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Retrotransposon "*Qian*" mediated segmental duplication in silkworm, *Bombyx mori*

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ABSTRACT

Transposable elements constitute a large fraction of the eukaryotic genomes. They have the potential to alter genome structure and play a major role in genome evolution. Here, we report a segmental duplication mediated by a novel long terminal repeat (LTR) retrotransposon as the cause of an egg-shell recessive lethal mutant ($l\text{-}e^m$ mutant) in silkworm (*Bombyx mori*). The segmental duplication resulted in the duplication of six genes and the disruption of two genes. Disruption of *BmEP80* (*B. mori egg protein 80*), a gene encoding a major egg-shell structure protein, is likely responsible for the lethal water-loss phenotype in the $l\text{-}e^m/l\text{-}e^m$ mutant. Our data revealed that BmEP80 is present in the inner egg-shell layer and plays important roles in resistance to water efflux form eggs. A novel LTR retrotransposon (named as "*Qian*") was identified and the model for the *Qian*-mediated chromosomal segmental duplication was proposed. Detail biochemical and genomic analyses on the $l\text{-}e^m$ mutant offer an opportunity to demonstrate that an LTR retrotransposon could trigger duplication of a chromosomal segment (~96.3 kb) and confer novel phenotype.

1. Introduction

Transposable elements (TEs) have been considered parasitic or selfish DNAs, but recent studies have revealed that they also have the potential to alter gene function and genome structure (Bennetzen, 2000; Kazazian, 2004; Kidwell and Lisch, 2000, 2001). One class of TEs is retrotransposons which amplify through an RNA intermediate. Retrotransposons can be further divided into long terminal repeat (LTR) and non-LTR elements. As the largest component of plant genomes, LTR retrotransposons have been shown to cause DNA fragment removal, gene-pair inversion, and rearrangement of genomic sequence (Bennetzen, 2005; Krom and Ramakrishna, 2012). Particularly, the work reported by Xiao et al. highlighted the role of LTR retrotransposons in gene duplication, which led to the variation of an agronomic trait that was selected during domestication (Jiang et al., 2009; Xiao et al., 2008). To our knowledge, such a duplication event has not been reported in animal genomes.

Silkworm (*Bombyx mori*) is one of the few insects with a long history of domestication by humans, and has served as a model organism for biological study of Lepidoptera (Goldsmith and Marec, 2009). TEs make up 35.4% of its genome and 79.9% of them are retrotransposons (Osanai-Futahashi et al., 2008). Previous studies have shown that the activity of retrotransposons was responsible for several types of mutations in the silkworm genome including insertions and retrotransposon-associated genomic deletions (Liu et al., 2010; Sakudoh et al., 2007). Among the >600 silkworm mutant strains maintained in China, $l\text{-}e^m$ is a recessive lethal egg-shell mutant which is inherited as a recessive type maternal trait (Chen et al., 2012, 2009). Homozygous female and male individuals ($l\text{-}e^m/l\text{-}e^m$) are able to develop and mate normally. However, the eggs laid by $l\text{-}e^m/l\text{-}e^m$ females collapse within 1 h due to the loss of water. For this reason, the $l\text{-}e^m/l\text{-}e^m$ strain is maintained by mating a heterozygous female ($+/l\text{-}e^m$) with a homozygous male ($l\text{-}e^m/l\text{-}e^m$).

The egg-shell has a well-defined extracellular architecture and is an important structure for insects. It is synthesized by the female

Abbreviations: TEs, transposable elements; LTR, long terminal repeat; VM, vitelline membrane; FCs, follicle cells; PBS, primer binding site; PPT, polypurine tract.

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parent and functions in preventing the embryo from physical injury, pathogen invasion, and loss of water (Margaritis et al., 1980; Regier et al., 1982). The insect eggshell consists of two major layers, the inner vitelline membrane (VM) and the outer chorionic layer. The structural proteins embedded in the insect egg-shell are secreted by the follicle cells (FCs) and assembled by a precise mechanism to build a functional network during the late stages of oogenesis (from late vitellogenesis to choriogenesis) (Manogaran and Waring, 2004; Yamauchi and Yoshitake, 1984). The vitelline membrane proteins (VMPs) are specifically synthesized and secreted prior to the choriogenesis stage in FCs (Fargnoli and Waring, 1982). The VMPs and their coding genes were predicted in Lepidoptera and Diptera (Mindrinos et al., 1985; Xu et al., 2012). In *Drosophila melanogaster*, a sterile eggshell mutant, *fs(2)QJ42*, failed to produce a major vitelline membrane protein sV23. The *fs(2)QJ42* mutant females laid collapsed eggs for the oozy eggshell phenotype and the function of sV23 was investigated in depth (Manogaran and Waring, 2004; Savant and Waring, 1989; Wu et al., 2010). In *B. mori*, two putative VMPs, BmVMP30 and BmEP80 (also named as BmVMP90) were identified (Kendirgi et al., 2002; Sdralia et al., 2012; Xu et al., 2011), but the functions of them were not clear.

The research presented here examines the genetic basis of the *l-e*^{m} egg-shell mutation and then demonstrates the function of BmEP80 in water retention for the egg-shell. The data suggest that a novel LTR retrotransposon triggers duplication of a large chromosomal segment and confers a novel phenotype in the silkworm egg.

2. Material and methods

2.1. Silkworm strain

The silkworm strain p50 (*Dazao*, $+^D/+^D$) was maintained at the State Key Laboratory of Silkworm Genome Biology at Southwest University and used for the silkworm Whole Genome Shotgun (WGS) sequencing project (Mita et al., 2004; Xia et al., 2004). The egg lethal mutant strain (*l-e*^{m}/*l-e*^{m}) and the wild-type strains (+/+, +/*l-e*^{m}) were maintained at the Jiangsu University of Science and Technology. Silkworm larvae were reared on fresh mulberry leaves at 25 °C.

2.2. Antibody preparation and immunofluorescence

The 3′ terminal of *BmEP80* (amino acid residues: 453–720) was amplified by an antibody-primer (S-Table 1, flanked by the restriction enzyme cutting sites of *Bam*HI and *Sal*I) and cloned into a pET-28a vector. The pET-28a vector was transformed into *E. coli* BL21 cells and the expression of a His-BmEP80 fusion protein was induced by IPTG. The His-BmEP80 fusion protein was separated on a Ni Sepharose™ column (GE Healthcare) and recovered by electroelution from SDS gels. For preparing the BmEP80 antibody, a total of 800 μg His-BmEP80 fusion protein was used to immunize New Zealand White male rabbits three times and the polyclonal antibodies were purified by a MAbTrap™ Kit (GE Healthcare) according to the manufacturer's instructions.

For immunofluorescence, the follicles from early choriogenesis stages (+1–+6) (Swevers and Iatrou, 2003; Xu et al., 2011) were dissected form Day-6 pupae of the *Dazao* strain and fixed in 4% paraformaldehyde overnight. The follicles were embedded in paraffin and four micrometer thick sections were cut with a microtome (LEICA RM2235, Germany). Sections were subsequently incubated with 1:800 and 1:800 dilutions of the primary and second antibody (Alexa Fluor 555-labeled Goat Anti-Rabbit IgG, Beyotime, China). The photos were collected under the laser scanning confocal microscope.

2.3. Genomic PCR

Genomic DNAs were extracted from the larval post gland on day-3 at 5th instar from *Dazao* ($+^D/+^D$), homozygous *l-e*^{m} mutant (*l-e*^{m}/*l-e*^{m}), heterozygous (+/*l-e*^{m}), and wild-type (+/+) strains. The primer sets (1–7, S-Table 1) for PCR were designed according to the genomic DNA sequence of the *Dazao* strain (http://silkworm.swu.edu.cn/silkdb/).

2.4. Inverse PCR

For inverse PCR, 10 μg genomic DNA of the *l-e*^{m}/*l-e*^{m} mutant strain was digested with restriction enzymes *Hae*III, *Xsp*I, or *Nde*I at 37 °C overnight. After denaturation at 65 °C for 15min, the digested DNA was precipitated by ethanol and dissolved in 20 μl ddH_2O. One microliter digested DNA was ligated by Solution I (TaKaRa, China) and the product was used as the template for inverse PCR. The elongation times for inverse PCR of digested DNA by *Hae*III, *Xsp*I and *Nde*I were set at 3min, 3min and 5min, respectively. The primer sequences of iPCR-primer 1–9 are listed in S-Table 1.

2.5. RNA preparation and RT-PCR detection

The genes *BmVMP23*, *BmEP80* and *BmVMP30.1* were predicted as VM structural proteins and expressed specifically in ovary tissue during the middle/late pupal stage (Xu et al., 2012). According to the microarray data in the larval stage (Xia et al., 2007), *BGIBMGA006586* and *BGIBMGA006585* were highly expressed in testis tissue. *BGIBMGA006758* and *BGIBMGA006584* were highly expressed in midgut/hemocyte and testis/malpighian tubule, respectively. *cJHBP* was expressed in all detected tissues. We then isolated total RNA from male individuals (*l-e*^{m}/*l-e*^{m} and +/*l-e*^{m}, and 5th instar Day-4 larvae) and ovary tissues (*l-e*^{m}/*l-e*^{m} and +/*l-e*^{m}, dissected from the Day-7 female pupae) using Trizol reagent (Invitrogen, USA). The first-strand cDNA was synthesized with AMV Reverse Transcriptase (Promega, USA) according to the manufacturer's protocol. RT-PCR was performed to detect the expression of *BmVMP23* and *BmEP80*, and 25 cycles were performed for each RT-PCR detection. The amplification of *BmActin 3* served as an internal control.

2.6. Genomic DNA quantitative PCR and quantitative RT-PCR

Quantitative PCR was carried out using an ABI PRISM 7000 sequence detection system with SYBR *Premix Ex Taq*™ II reagent (TaKaRa, China). For each primer set, PCR was repeated three times. For genomic DNA quantitative PCR, 30 ng of genomic DNA from *l-e*^{m}/*l-e*^{m} and +/+ strains were used as templates in each amplification. All primers were checked before synthesis against the *B. mori* genome to confirm their uniqueness. The amplification of *BmVMP23* (single copy gene) served as a control.

For quantitative RT-PCR, the cDNA from male individuals (5th instar Day-4 larvae), and ovary tissues (Day-7 pupae) was used as template. The amplification of *B. mori* translation initiation factor 4A (BGIBMGA003186, sw22934, a housekeeping gene) served as a control. The primer sets are listed in S-Table 1. The relative change of the copy number for genomic DNA sequences between *l-e*^{m}/*l-e*^{m} and +/+ was calculated using the $2^{-\triangle\triangle CT}$ method ($\triangle\triangle CT = [(C_T \text{ Target} - C_T \textit{ BmVMP23})(\textit{l-e}^m/\textit{l-e}^m) - (C_T \text{ Target} - C_T \textit{ BmVMP23})(+/+)]$) (Livak and Schmittgen, 2001). The value of $2^{-\triangle\triangle CT}$ corresponds to the relative level of the genomic DNA sequences between the *l-e*^{m}/*l-e*^{m} genomic DNA and the +/+ genomic DNA. For instance, "$2^{-\triangle\triangle CT}$ value = 2" means the copy number of the target sequence in *l-e*^{m}/*l-e*^{m} genomic DNA is twice that in +/+ genomic DNA, and the "$2^{-\triangle\triangle CT}$ value = 1" means the