in the midgut versus other tissues and cells, the cell-to-cell spread may be different yet. Techniques involving labeling of individual molecules to monitor their spread and next-generation sequencing may capture the nature of the RNAi molecules that spread to distal tissues. To conclude, WCR exhibits a robust response to environmental RNAi, while in the most common insect model, *Drosophila*, RNAi is not systemic. Therefore, WCR may be an opportune agricultural pest and model organism to investigate whether the same or different mechanisms govern the initial uptake and the spread of the RNAi effect.

3.5 RNAi competition

One of the key questions in applying RNAi for the management of WCR is whether two dsRNAs can act synergistically, produce a linear/additive response or be antagonistic. A recent study that established a pigmentation-based bioassay for RNAi response in WCR described possible competition between dsRNAs.[53] The authors observed that cofeeding of non-lethal dsRNA along with the reporter dsRNA suppressed the reporter phenotype. Those effects were dsRNA concentration dependent. An *ex vivo* experiment using WCR fat body also showed that unlabeled dsRNA can outcompete Cy3-labeled dsRNA.[77] The competition can also be interpreted as saturation of the dsRNA uptake. Potential competition of dsRNAs has implications on how RNAi traits may be implemented in the field. The benefit of stacking two RNAi traits has to be weighed against the potential for competition. However, high doses of dsRNA surface applied to or incorporated into artificial diet may not reflect the dsRNA amounts that can be supplied by plants transformed to express dsRNA. A study in *Tribolium* demonstrated that a 100× concentration of competing dsRNA was needed to outcompete the target dsRNA.[79] That work also suggests that the competition takes place at the level of dsRNA entry into the cell. Further investigations identifying the conditions under which two dsRNAs may compete, or how competition may be avoided, will aid in developing RNAi traits and IRM strategies for WCR.[79]

The potential for dsRNA competition also poses a question as to whether other environmental dsRNAs such as plant-, fungal-, bacterial- or virus-derived dsRNAs can compete with dsRNA transgenically expressed in corn targeting a specific WCR mRNA.[90] Ivashuta *et al.*[77] began to investigate this question by identifying plant-derived siRNAs in WCR that fed on plant material. That study showed that 12% of siRNAs (21 nt) found in WCR that fed on corn roots originated from corn; however, plant-derived siRNAs had little effect on the WCR transcriptome. These findings

suggest that siRNA competition is unlikely to occur at concentrations encountered through consuming host plants or associated non-plant flora, which serves as an approximation of the concentrations expected in the field. Nonetheless, initial findings underscore the need to further explore the uptake of environmental dsRNA by WCR and the potential for dsRNA competition.

3.6 Stability/processing in plants

One of the key elements for effective control of WCR in corn plants using RNAi is the stable expression and accumulation of dsRNA in the tissues consumed by WCR larvae, i.e. the roots. The need for accumulation of long dsRNA molecules is assumed from bioassays on artificial diet, which demonstrated that an effective RNAi response requires ingestion of 60 nt dsRNAs or longer,[24,78] and the observations that insects do not accumulate plant-produced siRNAs.[77] Two transgenic RNA populations are present in insect-resistant RNAi corn tissues expressing dsRNA. One population consists of intact long dsRNAs that initiate a lethal RNAi response when consumed by WCR; the second major population is a mixture of siRNAs 21–24 nt in length that are generated by plant Dicer-like processing of the long dsRNA.[22,77,78] When fed an artificial diet, the plant-derived siRNA sequences do not trigger RNAi in the insect.[78] Moreover, Ivashuta *et al.*[77] noted that plant-fed WCR and CPB accumulate predominantly siRNAs 21–23 nt in size, while the dominant siRNA species in plants are 24-mers. CPB and WCR mainly accumulated plant-derived 21-mer siRNAs; these abundant 21-mers correspond to plant dsRNA loci[77] and suggest that the 21-mers that accumulate in insects are processed from endogenous long plant dsRNA sequences.

Our understanding of active RNAi species in pest insects is important for transgenic RNAi trait design. As siRNA does not effectively initiate RNAi in WCR, it is important to maintain efficacious levels of intact hairpin dsRNA (hpRNA) in corn plants and minimize or overcome plant Dicer-like processing within the plants. This can be achieved through dsRNA trigger sequence selection and expression optimization (Dow AgroSciences, unpublished data). In addition to expression levels, the subcellular localization of hpRNA may also be important. Recently, dsRNA was stably expressed in potato chloroplasts, resulting in protection from CPB feeding damage. This approach exploits the lack of RNAi machinery in plant plastids.[91]

The fact that long dsRNAs are the initial RNAi triggers is also important for quantitative determination of the RNAi active molecules in transgenic plants.[92] While quantitation of dsRNA incorporated into the diet or applied to the diet surface may be relatively straightforward, correlation of those doses with plant-expressed hpRNA or the doses of dsRNA that insects receive from plants remain mostly unexplored.

4 RNAi Risk Assessment

4.1 Ecological risk assessment

4.1.1 Effects on non-target arthropods

Early characterization of the spectrum of activity of ingested insecticidal dsRNA has indicated a high degree of specificity to the target species.[22,28,93] Multiple studies suggest a sequence-specific response, with the response decreasing as the evolutionary distance between species and the divergence between the sequences increase. The first study to address the effect of RNAi on non-target arthropods evaluated species-specific and non-specific *V-ATPase* dsRNA in *T. castaneum*, the pea aphid *Acyrthosiphon pisum* and the tobacco hornworm *Manduca sexta* by feeding unprotected dsRNA, and the fruit fly *Drosophila melanogaster* by feeding dsRNA protected by liposomes.[93] Target species were selectively susceptible when fed species-specific *V-ATPase* dsRNA, and insignificant mortality was observed when fed non-specific dsRNA. Furthermore, feeding of *γ-tubulin* dsRNA targeting the more variable region of the gene selectively killed species within the genus *Drosophila*.[93] Initial studies of WCR target genes evaluated dsRNAs targeting putative *V-ATPase-A* and *V-ATPase-E* in the southern corn rootworm, *Diabrotica undecimpunctata howardi*, *L. decemlineata* and the boll weevil *Anthonomus grandis*.[22] Both WCR dsRNAs generated lower but significant mortality in *D. undecimpunctata howardi* and *L. decemlineata*. However, no effects of WCR dsRNA were observed in the boll weevil.[22] Lethal and sublethal effects of dsRNA targeting WCR *Snf7* were evaluated in insects representing ten families and four orders.[28] Results indicated that the insecticidal activity of WCR *Snf7* dsRNA was narrow. Effects were only observed in beetles within the Galerucinae subfamily of Chrysomelidae, predicting that the likelihood of adverse effects on non-target arthropods from a realistic exposure to WCR *Snf7* dsRNA is extremely low.[28] Given that the species more likely to be susceptible are those with the highest sequence similarity,[22,28] *in silico* evaluations (e.g. BLAST-based searches) could reduce animal testing for non-target impacts.[94]

Additional studies evaluating the effects of WCR dsRNA on non-target arthropods support the low risk of adverse effects. Field testing of corn expressing WCR *Snf7* dsRNA and Cry3Bb1 (event MON 87411) was performed to confirm the results obtained in the laboratory.[95] For that purpose, the abundance of non-target arthropods and plant damage from non-target pests were evaluated in a broad range of environmental conditions and agricultural ecosystems. These studies demonstrated the absence of adverse effects on non-target arthropod communities exposed to MON 87411 corn.[95] Studies that evaluated the effects of WCR dsRNA on honey bees showed similar results: experiments evaluating the effects of WCR *Snf7* dsRNA[96] and *V-ATPase-A*[97] in honey bee larvae and adults indicated no observable effects under high levels of exposure to dsRNA. More interestingly, no effects of high doses of *A. mellifera*-specific *V-ATPase-A* dsRNA were observed.[97] Similar results in other insect orders suggest that some taxonomic groups are inherently less susceptible to orally ingested dsRNA.[21,98] These results suggest that in addition to sequence specificity of dsRNA, there are inherent barriers to both target-specific and non-target RNAi responses.[18,21,97,99]

Currently, the ecological risk assessment used for the evaluation of insect-protected genetically engineered (GE) crops (e.g. plants expressing insecticidal proteins from *B. thuringiensis*) provides a basis for evaluating potential hazards for RNAi-mediated insect-protected crops.[100,101] However, because of the unique mode of action of RNAi, modifications to the current risk assessment framework have been suggested. For example, ecologically important non-target organisms that are closely related to the target species should be most closely evaluated, as they are more likely to be susceptible.[28,94] There is also a consensus among risk assessors that each dsRNA used for *in planta* RNAi should be tested for hazards to non-target organisms,[18,101] as is routinely done for other insecticidal GE traits.[94] Although there is much disagreement, some have raised concerns that off-target gene knockdown can occur owing to random sequence homologies,[102,103] as well as potential effects on immune viral response of non-target organisms. However, insects are continuously exposed to non-insect dsRNAs from a variety of sources under natural conditions. Therefore, dsRNA that is targeted at silencing genes from insect pests will have a similar likelihood of affecting off-target genes or arthropod immune response compared with

other occurring environmental dsRNA molecules naturally produced by plants or that have been genetically engineered into plants to provide viral defense or other traits. As mentioned in Section 3.5, WCR readily uptake and process endogenous dsRNA from wild-type plants, without noticeable changes to their transcript profiles.[77] These observations are yet to be replicated in other species, although given that RNAi IR traits are in the very initial stages of their deployment, generation of additional data that examine RNAi effects on non-target arthropods will further inform the assessments of environmental risks.

4.1.2 Interaction between Bt and RNAi

Regulators in the United States and other countries require studies to investigate the potential for synergistic interaction between insecticidal GE traits when they are combined in individual plants. If little or no interaction is detected between different traits, studies of the effects of the individual traits on non-target organisms can be used for the risk assessment of the combination.[104,105] Because dsRNA for WCR management will likely be expressed in corn in combination with *Bt* insecticidal proteins, the potential interaction between *Bt* and dsRNA is considered part of the environmental risk assessment. To date, only one study has evaluated the potential interaction between a Cry protein and a dsRNA (Cry3Bb1 and WCR *Snf7* dsRNA).[23] The potential interaction was evaluated with *D. undecimpunctata howardi* using two approaches: (1) evaluating each substance alone and in combination over three different response levels, and (2) testing the potential for a fixed sublethal concentration of one component to reduce the median concentration (LC^{50}) of the other. Both approaches demonstrated that there was no synergy between Cry3Bb1 and WCR *Snf7* dsRNA expressed in MON 87411, indicating that they act independently and supporting the testing of the two materials independently for non-target arthropod risk assessment purposes.[23] As the modes of action of *Bt* proteins (binding to midgut receptors, followed by pore formation and cell lysis)[106] and of dsRNA (depletion of target mRNA) [107] are not related, non-additive effects of combining *Bt* proteins with RNAi are not anticipated. Additional studies of RNA hairpins in combination with WCR-active *Bt* trait proteins would further confirm the independent action of these agents.

4.1.3 dsRNA environmental stability

An important part of the ecological risk assessment of insecticidal molecules is determining the potential for residues of the pesticidal substance to persist in the environment and potentially affect populations of non-target species.[94,108] The environmental stability of the active pesticidal molecule is examined to determine whether there are possible long-term risks to susceptible non-target organisms; for *Bt* crops, the analyses include testing of soil and crop residues for their activity against the target pest.[94] A laboratory degradation study was performed by Dubelman *et al.*[109] to determine the biodegradation potential of WCR *Snf7* dsRNA derived from the Monsanto corn line MON 87411. Researchers tested soil with different physicochemical properties, including silt loam, loamy sand and clay loam, and exposed *D. undecimpunctata howardi* to dsRNA from incubated soils to evaluate biological activity (i.e. insect mortality). That study demonstrated that *Snf7* dsRNA was not detectable after 48 h in the three soil types tested. The half-life of *Snf7* dsRNA was less than 30 h,[109–111] which is in the range of 1 day to several days half-life generally reported for *Bt* proteins.[112,113] Additionally, *D. undecimpunctata howardi* mortality was undetectable within 2 days.[109] Those results suggest that *Snf7* dsRNA and other dsRNAs are unlikely to persist and accumulate in the soil.[109] If soil persistence were to be demonstrated, it would be necessary to understand whether any exposed beneficial soil organisms are sensitive to the dsRNA, possessing both the necessary RNAi machinery to take up and process the molecule and a matching target gene sequence.

Laboratory studies with *Bt* insecticidal proteins have shown that, even though the half-life of the proteins in the environment ranges from less than one day to several days to more than a month,[112,113] depending on the protein and environmental conditions,[112,113] some proteins can bind to clay particles in the soil.[114] In its regulatory requirements, the EPA concluded that enhanced stability and buildup over continuous cultivation is not a concern for plants expressing *Bt* proteins.[94,115] Experiments evaluating dsRNA stability in honey bee diet indicated that dsRNA could bind to royal jelly components,[97] suggesting that results similar to those reported for *Bt* could occur with dsRNA. Interestingly, binding to other molecules could actually make the dsRNA unavailable for non-target species, thereby reducing the risk of exposure.[97]

4.2 Insect resistance management

Transgenic crops that produce substances that provide protection from insect feeding are vulnerable to the evolution of resistance in the target insect pest population, resulting in a reduction in the durability of the insect resistance substance(s) and the associated loss of benefits. Resistance can arise in a target pest population through the sequestering or degradation of the insecticidal substance, the disabling of any of the steps in the mode of action, reduction in the sensitivity of the target site or compensatory changes that circumvent the effects of the substance. In the case of RNAi, one can postulate any number of potential resistance mechanisms, although no resistance mechanism has yet been identified. For example, resistance to dietary dsRNA could arise from reduced uptake when feeding (perhaps by avoiding feeding on plant tissues with high levels of dsRNA), increased degradation of the molecule in the insect digestive system, barriers to absorption of dsRNA by cells, decreased production of or processing by Dicer ribonucleases, reduced recognition by the RISC complex of siRNA molecules, failure of the RISC complex to degrade the target mRNA or blocking of systemic spread of the RNAi. Insects could also develop compensatory mechanisms to circumvent the gene silencing by increasing transcription rates of the target gene sequence or upregulating other genes that can perform the same or similar functions of the target (silenced) gene. Adaptations could also involve point mutations in the target gene sequence so that the 21-mer matches with the mRNA are reduced or eliminated.

Target-site-mediated resistance seems less likely to occur given that relatively long dsRNA sequences are transgenically expressed for downregulation of WCR essential genes. In the nematode *C. elegans*, RNA-deficient mutants have been identified in core pathway genes[116] as well as genes involved in the systemic spread of RNAi.[117] Analogous RNAi-resistant mutants have yet to be identified in WCR. However, one can envision that mutations in RNA uptake mechanisms might occur, but it is as yet unknown in WCR whether multiple or compensatory routes to dsRNA uptake exist (e.g. SID-like systems and endocytosis).[53] Given that RNAi is involved in defense against exogenous dsRNA, perhaps another risk to the durability of RNAi traits to control rootworms might be selection pressure applied by persistent virus exposure resulting in mutations to core RNAi machinery.[118] Resistance mechanisms most likely to arise in insect

populations will likely depend on the number of mutations involved in gene sequences and regulatory elements, as well as the fitness costs entailed. Fitness costs associated with reduced RNAi uptake could be related to reduced ability to feed or uptake nutrients, reduced activity of the RNAi machinery could lead to increased susceptibility to viral diseases, changes in gene regulation could alter other cellular metabolic functions and changes in target gene sequence could reduce the activity or specificity of the protein produced.

Traditionally, delays in insect pest resistance evolution to insect-protected GE crops are achieved by (1) planting refuges [crops that do not contain the pesticidal substances and therefore allow survival of insects that are susceptible to the pesticidal substance(s)] and (2) combining multiple insecticidal substances with different modes of action.[119] Refuges are cropping areas where there is no selection pressure for resistance to a given trait and therefore that allow survival of insects that do not possess resistance alleles at high frequencies. These susceptible insects are intended to be available to mate with resistant insects that may survive in insect-protected GE crops so that their progeny are heterozygous for resistance alleles. If the heterozygotes are also controlled by the insect-protected GE crop, the spread of initially rare resistance alleles through a pest population can be greatly delayed. If the insect-protected GE crop simultaneously produces two or more insecticidal substances with different modes of action such that cross-resistance is less likely to occur, insects that carry resistance alleles to one of the substances will continue to be controlled by the other substance(s) and fail to pass resistance alleles on to the next generation. The combination of refuges and insect-protected GE crops that produce multiple insecticidal substances can be an effective resistance management strategy,[120] and there are many examples today where this strategy is implemented using different *Bt* proteins.[121] Combinations of *Bt* proteins for corn rootworm management in corn (e.g. Cry3Bb1 + Cry34Ab1/Cry35Ab1 and Cry34Ab1/Cry35Ab1 + mCry3A) have been deployed for this reason, although their long-term effectiveness is likely to be reduced in areas where resistance to one of the components is established in the target pest populations.[12]

It has been recognized that RNAi for corn rootworm control should be combined with other modes of action to promote durability. The first likely commercialized event, MON 87411, produces both WCR *Snf7* dsRNA and Cry3Bb1 *Bt* protein.[23] The commercial concept combines these two modes of action with Cry34Ab1/Cry35Ab1 to improve durability, considering that field resistance to Cry3Bb1 has been documented at many locations across the US Corn Belt.[12,122] There is no information yet on whether RNAi events provide high-dose protection in corn. High dose has been defined by EPA as causing 99.99% larval mortality under field conditions such that resistance to the event would be expected to be functionally recessive (US EPA 1998).[123] For corn rootworm, larval mortality is difficult to measure directly in the field, and relative adult emergence, which has been used as a proxy, is confounded by variable larval infestation rates and variable biotic and abiotic mortality factors.[124–126] Should high-dose RNAi events be developed for WCR, they would be expected to provide higher durability to the IRM stack.

Parental RNAi (pRNAi) that prevents oviposition or includes loss of egg viability has the potential to bring further durability benefits to transgenic crops that use RNAi and other mechanisms for insect protection. pRNAi prevents exposed insects from producing progeny and therefore from passing on to the next generation any alleles that confer resistance to the other pesticidal substance(s) (e.g. *Bt*). Therefore, pRNAi can extend the durability of insect-protected transgenic crops when combined with one or more *Bt* proteins (or other insecticidal substances) targeting the same pest populations. This benefit arises because insects that are resistant to the *Bt* protein will occur as a higher proportion of the population in the transgenic crop compared with the refuge crop. If the ratio of resistance alleles to susceptible alleles that are passed on to the next generation is lower in the presence of pRNAi than in the absence of pRNAi, the evolution of resistance will be slowed. Transgenic crops that produce parental active dsRNA in addition to an insecticidal protein can be much more durable compared with transgenic crops that produce only one insecticidal trait.

With *Snf7* and other gene targets being investigated for potential corn rootworm control applications, an important consideration is the potential for resistance to one RNAi to confer cross-resistance to other RNAi molecules. Resistance mechanisms that disrupt the RNAi machinery would seem more likely to lead to resistance throughout the whole class of dsRNA-mediated interference. Whereas, more specific mechanisms such as altered target gene sequence or upregulation of compensating genes would not confer cross-resistance to RNA interference of other target genes. Currently, it is not known what mechanisms of resistance will develop in the field, and therefore it is difficult to predict cross-resistance scenarios. It is possible that simultaneous expression of two or more RNAi molecules could represent multiple modes of action against target species, if resistance is sequence-specific and therefore cross-resistance will be low; however, if resistance involves common RNAi machinery, cross-resistance will be high.

5 Challenges and Future Directions

Western corn rootworm is a highly adaptive pest in its ability to overcome insect management practices, including transgenic *Bt* traits.[12,122,127] Currently, there are only two distinct modes of action, Cry3 and Cry34/35 proteins derived from *Bt*, commercialized as insect resistance traits targeting WCR.[10] Pyramided Cry3Bb and Cry34/35Ab1 corn hybrids have been commercialized in SmartStax® hybrids for resistance management of WCR; however, WCR field-evolved resistance to Cry3-based IR traits has likely increased selection pressure against WCR populations for resistance to Cry34/35. This situation has created an urgent need for new modes of action as alternatives to Cry3 and Cry34/35 traits. The successful demonstration of transgenic dsRNA to provide corn root protection against WCR feeding damage[22] has catalyzed industry-wide interest in RNAi as a novel mode of action to combine with *Bt* technology to reduce the probability of field-evolved resistance to currently marketed traits. Recent work targeting *Snf7*[24–26,109] as well as *vATPases*[22] provides reason for optimism that RNAi will soon be successfully deployed in commercial hybrids resistant to damage by WCR.

Several key uncertainties remain that represent potential hurdles to realizing the commercial application of RNAi as a mode of action to pyramid with traits based on *Bt* proteins:

1. RNAi trait performance over multiple field seasons and commercial hybrid yield potential has yet to be reported.
2. The regulatory framework to assess safety of dsRNA insecticidal traits may differ in certain regards from that established for *Bt*-based insecticidal traits. Perspectives have been published indicating that the current framework for environmental safety assessment of protein-based traits is appropriate for the assessment of RNAi crops.[18,101] In the context of

mammalian safety, RNA is a component of all food and feed, and is generally regarded as safe. Petrick *et al.*[128] recently reviewed human health safety studies designed to assess biotech traits and proposed that the currently recognized principles for the safety evaluation of biotechnology-derived crops are applicable to RNA-based traits such as RNAi. Recently, the US EPA issued a registration for transformation event MON 87411, which produces the *Snf7* dsRNA in addition to Cry3Bb1 *Bt* protein, following extensive review of mammalian toxicology and the environmental risk assessment. That event has been deregulated by the US Department of Agriculture and has completed review at the US Food and Drug Administration for food and feed safety. Event MON 87411 has also completed regulatory reviews for food, feed and cultivation in Canada. At the time of writing, regulatory approvals for food and/or feed use are also in place in Australia, New Zealand, and Taiwan.[129]

3. As with all insecticides, selection for field-evolved resistance is a major concern. Resistance to dsRNA traits could result from target-site mutation or mutations in RNAi pathways for dsRNA uptake, processing of dsRNA into siRNA and spread of the RNAi effect. More research on the potential for resistance to RNAi traits to be selected for in the laboratory or to evolve in a field setting will help us to understand the long-term value of RNAi in the context of insect resistance management and trait durability. Pyramiding RNAi-based traits with protein-based corn-rootworm-active traits will help to mitigate these resistance risks.

To conclude, first-generation RNAi traits for WCR control are likely to be available to growers in the near future. Opportunities to improve upon the first-generation of RNAi traits for control of corn rootworm will aim at trait performance attributes such as root protection, adult emergence and high dose potential. Other possibilities include use of parental RNAi for transgenerational control of rootworm populations, as well as innovation for topical applications or baits.

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