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In silico identification of off-target pesticidal dsRNA binding in honey bees (Apis mellifera)

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Background. Pesticidal RNAs silencing critical gene function have great potential in pest management, but the benefits of this technology must be weighed against non-target organism risks. Methods. Published studies that developed pesticidal dsRNAs were collated into a database. The target gene sequences for these pesticidal RNAs were determined, and the degree of sequence homology with the honey bee genome were evaluated statistically for each. Results. We identified 101 insecticidal dsRNAs sharing high sequence homology with genomic regions in honey bees. The likelihood of off-target sequence homology increased with the parent dsRNA length. Non-target gene binding was unaffected by taxonomic relatedness of the target insect to honey bees, contrary to previous assertions. Gene groups active during honey bee development had disproportionately high sequence homology with pesticidal RNAs relative to other areas of the genome. Discussion. Although sequence homology does not itself guarantee a significant phenotypic effect in honey bees, in silico screening may help to identify appropriate experimental endpoints within a risk assessment framework for pesticidal RNAi.
In silico identification of off-target pesticidal dsRNA binding in honey bees (Apis mellifera)

Short title: Identification of off-target RNAi in honey bees

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Abstract

Background. Pesticidal RNAs silencing critical gene function have great potential in pest management, but the benefits of this technology must be weighed against non-target organism risks.

Methods. Published studies that developed pesticidal dsRNAs were collated into a database. The target gene sequences for these pesticidal RNAs were determined, and the degree of sequence homologies with the honey bee genome were evaluated statistically.

Results. We identified 101 insecticidal dsRNAs sharing high sequence homology with genomic regions in honey bees. The likelihood of off-target sequence homology increased with the parent dsRNA length. Non-target gene binding was unaffected by taxonomic relatedness of the target insect to honey bees, contrary to previous assertions. Gene groups active during honey bee development had disproportionately high sequence homology with pesticidal RNAs relative to other areas of the genome.

Discussion. Although sequence homology does not itself guarantee a significant phenotypic effect in honey bees, in silico screening may help to identify appropriate experimental endpoints within a risk assessment framework for pesticidal RNAi.

Keywords: RNAi, non-target, risk assessment, transgenic crops
Introduction

The potential to silence critical gene function in pest species has led to the proposed application of RNA interference (RNAi) as a novel class of agricultural products (Price and Gatehouse 2008; Gu and Knipple 2013) that target several species of economically important pests (Baum et al. 2007; Maori et al. 2009; Desai et al. 2012; Hajeri et al. 2014; Marr et al. 2014). These RNAi-based pesticides may be delivered to the target pest via a number of methods, including transgenic plants and sprays of naked or encapsulated small RNAs, which elicit post-transcriptional gene silencing. Once ingested, the insect’s cellular machinery cleaves the double stranded RNA (dsRNA) molecule into small-interfering RNAs (siRNAs) that are 19-25 nucleotides in length; these serve as the functional unit of RNAi and govern the location of gene suppression through the degradation of complementary messenger RNA molecules (Fire et al. 1998; Martinez et al. 2002; Vermeulen et al. 2005). To date, this process has been investigated in the control of a number of pest groups, including parasites of medical importance, urban pests, pests and pathogens of honey bees, and agricultural pests of economic importance.

While the technology promises to be target specific (Whyard et al. 2009; Bachman et al. 2013), there is concern that the current risk assessment framework for genetically modified crops is not adequate to proactively assess the risks to non-target organisms (Lundgren and Duan 2013; FIFRA-SAP 2014). The risks associated with RNAi to non-target organisms include immune stimulation (Lu and Liston 2009), saturation of an organism’s RNAi machinery that could interfere with normal cellular processes (Grimm 2011; Flenniken and Andino 2013), and unintentional gene silencing. Unintentional gene silencing in non-target organisms is the primary risk posed by pesticidal RNAi; within a non-target species, this unintentional gene silencing can be of the targeted gene sequence (non-target binding) or occur elsewhere in the genome with
high sequence homology to the target gene (off-target binding) (Lundgren and Duan 2013; FIFRA-SAP 2014). Because pesticidal RNAi poses risks to non-target organisms that are unique from other pesticides, a risk assessment framework has been proposed to proactively assess these risks using a series of steps (FIFRA-SAP 2014; Roberts et al. 2015).

The hazard to non-target organisms should be predictable if the functional genome of a non-target organism is known, recognizing that numerous circumstances influence gene silencing even when sequence homology is identical between a small RNA and the non-target genome (Kerschen et al. 2004). Bioinformatic analyses have thus been advocated as an initial screen of potential risks posed by RNAi (FIFRA-SAP 2014; Roberts et al. 2015). In the present study, we used in silico searches to determine whether putative pesticidal dsRNAs share sequence homologies with off-target regions of the honey bee (Apis mellifera L.), a model non-target organism. We were specifically interested in testing the hypotheses that 1) longer dsRNAs increase the potential for off-target binding, 2) non-target silencing of the target gene is dependent on relatedness of the target and non-target species, and 3) certain gene groups in the honey bee are more prone to off-target sequence homologies with pesticidal dsRNAs.

Materials and Methods

Literature review

Published studies evaluating the effects of pesticidal dsRNAs were searched using the ISI Web of Knowledge database, using combinations of the search terms “pesticidal,” “insecticidal,” “siRNA,” “dsRNA,” “RNAi,” and “RNA interference.” Studies were included if they evaluated the pesticidal effects of a dsRNA and provided either the dsRNA sequence or primer sets that allowed the dsRNA sequences to be determined from the target species’ genome using the NCBI genome database (http://www.ncbi.nlm.nih.gov/genome/). A total of 24 studies were included,
with pesticidal qualities being evaluated for 74 dsRNAs and 21 siRNAs targeting 57 genes (Supplemental Data 1). These included species of medical importance (Hajdusek et al. 2009; Kwon et al. 2013), urban pests (Zhou et al. 2008; Itakura et al. 2009), parasites and pathogens of honey bees (Maori et al. 2009; Campbell et al. 2010; Desai et al. 2012), agricultural pests (Mutti et al. 2006; Baum et al. 2007; Whyard et al. 2009; Tang et al. 2010; Choudhary and Sahi 2011; Wuriyanghan et al. 2011; Gong et al. 2013; Ochoa-Campuzano et al. 2013; Yao et al. 2013; Christiaens et al. 2014; Chu et al. 2014; Han et al. 2014; Meng et al. 2014; Miyata et al. 2014; Yu et al. 2014), and others (Whyard et al. 2009; Kelkenberg et al. 2015; Petrick et al. 2015).

In silico sequence homology identification

Published pesticidal dsRNAs ranged from 19 to 2500+ nucleotides in length. These were queried against the annotated honey bee genome accessed through GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the BLAST nucleotide algorithm for somewhat similar sequences (blastn). Homologous regions were mostly less than 25 nt long, the length expected for resultant siRNAs randomly generated from the parent dsRNA molecule. Sequence homologies of 19/21, 20/21, and 21/21 nt were tallied for each dsRNA against the honey bee genome, and the off-target gene name was recorded. Each off-target gene was only tallied once per dsRNA, even when that dsRNA targeted multiple locations along that gene. Sequence similarity for the target gene (non-target binding) was also recorded. Low quality proteins (as defined by NCBI) and genes of unknown function were excluded from the analysis, as were any homologous regions that did not return any protein or gene information, such that the resultant database represents a conservative estimate of putative binding.

Statistical analysis
Because data violated parametric assumptions, the number of off-target homologies were log(x+1) transformed and dsRNA length were log transformed to uphold assumptions for analysis with linear regression (Systat v.13.1, San Jose, CA, USA). A chi-square test of independence was used to determine whether there was a significant effect of target taxa on the incidence of non-target binding in honey bees, and whether certain functional gene groups were targeted more frequently.

Results and Discussion
dsRNA length-suppression

Each of the 74 pesticidal dsRNAs shared at least one region of perfect or high sequence homology with the honey bee genome (average 28.6 ± 3.32 off-target homologies per dsRNA) (Supplemental Data 1). However, none of the published pesticidal siRNAs (21 total, 19-23 nt in length) found sequence homology within the honey bee genome at our specified level (19/21, 20/21, 21/21 nt matches), indicating that these much smaller sequences were more specific when focusing on a single non-target organism. This result was mirrored by Li et al. (2015), though siRNAs are not always this benign: Qiu et al. (2005) demonstrated that 5-80% of tested siRNAs resulted in off-target binding among diverse organisms.

Off-target sequence homology increased significantly as the parent dsRNA increased in length (linear regression: $F_{1,100} = 623, P < 0.001$) (Figure 1a), with every increase of 100 nt in the dsRNA resulting in 6 more predicted hits. This strong relationship between dsRNA length and potential off-target binding can be further demonstrated using only the genes described in Miyata et al. (2014), in which the authors evaluated the effects of dsRNA length on RNAi activity in vivo in western corn rootworms. Although the gene targets in this study were not
pesticidal specifically, and thus excluded from our overall analysis, the authors evaluated silencing of the same gene targets (*laccase 2* and *ebony*) using different sized dsRNAs to evaluate efficacy. When we examined this suite of genes from a risk assessment perspective using the same methodology as for the pesticidal RNAs, the longer dsRNAs returned significantly more regions of off-target sequence homology in the honey bee genome (*laccase 2*: $F_{1,5} = 181$, $P < 0.001$; *ebony*: $F_{1,2} = 103$, $P = 0.01$) (Figure 1b). While intuitive (Bolognesi et al. 2012), this is the first demonstration of the possibility for increased length-suppression in a non-target organism. Thus, optimizing dsRNA length to have maximum gene suppression efficacy in the target pest needs to be balanced against the non-target risks posed by longer molecules.

### Target-species specificity

Taxonomic relatedness of the target organism to honey bees had no effect on potential binding of siRNAs on the original gene target (non-target binding) ($\chi^2 = 9.4$, $df = 7$, $P = 0.23$) (Figure 2). Contrary to assertions of pesticidal specificity (Bachman et al. 2013), this implies that silencing of the target gene in a non-target organism may be more likely to occur from random sequence similarities than based on evolutionary relatedness to the target organism. Although the pool of available literature is limited to date with regards to targeted applications of RNAi against pest species, with certain species being more frequently researched (e.g. *Diabrotica virgifera*), our results suggest that non-target hazard assessments should focus on species of ecological relevance rather than strictly on phylogenetic relatedness to the target species.

Unfortunately, when conducting bioinformatics analyses for the purposes of a risk assessment, the availability of sequenced genomes from representative species becomes a limiting factor. Further, the potential non-target community will differ depending on the specific pest being targeted, making it difficult to have a standard suite of species to evaluate for non-
target effects. Bioinventories are crucial for identifying appropriate non-target species for each target pest. Supporting initiatives such as i5K (i5K Consortium 2013), which strives to sequence the genomes of 5000 representative invertebrates, and making these genomes freely available, will bolster the applicability of future *in silico* analyses aimed at identifying potential risks of gene-oriented pest control.

**Targeted gene groups**

The homeobox genes and other genes involved in embryonic and developmental pathways in honey bees frequently shared sequence homology with the pesticidal dsRNAs, particularly when vATPase subunits were the pesticidal targets ($\chi^2 = 10, \text{df} = 4, P = 0.03$). 67% of all tested dsRNAs had off-target binding with developmental genes in honey bees, and 33% of these shared homology with homeobox genes specifically (Supplemental Data 1). Although we have an incomplete picture of which genes are expressed in most genomes at any given time, many of these genes, while important during embryogenesis and development, perform additional critical functions such as cell proliferation and apoptosis, and are highly conserved across metazoans. In this instance, *in silico* analysis identified potential gene targets that could present a hazard requiring unique assessments across life stages to properly identify a phenotypic effect. If validated in future *in vivo* assessments, this screening method may prove useful in identifying appropriate experimental endpoints in non-target risk assessments.

**Conclusions**

Our bioinformatics-based *in silico* analysis provides a conservative assessment of potential off-target binding of pesticidal dsRNAs in the honey bee genome; the actual binding affinity of RISC is more nuanced than 100% or similar sequence homology for subsequent
mRNA degradation. While some have documented off-target gene knockdown with 20/21 nt similarity (Jarosch and Moritz 2012), others have found silencing with even less sequence similarity in certain study systems, particularly in the 2-8 nt seed region of the siRNA. For example, in experiments with cultured human cells, Saxena et al. (2003) found gene silencing with as many as 3-4 bp mismatches in addition to G.U wobbles (guanine and uracil have a slight affinity for each other), while Jackson et al. (2003) found mRNA degradation with only 11/21 contiguous nt. The locations of the mismatches along the siRNA are also important; perfect sequence homology of the seed region is particularly crucial for mRNA recognition (Jackson et al. 2006; Chu et al. 2014).

However, in silico identification of sequence homology between a pesticidal dsRNA and non-target organism’s genome does not imply that RNAi will occur in the non-target organism. Unintended gene silencing will depend on a number of factors. The organism would need to possess behavioral characteristics that would put it into contact with contaminated materials, e.g. leaf tissue versus pollen versus nectar feeding at a contaminated location. Other factors include the length of the dsRNA and whether the organism is exposed to siRNA or dsRNA, the identity of the target or off-target mRNA, the size of a non-target organism’s genome (more off-target binding would be expected when there are more potential gene targets), the necessary binding affinity of a particular siRNA, exposure concentration of the dsRNA, and the physiological state of the insect (Qiu et al. 2005; Baum et al. 2007; Huvenne and Smagghe 2010; Gu et al. 2014).

Ecological risk assessment is a complex and multi-stepped process, and no single piece of work is sufficient to fully quantify the risk of a toxicological event. We have demonstrated that an in silico analysis may be used as a first step in establishing whether off-target binding could pose a significant threat for a particular pesticidal dsRNA in a non-target organism such as the
honey bee. Future experiments to evaluate the usefulness of this tool are planned that would quantify up/down gene regulation of honey bees exposed to pesticidal dsRNA. Taken together, these data may provide a basis for designing biologically appropriate experiments to optimize hazard assessments for applications of this novel pesticidal technology in field settings where honey bees and other non-target organisms may be exposed.

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Supplemental Data 1. Database of putative off-target gene silencing.

Figure 1. The relationship between pesticidal dsRNA length and potential off-target binding in honey bees for pesticidal dsRNAs (a) and the non-pesticidal laccase 2 and ebony genes (data from Miyata et al. (2014)) (b).

Figure 2. Potential non-target binding of pesticidal dsRNAs in honey bees (y-axis, shaded area) versus the original target taxa (x-axis), in relation to the total number of examined pesticidal dsRNAs. Taxa are ordered by increasing relative divergence time from honey bees.
Figure 1

Pesticidal dsRNA length and potential off-target binding in honey bees

The relationship between pesticidal dsRNA length and potential off-target binding in honey bees for pesticidal dsRNAs (a) and the non-pesticidal laccase 2 and ebony genes (data from Miyata et al. (2014)) (b).
Figure 2

Pesticidal dsRNA target organisms and the likelihood of off-target binding in the honey bee genome.

Potential non-target binding of pesticidal dsRNAs in honey bees (y-axis, shaded area) versus the original target taxa (x-axis), in relation to the total number of examined pesticidal dsRNAs. Taxa are ordered by increasing relative divergence time from honey bees.
