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Bacterially expressed dsRNA induces *Varroa destructor* gene knockdown by honey bee-mediated oral administration

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ABSTRACT
The ectoparasite *Varroa destructor* causes serious losses of *Apis mellifera* colonies and negatively impacts the beekeeping industry around the world. New control methods have been proposed based on the RNA interference technique. Previous reports showed that parasitized honey bees fed with double-stranded RNA (dsRNA) synthesized *in vitro* reduce the transcription levels of target genes in Varroa mites. An efficient and inexpensive alternative to produce dsRNA is the use of bacteria capable of achieving high levels of *in vivo* synthesis. In the present study, dsRNA synthetized *in vivo* was used to induce gene silencing in *V. destructor* and evaluate their effect on the survival of both honey bees and the parasitic Varroa mites. The results evidenced that dsRNA fed to the bees engendered gene silencing in mites, inhibiting expression levels of target genes by 50%. Indeed, a reduction of 50% in Varroa survival was observed when bacterially expressed dsRNAs were administered to mite-parasitized bees. Worker bees that were fed with Varroa-targeted dsRNA by oral route showed no survival differences compared to control bees, fed with sucrose or dsRNA-GFP solutions. Our results demonstrated that specific dsRNA over-expressed in bacteria is capable of reducing mite survival by bee-mediated oral administration. This study provides an efficient and low-cost method for dsRNA production to control parasites and honey bee diseases.

Introduction

The ectoparasite *Varroa destructor* Anderson and Trueman is one of the most important pests of *Apis mellifera* Linnaeus, causing economic losses in the honey bee industry around the world. The Varroa mite produces damage mainly in the larval and pupal stages but also in adults (Rosenkranz et al., 2010). Although the mite does not directly kill the bees, it has strong negative effects by weakening brood and adults through feeding on them (Amdam et al., 2004; Ramsey et al., 2019; Zaobidna et al., 2017) and playing a key role in the replication of several honey bee viruses (De Miranda & Genersch, 2010; Di Prisco et al., 2011; Francis et al., 2013; Nazzi & Le Conte, 2016; Shen et al., 2005). Previous studies have demonstrated that the spread of *V. destructor* contributed to turning a widespread viral infection into a devastating global epidemic (Wilfert et al., 2016). The epidemic disease within the colony

triggered by the mite eventually results in colony death if untreated (Boecking & Genersch, 2008; Neumann & Carreck, 2010; Van Dooremalen et al., 2012).

Conventional *V. destructor* control in managed honey bee colonies is based on chemical acaricides (Haber et al., 2019; Lodesani, 2004). Both the reasonable price and easy application are still the main advantages of these pesticides and therefore, most beekeepers use chemical treatments on their colonies. Negative aspects of the use of these chemicals are honey contamination with human health implications due to toxicity, and the potential impact on the environment, bees' health and non-target organisms (Ansari et al., 2014; Gregorc et al., 2018). Moreover, in recent years the continued utilization of acaricides as fluvalinate, flumetrine, amitraz, coumaphos and cymiazole against *V. destructor* has resulted in the development of resistance to such chemicals in populations of the mite (Lipiński & Szubstarski,

2007; Maggi et al., 2009; 2011; Rinkevich, 2020; Stara et al., 2019). Therefore, alternative approaches for Varroa control are being evaluated.

RNA interference (RNAi) is a widely used technology that introduces double-stranded RNA (dsRNA) into organisms to reduce the transcription of a specific gene. This post-transcriptional gene silencing mechanism is also called "gene knockdown" (Fire et al.,1998). With the advent of RNAi-based methodologies, there has been an increasing interest in assessing their potential applications in controlling virus-mediated diseases and agricultural pests in both laboratory and field conditions (Di Lelio et al., 2014; Garbutt et al., 2013; Hunter et al., 2010). Studies performed in several insect orders have demonstrated that dsRNA oral administration is a convenient and practical way for controlling pests and pathogens of beneficial organisms (Whyard et al., 2009; Zotti & Smagghe, 2015). Specifically for *A. mellifera*, RNAi methods were evaluated to control honey bee viruses (reviewed by Yang et al., 2018) and Varroa mites (Campbell et al., 2010; 2016; Garbian et al., 2012; Huang et al., 2019). A pioneering study showed that dsRNAs ingested by honey bees are transferred to Varroa mites and *vice versa* (Garbian et al., 2012). These authors reported that feeding honey bees with dsRNA produced a lethal effect on mites. Moreover, through field bioassays using dsRNAs synthesized *in vitro,* they evidenced a reduction of the Varroa population in honey bee colonies.

The dsRNA *in vitro* production by commercial kits allows the synthesis of milligram amounts of RNA in a short time. An alternative to *in vitro* dsRNA production is using bacteria capable of achieving a high level of *in vivo* synthesis (Ahn et al., 2019). The production of bacterially expressed dsRNA is cheaper and more easily used in large-scale assays. Moreover, several studies have been carried out using bacterially expressed dsRNA to induce gene silencing by oral route in different groups of arthropods (Gamboa Cedeño et al., 2015; Lü et al., 2019; Salvador et al., 2021; Tian et al., 2009).

Here, we report that *V. destructor* gene expression can be modulated by dsRNA synthetized *in vivo* with a reduced number of target genes compared to previous studies (Garbian et al., 2012). The present finding confirms that the use of dsRNA is a potential biotechnological tool in mite control strategies, and that *in vivo* dsRNA synthesis is an inexpensive and efficient method for this purpose.

Materials and methods

RNA extraction and reverse transcription for complementary DNA synthesis

RNA extractions were performed on pools of five adult mites. The mite pools were macerated using a plastic pestle into a 1.5 ml Eppendorf-type with 500 μl of cold TRIZOL reagent (Invitrogen, USA) according to the manufacturer's instructions. The extracted RNA was stored in 20 μl of double distilled water and its concentration was determined using a spectrophotometer (NanoDrop Technologies, USA). Total RNA of extracted samples was treated with RQ1 RNase-free DNase (Promega) to eliminate any residual DNA. Reverse transcription (RT) reactions were performed with Random primers (Invitrogen), and 2 μg of RNA was used for complementary DNA (cDNA) synthesis with M-MLV reverse transcriptase enzyme (Promega, UK) according to the manufacturer's guidelines.

PCR amplification and cloning of Varroa gene fragments

Using specific primers, a total of three *V. destructor* gene fragments were amplified by polymerase chain reaction (PCR). The selected target fragments belong to genes involved in inhibition of apoptotic process (IAP), RNA polymerase I (RNA1), and Sodium-Potassium transporters (NaK) (Garbian et al., 2012). The expected lengths of PCR products in base pairs (bp) are 263 bp (IAP), 324 bp (RNA1) and 290 bp (NaK). PCRs were performed under the following reaction conditions: 92 °C for 5 min, 35 cycles of 92 °C for 1 min, specific annealing temperature for 40 sec, 72 °C for 1 min and 72 °C for 5 min. The obtained PCR fragments were purified using Inbio Highway Kit® and individually cloned in pGem-T vector (Promega), according to the manufacturer's guidelines. The cloned fragments were sequenced using T7 and Sp6 promoter primers to verify correct amplification. The fragments cloned in pGem-T vector were released by NotI restriction enzyme digestion (Thermo Fisher Scientific Co.) and individually ligated into the NotI restriction site of the dsRNA transcription vector pL4440 (Timmons et al., 2001). The pL4440 plasmid was provided by Addgene Inc. (Cambridge, USA). The resulting recombinant vectors (pL4440-NaK, pL4440-IAP, pL4440-RNA1) were introduced individually into HT115 (DE3) chemical competent bacteria (Caenorhabditis Genetics Center, MN, USA) (Timmons et al., 2001). A plasmid pL4440-GFP with green fluorescent protein (GFP) gene fragment was used as a control (Gamboa Cedeño et al., 2015).

DsRNA synthesis and feeding bioassays

Colonies of *E. coli* HT115 containing pL4440 recombinant plasmids were individually grown and IPTG-induced under the conditions described by Tian et al. (2009). The dsRNA expression in bacteria was verified by analyzing 1 ml of each cell culture. One

ml of each aliquot was centrifuged and resuspended in 200 μl of TRIZOL reagent (Invitrogen™) to obtain total RNA, according to the manufacturer's instructions. The extracted RNA was dissolved in 30 μl of distilled water and dsRNA expression product was confirmed by 1% agarose gel electrophoresis.

The production of dsRNA was achieved by growing *E. coli* HT115 colonies containing pL4440 recombinant plasmids in 100 ml IPTG-induced cultures, which were subsequently centrifuged at 10,000 rpm for 4 min. Pellets were resuspended individually in TRIZOL reagent (Invitrogen, USA) according to the manufacturer's instructions. The total RNA extracted from the pellets was diluted in distilled water and aliquots of 15 μl were separated by 1% agarose gel electrophoresis to verify dsRNA synthesis. The dsRNA concentrations were determined using image analysis software (Genetools, Synoptics Ltd). A 100-base pair (bp) molecular marker was used as a reference (Inbio Highway Co.).

Two bioassays were performed to evaluate separately the effect of different treatments on honey bee (bioassay I) and mite (bioassay II) survival. Worker bees and mites were obtained from colonies of the Italian bee, *Apis mellifera ligustica* Spinola, located at Instituto de Genética "E.A. Favret" (IGEAF-INTA), Hurlingham, Buenos Aires, Argentina. Both bioassays were prepared placing 30 newly-emerged worker bees in a flask (3 liters) where the bees were fed with 10 ml of solution containing water, 50% sucrose, and 400 μg/μl of mix dsRNA-NaK, dsRNA-IAP and dsRNA-RNA1 (dsRNA mix treatment). Two negative control treatments were performed: 1) a RNA control that consisted of 30 bees in a flask fed with 10 ml of solution containing water, 50% sucrose, and 400 ng/μl of dsRNA-GFP; and 2) a solution control, that consisted of 30 bees fed with 10 ml of solution containing water and 50% sucrose. Each of the three treatments (dsRNA-mix, dsRNA-GFP and sucrose) was performed in quintuplicate (5 flasks with 30 bees per flask) for bioassay I and in triplicate (3 flasks with 30 parasitized bees with 15 mites per flask) for bioassay II. All flasks contained small containers with honey. The flasks were kept in a breeding chamber (Sanyo, Versatile Environmental Test Chamber MLR-350) under controlled conditions at 34 ± 1 °C and 65 ± 5% relative humidity. Dead bees detected on the first day post-feeding were discarded. The treatment solutions were replaced every two days. The mortality of individual bees and mites were daily registered. The bioassays lasted four weeks. Comparisons of survival curves of Varroa and bees between treatments were performed by Log-rank (Mantel-Cox) Test and graphed using Graph Pad Prism version 5, GraphPad Software (San Diego California USA, www.graphpad. com). Mean longevity of mites was compared between treatments using one-way ANOVA (Infostat Software, version 2020) and significant differences were analyzed by Tukey Test (0.05% significance level).

Quantitative real-time RT-PCR

The effect of honey bee-mediated dsRNA administration on the steady-state transcriptional levels of Varroa target genes was evaluated on mites attached to bees fed with dsRNA solutions and compared to mites from control solutions. Total RNA was extracted from pools of five mites at 7 days post-feeding using Trizol reagent (Invitrogen, USA). Residual DNA was removed with RQ1 RNase-free DNase (Promega). The cDNAs were synthesized using the methodology described above. Standard curve calibration was generated using five-fold serial dilutions of already known cDNA controls to assess quantitative PCR (qPCR) performance. The cDNAs were diluted 1:50 in RNAse free water and qPCR was performed using 0.5 μl of diluted cDNA, 0.5 μl of each specific primer (10 mM), 5 μl of qPCR Master Mix (KAPA SYBR® FAST qPCR Kit) RNAse-free water. IAP primers were used to evaluate the gene expression level according to Garbian et al. (2012). Specific primers of house-keeping Varroa genes were q18S-F:5′AATGCCATCATTACCATCCT3′, q18-R: 5′CAAA AACCAATCGGCAATCT3′, qHSP 90-F 5′TTTGTAA CCGACACGAGCTG-3′ and qHSP90-R 5′TGTTGAGCGT GTGAAGAAGC-3′ (Campbell et al., 2016). The PCR program used for all samples was as follows: 94 °C for 2 min, 40 cycles of 94 °C for 15 sec, 55 °C for 15 sec, 72 °C for 20 sec followed by melting curve steps 95 °C for 15 sec, 60 °C for 15 sec and a final step at 95 °C for 15 sec. All qPCR experiments included three biological replicates per treatment (three pools of five mites each) and three technical repetitions per sample. The qPCRs were performed in Eppendorf Realplex2 equipment and the threshold cycle (Ct) values were calculated using Eppendorf software. The 18S and HSP90 were used as reference genes. The qPCR data were analyzed according to the method of Livak and Schmittgen (2001). Statistical analyses of the transcript's expression levels were performed using ANOVA followed by post-hoc Tukey's test with a 0.05% significance level for comparisons between treatments.

Results

In vivo dsRNA synthesis

To evaluate the effectiveness of dsRNA synthetized *in vivo* in regulating the Varroa gene target expression, we first amplified and cloned three DNA fragments into the plasmid pL4440 to synthesize the

specific dsRNA in the bacterial strain HT115 (DE3). Each HT115 synthesized the corresponding dsRNA in agreement with the expected molecular lengths from 500 to 600 bp, as evidenced by agarose gel electrophoresis and scanning digitization (Figure 1). The obtained dsRNA, whose concentrations ranged between 100 and 400 ng/μl, were used in subsequent feeding bioassays.

Survival analysis in bees and mites

To determine whether dsRNA synthesized *in vivo* produces unexpected effects on honey bees' survival, bioassays were performed by feeding individuals sucrose solutions containing dsRNA. As is shown in Figure 2 (left panel), differences in survival curves were detected between treatments (log-rank (Mantel-Cox) test: $\chi^2 = 28.70$, $p < 0.001$). Specifically, bees that received the dsRNA-GFP treatment exhibited a lower survival rate compared to bees exposed to the sucrose and dsRNA-mix treatments (log-rank (Mantel-Cox) test: control, $\chi^2 = 34.41$, $p < 0.001$; dsRNA-mix, $\chi^2 = 29.36$, $p < 0.001$). No significant differences in survival rates were observed between sucrose and dsRNA-mix treatments (log-rank (Mantel-Cox) test: $\chi^2 = 0.017$, $p = 0.894$), indicating

that dsRNA-mix administration has no off-target effect on bee survival.

Once we had demonstrated that the dsRNA-mix was not deleterious to bees, we proceeded to evaluate the effect of this and the control treatments on mite survival. The results showed that dsRNA-mix treatment decreased the survival rates of the Varroa mites (Figure 2, right panel). Specifically, survival rates of mites on bees fed with dsRNA-mix were significantly lower compared to those that received the sucrose treatment (log-rank (Mantel-Cox) test, $\chi^2 = 10.62$, $p = 0.001$). The same pattern was found when dsRNA-mix was compared with the dsRNA-GFP treatment, although the differences in mite survival were not significant (log-rank (Mantel-Cox) test, $\chi^2 = 1.31$, $p = 0.251$; Figure 2, right panel).

Results of Varroa longevity under different treatments were consistent with those obtained for survival curves (Figure 3). The results showed that the mean mite longevity was significantly lower for dsRNA-mix treatment (4.10 ± 0.40 days) than for sucrose treatment (7.05 ± 0.25 days) (post hoc comparisons from one-way ANOVA: $F_{2,111} = 5.78$, $p = 0.004$). Differences were not detected in mean mite longevity between dsRNA-GFP treatment (5.10 ± 0.30 days) vs. sucrose and dsRNA-mix treatments.

Gene level expression in the Varroa mites

To verify that mite survival differences registered in the bioassay II were associated with the Varroa genes knockdown, we performed a quantitative PCR (qPCR) experiment. This experiment consisted of assessing the relative expression level of one target gene in mites attached to bees that received the three feeding treatments: dsRNA-mix, dsRNA-GFP and sucrose. The relative expression level of IAP mRNA decreased 50% in Varroa mites attached to bees that ingested dsRNA-mix compared to the control treatments (Tukey's test, $p < 0.05$) (Figure 4).

The IAP mRNA transcript levels did not differ significantly between mites exposed to dsRNA-GFP and sucrose solution treatments. These results confirmed

Figure 1. Selected dsRNA expressed in bacteria. Total RNA was extracted from bacteria HT115 (DE3) containing the recombinant plasmids. MW: Molecular weight 100 base pairs (bp). Lines 2-3: RNA 1 dsRNA, line 4: pL4440 Plasmid empty (control), line 5: GFP dsRNA (control), lines 6-9: NaK dsRNA, line 10: IAP dsRNA. The asterisks indicate the dsRNA bands.
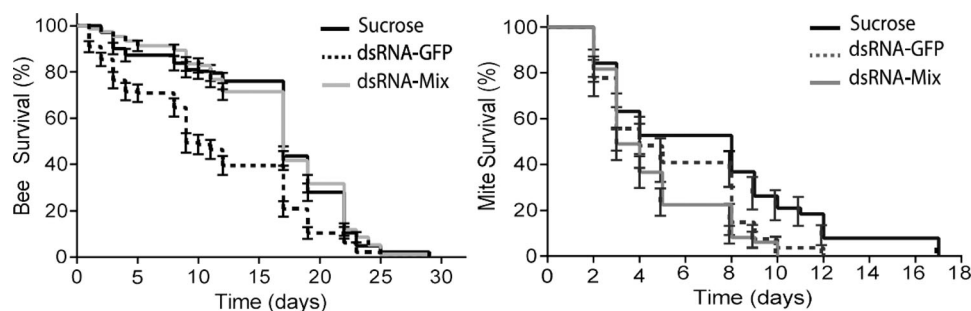

Figure 2. Left. Bioassay I: Survival curves of honey bees fed with the three different treatments: sucrose, dsRNA-GFP and dsRNA-mix. Right. Bioassay II: Survival curves of mites attached on bees that were fed with the three different treatments detailed above.

that bee-mediated transference of *in vivo* synthesized dsRNA is capable of inducing gene silencing in the parasitic Varroa mites.

Discussion

In a pioneer study, Garbian et al. (2012) demonstrated that dsRNA ingested by bees is transferred to the Varroa mite and from the mite to a parasitized bee. These authors evidenced that this reciprocal exchange of dsRNA between the bee and Varroa engendered targeted gene silencing in the latter, and resulted in an over 60% decrease in the mite population at the colony level. Here, we followed a similar protocol to those performed by Garbian et al. (2012), but using dsRNA synthesized *in vivo* and a lower number of Varroa target genes. We demonstrated that the ingestion of bacterially expressed dsRNA offers an efficient alternative to induce gene silencing in Varroa tissues and that the use of the present dsRNA mixture is sufficient to significantly reduce mite survival.
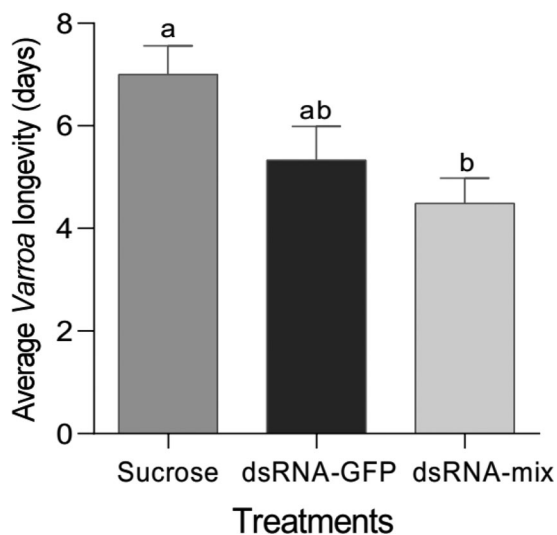

Figure 3. Average longevity of the Varroa mites parasitizing bee adults that were fed with three different treatments: Sucrose, dsRNA-GFP and dsRNA-mix. Different letters above columns indicate significant differences between treatments ($p < 0.05$) based on ANOVA analysis.
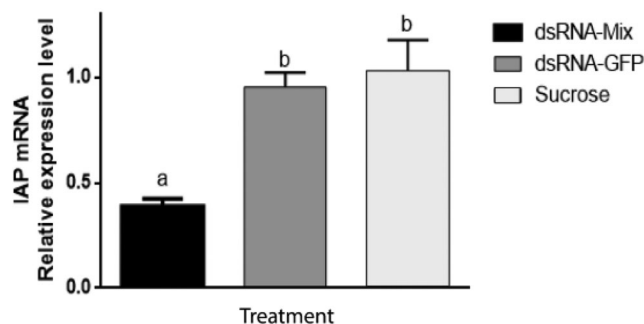
Previous reports have shown that using specific dsRNA synthetized *in vivo* by adapted bacteria is feasible to reduce the transcription levels of the target gene in different groups of organisms like plants (Yin et al., 2009) and insects (Leelesh & Rieske, 2020; Lü et al., 2021; Tian et al., 2009). In the honey bee *Apis cerana*, bacterially expressed dsRNAs were used to reduce viral infections (Zhang et al., 2016). Our experiments based on quantitative PCR and bioassays in *A. mellifera* evidence that dsRNAs synthetized in bacteria can induce an RNAi effect in Varroa, inhibiting expression levels of the target genes by 50%, and reducing mite survival by a similar percentage. Moreover, we demonstrate that oral administration of dsRNA synthetized *in vivo* to bee adults did not affect insect survival rate or longevity. Our results support that bacterially expressed dsRNA constitutes a simple, safe, less costly and efficient alternative to *in vitro* synthesis of dsRNA that could be used not only to control Varroa infestation in honey bee colonies but also for functional studies in mites by gene knockdown expression.

Contrary to expectations, we registered that the control treatment dsRNA-GFP affects the honey bee survival. A previous study showed that in specific cases, the application of dsRNA in honey bees is capable of producing unexpected expression knockdowns in non-targeted genes (Jarosch & Moritz, 2012). These authors evidenced gene silencing effects on transcript levels of off-target genes in bees treated with dsRNA-GFP. A posterior assay carried out by Nunes et al. (2013) demonstrated that the expression of target genes is affected by using dsRNA-GFP as control treatment. In our study and studies mentioned above, the dsRNA-GFP was specifically designed to have no sequence homology longer than 20 bp with genes described in the *A. mellifera* genome. Although dsRNA-GFP is not expected to trigger an RNAi response in treated bees, undesirable effects on gene expression, pupal pigmentation or developmental timing have been observed (Nunes et al., 2013). The mechanisms whereby dsRNA-GFP can produce a down-regulation

Figure 4. Relative gene expression of IAP mRNA in Varroa mites parasitizing adult bees that were fed with dsRNA-mix, dsRNA-GPF and sucrose treatments. The bars (± SE) headed by the same letter are not significantly different at $p < 0.05$ according to ANOVA analysis followed by Tukey's test.

on non-target genes remains unclear. Therefore, alternative dsRNA controls should be used in honey bee gene silencing experiments in order to avoid incorrect conclusions on RNAi-derived studies.

The selection of target genes to be silenced can significantly affect the efficacy of RNAi strategy in insects (Katoch et al., 2013). The use of dsRNA mixtures against multiple target genes offers an alternative to increase the lethal effect on prejudicial organisms. In a previous study, Garbian et al. (2012) evaluated the effect of dsRNA mixtures of 5 and 14 targeted Varroa genes that resulted in an increase of mite mortality and a reduction of their populations in honey bee colonies. We demonstrated that a dsRNA mixture against only 3 of these target genes is sufficient to produce a comparable decrease in survival rates of the treated mites. Moreover, knocking down of these three genes (IAP, RNA1 and NaK) caused a 50% reduction in Varroa survival, supporting the evidence that they play critical roles in mite survival. Further studies must be performed to evaluate the effect of this mixture on Varroa population at a colony level. It also remains to be tested whether a single gene, such as one of the three genes analyzed here, can be used to generate the same effect at individual and colony levels. For example, Campbell et al. (2016) demonstrated direct lethality to individual mites using a single target with dsRNA of a neural peptide (B-type allatostatin) that killed 54% mites compared to controls. This finding encourages future studies based on different target genes and strategies that will increase the efficacy of RNAi as a means of control.

The use of RNAi technology against *V. destructor* is a possible solution to reduce the negative effects of mite parasitism on honey bee populations. A practical or commercial use of mite control strategy based on gene silencing requires selecting the more effective target genes and an economical method for the large-scale production of dsRNA. Further studies, specifically at a colony level and throughout controlled field assays, that test different mixtures and doses, and monitor the mid-long effect of dsRNA treatment on Varroa and honey bee populations survival need to be performed in order to improve the use of RNAi strategy to potentially control this pest in the field.

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