Experimental Results (2021), 2, e11, 1–9
doi:10.1017/exp.2020.67

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NOVEL-RESULT

Effect of using *green fluorescent protein* double-stranded RNA as non-target negative control in *Nasonia vitripennis* RNA interference assays

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(Received 02 November 2020; Revised 09 December 2020; Accepted 09 December 2020)

Abstract

RNA interference (RNAi) is a technique used in many insects to study gene function. However, prior research suggests possible off-target effects when using *Green Fluorescent Protein* (*GFP*) sequence as a non-target control. We used a transcriptomic approach to study the effect of *GFP* RNAi (*GFP*-i) in *Nasonia vitripennis*, a widely used parasitoid wasp model system. Our study identified 3.4% of total genes being differentially expressed in response to *GFP*-i. A subset of these genes appears involved in microtubule and sperm functions. *In silico* analysis identified 17 potential off-targets, of which only one was differentially expressed after *GFP*-i. We suggest the primary cause for differential expression after *GFP*-i is the non-specific activation of the RNAi machinery at the injection site, and a potentially disturbed spermatogenesis. Still, we advise that any RNAi study involving the genes deregulated in this study, exercises caution in drawing conclusions and uses a different non-target control.

Keywords: RNA interference; *Nasonia*; RNA sequencing

1. Introduction

Nasonia vitripennis is a widely used parasitoid wasp model system to study major topics such as genetics, development, ecology, and behavior (Beukeboom & Desplan, 2003; Lynch, 2015). The first sequencing of its genome has been published in 2010 (Werren et al., 2010). Recently the development of new technologies has pushed the model forward. For example, knock out of genes by the CRISPR/Cas9 technology has been reported but has at the moment not been widely used (Li et al., 2017) due to low survivability of embryos. Therefore, knockdown of genes by RNA interference (RNAi) or by parental RNAi (pRNAi) is still the most popular method to study gene function and maternal transcript provision in *N. vitripennis* (Dalla Benetta et al., 2020; Lynch & Desplan, 2006; Wang et al., 2020; Werren et al., 2009). However, in other Hymenoptera such as the honeybee, RNAi has been reported to have undesirable off-target effects, for example when using *GFP* dsRNA as a control (Jarosch & Moritz, 2011; Nunes et al., 2013). Here we investigate the potential for off-target effects of using *GFP* double-strand RNA (dsRNA) as non-target control in *N. vitripennis* RNAi experiments.

2. Objective

As RNAi has become a widely used technique in *N. vitripennis* (Lynch, 2015), it is of importance to identify potential pitfalls generated by the technique. Also, with the now affordable cost of

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RNA-sequencing technologies, RNAi experiments can be coupled with genome-wide transcriptomic analyses. Therefore, identifying the potential off-target effects of RNAi in *N. vitripennis* will help researchers to design appropriate controls for their experiments and avoid confusing results or following false positive leads. To this aim, we tested the off-target effect of a non-specific RNAi response by comparing the effect of injecting dsRNA against the non-target *GFP* together with water-injected or uninjected controls at whole transcriptome level.

3. Methods

Second instar male larvae were injected with either *GFP* dsRNA (*GFP*-i), water, or were not injected but otherwise treated the same. Each condition was analyzed in triplicate using five males per sample. The treated males were collected approximately five days later at the white pupal stage and RNA was extracted with Quick-RNA Tissue/Insect Kit according to manufacturer's protocol (ZymoResearch – R2030). Libraries were prepared with a custom protocol and 150 bp paired-end sequencing was achieved on a HiSeqX (Illumina) by Novogene (Novogene, HK company limited). From the fastQ files, read counts per gene were retrieved using GeneCounts quantification method from STAR (Dobin et al., 2013) version 2.6.1b and the Nvit_psr_1.1 *N. vitripennis* genome version with RefSeq annotation GCF_009193385.2 as reference. Differential expression analysis was calculated with DESeq2 (Love et al., 2014) version 1.20.0. Gene Ontology analyses were performed using DAVID bioinformatics resources (Huang da et al., 2009) version 6.8. For comparison with the Nunes et al. microarray data from honeybee, only genes found affected in at least two experiments were used (Nunes et al., 2013).

Complete detailed description of the methods is available as supplemental material.

4. Results

Full statistics of bioinformatics analysis are presented in Table S1 and show consistent high quality data in all samples. Pearson correlation analysis shows that the samples have a high correlation coefficient and cluster in three different groups but not based on experimental conditions (Figure 1A). Principal component analysis also revealed no apparent clustering of the different samples based on experimental conditions, except for one replicate of uninjected control segregating away on the PC2 (Figure 1B). Altogether, these results suggest few differences between samples.

Differential expression analysis between uninjected and water-injected samples with an adjusted *P*-value threshold of (*P*-adj) < 0.05, revealed that only 46 genes are differentially expressed (Table S2).

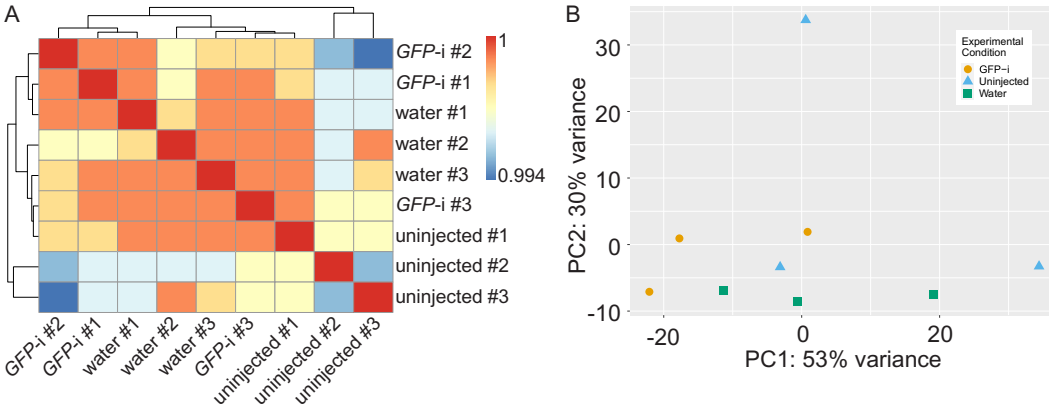

Figure 1. Analysis of RNA-sequencing sample reproducibility. (A) Heatmap of Pearson correlation coefficient (r) of *GFP*-i, water-injected, and uninjected samples. (B) Principal component analysis of *GFP*-i (orange dots), uninjected (blue triangles), and water-injected (green squares) samples.

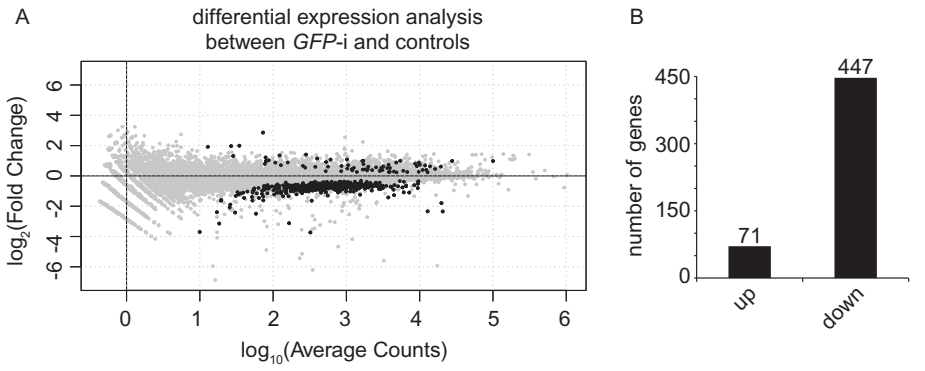

Figure 2. Injection of *GFP* dsRNA induces changes in gene expression when compared with controls. (A) MA plot showing the fold change (log_2-transformed) between gene expression in *GFP*-i and control (uninjected and water-injected combined) samples as a function of the normalized average count between the two conditions (log_{10}-transformed), as calculated with DEseq2. Genes with significantly differential expression (*P*-adj < 0.05) are showed in black. For *GFP*-i and control samples, three and six biological replicates were used, respectively. (B) Bar plot quantifying genes found more expressed ($log_2FC > 0$ and *P-adj* < 0.05) or less expressed ($log_2FC < 0$ and P-*adj* < 0.05) after differential expression analysis.

Therefore, we decided to pool these two conditions into one control group. Differential expression analysis between *GFP*-i and control samples with *P*-adj < 0.05 revealed 518 differentially expressed genes (DEG), of which 71 have a higher expression and 447 a lower expression (Figure 2 and Table S3). Gene Ontology analysis on these 518 DEGs identified enrichment for "microtubule-based process", "cytochrome-c oxidase activity" and "oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor".

To find regions in the *N. vitripennis* genome with a potential *GFP* sequence similarity causing the off-target effects, we aligned the *GFP* sequence used in our experiment against the *N. vitripennis* genome. Blastn identified 17 hits with an alignment length ranging from 18 to 46 nucleotides and between 78% to 100% identity (Table S4). Aside from *LOC100122071*, none of these genes aligned gapless for more than 17 nucleotides. Of these 17 genes, only *LOC103318004* is differentially expressed in our RNAseq experiments, with a reduced expression in both water-injected and *GFP*-i samples ($log_2FC = -0.76$ with *P*-adj = 0.013 and $log_2FC = -1.03$ with *P*-adj = 0.006 respectively). We also used the *Nasonia* specific RNAi off-target prediction tool (Davies & Tauber, 2015) and it did not find any matching 19-mers of *GFP* sequence.

Finally, we compared our results with microarray data obtained in another Hymenoptera, the honeybee *Apis mellifera.* Nunes et al. identified 1,416 differentially expressed genes after *GFP* dsRNA injection across three different experimental conditions, with high variations between samples and only 18 genes differentially expressed in at least two conditions (Nunes et al., 2013). None of these 18 genes were in our *N. vitripennis* DEG list (Table 1).

5. Discussions

Comparison of uninjected and water-injected samples did not reveal a major response caused by the injection procedure itself five days before sampling. However, among the 46 DEGs, *LOC100117489* (*pyrazinamidase/nicotinamidase*) and *LOC100120201* (*vanin-like protein 2*) have been previously linked to oxidative stress in *Drosophila* and human (Balan et al., 2008; Bartucci et al., 2019) and may suggest some level of stress response upon injection.

Our RNAseq experiments revealed that 518 out of 15,430 total genes (3.4%) are differentially expressed in whole pupae in response to *GFP*-i with *P*-adj < 0.05. Among the 447 lower expressed genes, 33 are coding for microtubules or microtubule-associated proteins, predicted to control sperm functions. Also, four cytochrome C oxydase subunits show a decrease in expression. In addition to be possible

Table 1. Comparison between the effect of *GFP* dsRNA injection in *Apis mellifera* and *Nasonia vitripennis* shows that none of the DE genes found in the two *A. mellifera* studies are differentially expressed in *N. vitripennis.*

Apis mellifera			*Nasonia vitripennis*			
honeybee gene (Amel_4.0)	Annotation	effect of *GFP* dsRNA	reference	RefSeq_ID	log_2FoldChange (*GFP* dsRNA/control)	*P*-adj
GB11613	Gpdh	downregulated	Jarosch et al., 2011	LOC100123687	0.167	0.054
GB13214	helicase at 25E ortholog	downregulated	Nunes et al., 2013	LOC100113785	0.082	0.771
GB15172	fumarate hydratase, mitochondrial-like	downregulated	Nunes et al., 2013	LOC100123162	0.014	0.973
GB15245	thioredoxin-related transmembrane protein	downregulated	Nunes et al., 2013	LOC100122126	0.014	0.950
GB18969	60 kDa heat shock protein	downregulated	Nunes et al., 2013	LOC100114031	−0.123	0.543
GB20002	farnesoic acid o-methyltransferase-like	downregulated	Nunes et al., 2013	LOC100114909	0.043	0.888
GB10133	superoxide dismutase 1 (Sod1)	upregulated	Nunes et al., 2013	LOC100116946	0.203	0.464
GB10398	ninjurin-1-like	upregulated	Nunes et al., 2013	LOC100123713	0.228	0.480
GB11103	translocator protein-like	upregulated	Nunes et al., 2013	LOC100120467	0.012	0.969
GB13473	apidaecin 1 (Apid1)	upregulated	Nunes et al., 2013	LOC100123721	−0.079	0.729
GB13879	hypothetical protein LOC725128	upregulated	Nunes et al., 2013	LOC100679911	0.016	0.969
GB15855	thioredoxin 2 (Trx-2)	upregulated	Nunes et al., 2013	LOC100118754	0.139	0.587
GB16277	histidine triad nucleotide-binding protein 3-like	upregulated	Nunes et al., 2013	LOC100114446	−0.200	0.408
GB17782	apidaecins type 22 precursor	upregulated	Nunes et al., 2013	LOC100123721	−0.079	0.729
GB18760	L-xylulose reductase	upregulated	Nunes et al., 2013	LOC107980890	0.116	0.821
GB10708	immume responsive protein of 30 kDa (IRP30)	upregulated	Nunes et al., 2013	LOC103316706	0.071	0.790
GB10428	hypothetical protein LOC100579019	upregulated	Nunes et al., 2013	LOC103315726	−0.369	0.297
DB780023	no significant similarity found	upregulated	Nunes et al., 2013	LOC100120051	−0.122	0.673
GB18966	cytochrome b5 type B-like	upregulated	Nunes et al., 2013	LOC107980965	1.269	1.000

markers of apoptotic stress, cytochrome C oxydases have been linked to sperm differentiation in *Drosophila* (Arama et al., 2003). Finally, *LOC103318004*, which is predicted to encode a kelch-like protein 10, showed some sequence similarity with the *GFP* sequence used in this study. However these two sequences aligned on less than 19 nucleotides without gap, which was described as the minimum consensus length for RNAi (Elbashir et al., 2001). *LOC103318004* homologs have been linked to gamete development in both *Drosophila* and mice (Hudson & Cooley, 2010; Yan et al., 2004). *LOC103318004* is less expressed in *GFP*-i samples compared to controls, but, surprisingly, also in water-injected samples when compared to uninjected controls. Therefore, it is unclear whether the decrease in expression of this gene is due to injection stress or is a sequence-specific *GFP*-i effect, or both. As dsRNA was injected in the abdomen, activation of the RNAi response in this region could affect spermatogenesis. Similar observations have been made in female *N. vitripennis*, which show some reduced fertility after water or dsRNA injection at pupal stage (Geuverink et al., 2017).

6. Conclusions

Our transcriptomic comparison of *GFP* non-specific RNAi with water and uninjected controls revealed moderate off-target effects in male *N. vitripennis*, potentially affecting spermatogenesis. Only in one case we were able to relate this effect to endogenous *GFP* sequence similarity in *N. vitripennis*, and this leads us to assume that *GFP*-i does not cause specific off-target effects, but instead results in a general response to dsRNA. As we injected into the abdomen, close to the reproductive organs, it could additionally lead to reduced fertility in males. It should be noted that our study focuses on only one sex at one developmental time and results may differ under different experimental conditions.

The lack of overlap between DEGs after RNAi activation in *Nasonia* and honeybees suggests that off-target effects cannot be generalized across Hymenoptera. The specific effects of RNAi activation and off-target effects should be studied on a species-specific basis. According to our results, *GFP* seems a suitable non-targeting negative control for RNAi experiments in *N. vitripennis* as it appears to elicit a general non-specific response against dsRNA, and sets a baseline reference for the target-specific knockdown. However, any RNAi study involving the genes we show to be affected in this study should exercise caution in drawing conclusions and may be safer by using a different non-target control.

Author Contributions. Y.W., J.R. and E.C.V. designed the study. Y.W. performed experiments. J.R. analyzed data. J.R. wrote the initial manuscript draft, all authors revised the draft and approved the final version. E.C.V. acquired funding.

Funding Information. Part of the work was funded by the Innovative Research Scheme of the Dutch Research Council (NWO) granted to ECV (016.Vidi.189.099).

Publishing Ethics. The authors confirm that

1. the manuscript has been submitted only to the journal – it is not under consideration, accepted for publication or in press elsewhere. Manuscripts may be deposited on pre-print servers;
2. all listed authors know of and agree to the manuscript being submitted to the journal;
3. the manuscript contains nothing that is abusive, defamatory, fraudulent, illegal, libelous, or obscene.

Conflict of Interest. The authors declare no conflict of interest.

Data Availability. The sequencing data have been submitted to GEO under accession number GSE153268.

Supplementary Materials. To view supplementary material for this article, please visit http://dx.doi.org/10.1017/exp.2020.67.

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Cite this article: Rougeot J, Wang Y, Verhulst EC (2021). Effect of using *green fluorescent protein* double-stranded RNA as non-target negative control in *Nasonia vitripennis* RNA interference assays *Experimental Results*, 2, e11, 1–9. https://doi.org/10.1017/exp.2020.67

Peer Reviews

Reviewing editor: Dr. Michael Nevels

University of St Andrews, Biomolecular Sciences Building, Fife, United Kingdom of Great Britain and Northern Ireland, KY16 9ST

This article has been accepted because it is deemed to be scientifically sound, has the correct controls, has appropriate methodology and is statistically valid, and has been sent for additional statistical evaluation and met required revisions.

doi:10.1017/exp.2020.67.pr1

Review 1: GFP double-strand RNA injection reveals no off-target effect in Nasonia vitripennis

Reviewer: Dr. Francis M. F. Nunes

UFSCar, Sao Carlos, Brazil, 13565-905

Date of review: 10 November 2020

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Conflict of interest statement. Reviewer declares none.

Comments to the Author: Using RNA-Seq, the authors analyzed the potential undesired effects of dsRNA-GFP as a control in RNAi experiments on the Nasonia vitripennis wasp species. Compared to the previous version of the manuscript, there are many improvements, especially with the use of a less stringent p-value that made it possible to perceive a greater number of genes impacted by GFP-i. Overall, I appreciate the current version of the manuscript and I just have a few questions/ suggestions about it.

In the files I received for analysis there are two titles, but I believe that the correct and most appropriate one is: Effect of using Green Fluorescent Protein double-stranded RNA as non-target negative control in Nasonia vitripennis RNA interference assays.

Based on the GFP-primers sequences, the reported dsRNA size (460 bp) and the Emerald GFP CDS sequence, I believe the target fragment used for dsRNA-GFP synthesis is the one below and should be included in the supplementary material:

5 ' - GTGACCACCTTGACCTACGGCGTGCAGTGCTTCGCCCGCTACCCCGACCACATGAAG-
CAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTT-
CAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGT-
GAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCA-
CAAGCTGGAGTACAACTACAACAGCCACAAGGTCTATATCACCGCCGACAAGCAGAA-
GAACGGCATCAAGGTGAACTTCAAGACCCGCCACAACATCGAGGACGGCAGCGTG-
CAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCC-
GACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGA - 3'

Lines 28-29: replace “for example when using GFP coding sequence as a control” by “for example when using GFP dsRNA as a control”.

The authors did not report in the main text or supplementary material how many micrograms were used to prepare the RNA-Seq libraries (for the enrichment of mRNAs and first strand cDNA synthesis).

Score Card

Presentation

Is the article written in clear and proper English? (30%)	5/5
Is the data presented in the most useful manner? (40%)	5/5
Does the paper cite relevant and related articles appropriately? (30%)	5/5

Context

Does the title suitably represent the article? (25%)	4/5
Does the abstract correctly embody the content of the article? (25%)	4/5
Does the introduction give appropriate context? (25%)	4/5
Is the objective of the experiment clearly defined? (25%)	5/5

Analysis

Does the discussion adequately interpret the results presented? (40%)	4/5
Is the conclusion consistent with the results and discussion? (40%)	4/5
Are the limitations of the experiment as well as the contributions of the experiment clearly outlined? (20%)	5/5

doi:10.1017/exp.2020.67.pr2

Review 2: GFP double-strand RNA injection reveals no off-target effect in Nasonia vitripennis

Reviewer: Dr. Peter Dearden

Date of review: 03 December 2020

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Conflict of interest statement. Reviewer declares none

Comments to the Author: This is an excellent contribution to the literature. I suggest it is accepted. The only thing I might add is that if the authors collected phenotypes from their experiemnst then it might be useful to put these in. If not there is no problem- the paper should be published anyway.

Score Card

Presentation

4.4 /5

Is the article written in clear and proper English? (30%)	4/5
Is the data presented in the most useful manner? (40%)	5/5
Does the paper cite relevant and related articles appropriately? (30%)	4/5

Context

4.5 /5

Does the title suitably represent the article? (25%)	5/5
Does the abstract correctly embody the content of the article? (25%)	4/5
Does the introduction give appropriate context? (25%)	4/5
Is the objective of the experiment clearly defined? (25%)	5/5

Analysis

5.0 /5

Does the discussion adequately interpret the results presented? (40%)	5/5
Is the conclusion consistent with the results and discussion? (40%)	5/5
Are the limitations of the experiment as well as the contributions of the experiment clearly outlined? (20%)	5/5