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Gene Silencing of *laccase 1* Induced by Double-Stranded RNA in *Callosobruchus maculatus* (Fabricius 1775) (Coleoptera: Chrysomelidae) Suggests RNAi as a Potential New Biotechnological Tool for Bruchid's Control

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Abstract: Bruchids are the most important pests of leguminous seeds in the world. In this study, the focus was done on *Callosobruchus maculatus*, a serious pest of *Vigna unguiculata* seeds. As no efficient control methods preventing collateral effects on beneficials currently exist, this study investigated whether RNA interference (RNAi) could provide a new biotechnological and selective tool for bruchids control. Three principal objectives were followed including (i) the identification of all RNAi machinery core components and a key protein to silence in *C. maculatus* genome (*c.f.*, *dicer-2*, *argonaute-2*, *R2D2*, and *laccase 1*), (ii) the identification of suitable reference gene for RT-qPCR analyses, and (iii) the micro-injection of dsRNA coding for *laccase 1* to adults of *C. maculatus* to assess gene expression levels by RT-qPCR and potentially related mortalities. Phylogenetical analyses performed from transcriptomic information successfully identified all necessary proteins in the RNAi mechanism and also the open reading frame of *laccase 1* in *C. maculatus*. A new reference gene was identified (i.e., *alpha-tubuline 1*) and coupled with *glutiathone S transferase* for RT-qPCR analyses. Double-stranded RNAs coding for *laccase 1* and *green fluorescent protein* (control) were produced and 400 ng of each dsRNA were micro-injected into *C. maculatus* adults. RT-qPCR analyses revealed a stable significant decrease in *laccase 1* expression in about 80% of adults treated with *laccase 1* dsRNA after three days post-injection. No significant mortalities were observed which is probably related to the non-exposure of adults to anti-nutritional factors that are usually regulated by laccase. Further research should focus either on the feeding larval stage which is directly exposed to anti-nutritional factors, or on other target genes to induce dead phenotypes. This study is the first gene silencing report on a bruchid species and supports RNAi as a potential future method of control.

Keywords: RNAi; Bruchidae; Bruchinae; reference gene; RT-qPCR; RNA interference; dicer; argonaute; RISC

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1. Introduction

Bruchids (also commonly named "seed beetles") are severe pests of leguminous seeds causing important economic losses. These pests correspond to small Coleoptera belonging to the family of Chrysomelidae and to the subfamily Bruchinae, which encompass 1700 recorded species, out of which 30 species are of economic concern and nine species are distributed worldwide (including genera *Acanthoscelides*, *Bruchus*, *Callosobruchus*, *Caryedon*, and *Zabrotes*) [1–3]. *Callosobruchus maculatus* (Fabricius 1775), commonly named "the cowpea weevil", is a multivoltine species that infests seeds of Cowpea crops (*Vigna unguiculata* ((L.)Walp.—Fabaceae). This pest is particularly harmful in West Africa where 20 to 90% of beans are ravaged while constituting an essential food resource for local populations [4]. Damage is caused by seminovorous larvae which entirely develop inside seeds [5]. Females are able to oviposit on ripening pods in the field (i.e., primary infestation) and also on

stored seeds (i.e., secondary infestation). Howe and Currie (1964) reported a maximal fecundity of 97.2 eggs per female on average and the shortest development period lasts 23 days at 35 °C [6]. The thermal development of this pest is 526.3 degree-days with a developmental threshold of 10.4 °C [7]. Such characteristics are highly detrimental to the stored commodities because only two percent of primary infestation in the field leads to the total destruction of crop products after 6 months of storage [8].

The current management of *C. maculatus* in storage mostly relies on the use of chemical insecticides by fumigation, but technical and financial locks impede this technique which also presents well-known impacts on the environment, human health, non-target organisms (NTOs), and is faced with the emergence of insect resistances [9–12]. Alternatives such as essential oils and plant extracts were widely investigated but most studies demonstrated that these treatments were impacting beneficial insects such as chalcidoid parasitoids. In addition, their use was effective for a short duration which required repeated applications [13–16]. Innovative and selective methods of control must be investigated to find effective management strategies to minimize collateral effects on NTOs.

RNA interference (RNAi) is an emerging pest control method in integrated pest management (IPM) [17]. This biotechnological method relies on the introduction of exogenous double-stranded RNA (dsRNA) in insects that will decrease the expression level of a target gene via three possible pathways including the microRNA (miRNA), the piwiRNA (piRNA), and the small interfering RNA (siRNA) [17,18]. In the siRNA pathway, dsRNAs enter in insect cells (Figure 1) and are cleaved into siRNAs duplexes of 21–24 nucleotides (nt) by the enzyme *dicer-2* (*dcr-2*) [19,20]. SiRNAs are then incorporated into a multi-protein complex named the *RNA-induced silencing complex* (*RISC*) via the RNA-binding protein called *R2D2* [21,22]. One strand is degraded and the other strand guides the complex for homological recognition with the target gene transcript, i.e., the messenger RNA (mRNA) [23]. *Argonaute-2* (*Ago-2*) proteins, the core catalytic components of *RISC*, then degrade the mRNA preventing the translation into protein [24–26]. The prevention enzymes or protein production is expected to cause lethal effects of the targeted pest.

RNAi technology has been widely applied to insects [27]. The principal asset of this biotechnological control tool is the absence of collateral effects in NTOs as at least 19 nucleotide homology is required for activation of the RNAi pathway [28,29]. However, its success varies considerably among pests [27] and depends on intraspecific factors such as the presence of RNAi core machinery, i.e., *dicer-2, argonaute-2, and R2D2* [18], the length of dsRNAs [30], the presence of transmembrane proteins that allow entry of dsRNAs into cells [31], the targeted stage in insect life cycle [32,33], the targeted gene [34], and the method of dsRNA delivery [35]. Molecular mechanisms driving cellular uptake and systemic spread of silencing are the most impacting components on RNAi success [36].

Many herbivorous beetles have already been subject to RNAi studies including species from Chrysomelidae, Tenebrionidae, Coccinelidae, Curculionidae, Nitidulidae, Buprestidae, and Cerambycidae families [37]. Studies led on leaf beetles (Chrysomelidae) such as *Diabrotica virgifera* (J. L. LeConte 1868), *Leptinotarsa decemlineata* (Say 1824), *Phaedon cochleariae* (Fabricius 1792), or *Plagiodera versicolora* (Laicharting 1781) showed high RNAi efficiency via feeding on transgenic plants, diets containing naked dsRNA or bacterially expressed dsRNA [38,39]. However, no study has yet broached RNAi with bruchid species while the cancellation of key proteins and enzyme production in bruchids with RNAi could provide a pledging alternative facing limitations or current methods of control. Recently, Zhang et al. (2018) demonstrated that *laccase 1* (*lac 1*) was a polyphenol oxidase involved in the detoxification process of phenolic compounds in *Sitobion avenae* (Fabricius 1775) (Hemiptera: Aphididae) [40]. In the case of cowpea weevils, tannin content is also a potential biochemical defense against feeding larvae [41]. Avoiding the production of enzymes involved in bruchid polyphenol detoxification mechanisms, such as *lac 1*, would provide a new tool supporting a new biotechnological method of control.

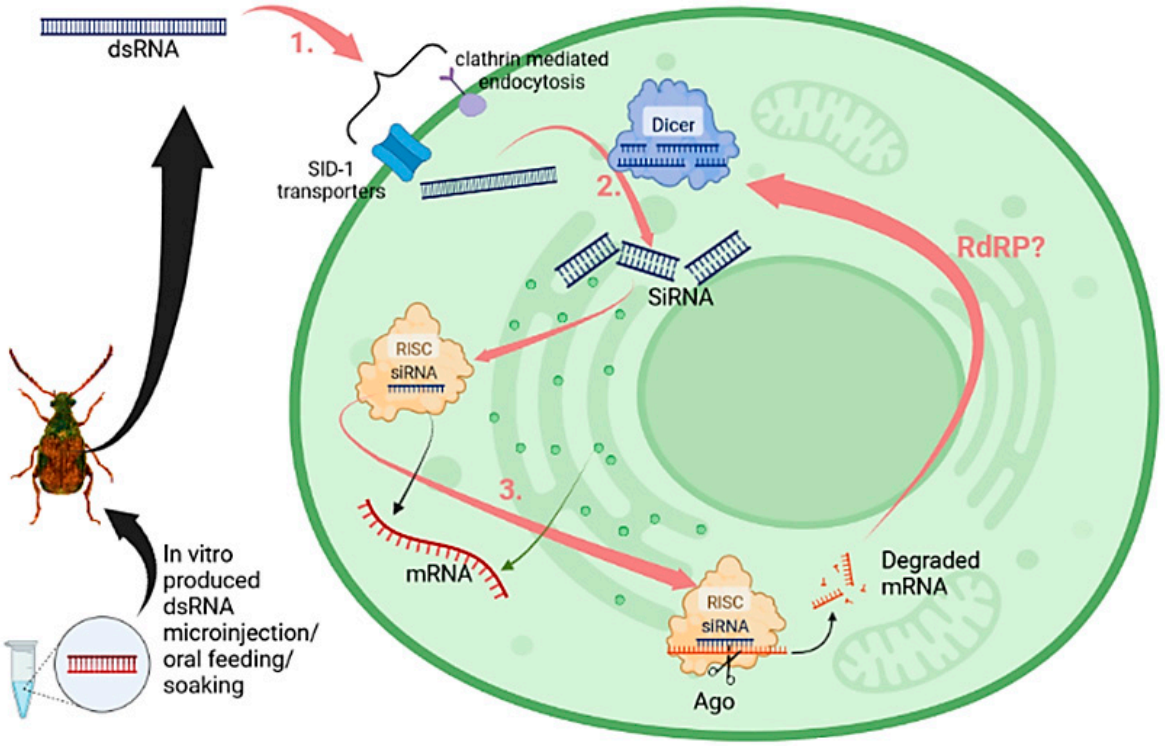

Figure 1. RNAi mechanism by siRNA pathway in insect cells and proteins involved in the degradation of double-stranded RNA into small interfering RNAs. The proteins involved in this pathway include *dicer-2* proteins that cleave exogenous dsRNAs, and the *RNA induced silencing complex* (RISC) containing *argonaute-2* and *R2D2* proteins for the homological degradation of the messenger RNA (mRNA). In some insects, the presence of *RNA-dependent RNA polymerases* (RdRp) is reported to retroactively amplify the siRNA mechanism. © A. Segers (created with biorender).

In this study, the RNAi mechanism and the gene silencing of *lac 1* were investigated as a potential new biotechnological tool of bruchid's control. As the RNAi systemic mechanism could not be conserved across organisms [17], three principal objectives were followed including (i) the identification of all RNAi machinery core components and a key protein to silence in the *C. maculatus* genome (*c.f.*, *dicer-2*, *argonaute-2*, *R2D2*, and *lac 1*), (ii) the identification of suitable reference gene for RT-qPCR analyses, and (iii) the micro-injection of *lac 1* dsRNA to adults of *C. maculatus* to assess gene expressions levels by RT-qPCR and potentially related mortalities. Results provided identifications and descriptions of all investigated proteins or protein complexes. A new reference gene for RT-qPCR analyses in adults of *C. maculatus* was highlighted (i.e., *alpha-tubuline 1*) and micro-injection experiments demonstrated that RNAi decreased significantly in the *lac 1* expression. This study is the first report of RNAi in a bruchid species which provides new insight into a potential method of control of these pests.

2. Materials and Methods

2.1. Identification of lac 1, dcr-2, ago-2, and R2D2 in C. maculatus

Sequences of *Dcr-2*, *Ago-2*, *R2D2*, and *lac 1* were identified in *C. maculatus* following a quite similar methodology as Zhao et al. (2015) [18]. First, protein queries in *Tribolium castaneum* (Herbst 1797) (Coleoptera: Tenebrionidae), a reference model of beetle, were performed and followed by translated basic local alignment search with tBLASTn on transcriptomic information of *C. maculatus* provided by El Sayadi et al. (2016) (E-value threshold: 1×10^{-10}) [42]. The sequence *Tcas-Dcr-2* (NP_001107840.1), *Tcas-Ago-2a* (NP_001107842.1), *Tcas-R2D2* (NP_001128425.1), and *Tcas-Lac-1* (NP 001034514.1) were searched against the Transcriptome Shotgun Assembly (TSA) database limited by *C. maculatus* (taxid: 64391). The

RNA sequences obtained were identified according to the E-value and the percentage identity and were subsequently assigned as *Dcr-2* (GEUD01209535.1), *Ago-2* (GEUH01006697.1), *R2D2* (GEUD01188481.1), and *lac 1* (GJDX01063393.1). These sequences were downloaded for manual trimming and truncation with Serial Cloner software (version 2.6.1). On the basis of these RNA sequences, corresponding *C. maculatus* proteins were generated.

Then, protein sequences of *C. maculatus* were submitted for the detection of domain architecture using the ScanProsite tool [43]. The proteins involved in the RNAi mechanism (*c.f.*, *Dcr-2*, *Ago-2,* and *R2D2*) were tested with the amino acid sequences from *T. castaneum*, *Agrilus planipennis* Fairmaire 1888 (Coleoptera: Buprestidae), and *Drosophila melanogaster* Macquart 1843 (Diptera: Drosophilidae). As there may be several forms of *dicer* (two forms) or *argonaute* proteins (three forms) [18], all possible forms were considered and phylogenetic analyses were performed to confirm that the sequence corresponded to *Dcr-2* or *Ago-2* following the clustering. Amino acid sequences used in these analyses are provided in Table 1.

Table 1. Accession number of amino acid sequences used for the analyses of the protein domain architecture concerning *Dcr-2*, *Ago-2,* and *R2D2* in *C. maculatus*.

	Callosobruchus maculatus		
	Cmac-Dcr-2 ***GEUD01209535.1***	***Cmac-Ago-2*** **GEUH01006697.1**	***Cmac-R2D2*** **GEUD01188481.1**
Tribolium castaneum	*Tcas-Dcr-1*, XP_968993.2 *Tcas-Dcr-2*, NP_001107840.1	*Tcas-Ago-1*, EFA09197.2 *Tcas-Ago-2a*, NP_001107842.1 *Tcas-Ago-2b*, NP_001107828.1 *Tcas-Ago-3*, XP_968053.2	*Tcas-R2D2*, NP_001128425.1
Argillus plannipennis	*Apla-Dcr-1*, AJF15702.1 *Apla-Dcr-2*, AJF15703.1	*Apla-Ago-1*, AJF15704.1 *Apla-Ago-2*, AJF15705.1 *Apla-Ago-3*, AJF15706.1	*Apla-R2D2*, AJF15707.1
Drosophila melanogaster	*Dmel-Dcr-1*, AAF56056.1 *Dmel-Dcr-2*, NP_523778.2	*Dmel-Ago-1*, AAF58314.1 *Dmel-Ago-2*, AGB94575.1 *Dmel-Ago-3*, NP_001163498.1	*Dmel-R2D2*, NP_609152.1

Concerning the identification of *lac 1*, the two forms of insect laccases (*laccase 1* and *laccase 2*) were considered in seven species for which sequences have been previously listed. A phylogenetic analysis was performed to confirm whether the putative sequence considered in *C. maculatus* fitted with *laccase 1* [44]. The sequence *Cmac-Lac1* (GJDX01063393.1) was tested with *T. castaneum* (*Tcas-Lac1*, NP 001034514.1; *Tcas-Lac-2*; NP 001034487.2), *Absolus verrucosus* (LeConte 1852) (Coleoptera: Tenebrionidae) (*Aver-Lac1*, RZB39173.1; *Aver-Lac-2*, RZC35935.1), *Monochamus alternatus* Hope 1843 (Coleoptera: Cerambycidae) (*Malt-Lac1*, ATI08981.1; *Malt-Lac-2*, ABU68466.1), *Apis mellifera* L. 1758 (Hymenoptera: Apidae) (*Amel-Lac1*, XP 026295929.1; *Amel-Lac-2*, BAJ06133.1), *Manduca sexta* L. 1763 (Lepidoptera: Sphingidae) (*Msex-Lac1*, AAN17506.1; *Msex-Lac-2*, AAN17507.1), *Nephotettix cincticeps* Uhler 1896 (Hemiptera: Cicadellidae) (*Ncin-Lac1*, BAJ06132.1; *Ncin-Lac-2*, BAJ06133.1) and *Anopheles sinensis* Wiedemann 1828 (Diptera: Culicidae) (*Asin-Lac1*, KFB43437.1; *Asin-Lac-2*, ARG47519.1). After the identification of *lac1*, pair of primers were designed for the amplification of the gene from the cDNA of *C. maculatus* (Table 2). Electrophoresis gel migrations and a Sanger sequencing were performed on PCR products by Eurofins Genomics (Germany) and confirmed the *lac 1* gene identity (data not shown).

The ORF of *C. maculatus lac 1* was deduced with the *ORF finder* (https://www.ncbi.nlm.nih.gov/orffinder/, accessed on 24 November 2022) tool. The deduced protein sequence of amino acids was analyzed with the *Protein Molecular Weight* (https://www.bioinformatics.org/sms/prot_mw.html, accessed on 24 November 2022) tool and the Cu-oxidase Pfam domains were predicted with the *SMART* (http://smart.embl-heidelberg.de/, accessed on 24 November 2022) tool.

Table 2. Primers used for laccase 1 and GFP gene amplification.

Gene	Primers	Sequence (5′ to 3′)	Amplicon Size (bp)
lac 1	Lac1-CM-F	ATT CCT GTT TTA AAT AAT TTG ATG ACA TG	2433
	Lac1-CM-R	TTG ATG TGT CAC TGT GTT TCT	
T7—*lac 1*	Lac1-CM-T7-F′	TAA TAC GAC TCA CTA TAG GG TGT CTT TGC TTC CGT TCC C	588
	Lac1-CM-T7-R′	TAA TAC GAC TCA CTA TAG GG CGT GAT GCT CTA TTG CTT TCC	
T7—*gfp*	GFP-T7-F′	TAA TAC GAC TCA CTA TAG GG GCC AAC CTT AGT CAC TAC TTT C	542
	GFP-T7-R′	TAA TAC GAC TCA CTA TAG GG TGG GTA ATA CCA GCA GCA G	

Phylogenetic analyses were performed using MEGA *version X* and MUSCLE alignment [45,46]. The appropriate model was determined for each of the sequence sets prior to the tree construction and were « LG+G+F » for *Dcr-2*, « LG+G+I » for *Ago-2*, and « LG+G » for *lac 1*. The Maximum Likelihood (ML) method with partial deletion was used to construct phylogenetic trees. The Bootstrap value was fixed at 1000 replicates.

2.2. DsRNA Synthesis

Two types of dsRNA were produced for micro-injection experiments. The DsRNA coding for the *C. maculatus laccase 1* was synthesized to induce the decrease in *lac 1* expression and dsRNA coding for the *green fluorescent protein* (*gfp*), was synthesized to ensure the specificity of the RNAi mechanism on the target (i.e., cellular mRNA of *lac 1*) that should not impact the expression level of *lac 1*. *Green fluorescent protein* amplicons of 542 nt containing a T7 promoter sequence were obtained by PCR using star plasmids (see primers in Table 2) and the kit Q5® High-Fidelity PCR (New England Biolabs, Inc). *laccase 1* dsRNAs were produced by extracting total RNA from adults of *C. maculatus* with the kit RNeasy® (Qiagen, Chats-worth, CA, USA). Total RNA was checked for quality and quantity with a Nanodrop spectrophotometer. Retrotranscription was then performed with the kit High-Capacity cDNA reverse Transcription (Applied Biosystems, CA, USA) to obtain total cDNA. *laccase 1* amplicons of 588 nt containing a T7 promoter sequence were finally obtained by PCR using a Q5® High-Fidelity PCR kit (see primers in Table 2). PCRs were performed with an initial denaturation cycle of 30 s at 98 °C, followed by 35 cycles of denaturation at 98 °C for 10 s, annealing for 30 s at 54 °C (amplification of *gfp*) or 64° (amplification of *lac 1*) and extension at 72 °C for 30 s. The final extension was maintained at 72 °C for 2 min. The size and the quality of the amplicons were checked by electrophoresis gel migration (1% agar), then purified with the Nucleospin® kit (MACHEREY-NAGEL GmbH & Co. KG, Valencia). The synthesis of dsRNAs was carried out from purified PCR products with the MEGASCRIPT® RNAi kit (Invitrogen, Waltham, MD, USA). Around 1 µg of template DNA was used in 20 µL of the in-vitro transcription mix that was then incubated for 6h at 37 °C, before being treated for 15 min with DNAse/RNase and purified. DsRNAs were stored in an elution buffer (10 mM Tris-HCl pH 7, and 1 mM EDTA). The quality, size, and quantity of dsRNAs were also checked by NanoDrop spectrophotometer, then by electrophoresis gel migration (1% agar), and stored at −20 °C until micro-injection.

2.3. Micro-Injection Experiments

Adults of *C. maculatus* that were subjected to dsRNA micro-injections were reared on *V. unguiculata* seeds at 27 ± 2 °C and 60 ± 10% relative humidity in total darkness. These insects came from Lincoln University (UK). Emerging adults were immediately collected from the rearing and were distributed in 10 × 10 × 5 cm boxes for micro-injection experiments.

Micro-injections were performed under a dissecting stereomicroscope (Euromex DZ series, Euromex microscope bv, Arnhem, The Netherlands) using capillaries with a 10 mm

long tip and 500 µm outer diameter designed from 1.0 mm × 0.50 mm BF100-50-10 silica tubes (model P-97 Flaming/Brown Micropipette Puller, Sutter Instrument Company; program 0: heat = 555, pull = 150, time = 250, pressure = 500). These capillaries were mounted on a micropump (Nanoliter 2010, World Precision Instruments, Inc.) that was connected to a flow controller (Micro4™, World Precision Instruments, Inc.). Insects were microinjected at the pygidium with 400 nL of dsRNA solution at a rate of 200 nL/s and a concentration of 1000 ng/µL, i.e., 400 ng of dsRNA were administered per insect. These insects were anesthetized for 30 s with CO_2 and then maintained on an adhesive surface cooled on crushed ice to maintain them immobile during micro-injections (Figure 2). Three treatments were performed on 50 insects: dsRNA of *laccase 1*, dsRNA of *gfp*, and a control consisting of micro-injection of the elution buffer (10 mM Tris-HCl pH 7, and 1 mM EDTA) to correct mortalities induced by micro-injections and to compare the gene expression profiles of *lac 1*. Three additional batches of 20 insects were subjected to the same micro-injection treatments to assess mortalities. Dead adults were recorded every day of post-injection for seven days.

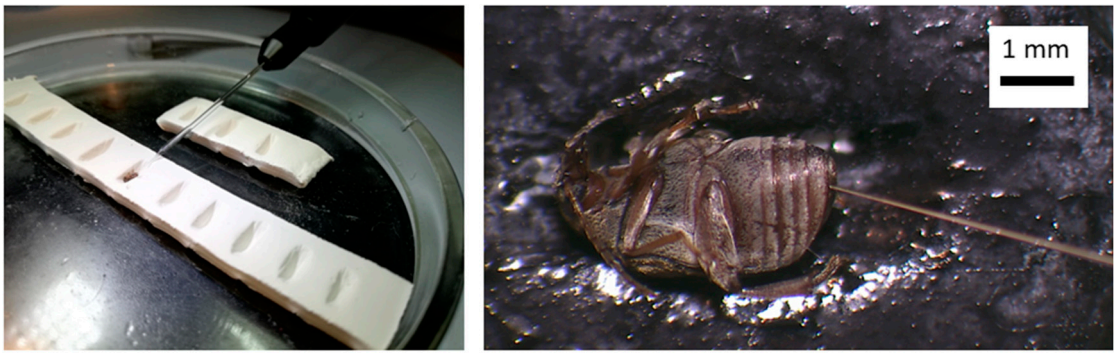

Figure 2. Micro-injection dispositive for the administration of 400 nL of dsRNA/elution buffer at the pygidium of *C. maculatus*.

Micro-injected insects were maintained at 23 ± 0.1 °C during the entire experimental period. Samples of three adults were polled as one biological replicate every 24 h after the injection (i.e., day post-injection, dpi). Three biological replicates were sampled in each treatment per dpi. Sampled insects were flash-frozen in liquid nitrogen and stored at −80 °C for RNA extraction and retrotranscription. A summary diagram of the methodology used in the micro-injection experiments and the statistical analyses performed is presented in Appendix A.

2.4. Reference Gene and RT-qPCR Analyses

Gene expression analyses were performed from reverse transcripted RNA by RT-qPCR to examine the gene expression profiles of *lac 1*. First, validation of reference genes, also named endogenous control or housekeeping gene (HKG), was necessary to normalize gene expression across samples that may present different levels of mRNAs due to methodological variations instead of biological causes [47]. These reference genes are generally related to cellular functions such that their expression level is constant and independent of biotic variations such as the sex, the developmental stage, or the age of individuals and independent of abiotic factors such as temperature, photoperiod, or dsRNA treatments [48].

As no reference genes were already identified in *C. maculatus* at the setting up of experiments, the stability of three reference genes in adults was investigated: *arginine-kinase* (*arg-K*; GEUF01011058.1), *alpha-tubulin1* (*tuba1*; GEUH01049608.1), and *beta-actin* (*bactin*—GEUH01052590.1). Their validation followed MIQE guidelines and a stepwise process for producing quality and reproducible data [49,50]. Principal criteria necessary for the primer validation of HKGs and the target gene were (i) the relative efficiency of the primers (determined according to a regression line conducted on serial dilutions) must be between 90 and

110% [51], (ii) the absence of primer dimer in the melting curves to confirm the specificity of amplification. The validation of HKGs was then performed after stability tests by comparing the gene expression in seven samples that differed from age (*c.f.,* biotic condition) and from dsRNA exposure (*c.f.,* abiotic condition). The sample for stability test according to the age consisted in the pooling of three adults of the same age, from one to seven days old. Samples for stability test according to dsRNA exposure were three pooled adults from different dsRNA exposures (no dsRNA, 100 ng, 200 ng, 400 ng of microinjected *lac 1* dsRNA; 100 ng, 200 ng, 400 ng of microinjected *gfp* dsRNA). The GeNorm algorithm was used to characterize the stability of HKG expression [52]. Brar et al. (2022) completed the ongoing research in the selection of reference genes by suggesting *gluthiatone S-transferase* (gst) and *tata binding protein* (*tbp*) as stable HKG in adults of *C. maculatus* [53].

Quantitative PCRs were performed with TB Green® Premix Ex Taq™ kit (Takara Bio Inc., Mountain View, CA, USA) and were analyzed with the cfxMaestro™ software of the Bio Rad C1000 touch™ thermocycler, with two technical replicates. One RT-qPCR reaction contained 12.5 µL of TB Green Premix Ex Taq, 0.75 µL of each primer (10 µM), 2 µL of 25 ng/µL retro-transcripted cDNA, and 9 µL of nuclease-free ddH_2O. Thermal cycles consisted of initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 5 s, annealing at 60 °C for 30 s, elongation at 72 °C for 30 s, and finalized at 95 °C for 30 s before generating melting curve to check the presence of non-specific products or primer dimers in *No Template Controls* (60 °C for 5 s to 95 °C with an increment of 0.5 °C/s).

2.5. Statistical Analyses

Relative gene expression analyses were based on the method of Vandesompele et al. (2002) using the geometric mean of two HGK Cq values for normalization [52]. For statistical analyses, differences in the *lac 1* expressions (from *gfp* and *lac 1* treatments) were assessed with an unpaired *t*-test relative to the control group (*p*-value of 0.05). This test was based on the log2 converted expressions that were tested for normality with a Shapiro–Wilk Normality test.

Mortalities of each treatment were assessed with median lethal times (LT_{50}) that were estimated with a non-parametric method (Kaplan–Meier) attributing survival probability from observed survival time [54]. Survival curves obtained were then compared with a non-parametric equality test approximately distributed as a Chi-squared test (log-rank test), to check for significant differences between treatments and control. All these statistical treatments were performed with RStudio software *version 1.3.959*, using packages "*survival*" and "*survminer*".

3. Results

3.1. Identification and Description of RNAi Core Machinery (dcr-2, ago-2, and R2D2) in C. maculatus

Putative sequences of RNAi proteins were identified in *C. maculatus* after tBLASTn of *dcr-2*, *ago-2,* and *R2D2* proteins with *T. castaneum* followed by phylogenetic analyses considering all potential forms of these proteins (i.e., homolog sequences) in *A. plannipennis*, *D. melanogaster,* and *T. castaneum* to confirm that the putative sequences match to the RNAi proteins.

Phylogenetical trees showing similarities of the putative *C. maculatus* sequence with *dcr-2* and *ago-2* proteins of other insect species are presented in Figure 3. These trees highlight that the predicted *C. maculatus dicer* protein sequence is clustered with the *dcr-2* protein of *T. castaneum*, *A. planipennis,* and *D. melanogaster* with the maximal bootstrap value support, confirming the *dcr-2* homology (Cmac-Dcr-2). Concerning *ago- 2*, the sequence Cmac-Ago-2 is distantly related to the homolog sequences of *ago-1* and *ago-3*. Moreover, it forms a subclade with the *ago-2* sequences from the Coleoptera species. These phylogenetic analyses are in line with the tBLASTn search results.

Alignment and architecture analyses of protein domains performed with the scan Prosite tool are presented in Figure 4. Domain architecture of the putative *R2D2* highlighted a protein sequence of 321 amino acids including two dsRNA binding domains (DSRB). The prosite profile hit score and the positions of DSRB domains are more similar to Coleoptera species rather than *D. melanogaster*. Concerning the protein complex *dcr-2*, a complex of 1597 amino acids was

identified which includes two amino-terminal helicase domains, a *dicer* dsRNA binding fold domain, two carboxy-terminal RNAseIII, and a *PAZ* domain. This architecture is also like other Coleoptera species and differs from *D. melanogaster* which includes an additional domain of carboxy-terminal dsRNA binding. The *ago-2* protein complex identification corresponded to a sequence of 803 amino acids including a *PAZ* domain and a *PIWI* domain.

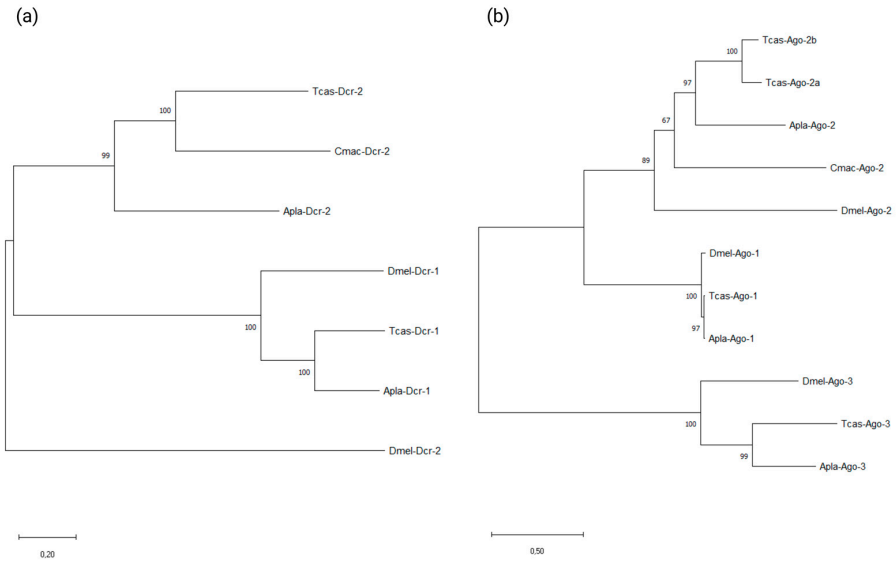

Figure 3. Maximal likelihood inferred phylogeny of *dicer* proteins (**a**) and *argonaute* proteins (**b**) and clustering of putative sequences of Cmac-Ago-2 (accession n° GEUH01006697.1) and Cmac-Dcr-2 (accession n° GEUD01209535.1) with RNAi protein complex of other insects.

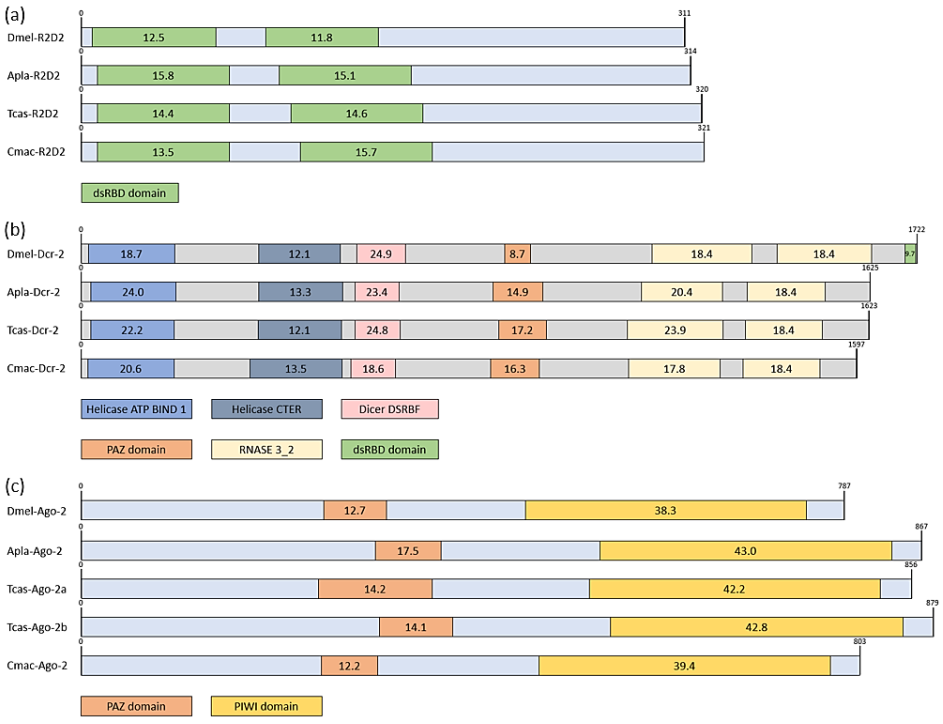

Figure 4. Domain architecture analyses of RNAi core components according to the putative proteins complex identified in *C. maculatus* and alignments performed with *D. melanogaster*, *A. plannipennis*, and *T. castaneum*. (**a**) *R2D2* proteins with localization of *dsRBD* domains; (**b**) *dicer-2* protein complex including multiple proteic domains of *helicase ATP Bind1*, *helicase CTER*, *DSRBF*, *PAZ*, *RNAse*, *and dsRBD*; (**c**) *argonaute-2* protein complex including domains *PAZ* and *PIWI*.

3.2. Identification and Description of the laccase 1 Protein in C. maculatus

The complete cDNA sequence of the *lac 1* gene identified in *C. maculatus* (accession number GJDX01063393.1) corresponded to a gene of 2803 *bp* including a coding sequence of 2064 *bp* which corresponds to a protein of 688 amino acids with a predicted weight of 79.01kDa (Figure 5).

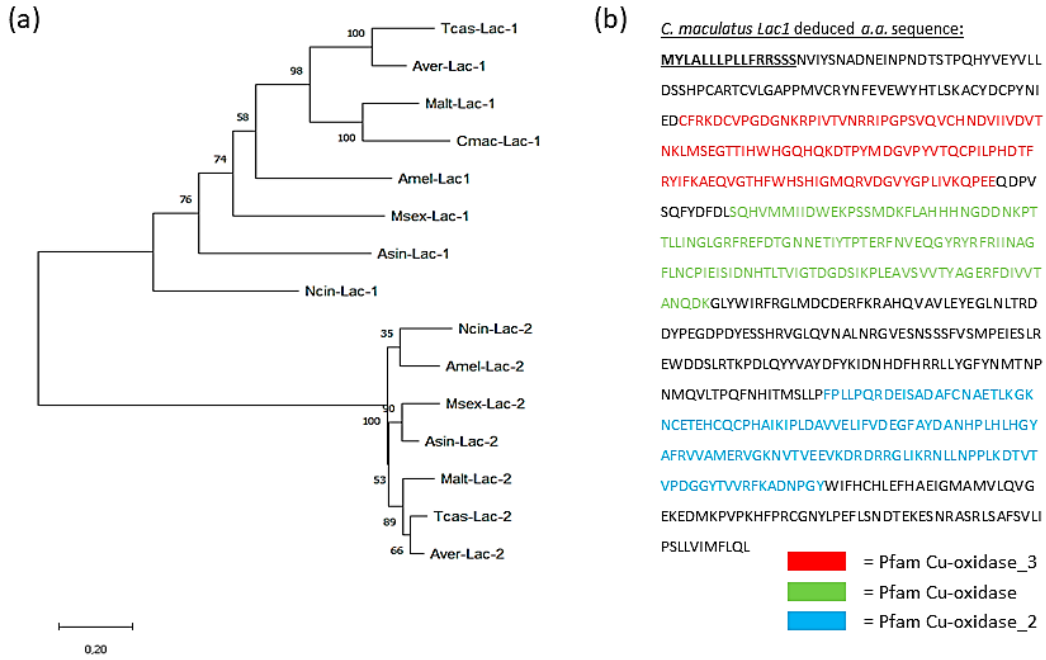

Figure 5. Maximal likelihood inferred phylogeny of *lac 1* (**a**); and *lac 1* amino-acids sequence (**b**) deduced from GJDX01063393.1 highlighting three copper domains TI (*Pfam Cu-oxidase* in green), T2 (*Pfam Cu-oxidase_2* in blue), and T3 (*Pfam Cu-oxidase_3* in red) and the putative signal peptide predicted (bold and underlined).

Phylogenetic analyses following the maximum likelihood method led on this sequence (Figure 5a) confirmed that it corresponded to *la c1* with the discrimination of two clades corresponding to the two forms of laccases as expected. In Coleoptera, Tcas-Lac-2 and Aver-Lac-2 form a subclade corresponding to the Tenebrionidae family while Malt-Lac-2 form another subclade. The same pattern is observed concerning the clade of *laccase 1*. Cmac-Lac-1 and Malt-Lac-1 are clustered together with the maximal bootstrap value.

The full length of the deduced amino acid sequence and domain architecture are provided in Figure 5b. The architecture of protein domain analyses performed on the *C. maculatus lac 1* revealed that three typical Cu-oxidase domains were present, including a type 1 (T1), a type 2 (T2), and a type 3 (T3) copper domain of respectively 158 amino acids (from 219 to 376), 152 amino acids (from 485 to 636) and 118 amino acids (from 89 to 276). The predicted N–terminal signal peptide identified included 16 amino-acid residues. No transmembrane domain was found.

3.3. Validation of Primers and Reference Gene for qPCR Analyses

Several sets of primers were evaluated during the validation tests to select gene for RT-qPCR analyses, including the target gene (*lac 1)*, the potential HKG (*tuba1*, *arg-K,* and *bactin*) and the two HKG identified by Brar et al. (2022) (*tbp* and *gst*). Validated primers and relative efficiency results are presented in Table 3. Melting curves highlighting their specificity are presented in Figure 6. Validated HKGs primers corresponded to genes of *tuba1*, *arg-K,* and *gst*. The primers of *tbp* presented primer dimer in melting curves and a relative efficiency out of the acceptance range (RE > 110%). None of the primers tested for *bactin* were validated because of primer dimer and/or relative efficiencies out of the acceptable range.

Table 3. Description of validated primers for the target gene (*lac 1*) and for the reference gene (*tuba1*, *arg-K*, *tbp*, and *gst*). Parameters of the standard curve generated from amplifications on serial dilutions are presented. RE = relative efficiency; R^2 = determination coefficient of the linear regression.

Gene Name	Gene Symbol	Accession Number	Primers	Sequence (5′ to 3′)	Amplicon Size (bp)	Melt. Temp.	RE	R^2	Slope	y Intercept	Primer Dimer
Alpha-tubulin1	*tuba1*	GEUH01049608.1	Tuba1 F1 Tuba1 R1	TGC ATC ACT AGC TTT TCT GAA CAA TTC CCA GCA GGC ATT AC	149	80.5 °C	97.8%	0.997	−3.375	22.147	No
Arginin-kinase	*arg-K*	GEUF01011058.1	ArgK F23 ArgK R2	ATT TGA CCT TCT GCC CGA CC CCT GCA AGT TGA ACT GTC CC	123	84 °C	108.5%	0.993	−3.113	30.981	No
Tata binding protein	*tbp*	GEUH01047165.1	TBP F1 TBP R1	TTG CTC ACA ACG CAA GTA GG TCG CCT GCA AGT CTT TCA TA	103	83 °C	**117.6%**	0.991	−2.962	39.977	Yes
Gluthiatone-S-transferase	*gst*	GEUE01064616.1	GST F1 GST R1	CAG TCC CTG TCA AGA GCA CA TGC ATG GAG TGC AAT TCC TA	120	82 °C	108.7%	0.999	−3.129	40.815	No
laccase 1	*lac 1*	GJDX01063393.1	Lacc F3 Lacc R3	ACA CAA GCA CCC CTC AAC AT GAA GCT GTA CCG ACA CAC CA	110	84.5 °C	107.5%	0.998	−3.154	43.238	No

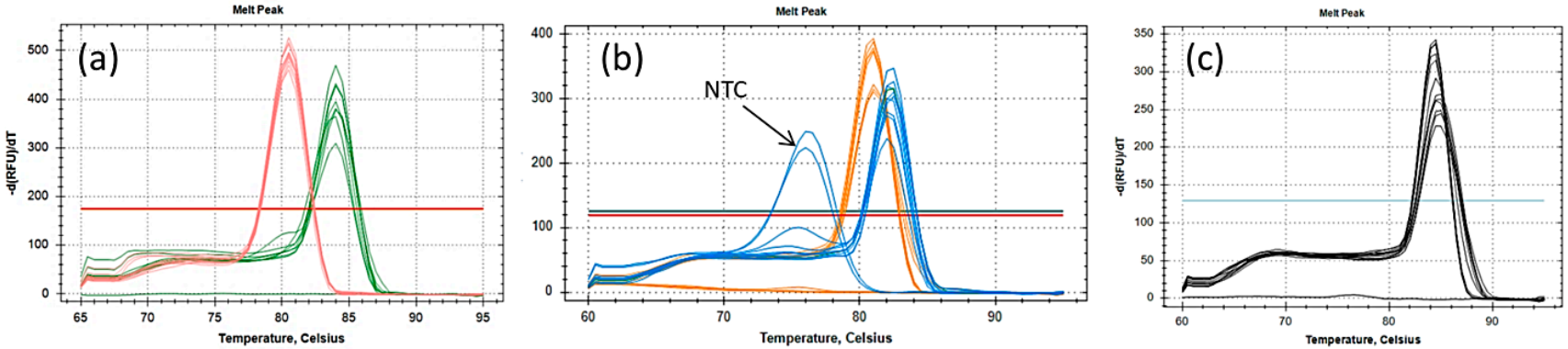

Figure 6. Melting curves associated with the amplifications performed in the serial dilutions during the primer validation showed amplification in *No Template Control* (NTC). (**a**) Melting curves of the gene *tuba1* (pink) and *arg-K* (green); (**b**) melting curves of the gene *tbp* (blue) and *gst* (orange); and (**c**) melting curves of the gene *lac 1*.

Based on the two identified HKGs (*c.f.*, *tbp* and *gst*) and validated primers of *tuba1* and *arg-K*, further tests were performed to assess the stability of their expression in samples of different ages and different dsRNA treatments. Figure 7 provides the mean coefficient of stability (M-value) based on the cq-values of each tested sample (n = 7). Genes of *tbp*, *gst*, and *tuba1* presented ideal stability with an M coefficient <0.5 which means that these genes of reference present a minimal variation across tested samples (in both biotic and abiotic variations) and may therefore be selected for RT-qPCR analyses. The *arg-K* gene was less stable (0.5 > M-value > 1) and presented moderate variations in the tested samples. Following these results of primer validation and of stability assessment, two reference genes were selected for RT-qPCR analyses, including the *tuba1* gene, a new HKG in *C. maculatus*, and the *gst* gene identified by Brar et al., 2022.

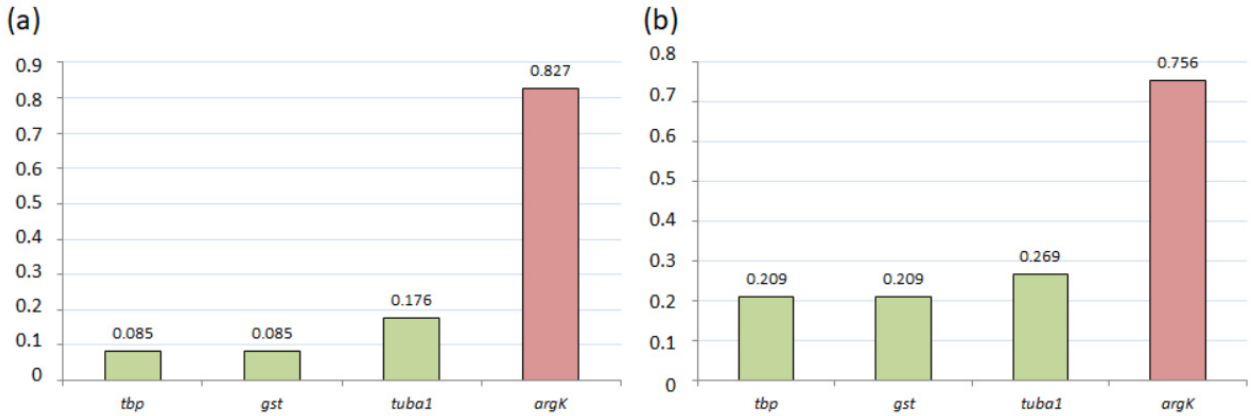

Figure 7. Average M values computed from stability tests (algorithm GeNorm) according to the age (**a**) and according to dsRNA treatments. (**b**) Ideal reference genes (green) are *tbp*, *gst*, and *tuba1*. The acceptable reference gene (red) is *arg-K*.

3.4. Gene Expression Analyses and Survival Curves

To test the RNAi-mediated gene silencing of *lac 1* in *C. maculatus*, 400 ng of dsRNA (400 nL at 1 µg/µL of dsRNA) coding for a fragment of 588 bp of *lac 1* were micro-injected in freshly emerged adults. The same amount of dsRNA coding for a fragment of 542 bp of *gfp* was injected in parallel to check the specificity of the gene silencing mechanism as this dsRNA treatment would not impact the expression of *lac 1*. The effect of micro-injections on mortalities as well as the consideration of a reference group for the quantification of the *lac 1* expressions (*c.f.*, *lac 1*, and *gfp* dsRNA treatments) was assessed via the micro-injection of 400 nL of elution buffer without dsRNA (i.e., the control group). The expression profiles of these three treatments and the statistical test for the assessment of differences in *lac 1* expression are respectively presented in Figure 8 and in Table 4.

A decrease in the *lac 1* expression is observed in both *gfp* and *lac 1* dsRNA treatments when compared with the control group during the three days of post-injection (dpi). Significant differences are observed in *lac 1* dsRNA treatment at two dpi (p-value = 0.022) and at three dpi (p-value < 10^{-6}). The expression of *lac 1* in *gfp* dsRNA treatment is also statistically different from the control group at three dpi (p-value = 0.004), where the expression of *lac 1* is decreased by around 40%. However, the *lac 1* expression in the *gfp* dsRNA treatment increases at five dpi (115%) while the expression in the *lac 1* dsRNA treatments remains stably decreased at around 80% during the rest of the experiment. This highlights a stable and specific decrease in the *lac 1* expression, which was induced by micro-injected dsRNA on adults of *C. maculatus*.

Table 4. Relative expression of *laccase 1* in different treatments (*c.f.*, biological groups) and statistical comparison with the control group ("*" indicates significance levels).

Dpi	Target Gene	Biological Group	N Samples	Expression	Lower Error Bar	Upper Error Bar	*p*-Value (*t*-Test)
1 day	*lac 1*	Control	3	1.00	0.85	1.18	
1 day	*lac 1*	gfp	3	0.78	0.69	0.89	0.109
1 day	*lac 1*	Laccase	3	0.85	0.59	1.23	0.534
2 days	*lac 1*	Control	3	1.00	0.68	1.47	
2 days	*lac 1*	gfp	3	0.67	0.56	0.79	0.245
2 days	*lac 1*	Laccase	3	0.35	0.22	0.56	0.022 *
3 days	*lac 1*	Control	3	1.00	0.98	1.02	
3 days	*lac 1*	gfp	3	0.67	0.59	0.75	0.004 **
3 days	*lac 1*	Laccase	3	0.24	0.23	0.25	$<10^{-6}$ ***
4 days	*lac 1*	Control	3	1.00	0.64	1.56	
4 days	*lac 1*	gfp	3	0.60	0.47	0.77	0.163
4 days	*lac 1*	Laccase	3	0.21	0.19	0.23	0.004 **
5 days	*lac 1*	Control	3	1.00	0.77	1.30	
5 days	*lac 1*	gfp	3	1.15	1.04	1.27	0.646
5 days	*lac 1*	Laccase	3	0.27	0.23	0.31	0.012 *

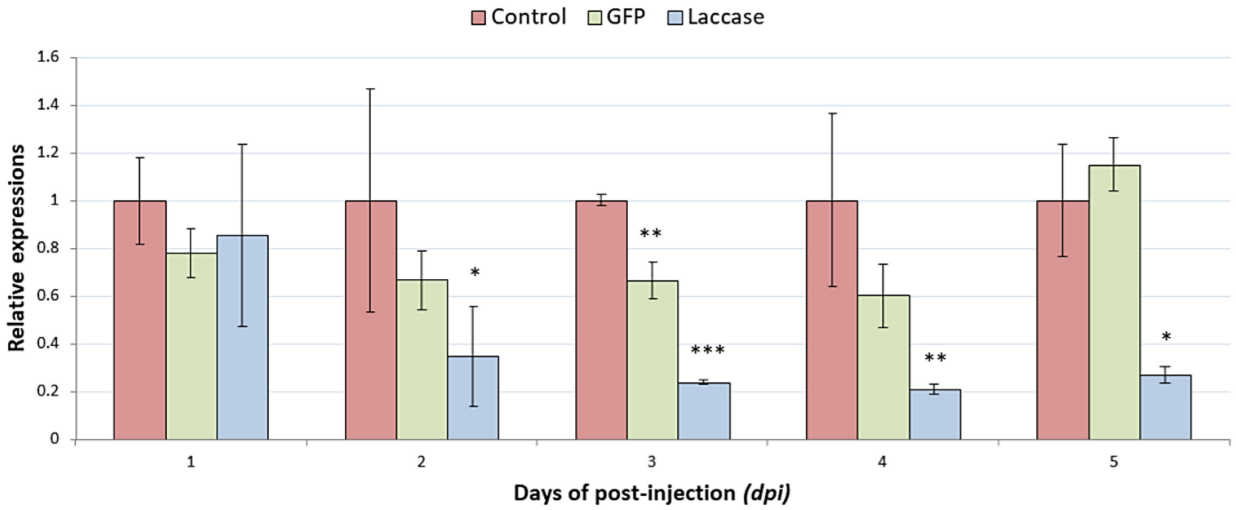

Figure 8. Evolution of the *lac 1* expression in different treatments of the micro-injection experiment. Red = control group; green = *gfp* dsRNA treatment; blue = *lac 1* dsRNA treatment.

No significant mortality was observed in *lac 1* treatments despite the decreasing *laccase* expression (Figure 9). The LT50s were not reached in all treatments, and survival curves led to Kaplan–Meier estimations of survival probabilities that did not differ statistically according to the log-rank test (Pval = 0.44).

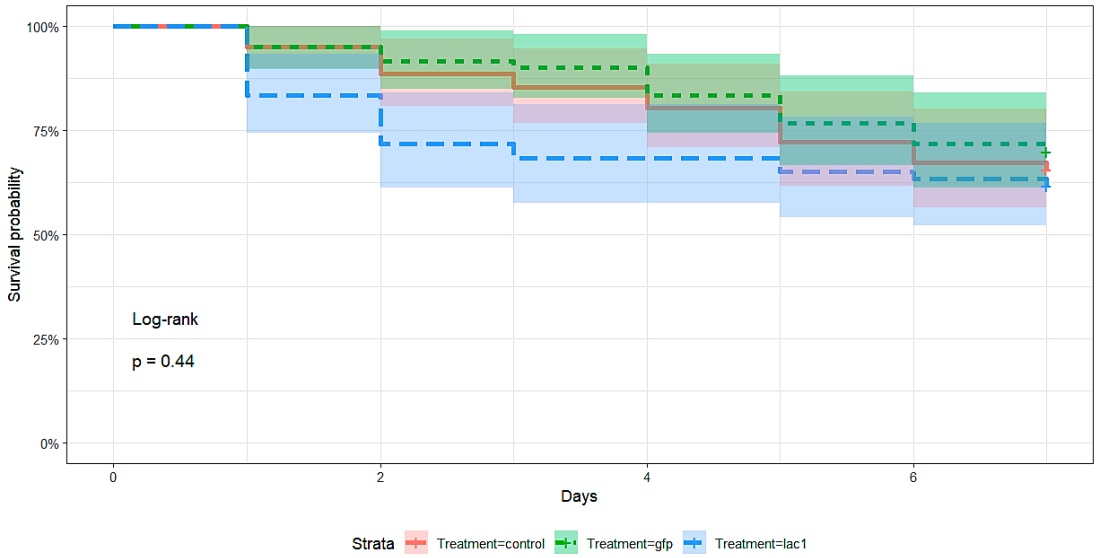

Figure 9. Survival curves highlighting survival probabilities (%) with days of post injection (*dpi*) of *C. maculatus* and log-rank test computed from mortalities recorded in the control (red), *gfp* dsRNA treatment (green) and the *lac 1* dsRNA treatment (blue).

4. Discussion

4.1. Identification of RNAi Machinery Core Components and lac 1 in C. maculatus

Since the discovery of the ability of dsRNA to silence gene expression in *Caenorhabditis elegans* thirty years ago [55,56], RNAi mechanisms were described in several species and were widely used to knock down genes and analyze their functions [57]. RNAi-based control of pests relies on the introduction of dsRNA into insect bodies to silence a gene of interest via the siRNA pathway that depends on key protein complexes named *dicer-2* and *RISC* including *argonaute-2 and R2D2*. In this study, RNAi core components were identified in *C. maculatus* using phylogenetical analyses and tBLASTn followed by protein architecture description with prosite tool. Phylogeny inference followed the Maximum Likelihood (ML) method which is reported to be more robust than the neighbor-joining (NJ) methods when appropriate models of nucleotide substitution are used [18,40,58–60]. The RNAi genes identified in *C. maculatus* presented similarities with other insect species, supporting that bruchids would have conserved the same cellular mechanism. Phylogenetic clustering gathered Coleoptera species together, more precisely Chrysomelidae species that are reported to be highly sensitive to RNAi [37,61]. These identifications provided a first indication that the RNAi mechanism via the siRNA pathway would be functional in *C. maculatus* and should be further assessed by exposing insects to specific dsRNAs for confirmation.

The identification of *laccase 1* in *C. maculatus* was based on the two types of *laccases* identified in insects, *lac 1* and *lac 2* [62]. These enzymes are multicopper oxidases (MCOs) able to catalyze the oxidation of numerous phenolic and non-phenolic compounds [63]. *laccase 1* is playing a protective role against a plant-based diet while *lac 2* is involved in the tanning of the insects' cuticle (sclerotization and pigmentation) [64,65]. In *C. maculatus*, the *lac 1* inferred phylogeny showed that sequences are similar among different orders suggesting that the *lac 1* protein is evolutionarily conserved. Molecular architecture descriptions identified three copper domains fitting with orthologous *lac 1* architecture described in Hemipteran insects such as *Bemisia tabaci* (Gennadius,1889) (Hemiptera: Aleyrodidae), *Nephotettix cincticeps* Uhler 1896 (Hemiptera: Cicadellidae), *Acyrtosiphon pisum* (Hemiptera: aphididae), and *S. avenae* [40,58,66,67]. These cupredoxin-like domains were reported to include four copper atoms and were named type-1 or blue copper center, type 2 or normal copper center, and the type 3 coupled binuclear copper centers. They allow the enzyme to reduce oxygen in the water without producing harmful byproducts [44]. Such architectural indications suggest that *lac 1* in *C. maculatus* has also oxidative activity. The whole enzyme

size as well as the locations/sizes of copper domains in *C. maculatus* was similar to the *lac 1* of *S. avenae* [40]. However, the absence of a transmembrane domain suggests that *lac 1* is a secreted enzyme in *C. maculatus* as it was reported with *B. tabaci* [58]. No indication about the site of the enzyme production can be highlighted in the present study.

4.2. Identification of Reference Genes for RT-qPCR

The prior identification of at least two reference genes is essential to perform viable gene expression studies as they serve for the normalization in relative gene quantification [68]. The validation of these genes should follow an appropriate stepwise approach to get reliable results in RT-qPCR analyses [50,52,69]. In this work, it was investigated whether *arg-K*, *tuba 1* and *bactin* genes could constitute new reference genes. Following primer validation, these genes were tested for stability under biotic and abiotic variations that are likely to interfere with the expression patterns among samples of subsequent micro-injection experiments (*c.f.*, age and exposure to dsRNA). *Alpha-tubulin 1* emerged as the most promising candidate that presented all necessary criteria for validation [50]. Primers of the *Arg-K* gene presented a moderate stability coefficient which was not optimal for subsequent experiments. The results of Brar et al. (2022) provided two new reference genes (*gst* and *tbp*) that were tested for validation in parallel with *arg-K* and *tuba1*. Primers of the *gst* gene presented a relative efficiency of 108.7% and ideal stability while the relative efficiency of *tbp* primers was out of the validation range (117.6%). These results are consistent with the study of Brar et al. (2022) that highlighted respective efficiency of 102.25% and 118.9% for *gst* and *tbp*. The high relative efficiency of *tbp* primers was probably explained by unspecific amplification and a fluorescence emission that is not directly linked to the target gene. Non-specific elements such as primer dimers may also induce non-specific fluorescence as suggested by melting curves (*c.f.*, Figure 6b) but no melting curve was provided in the study of Brar et al. (2022). Consequently; the consideration of *tbp* gene as reliable reference gene was not emphasized in this study that used *tuba1* and *gst* genes in RT-qPCR analyses.

Alpha-tubuline 1 is involved in the cytoarchitecture of cells and is stably expressed in several organisms [70,71]. The *gst* gene codes for an enzyme involved in the detoxification of endogenous/exogenous compounds and is involved in the intracellular transport and biosynthesis of hormones [53,72]. This work is the first study suggesting *tuba 1* as a new reference gene in *C. maculatus* but it should be relevant to further test the stability of the gene expression in more biotic and abiotic variations, such as different developmental stages of *C. maculatus*, different temperatures, or different photoperiods. Other algorithms could also be used in the calculation of the gene expressions stability such as Normfinder [73], bestkeeper [74], delta-cq [75], or RefFinder (a web-based tool combining the latter algorithms).

4.3. Gene Knockdown and Mortalities

Micro-injection experiments and RT-qPCR analyses highlighted a stable and significant decrease in the *lac 1* gene expression in the *lac 1* treatment after two *dpi* that was stabilized until the end of the experiment at about 20% (i.e., an expression decrease in about 80%). The *lac 1* gene expression in the *gfp* treatment did not differ statistically from the control, except at three *dpi.* The *lac 1* expression in the *gfp* treatment stabilized at around 65% and became higher in the control group (115%) at the end of the experiment. This observation completes the previous identification of RNAi core machinery and supports that RNAi via siRNA pathway would be specifically triggered by dsRNA exposure in *C. maculatus*. The extent of gene knockdown ranging at ~80% following the micro-injection of 400 nL of dsRNA also suggests that *C. maculatus* would be a sensitive species to RNAi, such as other chrysomelid species [36]. Moreover, the RNAi would be expected to be systemic in *C. maculatus* as the RNAi affected the whole insect body (*c.f.*, the pooling of three complete adults in RNA extractions) [65]. The stability of the *lac 1* expression decrease also suggests that no compensation mechanism would be observed to rebalance the decrease in *lac 1*

expression as it could be observed with *T. castaneum* [76]. All these observations contribute to the first report of gene silencing induced by dsRNA in *C. maculatus* and support that RNA interference via the siRNA pathway would provide a pledging alternative method of control against *C. maculatus.* However, no subsequent mortalities were observed with the *lac 1* gene knockdown which differs from results obtained in *S. avenae* [40]. This was probably due to the low feeding of *C. maculatus* adults on water or nectar [77]. Further studies should focus on other target genes or should focus on larval life stages that are feeding on seeds and that are more susceptible to the intake of anti-nutritional factors such as tannins or antitrypsic factors for which a decrease in *lac 1* expression should induce mortalities [41,77].

4.4. Perspectives for Future Research in the Development of RNAi Pesticide against Bruchids

The systematic identification of suitable RNAi target genes that lead to dead phenotype insects is a challenge in the development of efficient RNAi-based control methods because some insects are difficult to rear in controlled conditions and because the whole genomic information is often lacking [78]. In this study, the first demonstration of systemic RNAi in *C. maculatus* was mainly limited by the difficulty of reaching larvae that develop inside seeds, which restrained micro-injection experiments on emerging adults. Further bioassay should test other delivery methods of dsRNA such as feeding bioassay led on larvae and should assess if the systemic RNAi could be transmitted to the next generation [79].

The identification of new genes that would present a combined lethal effect with gene silencing is also needed to develop new selective pest management tools for *C. maculatus* [34]. Such genes could already be identified in *T. castaneum,* including eleven genes (NCBI referred gene symbols *cact, srp54k, rop, alpha snap, shi, pp1alpha-96a, inr-a, hsc70-3, rpn7, gw, rpt3, copi coatomer, vATPased, vATPase a*) [78]. Other research led on larvae of *P. cochleariae* has identified that five of these genes were highly lethal when sprayed at very low doses (300 ng/leaves), *srp54k, rop, alpha snap, rpn7, and rpt3* [80]. These genes could be pledging genes to efficiently induce mortality in *C. maculatus*.

The use of insecticidal dsRNA products in large-scale pest management is always a challenge Most recent suggested approaches include (i) dsRNA encapsulation into nanoparticles [81], (ii) host-induced gene silencing (HIGS) technologies via the use of transgenic cultivars containing RNAi traits, (iii) spray induced gene silencing [37], or (iv) bacterial expressing RNAi traits ingestion [82]. If foliar spraying of dsRNA could be efficiently developed for the control of leaves chrysomelid pests such as *L. decemlineata* or *D. virgifera* [39,83], the consideration of endophytic larval development of bruchids would restrict possibilities of RNAi pesticides applications to HIGS through transgenic cultivars or endophytic bacterial deliveries.

5. Conclusions

This study provided a complete description of the necessary protein involved in the RNAi mechanism via the siRNA pathway and also described the architecture and the deduced function of *laccase 1* in *C. maculatus*. The administration of dsRNA coding for this protein confirmed a systemic and constant gene knockdown after two dpi. This evidence of gene silencing in *C. maculatus* offers a new perspective for a specific control. Although no lethal effect could be demonstrated, future studies should focus on other promising proteins to develop an effective control method with high specificity as has been done in many species of beetles of the Chrysomelidae family.

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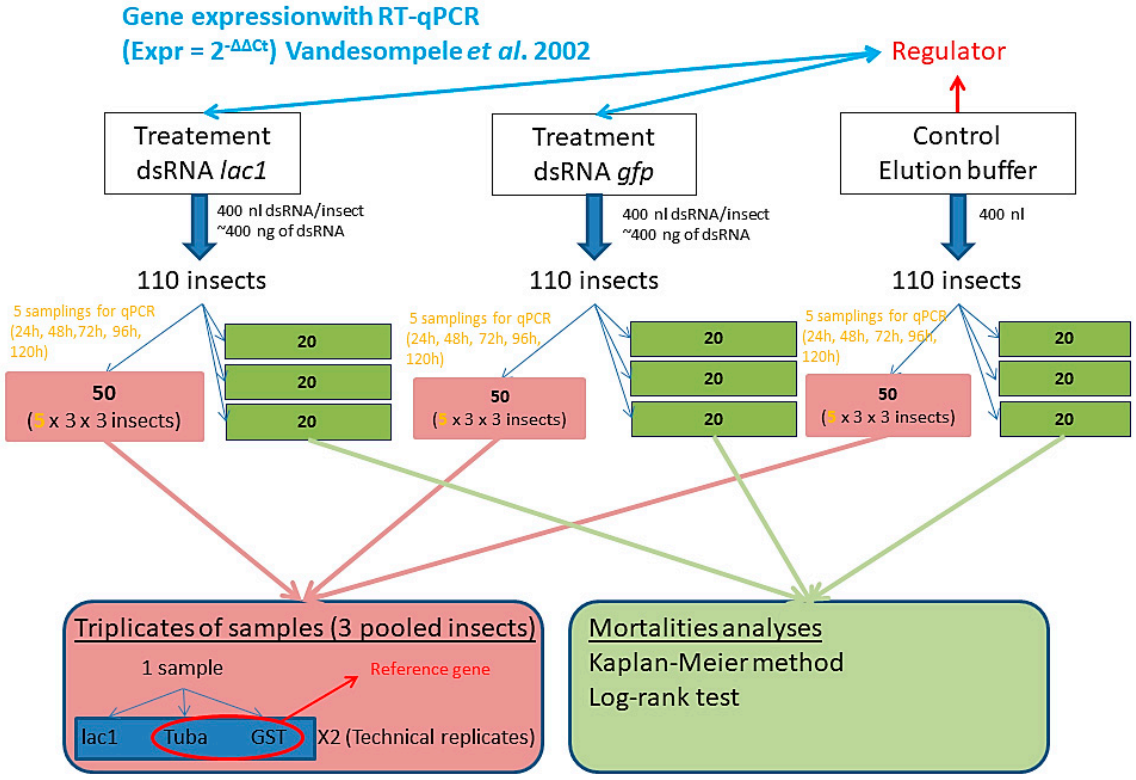
Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Summary diagram of the methodology and statistical analyses used in the micro-injection experiments for the assessment of gene expression by RT-qPCR and survival analyses.



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