An Adaptive Transposable Element Insertion in the Regulatory Region of the *EO* Gene in the Domesticated Silkworm, *Bombyx mori*

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Abstract

Although there are many studies to show a key role of transposable elements (TEs) in adaptive evolution of higher organisms, little is known about the molecular mechanisms. In this study, we found that a partial TE (*Taguchi*) inserted in the *cis*-regulatory region of the silkworm ecdysone oxidase (EO) gene, which encodes a crucial enzyme to reduce the titer of molting hormone (20-hydroxyecdysone, 20E). The TE insertion occurred during domestication of silkworm and the frequency of the TE insertion in the domesticated silkworm (*Bombyx mori*) is high, 54.24%. The linkage disequilibrium in the TE inserted strains of the domesticated silkworm was elevated. Molecular population genetics analyses suggest that this TE insertion is adaptive for the domesticated silkworm. Luminescent reporter assay shows that the TE inserted in the *cis*-regulatory region of the *EO* gene functions as a 20E-induced enhancer of the gene expression. Further, phenotypic bioassay indicates that the silkworm with the TE insertion exhibited more stable developmental phenotype than the silkworm without the TE insertion when suffering from food shortage. Thus, the inserted TE in the *cis*-regulatory region of the *EO* gene increased developmental uniformity of silkworm individuals through regulating 20E metabolism, partially explaining transformation of a domestication developmental trait in the domesticated silkworm. Our results emphasize the exceptional role of gene expression regulation in developmental transition of domesticated animals.

Key words: **ecdysone oxidase, transposable element, selective sweep, regulatory region, domestication, silkworm.**

Article

Introduction

Transposable elements (TEs) were once considered as "junk DNA" because their effect or apparent role in the genome structure, function, and maintenance was rather unknown. However, more and more studies suggest that TEs sometimes play major roles in genome evolution and function, due to the fact that TEs can proliferate and reach high copy numbers, donate regulatory sequences, and change gene structure of nearby genes (Britten 1996; Brosius 2003; Kazazian 2004; Naito et al. 2006; Feschotte 2008; Bourque 2009). Indeed, several studies have demonstrated that TEs were involved in adaptive and functional evolution in different species. A comprehensive genome-wide screen for recent adaptive TE insertions in the *Drosophila melanogaster* genome showed that TEs are a major source of adaptive mutations in this species (González et al. 2008). Further, the researchers also found a recent adaptive TE insertion near highly conserved developmental loci in *D. melanogaster* (González et al. 2009; Guio et al. 2014). A short interspersed nuclear element insertion in the second intron of the insulin-like growth factor 1 (*IGF1*) gene has contributed to the domestication of small dog breeds (Gray et al. 2010), and a retrotransposon (*Hopscotch*) inserted upstream of the *tb1* gene is associated with the domestication of maize (Studer et al. 2011). Thus, TE mobilization might sometimes benefit host genomes by increasing genetic diversity.

The domesticated silkworm, *Bombyx mori*, is an important economic insect and also the only thoroughly domesticated insect. It is generally believed that *B. mori* was domesticated from the Chinese wild silkworm, *B. mandarina* (chromosome number $2n = 56$, as in *B. mori*), about 5,000 years ago (Underhill 1997). During domestication, silkworm experienced a dramatic transition in morphological, behaviour, and developmental traits as compared with its wild progenitor. Unlike its wild relative *B. mandarina*, the domesticated silkworm has very strong crowded tolerance and is usually reared in a very large population (Goldsmith et al. 2005). Of economically important traits, therefore, developmental uniformity of individuals is a crucial target of artificial selection for silkworm. However, the mechanism underlying developmental uniformity of individuals in the domesticated silkworm is still unknown.

A previous study suggested that wild silkworm is much sensitive to environmental changes (Shen et al. 2003). For example, *B. mandarina* showed rapid metamorphosis to adapt to food shortage. This phenomenon is also found in other insects including *D. melanogaster* and *Onthophagus taurus* (Shafiei et al. 2001; Shen et al. 2003; Terashima et al. 2005). Terashima et al. (2005) showed that starvation or food shortage might elevate the titer of molting hormone, 20-hydroxyecdysone (20E), and then induce the accelerated development in *D. melanogaster*. The fluctuation of the 20E

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titer, thus, is crucial to regulate the insect development (Warren et al. 2011).

In lepidopteran insects, ecdysteroid C3-epiecdysteroid pathway is a major route to degrade the 20E, and ecdysone oxidase (EO) is a rate-limiting enzyme in this pathway (Warren et al. 1988; Sakurai et al. 1989). Therefore, changes in the expression of *EO* gene are likely to affect 20E titer and consequently any of the processes controlled by this hormone. In this study, we found a TE insertion in the *cis*-regulatory region of the *EO* gene in some domesticated silkworm strains. Molecular population genetics analyses suggest that this TE insertion is adaptive for the domesticated silkworm. Functional assay evaluates that the inserted TE functions as a 20E-induced enhancer of the *EO* gene expression. Phenotypic bioassay reveals that the TE insertion partially explains the increased developmental uniformity of individuals, a domestication developmental trait, in the domesticated silkworm.

Results

Higher Frequency of the TE Insertion in the Upstream of the *EO* Gene in the Domesticated Silkworm

We found insertion-deletion (indel) polymorphisms in the *cis*-regulatory region of the *EO* gene in silkworm. Using the specific primers (PGL-F and PGL-R, supplementary fig. S1, Supplementary Material online), we amplified the upstream region (−1,479 to 0 bp) of the *EO* gene from 59 domesticated silkworm strains (*B. mori*). Thirty-two (54.24%) domesticated silkworm strains generated a large fragment (1,500 bp) and 27 (45.76%) samples generated a small fragment (1,000 bp). We then cloned and sequenced the polymerase chain reaction (PCR) products from these 59 strains. The biggest difference between the sequences from two types of PCR products is the presence of a 512 bp fragment in the large one (supplementary fig. S2, Supplementary Material online). This fragment located 462 bp upstream of the transcription start site of the *EO* gene was predicted to be a fragment of the TE *Taguchi*, a long interspersed nuclear element (LINE). For the Chinese wild silkworm (*B. mandarina*), the progenitor of the domesticated silkworm, three samples (W1, W5, and W14) produce a large PCR fragment. By sequencing these PCR products, we found that these three sequences all have unique insertions, respectively. However, the inserted regions do not have any sequence similarity with each other and with the *Taguchi* from the domesticated silkworm. The alignment of the sequences is shown in supplementary figure S3, Supplementary Material online. Therefore, based on our observations, there is no the *Taguchi* insertion on the upstream of the *EO* gene in the Chinese wild silkworm. In addition, the Japanese wild silkworm (*B. mandarina*, chromosome number: $2n = 54$), which separated from the Chinese wild silkworm before domestication of *B. mori* (Sun et al. 2012), also has no TE insertion. These results indicate that the TE insertion occurred during domestication of *B. mori*. Most of the TE insertions are found at low frequencies, because most insertions may be deleterious (Charlesworth and Langley 1989; Charlesworth et al. 1994). In this study, we found that 54.24% of *B. mori* strains have the *Taguchi* insertion, whereas *B. mandarina* does not have. Thus, a high proportion of this TE insertion in the domesticated silkworm indicated that it might be adaptive during silkworm domestication.

DNA Polymorphism and Linkage Disequilibrium

To determine whether the TE insertion is truly adaptive in the domesticated silkworm, we seek to search for the signatures of a selective sweep in the regions flanking the TE insertion. To this end, we sequenced an average 11, 440 bp length region including the 5′-flanking region, exon, intron, and 3′-flanking region of the *EO* gene for 18 *B. mori* strains (nine TE inserted strains and nine non-TE inserted strains) and nine *B. mandarina* samples. For the entire sequence region, the nucleotide diversity (π) is 0.0113 in the non-TE inserted strains, which is similar to previous estimates from multiple nuclear loci in the domesticated silkworm (0.0117 in Guo et al. 2011; 0.0134 in Yu et al. 2011). The π value of the entire region from the TE inserted strains is much lower than that in the non-TE inserted strains and the wild silkworm (0.00763, 0.0113, and 0.01461, respectively) (table 1). According to the sliding window analysis of the whole sequence, the biggest difference between the two types of the domesticated silkworm strains is the 5′-flanking region (fig. 1A). In this region, the lower level of polymorphism was present in the TE inserted strains (0.00881 vs. 0.01578), particularly, the sequences around the TE inserted site (from −973 to −462) are devoid of nucleotide polymorphism (fig. 1A). Absolute levels of diversity are also expected to vary according to levels of functional constraints on sequence evolution (e.g., coding region vs.

Table 1. Nucleotide Diversity and Neutral Test.

Test Set	Entire Sequence Regions (11,440 bp[a])					Coding Region (2,007 bp[a])					5′-Flanking Region (7,218 bp[a])				
	S	π	θ_w	Tajima's *D*	FL *D*	S	π	θ_w	Tajima's *D*	FL *D*	S	π	θ_w	Tajima's *D*	FL *D*
Domestic ($n = 18$)	344	0.01558	0.01006	2.34053*	1.46663*	31	0.00755	0.00450	2.68232*	1.47170*	290	0.02294	0.01472	2.37800	1.46453
With TE ($n = 9$)	224	0.00763	0.00778	−0.09997	−0.20309	30	0.00801	0.00551	2.27986*	1.47331*	174	0.00881	0.01004	−0.63688	−0.57781
Without TE ($n = 9$)	257	0.01132	0.00943	1.04077	1.30956	30	0.00715	0.00551	1.44682*	1.47331*	208	0.01578	0.01318	1.02605	1.31970
Wild ($n = 9$)	326	0.01461	0.01190	1.01522	1.41968*	36	0.00858	0.00660	1.51591	1.50627*	263	0.01965	0.01633	0.87352	1.37332

NOTE.— π, nucleotide diversity per site; θ_w, Watterson's estimator of $4N_e\mu$; FL *D*, Fu and Li's *D* test; S, number of segregating sites.

[a]The number of sites aligned sequences.

*Significant at a probability level 0.05.

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noncoding region) (Clark et al. 2004). To examine this scenario, the ratio of the nucleotide polymorphism for those domesticated silkworm strains with and without the TE insertion was calculated (fig. 1C). Along the entire sequenced region, there is a distinct valley of reduced nucleotide variation around the TE inserted site (fig. 1C). This valley pattern is expected to be the signature of the selective sweep in populations and also detected at several domestication loci in maize and rice (Clark et al. 2004; Olsen et al. 2006).

In addition, the closer relationship of these strains with the TE insertion may also reduce the polymorphism. In order to identify whether this factor may affect the nucleotide polymorphism of the TE inserted strains, we performed network analysis using the 5′-flanking sequence and the *EO* coding sequence, respectively. For the 5′-flanking sequences, the relationship of haplotypes among the TE inserted strains was very close, and the haplotypes of non-TE inserted strains was grouped together (fig. 2A). If all the TE inserted strains are descended from a very recent common ancestor, and then these strains in the haplotype analysis should be in one group. However, the *EO* coding sequences of the TE and non-TE inserted strains have similar haplotype patterns (fig. 2B). Moreover, we also sequenced a gene (Endo-β-n-acetylglucosaminidase, *ENGase*), approximately 11 kb upstream of the TE inserted site. The π ratio of the TE and non-TE inserted strains was close to 1 (π: 0.01268 vs. 0.01297; ratio: 0.98) at this locus (fig. 1C and supplementary fig. S4, Supplementary Material online). These results indicate that the strains with the TE insertion are not very closely related.

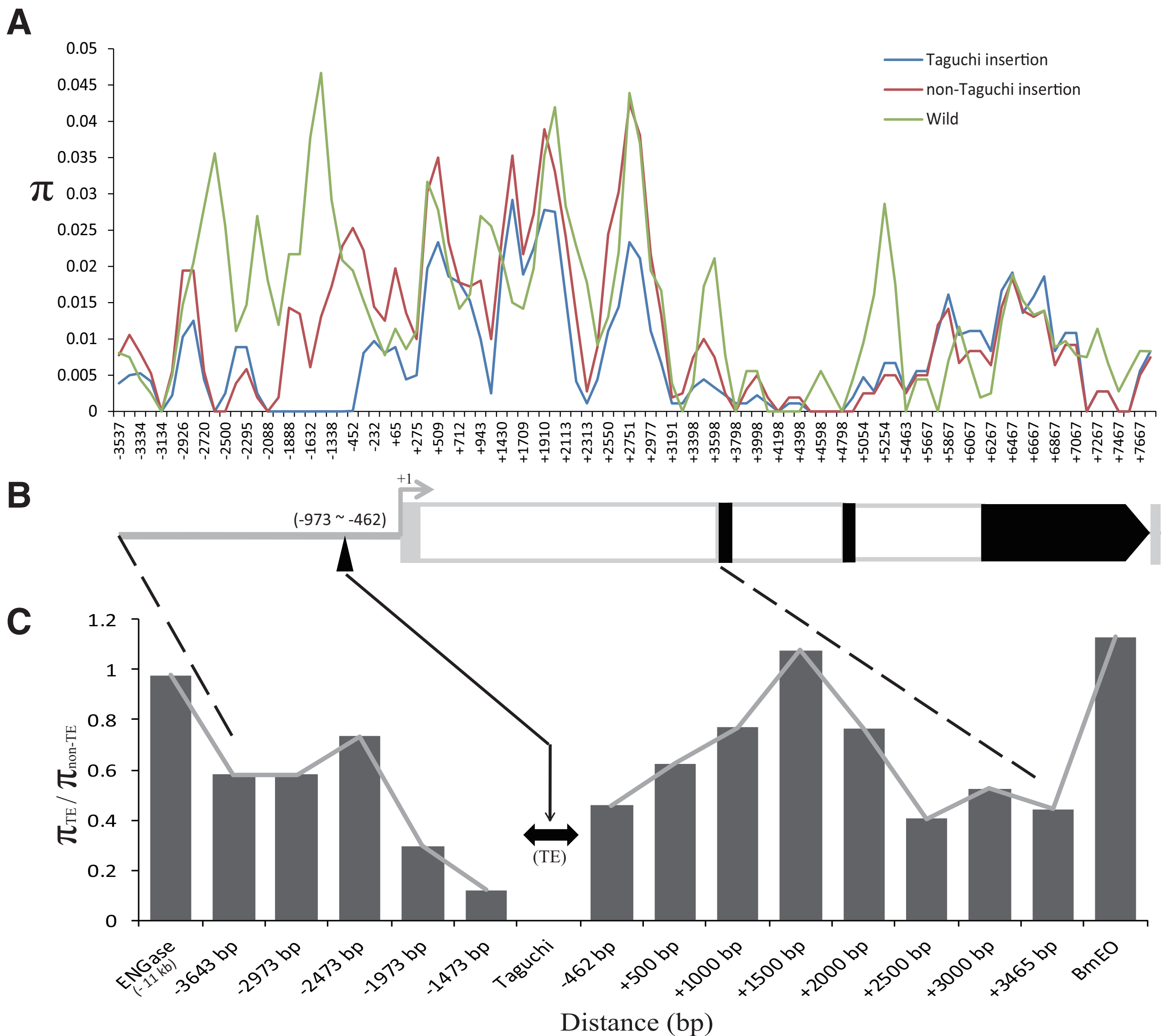

Fig. 1. Genetic diversity analysis around the *EO* region in the domesticated silkworm. (*A*) Sliding window analysis of nucleotide diversity (π) in the *EO* gene. Window and step size are 200 and 100, respectively. Blue line means the domesticated silkworms with the TE insertion; the red line means the domesticated silkworms without the TE insertion; the green line means the Chinese wild silkworm; the *x* axis represents the relative distance to the transcription start site of the *EO* gene. (*B*) The structure of the *EO* gene. Black box indicates exon; white box indicates intron; gray box indicates untranslated region; gray line indicates intergenic region; and black triangle indicates the location of the TE insertion. (*C*) The relative ratio of π in the strains with the TE insertion to strains without the TE insertion; the *x* axis represents the relative distance to the transcription start site of the *EO* gene.

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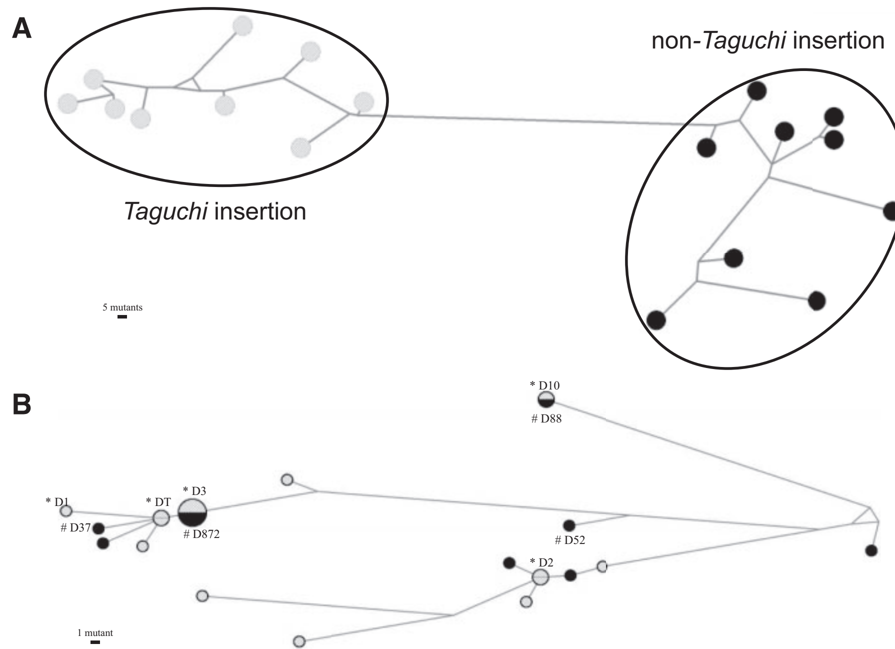

Fig. 2. Parsimonious median-joining haplotype network analysis. (*A*) The network based on the 5′-flanking region sequences of the *EO* gene in the domesticated silkworm. The scale bar means five mutants. (*B*) The network based on the coding region sequences of the *EO* gene. The sizes of the circles are proportional to the haplotype frequencies in the data sets. The scale bar means one mutant. The lengths of branches between haplotypes are proportional to the numbers of mutations. Oblique line circles represent the domesticated silkworm with the TE insertion; solid circles represent the domesticated silkworm without the TE insertion. The "*" labeled names mean the starvation treated domesticated silkworm strains with the TE insertion and the "#" labeled names mean the starvation treated strains without the TE insertion.

The linkage disequilibrium (LD) with nucleotide pairwise distance from the 5′-flanking region is summarized in supplementary figure S5, Supplementary Material online. r^2 refers to squared allele frequency correlation and was used to fit a logarithm curve across distances for the wild silkworm, TE, and non-TE inserted domesticated silkworm strains, respectively. All the curves of LD decay rapidly within 200 bp and drop slowly when distance is >200 bp. It is obvious that fitting curve in *B. mori* was above that *B. mandarina* which decays to less than 0.2 within 1,000 bp ($ZnS = 0.1486$). For the domesticated silkworm, the curve for non-TE inserted strains drops to 0.2 within 3,000 bp ($ZnS = 0.3356$), whereas the value in the TE inserted strains declines much slowly to 0.2 by almost 4,000 bp ($ZnS = 0.4984$). Low level of polymorphism and elevated level of LD is the indicator of the selective sweep (Kim and Nielsen 2004). Thus, we can predict that the lack of polymorphism and the elevated level of LD in the TE inserted strains is the result of this region being under abnormally high selective constraint.

Test for Selective Sweep

In order to identify whether selective sweep may affect the nucleotide polymorphism in the 5′-flanking region in the TE inserted strains, we first performed the integrated haplotype score (iHS) test in which the bottleneck scenario was incorporated. About 54% of the frequency of the TE insertion on upstream of the *EO* gene indicated that this insertion has not been fixed in the domesticated silkworm yet. iHS statistic is better at detecting incomplete selective sweep (Voight et al. 2006). Indeed, the iHS test showed significant deviations in the direction expected under a partial selective sweep. Pickrell et al. (2009) pointed out that iHS test has a limited statistical power for sample sizes smaller than 20. Because only nine samples with TE insertion were used in this study, the power of the iHS test may be reduced. Thus, several other methods were used to further detect selective sweeps. The value of f_{TE} was calculated and compared with those obtained by coalescent simulations (see Materials and Methods). Similarly, the f_{TE} value for the TE insertion strains also significantly deviates from neutrality (table 2).

We also detected the selective sweep using the composite likelihood ratio (CLR) test. This method evaluates the local reduction of variation and skew of the frequency spectrum. We calculated statistical significance of LR values in two ways: Distinguishing ancestral from derived alleles (test 1) and not distinguishing allele states (test 2) (Kim and Stephan 2002). The results were shown in table 3. Both the tests rejected neutral evolution for the TE inserted strains. Given that CLR test is not robust to demographic history, the goodness-of-fit (GOF) test was also performed. This test examines how well a selective sweep model fits the data, as opposed to a generalized alternative model (Jensen et al. 2005). Thus, nonsignificant P values represent a good fit of the sweep model to the data. Our results showed that the GOF test is not significant, suggesting that the rejection of neutrality by the CLR test is more likely due to a selective sweep. The strength of the selection (α) is also calculated in the CLR test. The value of α was 216.82 (test 1) or 167.80 (test 2), indicating a fairly modest selective advantage, and consistent with the narrow width of the swept region (~1,500 bp around the TE inserted

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Table 2. Neutrality Tests for the 5′-Flanking Region of the Silkworm *EO* Gene.

	$f_{TE} = \pi_{TE}/\pi_{TE} + \pi_{non\text{-}TE}$	iHS
Actual value	0.317*	−4.813*
Simulation	0.456	−2.83
95% CI	(0.368, 0.545)	(−4.806,−0.009)

NOTE.—CI, confidence interval.
*Significant at a probability level 0.05.

Table 3. CLR Test for the 5′-Flanking Region of the Silkworm *EO* Gene.

	Test 1			Test 2		
	LR	GOF	α	LR	GOF	α
Insertion	46.955	424.686	216.82	30.519	464.259	167.8
P value	$P < 0.001$	0.106		0.001	0.359	
Deletion	15.674			16.891		
P value	0.228			0.237		

NOTE.— α, selection.

site, fig. 1) (Pool et al. 2006). In addition, the inferred site of selection was position 2,791 bp (−848 bp, test 1) or 2,748 bp (−891 bp, test 2), which falls in the sequence of the TE *Taguchi*.

The Effect of the TE on the Response of the *EO* Gene to 20E

As shown above, an adaptive TE inserted just 462 bp upstream of the transcription start site of the *EO* gene. To assess whether this TE insertion has an effect on expression of nearby gene, we created several reporter gene systems containing the *EO* gene upstream sequences from some domesticated silkworm strains with or without the TE insertion (supplementary fig. S1, Supplementary Material online). The average length of the amplified products was 1,558 bp from strains with the TE insertion and 977 bp from strains without the TE insertion. There are a number of SNPs and indels in the upstream region of the *EO* gene between the two types of the silkworm strains but the main difference is a 512 bp TE insertion (supplementary fig. S2, Supplementary Material online). The luciferase activities were examined at 48 h post transfection (please see the details in Materials and Methods). Under normal condition, the regulatory regions from the two types of the silkworm strains drove the different levels of the luciferase activity (average value: 7.99 ± 2.09 vs. 4.60 ± 0.49, $P = 0.038$) (fig. 3*A*). After 20E treatment, two types of regulatory region significantly increased the luciferase activity (the TE insertion: 25.61 ± 7.11, $P < 0.001$; non-TE insertion: 6.85 ± 1.22, $P = 0.034$) compared with the control. However, the regulatory region with the TE insertion showed an increase of 3.21-fold over the control after 20E treatment, whereas the regulatory region without the TE insertion showed an increase of 1.50-fold after 20E treatment. In addition, we used the same regulatory sequence from one wild silkworm without the TE insertion to measure the luciferase activity and the result is similar to that obtained by using the corresponding regions from the domesticated silkworm without the TE insertion.

In order to detect the core regulatory region, we constructed several 5′-truncated regulatory regions of the *EO* gene from the silkworm strain DT with the TE insertion (fig. 3*B*). The constructs were then transfected and treated as shown above. After 20E treatment, we found no significant reduction of promoter activity by the fragment deletion from −1,565 to −539 nt (fig. 3*B*). However, the luciferase activity was dramatically reduced when the region between −539 and −462 nt was deleted (from 19.63 ± 0.40 to 2.83 ± 0.27, $P < 0.001$), suggesting that critical regulatory elements are located in this region. From figure 3*B*, this region belongs to the fragment of the TE *Taguchi*. Further, sequence analysis showed that a putative 20E-related transcript factor binding site (E74A) is located in this region (−539 and −462 nt) (fig. 3*B*).

The above results showed that the regulatory regions of the *EO* gene from the TE and non-TE inserted strains can be significantly activated by 20E. However, the regulatory regions of the *EO* gene with the TE insertion could significantly enhance 20E-induced response compared with the regions without the TE insertion.

The Phenotypic Effect of the TE Insertion

To survey the effects of the TE insertion in silkworm, we chose nine tetra-molting domesticated silkworm strains (five strains with the TE insertion and four strains without the TE insertion). The strains with the TE insertion or without the TE insertion are not closely related to each other genetically (fig. 2*B* and supplementary fig. S4, Supplementary Material online). The relative expression level of the *EO* gene in silkworm larvae was fluctuation among the strains and no difference between the two types of the strains with or without TE insertion under normal feeding condition (supplementary fig. S6, Supplementary Material online). However, when larvae were starved beginning on the Day 4 of the last instar, the transcript level of the *EO* gene from these two types of silkworm was significantly different (48 h after treatment, $P = 0.007$; 60 h after treatment, $P = 0.004$). The expression ratio of starved larvae to feeding larvae in the strains with the *Taguchi* insertion was significantly higher than that in the strains without *Taguchi* insertion (fig. 4*A*). Chen and Gu (2006) showed that starvation may increase the 20E titer and then accelerate the silkworm larval-pupal transformation. Indeed, the 20E titer was elevated for all silkworm strains after starvation treatment (supplementary fig. S7, Supplementary Material online). In order to measure the relative changes of the 20E titer for each silkworm strain, we used the ratio of the 20E equivalents in the starved silkworm to that in the feeding silkworm. From figure 4*B*, we found that the mean fold increase of the 20E equivalents from the silkworm strains with the *Taguchi* insertion was significantly lower than that from the strains without the *Taguchi* insertion (48 h after treatment, 1.18 ± 0.04 vs. 1.77 ± 0.02, $P < 0.001$; 60 h after treatment, 1.13 ± 0.04 vs. 1.68 ± 0.06, $P < 0.001$). As the result of

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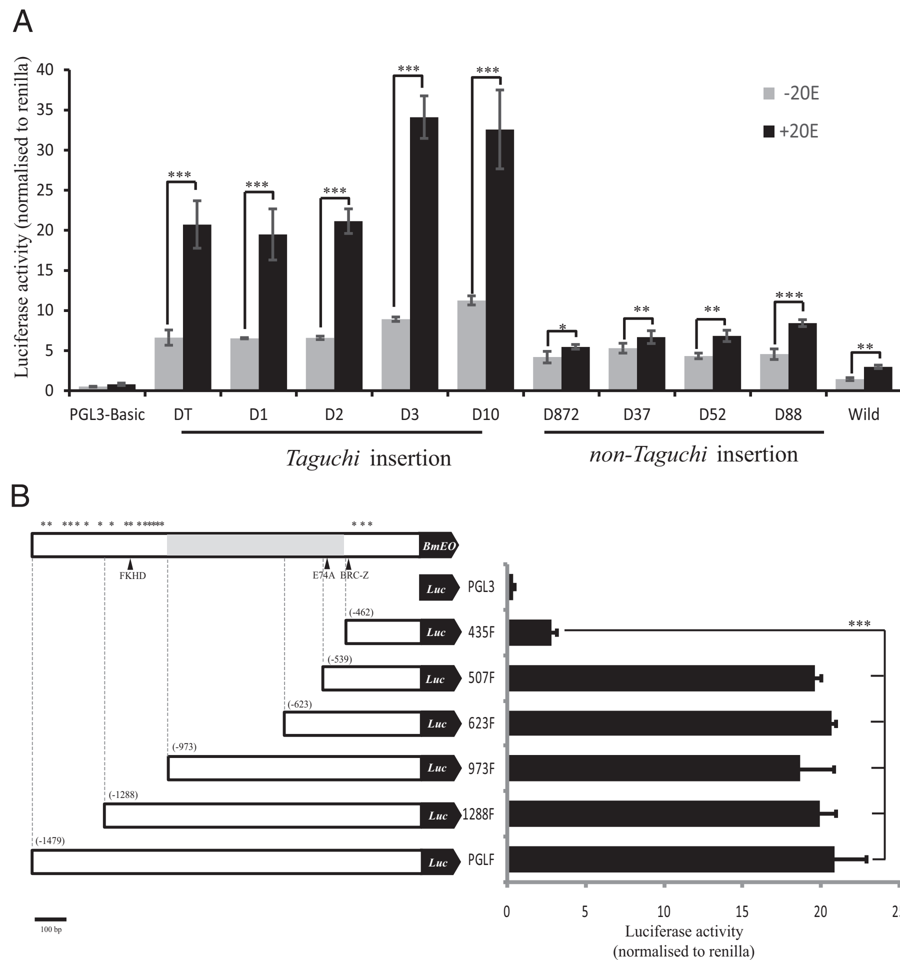

Fig. 3. The relative luciferase expression activity of the upstream region of the *EO* gene in the domesticated silkworm. (*A*) Gray columns indicate the DMSO treatment as control; the black columns indicate the 20E treatment. (*B*) Luciferase activity for 5′-stepwise deletion series of constructs for the *EO* regulatory region after 20E treatment; the left of the figure *B* shows the schematic structure of the stepwise deleted regulatory region, the gray box represents the TE region; the numbers in the brackets mean the distance between truncated sites and the transcription start site; the asterisks in the left figure means fixed SNPs/indels in the TE insertion strains, please see the details in the supplementary figure S2, Supplementary Material online; the black triangles in the left figure represent the predicted 20E-related TFBS; FKHD, fork head factor; BRC-Z, broad complex zinc finger. The right of the figure *B* shows the relative luciferase activity after 20E treatment. *t*-test was used for the statistical analyses.

the changing 20E titer after starvation, the silkworm strains without the TE insertion showed significantly accelerated development (fig. 4C). This is consistent with the observations obtained by Chen and Gu (2006). However, for the silkworm strains with the TE insertion, food shortage has no effect (fig. 4C). This suggests that the two types of the silkworm strains with and without the TE insertion in the *cis*-regulatory region of the *EO* gene had different developmental phenotypes when they suffered from food shortage. The silkworm with the TE insertion showed more stable developmental phenotype than the silkworm without the TE insertion.

Discussion

In nature, wild silkworm's development is extremely sensitive to environmental changes because this can avoid the survival harm of stress factors, such as food shortage (starvation). Food shortage is common in wild, and different insects have evolved different mechanisms to respond to food shortage (Shafiei et al. 2001; Terashima et al. 2005). Shen et al. (2003) found that wild silkworm could accelerate development to adapt to nutritional shortage. However, this mechanism is disadvantageous for sericulture. During the rearing process, the domesticated silkworm is always fed in a mass population. Not all of silkworm in one batch can get enough food (mulberry leaves) in this rearing manner; and some individuals may suffer from food shortage. In practice, therefore, to make sure that the domesticated silkworm grows at a similar rate, humans always do not feed the newly molted larvae until all individuals in one batch have completed molting. If the domesticated silkworm responds to food shortage by accelerating development like its wild progenitor, the domesticated silkworm in one batch will be developmentally nonuniform. Thus, in sericulture, developmental uniformity is one of crucial criteria to evaluate good domesticated silkworm varieties and is also an important domestication trait. However, little is known about the molecular mechanisms of the silkworm developmental uniformity.

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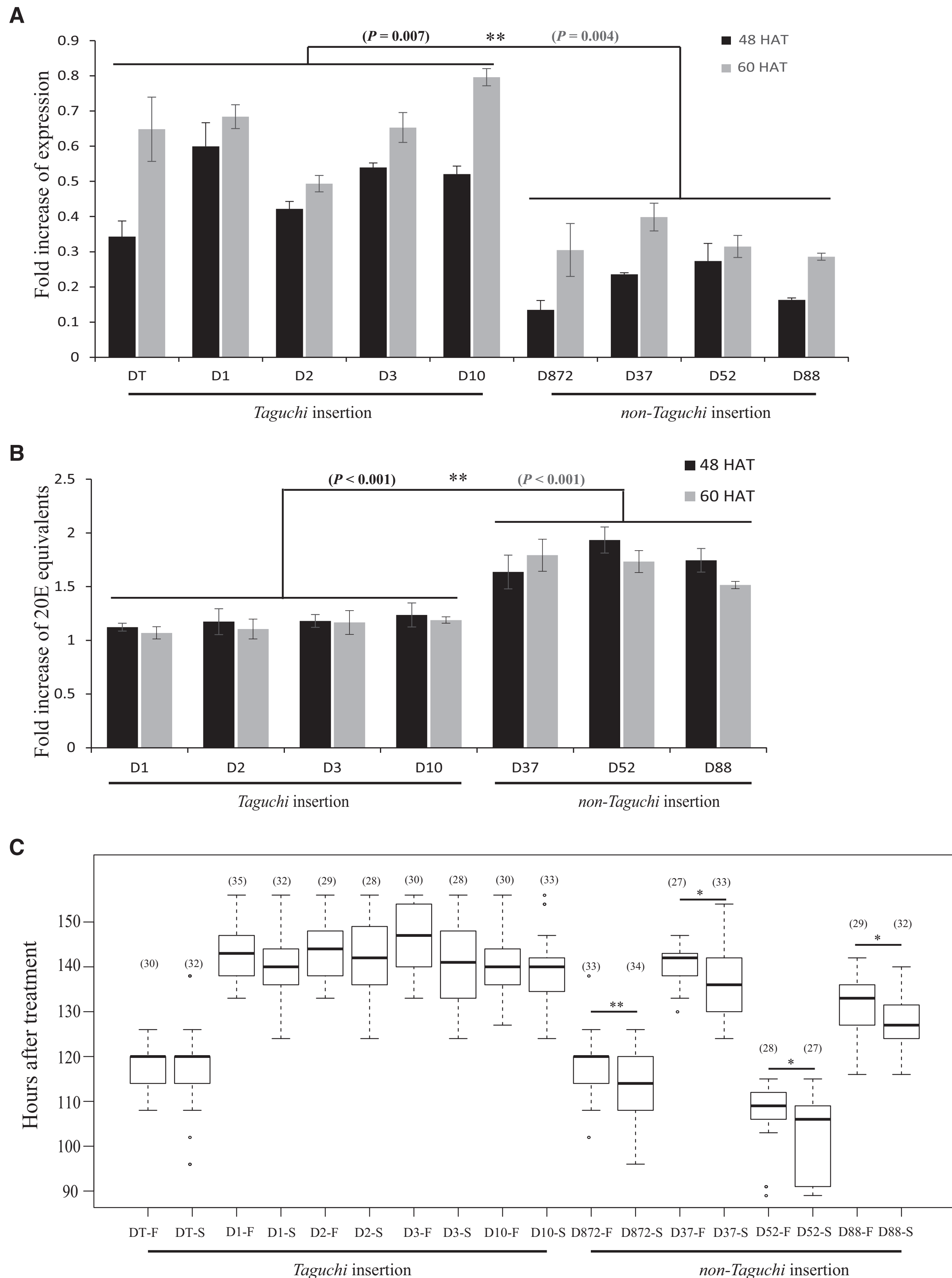

Fig. 4. Effects of starvation on the silkworm *EO* gene expression (*A*); the 20E equivalents (*B*) and the developmental time (*C*). Larvae were starved from Day 4 of the last instar. From the treated time point, we counted the number of silkworm that went to the wandering stage every 6 h. In (*A*), the *y* axis represents the expression ratio of starved larvae to feeding larvae. In (*B*), the *y* axis represents the ratio of the 20E equivalents in the starved silkworm to that in the feeding silkworm. The dark column means the fold increase of the expression in 48 h after treatment (HAT), and the gray column means that in 60 HAT. *t*-test was used for the statistical analyses. The *P* value with the dark color represents the statistic test from the data in 48 HAT, and the gray *P* value represents the statistic test from the data in 60 HAT. In (*C*), *F* means that the larvae were reared on leaves without starvation; *S* means that the larvae were starved from Day 4 of the fifth instar. The numbers above the boxes means the sample size. Wilcoxon signed-rank test was used for the statistical analyses.

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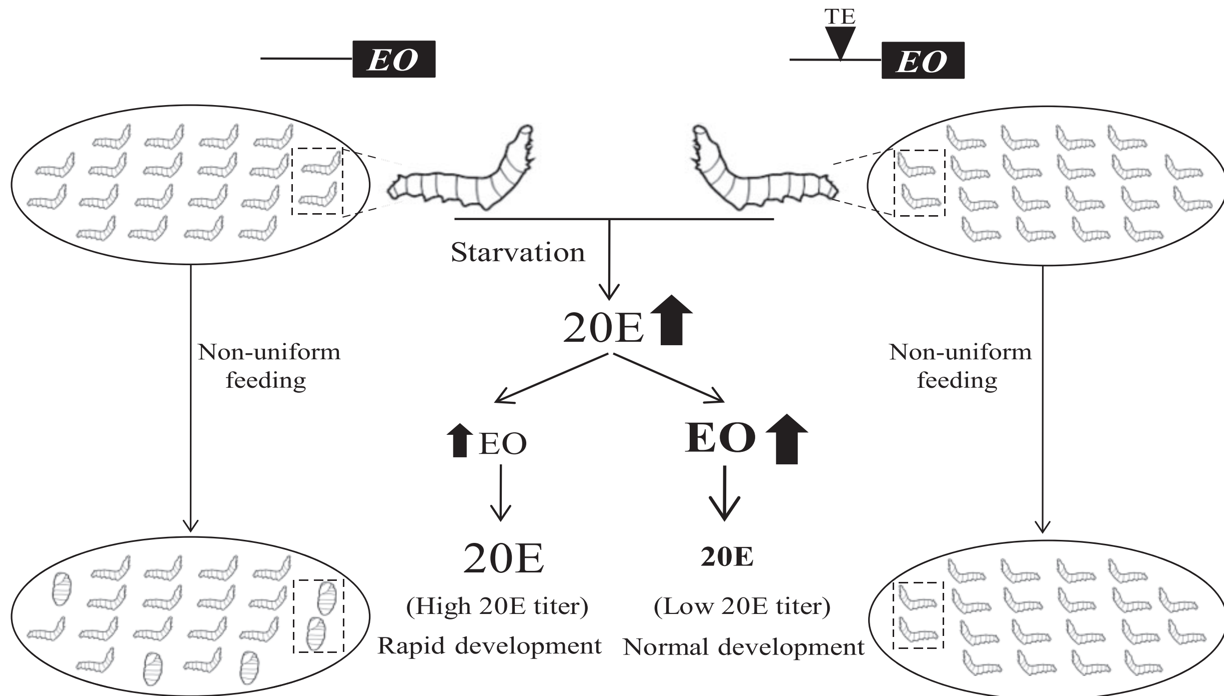

Fig. 5. A model for relationship between the TE insertion and domesticated silkworm development. The left of figure represents the domesticated silkworm strain without the TE insertion. The right of figure represents the domesticated silkworm strain with the TE insertion. The circles represent the domesticated silkworm rearing in one batch. The inner figure explains how the TE insertion affects the *EO* gene expression after starvation. In the inner figure, the size of the fonts represents the 20E titer level or the expression level of the *EO* gene.

In this study, we found that a TE (*Taguchi*) belonging to the LINE family inserted in the *cis*-regulatory region of the silkworm *EO* gene. Strikingly, the nucleotide diversity was found to be greatly reduced around the TE insertion region of the *EO* gene in the domesticated silkworm (table 1). Selective sweep can not only increase the frequency of the adaptive site, but also affect the regions hitchhiking the site. Therefore, it is possible that the TE may be in close linkage with an adaptive mutation. By sequencing the flanking region of the insertion, it was found that the TE appears to be completely linked to the partial sweep and the sweep decays on both sides of the TE insertion (fig. 1). The silkworm *ENGase* gene located about 11 kb upstream of the TE inserted site was also analyzed. This gene showed similar polymorphism patterns between the TE and non-TE insertion strains (fig. 1). In addition, the CLR test also showed that the center of the sweep is located in the TE *Taguchi*. These results strongly indicated that the TE is likely to be the causative mutation generating the selective sweep.

TEs have been shown to adapt to the host genome by providing novel regulatory motifs that control the expression of the nearby genes (Jordan et al. 2003; van de Lagemaat et al. 2003). For example, *Accord* retrotransposon carrying regulatory sequences can increase the expression of *Cyp6g1* gene in several *D. melanogaster* tissues (Chung et al. 2007). Also in *Drosophila*, the roles of the adaptive TEs are expected to regulate the adjacent genes (González et al. 2008; Guio et al. 2014). In this study, using luminescent reporter assay, we found the TE insertion could greatly enhance the transcription of its flanking genes after 20E treatment (fig. 3*A*). Further, the truncated experiments showed that a 77 bp fragment (from −539 to −462, fig. 3*B*) located in the 3′-terminal of the TE was the core regulatory region, confirming that the TE insertion is the center of the selective sweep. Besides, this core region houses a putative 20E-related transcription factor binding sites (TFBS)-E74A (Urness and Thummel 1990). All these results show that the TE insertion rather than other fixed SNPs/indel differences between the two types of the domesticated silkworm strains is the causative polymorphism that enhanced the 20E-induced response of the *EO* gene. EO is a critical enzyme to reduce the 20E titer which could regulate the insect development. Thus, any processes controlled by this hormone (20E) in the domesticated silkworm development and life history could be affected by the TE insertion.

However, we did not find significantly different phenotypes between the strains carrying and lacking the TE insertion in normal feeding condition. Moreover, the expression pattern of the *EO* gene among the strains with or without the TE insertion is also irregular under normal feeding condition. After starvation, the domesticated strains with the TE insertion in the *EO* gene showed normal development, whereas the strains without the TE insertion in the *EO* gene accelerated the development. Starvation could elevate the 20E titer which may up-regulate the expression of *EO* gene in insects (Takeuchi et al. 2001, 2005; Chen and Gu 2006). After starvation treatment, the domesticated silkworm with the TE insertion in the *EO* gene had a higher expression level of the gene compared with the strain without the TE insertion in the *EO* gene (fig. 4*A*). This observation is consistent with the known higher luciferase activity driven by the TE (fig. 3).

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Combined with the above results, it can be speculated that when the domesticated silkworm suffers from food shortage, the strains with the TE insertion in the *EO* gene can enhance the expression level of the *EO* gene which may more efficiently reduce the 20E titer elevated by starvation (fig. 4*B*). For the strain without the TE insertion, after starvation, the elevated 20E titer can only upregulate minor expression level of the *EO* gene which cannot degrade the massive 20E. Thus, the remained high level of molting hormone could accelerate the silkworm pupation (fig. 5, inner).

In conclusion, a TE insertion in the *cis*-regulatory region of the *EO* could enhance the expression level of the gene, which could rapidly reduce higher 20E titer in silkworm body induced by starvation. As a result, the silkworm exhibits normal development even if it suffers from some food shortage (fig. 5, right side). Indeed, several lines of evidence support that the TE insertion in the *cis*-regulatory region of the *EO* gene is adaptive (fig. 1 and tables 2 and 3). Thus, the increased expression level of the *EO* gene due to the TE insertion in the *cis*-regulatory region partially explains the increased developmental uniformity of individuals, a domestication developmental trait, in the domesticated silkworm. It has been proposed that morphological evolution relies predominantly on changes in the architecture of gene regulatory networks and in particular on functional changes within *cis*-regulatory elements (Carroll 2000, 2005). Recent studies have provided direct evidence for the role of *cis*-regulatory element change in morphological evolution (Gompel et al. 2005; Prud'homme et al. 2006; Williams et al. 2008; Wittkopp et al. 2008). Our results emphasize the exceptional role of gene expression regulation in developmental transition of domesticated animals.

Materials and Methods

Taxon Sampling and DNA Extraction

Fifty-nine *B. mori* strains representing four major geographic strains (Chinese, Japanese, European, and Tropical) were obtained from the silkworm resource pool at the Institute of Sericulture and Systems Biology, Southwest University, China. Sixteen Chinese wild silkworms were collected from different geographic regions in China and four Japanese wild silkworms were provided by Dr Y. Banno (Kyushu University, Fukuoka, Japan) (supplementary table S1, Supplementary Material online). Genomic DNA was extracted from individual pupae or moths using a standard phenol-chloroform extraction method (Nagaraja and Nagaraju 1995).

Loci Amplification and Sequencing

Based on the genome sequence of the silkworm, we designed primers in an overlapping fashion to amplify the 5′-flanking region, exon, intron, and the 3′-flanking region of the silkworm *EO* gene (supplementary fig. S1, Supplementary Material online). All the primers were listed in the supplementary table S2, Supplementary Material online. PCR reactions were performed to amplify the target sequences. The PCR products were cloned into PEASY-T1-Clone vectors (TransGen Biotech, China). The positive clones were selected and sequenced. In addition, the silkworm *ENGase* gene, approximately 11 kb upstream of the TE inserted site was also sequenced. *ENGase* gene has four exons. The PCR products for this gene cover it's the second exon and the second intron (Silkworm genome database: nacaf2829: 4310442–4311390). All the sequences have been deposited in GenBank (Accession nos: KF717651-KF717668).

Statistics of Sequence Polymorphism and LD

All the sequences for each silkworm strain were combined together. The sequences were aligned using MUSCLE 3.6 and then checked by hand (Edgar 2004). DnaSP 5.10 was utilized to calculate polymorphism parameters including *S* (the number of segregating sites), π (the mean number of nucleotide differences per site), π_a (the average pairwise difference for nonsynonymous sites), π_s (the average pairwise difference for synonymous sites) and θ_w (Watterson's estimator of $4N_e\mu$) (Librado and Rozas 2009). The minimum number (R_m) of recombination events was also obtained by DnaSP 5.10. LD was measured as r^2 between pairwise sites over physical distance. r^2 was plotted against pairwise distance and two logarithm curves fitting the data were drawn for the TE inserted or deleted domesticated silkworm strains, respectively.

In addition, a haplotype network was also reconstructed using the Median-Joining model implemented in Network 4.516 program (Bandelt et al. 1999).

Neutrality Tests

To examine deviation from the neutral evolution model and to reveal evolutionary history between the TE inserted or deleted domesticated silkworm strains, Tajima's *D* test and Fu and Li's *D* test were performed using DnaSP 5.10.

Selective Sweep Test

To test the selective sweep, we computed the CLR, the iHS and the proportion of nucleotide diversity within the haplotypes linked to the TE to the total nucleotide diversity in the sample ($f_{TE} = \pi_{TE}/[\pi_{TE} + \pi_{non\text{-}TE}]$). In the CLR test, the maximum likelihood of observing derived variants at a polymorphic site under the selective sweep model (H_A) is compared with what is expected under the standard neutral model (H_0) (Kim and Stephan 2002). The resulting maximum LR from the observed data is compared with the distribution of ratios obtained from 1,000 simulations of neutral data sets. Neutrality can be rejected if the LR value of the observed data is significantly larger than the LR values generated under neutrality. The one-tailed *P* value corresponds to the proportions of simulated neutral data sets having larger LR values than the observed data set (Kim and Stephan 2002). In the case the neutral model was rejected by the CLR test, the GOF test was performed with the same data (Jensen et al. 2005). This test compares the fit of a selective sweep model to the data against that of a generalized alternative model, with the goal of differentiating rejections due to selective sweeps from those potentially due to demographic history. GOF values obtained from the observed polymorphism data

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were compared with those estimated from 1,000 data sets simulated under a selective sweep scenario.

To further assess the statistical significance of the nucleotide polymorphism patterns of the *EO* gene in the TE insertion strains, we compared the estimates of iHS and f_{TE} with the distributions of these statistics obtained by neutral coalescent simulations (Voight et al. 2006; Macpherson et al. 2007; González et al. 2009). Guo et al. (2011) and Yu et al. (2011) have sequenced 16 loci from the domesticated and wild silkworms, respectively. Recently, Yang et al. (2014) used the Bayesian inference methods to deduce the demographic histories of the domesticated and wild silkworms using these loci and other four genes. In this study, we utilized the parameters drawn from the posterior distributions of the demographic models inferred by the 20 loci sequences to perform the coalescent simulation using the program msABC (supplementary fig. S8, Supplementary Material online) (Pavlidis et al. 2010). We simulated the 5′-flanking region of the *EO* gene (upstream of the start codon) in the strains with and without the TE insertion, respectively. The simulation samples and sequence length were set to be the same as the observed ones. iHS value was calculated by the iHS calculator under the WHAMM working directory. The method is carried out according to the WHAMM website (http://coruscant.itmat.upenn.edu/whamm/ihs.html, last accessed September 19, 2014).

Reporter Gene Assay

The upstream regulatory regions (−1,479 bp to 0 bp) of the *EO* gene from the different domesticated silkworm strains were amplified using primers PGL-F and PGL-R (supplementary fig. S1, Supplementary Material online). The primer PGL-F is located on the upstream of TE inserted site, and the primer PGL-R is within the 5′-UTR of the *EO* gene. A series of stepwise deletion fragments of the *EO* regulatory sequences from the silkworm strain DT were also amplified by the specific primer pairs. The PCR products were cloned into the PGL3-Basic vector (Promega), and the sequence and orientation were confirmed by sequencing. The recombinant plasmids were cotransferred with the reference plasmids (containing *Renilla luciferase* gene driven by ie1 promoter) into a *B. mori* cell line *BmN* using X-tremeGENE HP DNA Transfection Reagent (Roche). The transferred method was used according to the manufacturer's instruction. At 12 h posttransfection, the cells were treated with dimethyl sulphoxide (DMSO) or 20E (Sigma) at 1 μM. Twenty-four hours after 20E treatment, the cells were collected for luciferase assays.

Silkworm Strains and Treatment

Nine domesticated silkworm strains were reared on fresh mulberry leaves at 25 °C under a 12 h-light: 12 h-dark photoperiod. The larvae were starved beginning on the Day 4 of the last instar, and those that were reared on leaves without starvation were used as controls. All the statistical analyses were done using R software.

Expression Profile Analysis of the *EO* Gene in the Domesticated Silkworm

Two time points after starvation treatment were chosen. For each time point, three to five larvae were collected and grinded in liquid nitrogen to powders and stored in liquid nitrogen. Total RNA was extracted by the Ultrapure RNA Kit (Beijing CoWin Biotech, China) and treated with *DNase I* (Takara, Japan) to remove the genomic DNA contamination. The RNA was quantified by the ultraviolet spectrophotometer, and then 5 μg RNA was reverse-transcribed to the first strand of cDNA by the M-MLV Reverse Transcriptase Kit (Invitrogen). The specific primers were designed and used in the quantitative real-time PCR (qRT-PCR) analysis. The qRT-PCR was performed using real-time PCR Detection System (CFX96, Bio-Rad) with a SYBR Premix EX Taq kit (Takara, Japan). The PCR was carried out as follows: 30 s at 95 °C, followed by 40 cycles of 5 s at 95 °C, 1 min at 60 °C. The silkworm translation initiation factor 4A gene was used as the reference gene.

Ecdysteroid Measurements

For ecdysteroid measurements, ecdysteroids were extracted from the silkworm hemolymph. Birefly, 100 μl hemolymph was collected from the treated silkworm (3–5 individuals), and added with nine volumes of methanol. After centrifugation at 12 000× g for 10 min, an aliquot of supernatant was combined and dried at 70 °C, and then the dried extract was dissolved with 150 μl enzyme immunoassay (EIA) buffer (0.4 M NaCl, 1 mM EDTA [ethylene diamine tetraacetic acid], 0.1% BSA [bovine serum albumin] in 0.1 M phosphate buffer) (Sangon, China). Ecdysteroid levels were quantified via competitive EIA (Cayman Chemicals) using anti-20E rabbit antiserum (Cayman Chemicals), 20E acetylcholinesterase (AchE) tracer (Cayman Chemicals), and standard 20E (Sigma). The AchE activity was quantified by Ellman's Reagent (Cayman Chemicals), and the absorbance at 405 nm was detected with ELx800 absorbance microplate reader (Biotek). All assays were performed in triplicate.

Supplementary Material

Supplementary tables S1–S2 and figures S1–S8 are available at *Molecular Biology and Evolution online* (http://www.mbe.oxfordjournals.org/).

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