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Comparison of Two *In vivo* Models for Evaluation of B52 Function

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Abstract

B52/SRp55 is a *Drosophila* pre-mRNA splicing factor that affects the splicing of some genes in animals. A B52 deficiency in fruit flies is lethal at the larval stage, which indicates the essential and non-redundant functions of B52 *in vivo*. The genome-wide gene expression profile of B52 null mutant embryos using DNA microarray was previously analyzed to determine the molecular basis for the requirement B52 in normal fly development. In this study, the genes whose expression is altered by B52 were examined by analyzing the expression of several genes in two *in vivo* animal models, anti-B52 aptamer-expressed fly and B52 null mutant fly. The results confirmed that the Osiris genes are genuine B52-dependent genes. Overall, an inhibitory aptamer-expressed animal is a useful alternative knockout animal model for evaluating B52 loss-of-function.

Keywords: B52/SRp55, Gene knockout, RNA aptamer, *Drosophila melanogaster*

Introduction

B52 is an essential splicing factor in *Drosophila melanogaster* both *in vitro* and *in vivo*. B52/SRp55, which was originally identified as a bracketing factor of heat shock puffs in polytene chromosomes[1], was later found to be a member of the SR protein family. SR proteins are regulatory protein factors in splicing, which act as both essential splicing factors and modulators of alternative splicing that function in the early stages of spliceosome assembly[2]. SR proteins share a similar structure to the RNA recognition motifs (RRM) at the N-terminus and a region rich in Arg-Ser dipeptides (RS domain) at the C-terminus. While several studies have clearly demonstrated the critical requirement of SR proteins in normal cell growth and animal viability, there are few reports on the cellular genes regulated by SR proteins *in vivo*[3-10].

In this study, B52 knock-out flies were used to gain insight into the essential role of B52 in fly embryonic development[11]. Briefly, a B52 null mutant was generated by remobilizing a P element residing near the B52 gene. This mutant is a deletion extending from the 5' Hrb87F noncoding region to the 5' half of B52 coding region, and disrupts both the B52 and Hrb87F genes. Homozygous ($B52^{28}/B52^{28}$) mutants can be distinguished from heterozygous ($B52^{28}$/*TM6b Tb e*) offspring based on their normal body shape. The Tb phenotype first becomes distinguishable from the wild type at the end of the first larval instar and can be identified unambiguously through the second and third instars (Figure 1). From recent microarray analysis using this animal model system, differentially-expressed genes (DEGs) whose expression level was affected by B52 depletion were identified, and it was found that an impairment of B52 function affects the expression of the genes engaged in specific cell lineage differentiation and inner cellular structure organization (Hong *et al*., In press).

This paper reports that some of the B52 deficiency-related DEGs selected from microarray analysis are expressed in a larval-stage dependent manner and their change in expression pattern results in larval growth arrest. This suggests that this knock-out fly has some limitations as an animal model for a functional study of B52. Therefore, the expression of these DEGs was analyzed in another B52-impaired animal model that can conditionally overexpress anti-B52 RNA aptamers[12]. The induction of the B52 inhibitory aptamer, known as BBS, is controlled by the GAL4-UAS expression system (Figure 1). Using this system, the expression of the Osiris gene family members were found to be affected directly by the transient inhibition of B52 activity. Moreover, their expression patterns in inhibitory aptamer-expressed larvae were similar to those of B52-knockout larvae. These results demonstrate that an inhibitory aptamer-expressed fly is a useful model system for evaluating B52

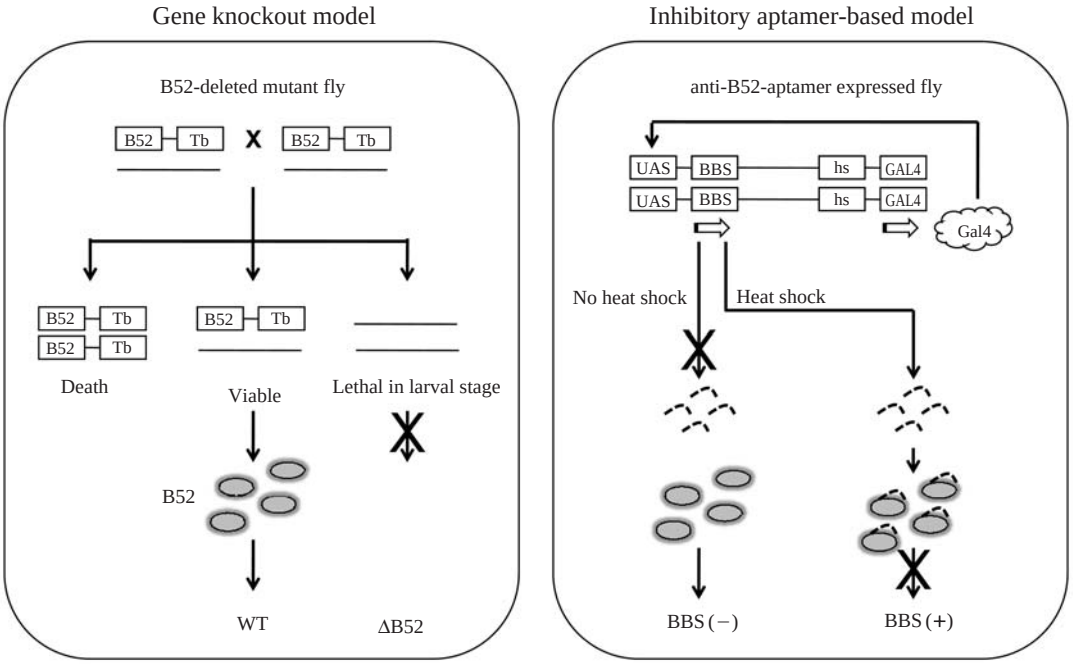

Figure 1. Two *in vivo* experimental model for an evaluation of the B52 loss-of-function. The action mechanism of each *in vivo* model system is presented. In the B52 knockout model, the homozygous B52 mutants (ΔB52, $B52^{28}/B52^{28}$) and heterozygous B52 wild type larvae (WT, $B52^{28}$/*TM6b Tb e*) are the progeny of $B52^{28}$/*TM6b Tb e* flies. In the B52 aptamer-based model, the overexpression of anti-B52 aptamer was induced using the GAL4-UAS expression system after the heat shock treatment.

loss-of-function.

Results and Discussion

Characterization of B52-related DEGs Identified in B52 Knockout Fly Model

First, three up-regulated genes (CG4116, CG14423, and Osi7) and three down-regulated genes (Lsp2, Obp99b, and CG4439) were selected to reconfirm the expression of the DEGs selected from our previous microarray experiment of a B52 null mutant. In this validation test, the expression of these genes changed dramatically, and correlated with DNA microarray analysis (Figure 2A).

As previously reported[11], the B52-deficient fly has a prolonged a 2nd instar larval stage that is lethal. In this study, B52-deficient larvae growing up for 7 days had a similar shape to the 2nd instar larvae while the wild-type larvae grew into the 3rd instar larvae. From these results, it was hypothesized that the B52 deficiency-specific expression profile may have resulted from a prolonged 2nd instar larval stage. In order to confirm this hypothesis, RT-PCR analysis was carried out on the total RNA from the wild-type larvae collected in different larval stages, and the result was compared with that of B52-deficient larvae. Interestingly, all the genes tested were expressed in a larval stage-dependent manner and the expression status of the B52 null mutant larvae was similar to that of the wild-type 2nd instar larvae (Figue 2B). The genes whose expression was regulated in a larval stage-independent manner could not be identified.

Expression Pattern of B52-related DEGs in B52-impaired Fly Model using Inhibitory Aptamer

RT-PCR analysis was also performed on another B52-impaired fly model to examine the change in expression of these DEGs by a B52 loss-of-function at the same larval stage [BBS (5.12)]. This transgenic fly was manipulated genetically to overexpress BBS (B52 binding sequence), which acts as an inhibitory RNA aptamer by binding B52 with high affinity and specificity. This aptamer interacts with two RNA recognition motifs in B52 and inhibits B52-stimulated pre-mRNA splicing both *in vitro* and *in vivo*[12]. BBS expression reached the maximum level one hour after the heat shock treatment and then decreased to the basal level (Figure 3, panel BBS). The genes tested were unaffected by the heat shock treatment (data not shown). The gene expression pattern in the 3rd instar larvae of BBS (5.12) prior to BBS induction was also similar to that in the 3rd instar larvae of the wild-type

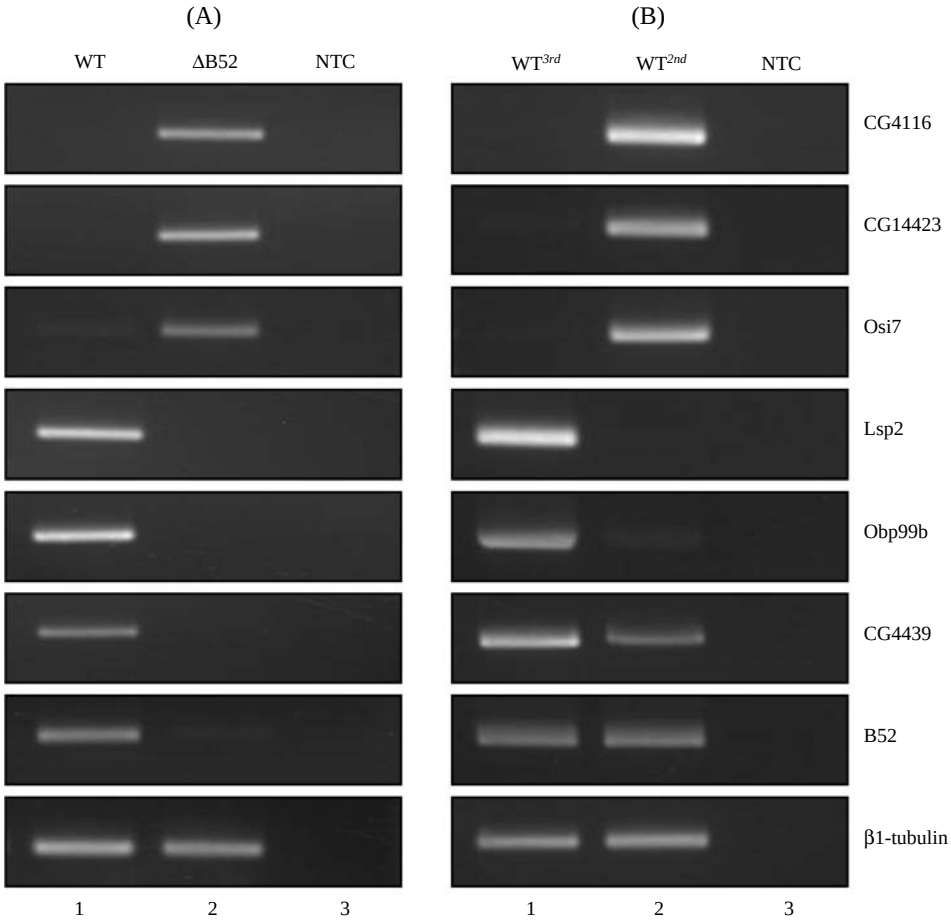

Figure 2. RT-PCR validation of the genes identified as DEGs in microarray analysis using B52 knockout flies. Six DEGs (up-regulated genes : CG4116, CG14423, and Osi7; down-regulated genes : Lsp2, Obp99b, and CG4439) were used for RT-PCR validation. β1-Tubulin was used as an internal control and NTC (No Template Control, lane 3) was used as a negative control. (A) To test the effect of B52 deletion, the wild-type (tubby 3rd instar larvae of heterogeneous B52 wild type, WT, lane 1) and B52-null mutant (non-tubby 2nd instar larvae of homozygous B52 mutants ($B52^{28}/B52^{28}$, ΔB52, lane 2) were used. (B) 3rd instar larvae (WT^{3rd}, lane 1) and 2nd instar larvae (WT^{2nd}, lane 2) of Oregon R were used to analyze the expression pattern of the selected DEG at each larval stage.

[compare Figure 2B, lane WT^{3rd} with Figure 3, lane BBS (−)]. The level of B52 mRNA was unaffected (Figure 3, panel B52), and five of the six genes did not show any change in expression after BBS overexpression (Figure 3). In contrast, the expression of the Osi7 gene was up-regulated dramatically in the BBS-overexpressed larvae, similar to the B52 null mutant (Figure 3, panel Osi7). This shows that the anti-B52 aptamer-expressed fly is a useful experimental system for validating the B52-related genes differentially expressed at each stage during *Drosophila* development. Moreover, these results suggest that Osi7 may be a good target for assessing the inhibitory aptamer-based *in vivo* validation system.

Comparison of Osiris Gene Expression in Two Animal Models for Evaluation of B52 Loss-of-function

From previous microarray analysis, eight out of 23 Osiris gene family members were up-regulated DEGs in the B52 null mutants. All eight genes were also expressed in the larval stage-dependent manner and down-regulated at the 3rd instar larval stage of the wild-type (Figure 4, lane 1-2). The expression patterns of these genes under B52 depletion were similar to that in the wild-type 2nd instar larvae. While all eight Osiris genes were up-regulated in the B52 knockout fly, the expression of the Osiris genes was affected differentially under B52 inactivation using the anti-B52 aptamer. RT-PCR showed that while Osi7, 9, 14, and 15 showed significantly higher expression, the changes in Osi18 and 20 expression were undetectable in the BBS-expressed 3rd instar larvae (Figure 4, lane 5-6). Therefore, more information can be obtained from an anti-B52 aptamer-expressed fly than a B52 knockout fly model by investigating the differential effect of a B52 loss-of-function on the expression of each Osiris gene.

The gene expression patterns change remarkably during the life cycle of *Drosophila melanogaster*[13-15]. This paper reports that six genes (CG4116, CG14423, Osi7, Lsp2, Obp99b, and CG4439), which were identified as B52-related DEGs, had similar expression patterns to the 2nd instar larvae of the wild-type and their expression levels were altered dramatically during larval development. Overall, these results suggest

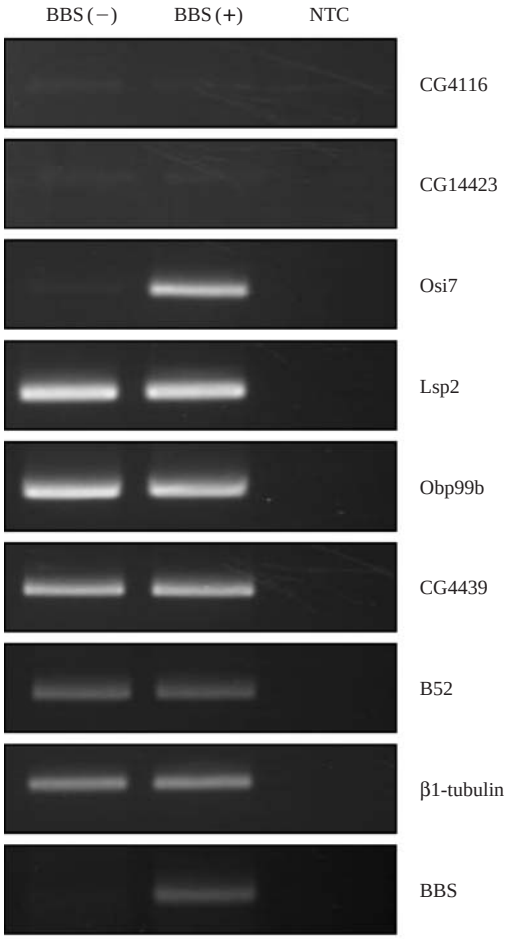

Figure 3. RT-PCR analysis of the DEGs in an anti-B52 aptamer expressed fly. Six DEGs selected from microarray analysis of a B52 knockout fly were analyzed. The 3rd instar larvae of BBS (5.12) without the heat shock treatment (BBS (−), lane 1) and the 3rd instar larvae of BBS (5.12) with the heat shock treatment (BBS(+), lane 2) were used to test the effect of BBS overexpression. β1-Tubulin was used as an internal control and NTC (No Template Control, lane 3) was used as the negative control.

that B52 plays a key role in regulating the change in expression between the 2nd and 3rd instar larval stage during *Drosophila* metamorphosis.

However, there were some limitations in the validation test using B52 knockout fly. The continued absence of B52 induced growth arrest in the 2nd instar larval stage. Therefore, it is difficult to distinguish between normal 2nd instar-specific gene expression and 2nd instar-like gene expression triggered by B52 depletion. In addition, in the B52 knockout model, it was impossible to analyze the change in gene expression at each larval stage caused by a B52 deficiency.

On the other hand, because controlled aptamer expression is possible in an inhibitory aptamer-based fly model, this model can be used to evaluate B52 loss-of-function under various developmental stages or conditions. In RT-PCR analysis, some of the Osiris gene family members were identified as candidate genes that are regulated directly by B52. In particular, B52 inactivation by the RNA aptamer altered the 3rd instar larva-specific expression pattern to the 2nd instar larval-specific expression pattern. This suggests that the expression of the Osiris gene family is tightly regulated during larval development. Moreover, B52 plays an important role in this regulation of larval stage-specific gene expression. Of the 23 Osiris gene family members, 20 genes named Osi 1 through Osi 20, based on their position in the cluster, were located in the cytological region corresponding to the triplo lethal gene region (Tpl) identified as a dosage-sensitive region in the *Drosophila* genome[16]. Three other members of this family were located elsewhere in three different sites in the genome (Osi 21, 22, and 23), and none of these loci are triplo-lethal or located within haplo-insufficient regions. Homologous genes are found only in insects, and the biochemical function of this family in any other insect species is unknown. All the proteins appear to have endoplasmic reticulum signal peptides and three conserved domains. Several studies reported that some of the Osiris gene family members or homologs of other inset species are expressed differentially during development[13,14,17]. In particular, some of the Osiris gene family showed a >50-fold increase in expression during wing differentiation[14]. All were also expressed differentially at each larval stage. These results provide additional evidence to explain the relationship between fruit fly development and Osiris expression.

Conclusions

In conclusion, the DNA microarray data obtained from a B52-knock-out animal was validated using another animal model that conditionally expresses B52-inhibiting aptamers. It is believed that the aptamer-based in vivo protein inhibition technology will be a useful complementary strategy to genetic knockout or knock-down approaches, and is expected to provide valuable information at the proteomic level.

Materials and Methods

Fly Preparations

The B52 depleted mutant flies were described previously[11]. Briefly, homozygous B52 mutants (*B52^{28}*/

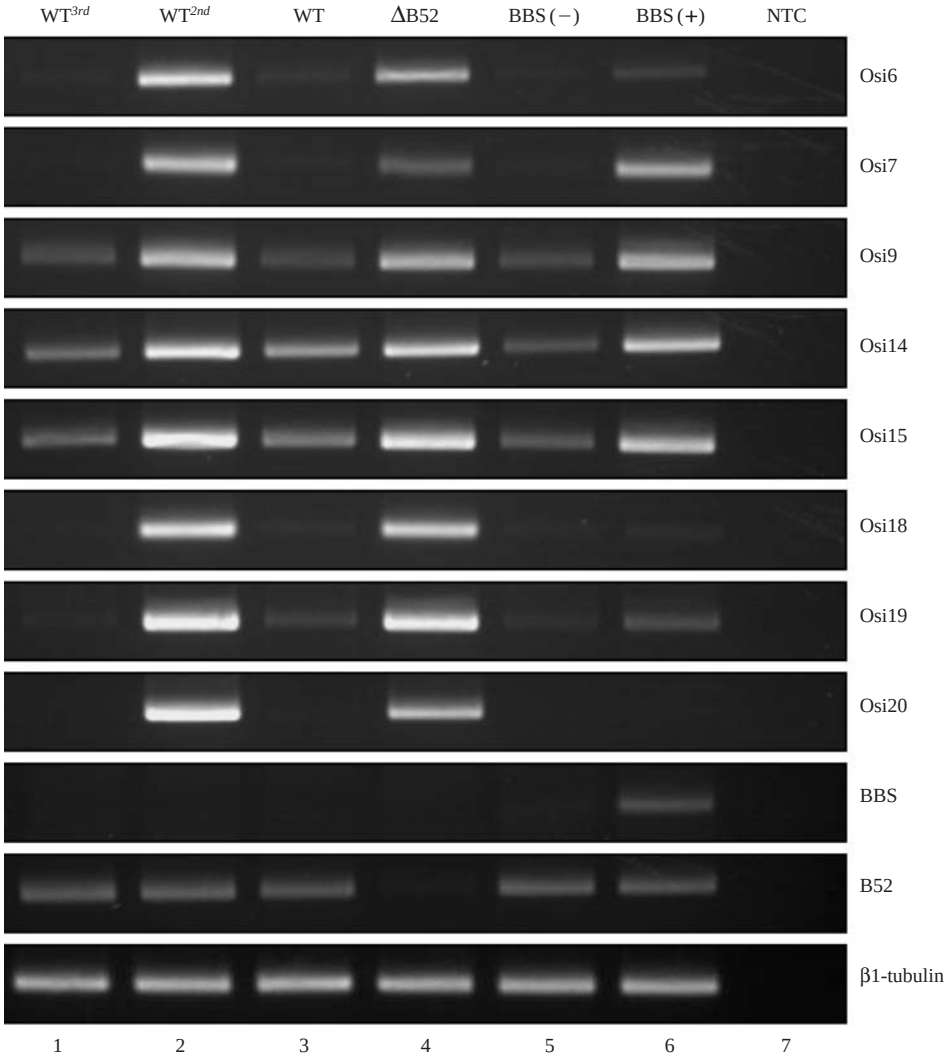

Figure 4. Expression pattern of the Osiris gene family members in the B52 knockout fly and anti-B52 aptamer expressed fly. Eight Osiris genes identified as upregulated genes in the B52 null mutant were analyzed by RT-PCR. The same samples and conditions shown in Figures 2-3 were used for RT-PCR analysis. β1-Tubulin was used as an internal control and NTC (No Template Control, lane 7) was used as a negative control.

B52^{28}) and heterozygous B52 wild type larvae (*B52*^{28} */TM6b Tb e*) were picked 7 days after laying eggs. The homozygous B52 larvae were distinguished from heterogeneous B52 wild type by their non-tubby and tubby shapes. Approximately, 150 non-tubby and 10 tubby larvae were hand-picked and frozen with the TRI reagent (SIGMA, USA) in −70˚C. The secondary -and third-instar larvae of Oregon R were used to test for larval stage-related gene expression. The BBS (5.12) transgenic fly line previously reported[12] was used as an inhibitory aptamer-based animal model.

Heat Shock Treatment for BBS Overexpression

Heat shock treatment was carried out in a water bath and the third-instar larvae of BBS (5.12) were used. The larvae were exposed four times to 15 minutes of heat shock at 36.5˚C with a 5 minutes interval between shocks. After the 4th heat shock, the larvae were incubated at room-temperature for 1 hour and then frozen with TRI reagent (SIGMA, USA) in −70˚C.

RNA Isolations

The frozen larvae were homogenized in TRI reagent using Pellet Pestle® Motor (KONTES) and a disposable grinder. The total RNA from the larvae were purified according to the manufacturer's protocol and treated with DNase I to remove the genomic DNA. The concentration of total RNA prepared was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA), and the RNA integrity was verified by 1% agarose gel electrophoresis. The RNA samples for microarray analysis were cleaned using a RNeasy Mini Kit (Qiagen,

Germany) and stored at −70˚C prior to use.

cDNA Synthesis and PCR Reactions

1 μg of the total RNA per 20 μL reaction was reverse-transcribed with ThermoScript Reverse Transcriptase (Invitrogen, USA). The RT reactions were carried out in 50˚C for 2 hours and treated with RNase H (Invitrogen) at 37˚C for 30 minutes. 1 μL of the cDNA was used as a template for each PCR. PCR (in 25 μL) was carried out as follows: 30 seconds at 95˚C, 30 seconds at 58˚C, 30 seconds at 72˚C for 25-35 cycles, followed by 72˚C for 7 minutes. PCR was performed using the primer sets shown in Supplementary Table 1.

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Supplementary Data

Supplementary Table 1. Primer sets for RT-PCR.

Primer	Primer sequence	Produt size
β1-tubulin F	CTGGAGCGCATCAATGTCTA	155 bp
β1-tubulin R	GACTGGCCGAACACAAAGTT	
B52 F	GTCCAAGTCGCCAGTCAAGT	296 bp
B52 R	GTGAGGCGTTTCCATTTTTG	
BBS F	CAGTACTCTCGACGATCAACCA	131 bp
BBS R	TCTCGACTGACCGAAGTCAA	
CG4116 F	CCGGCATCTATGTGAAGGAT	237 bp
CG4116 R	CCGAAGTCGTTCCTGTTGTT	
CG14423 F	GAGTGCCTGCCAAGAAAATC	211 bp
CG14423 R	CAGGAAGTGTGGCAGTAGCA	
Lsp2 F	GACTATGTGCACCCGGAGAT	176 bp
Lsp2 R	CAGTAGACGACGTGCTGGAA	
Obp99b F	CCAGAAGTTCGGCTTCTACG	249 bp
Obp99b R	CTGGATCCATGCTCCCTTTA	
CG4439 F	GAGTATCGCGGAGCAGTTTC	251 bp
CG4439 R	GGCTTGAAAGTCGATTGAGC	
Osi6 F	CTCCTGGCAGCAGGTATCTC	221 bp
Osi6 R	AGGGAGTTGTCCAGGGACTT	
Osi7 F	CTGTTCAGCTTCGTGGACAA	190 bp
Osi7 R	CCACCTTGATGGTGTGTGAG	
Osi9 F	CATGGTCTTGTGCATGAAGG	257 bp
Osi9 R	GCATGTCCTGGATGGAGTCT	
Osi14 F	GTGGAGTCACCTTCCAGAGG	256 bp
Osi14 R	GGTCCCAGCATCTTCTTGAT	
Osi15 F	TGGAGACCCACGAGCTAAAC	269 bp
Osi15 R	GGCCAACAGATCCTTGAAGA	
Osi18 F	GTCGAATCCGCTTACTCAGG	161 bp
Osi18 R	TCCAGGTCTTTAGGGCAATG	
Osi19 F	AGGGGTAAGAAGGGCAACAT	292 bp
Osi19 R	CAGGAACGGAGCTAAAGGTG	
Osi20 F	CAGACCTTCTTCGCTGGTTC	282 bp
Osi20 R	CATCTTCATGCCTCCCAACT	