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Mutation of a vitelline membrane protein, *BmEP80*, is responsible for the silkworm "Ming" lethal egg mutant

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ABSTRACT

The egg stage is an important stage in the silkworm (*Bombyx mori*) life cycle. Normal silkworm eggs are usually short, elliptical, and laterally flattened, with a sometimes hollowed surface on the lateral side. However, the eggs laid by homozygous recessive "Ming" lethal egg mutants (*l-e*^{m}) lose water and become concaved around 1 h, ultimately exhibiting a triangular shape on the egg surfaces. We performed positional cloning, and narrowed down the region containing the gene responsible for the *l-e*^{m} mutant to 360 kb on chromosome 10 using 2287 F_2 individuals. Using expression analysis and RNA interference, the best *l-e*^{m} candidate gene was shown to be *BmEP80*. The results of the inverse polymerase chain reaction showed that an ~1.9 kb region from the 3′ untranslated region of *BmVMP23* to the forepart of *BmEP80* was replaced by a >100 kb DNA fragment in the *l-e*^{m} mutant. Several eggs laid by the normal moths injected with *BmEP80* small interfering RNAs were evidently depressed and exhibited a triangular shape on the surface. The phenotype exhibited was consistent with the eggs laid by the *l-e*^{m} mutant. Moreover, two-dimensional gel electrophoresis showed that the BmEP80 protein was expressed in the ovary from the 9th day of the pupa stage to eclosion in the wild-type silkworm, but was absent in the *l-e*^{m} mutant. These results indicate that *BmEP80* is responsible for the *l-e*^{m} mutation.

1. Introduction

The silkworm, *Bombyx mori*, is a model lepidopteran and has been one of the animals best suited for physiological, biochemical, developmental, genetics, toxicological and mutational studies (Tamura et al., 2000; Yamamoto et al., 2008). The numerous genetic variations in the traits of this moth at every life stage have been discovered, and more than 1000 *B. mori* genetic materials including mutant genes, chromosomal variation strains, and genes for exceptional qualities are currently maintained as genetic resources (Lu et al., 2003).

Mutant genes are important genetic materials that have long held the interest of biologists. Genetic mutations occur in every developmental stage (egg, larva, pupa, and moth) of the silkworm. A relatively comprehensive amount of mutant genes has been collected as early as 1950s (Aruga et al., 1951). An extensive explanation of the mutant genes was presented by Goldsmith (1995). More recently, studies on mutations at the molecular level involving sex determination, genomic studies, and functional genomics (Wang et al., 2005; Goldsmith et al., 2005) have rapidly increased with the completion of the *Bombyx* genomic project (Xia et al., 2004; Mita et al., 2004), with which genomic database was developed (Yamamoto et al., 2008).

Egg mutant is an important type of silkworm mutants because of its special developmental stage. Normal eggs are usually short, elliptical and laterally flattened with a dark brown color. However, the mutant eggs tend to exhibit abnormal phenotypes that differ from those of normal eggs. For example, the eggs laid by the "small egg" (*sm*) mutant are smaller than normal and invariably lethal (Takeda et al., 1996), whereas those deposited by the "Giant egg" (*Ge*) mutant are bigger than normal because of the increased number of follicle cells in the ovary (Kawaguchi et al., 1987, 1991). With the development of molecular biotechnology, some genes related to these egg mutants have been discovered and identified by positional cloning, mutation analysis and RNA interference (RNAi) experiments. For example, *Bm-re* is responsible for the *red egg* mutant (Osanai-Futahashi et al., 2012). *Bmwh3* is responsible for the *w-3*^{oe} mutant (Komoto et al., 2009). *Bm-w-2*, which

Abbreviations: BC_1F, back-cross inbred population; Bm, *Bombyx mori*; CDS, coding DNA sequence; EGFP, enhanced green fluorescence protein; F_1, the first filial generation; F_2, the second filial generation; *Ge*, Giant egg; *l-e*^{m}, the lethal egg of "Ming"; ORF, open-reading frame; P, parent; PCR, polymerase chain reaction; RNAi, RNA interference; Scaf, scaffold; siRNAs, small interfering RNAs; *sm*, small egg; SSR, simple sequence repeat; UTR, 3′ untranslated region; WT, wild-type; 2-DE, two-dimensional gel electrophoresis.

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causes a premature stop codon, is responsible for *w-2* mutant (Tatematsu et al., 2011).

The *l-e*^{m} mutant is a new lethal egg mutant from the silkworm variety Ming. Eggs laid by the recessive homozygote of the *l-e*^{m} mutant lose water and become concaved around an hour. Genetic analysis reveals that the *l-e*^{m} mutation is controlled by a single recessive gene and follows the pseudo-maternal mode of inheritance (Chen et al., 2009). In the present study, we fine-mapped the candidate gene involved in the *l-e*^{m} mutant at a 360 kb area on chromosome 10 and identified *BmEP80* as the gene likely responsible for the *l-e*^{m} mutation. In addition, we found differences in gene sequence, transcriptional level of *BmEP80* and expression level of BmEP80 protein between the wild-type (WT) and *l-e*^{m} mutant. The results indicated that the abnormal termination of BmEP80 protein expression is responsible for the *l-e*^{m} mutation, thereby confirming the importance of the vitelline membrane in maintaining the integrity of the egg structure.

2. Materials and methods

2.1. Silkworm strains

The lethal egg mutant strain Ming-*l-e*^{m}, WT strain Ming and p50 were obtained from the Sericultural Research Institute, Chinese Academy of Agricultural Sciences. The silkworms were reared on mulberry leaves at 25 °C.

2.2. Establishment of positional cloning group

The female parent (P_1) was selected from an inbred line of p50, and the male parent (P_2) was selected from the *l-e*^{m} mutant. A single-pair cross between p50 and *l-e*^{m} mutant produced the F_1 offspring. Twenty BC_1F progenies selected from the cross (p50×*l-e*^{m}) ♀×*l-e*^{m} ♂ were used for the linkage analysis, and 2287 F_2 female progenies from the cross (p50×*l-e*^{m}) ♀×(p50×*l-e*^{m}) ♂ that laid dead eggs were used for the recombination analysis.

2.3. Genomic DNA extraction

Genomic DNA of the parents (P_1 and P_2), F_1 individuals, BC_1F individuals, and F_2 individuals were isolated from the abdominal skin of the moths. DNA was extracted according to previously described methods (Zhao et al., 2000). The quality of the genomic DNA was determined at a 260/280 absorbance ratio, and the concentration of the genomic DNA was diluted to 10 ng/μL and stored at −20 °C.

2.4. Linkage and recombination analysis

Simple sequence repeat (SSR) markers were obtained from the published SSR linkage map (Miao et al., 2005). Polymorphisms of these SSR markers were detected using ABI377 (ABIPRISM). Twenty BC_1F progenies were used for linkage analysis and 36 F_2 female progenies that laid dead eggs were used for preliminary SSR mapping (given that the F_2 female progenies that laid normal eggs included two genotypes (dominant homozygous and heterozygous), and we could not distinguished them from the phenotype, so we used F_2 female progenies that laid dead eggs for SSR mapping). Segregation patterns were analyzed using MapMaker 3.0.

We downloaded the up- and downstream sequences (http://sgp.dna.affrc.go.jp/) close to the tightly linked SSR marker of the *l-e*^{m} locus and searched for new SSR markers using SSRHunter 1.3 based on the results of the preliminary SSR mapping. A total of 2278 F_2 female progenies and new markers that exhibited polymorphism among the parents and F_1 were used for fine mapping. The primers for the SSR markers are listed in Supplemental Table S1.

2.5. Expression analysis of the candidate genes

Total RNAs were extracted from the ovaries of the WT and *l-e*^{m} mutants using RNAiso Plus (TaKaRa). The quality of the total RNAs was determined at a 260/280 absorbance ratio as well as electrophoresis method, after which the RNA was stored at −80 °C. After treatment with DNaseI, 1 μg of the total RNA was used to synthesize the first strand cDNA using Primerscript Reverse Transcriptase kit (TaKaRa) according to the manufacturer's protocol. The primers of the 24 candidate genes for semi-quantitative reverse-transcription polymerase chain reaction are listed in Supplemental Table S2.

2.6. RNAi against the candidate genes

Small interfering RNAs (siRNAs) of the two candidate genes and enhanced green fluorescence protein (EGFP) were designed and synthesized by the RiboBio Company (http://www.sirna.cn/index.aspx). Three optimum target positions of each gene were selected to synthesize siRNAs, namely, GCATGAGAATCAAGGGAAT, GACCAATACATGCAACATA, and CCAGAAATGCTTGGATATT of *BmVMP23*, and GAACGAAGGATTAA TGAAA, CATCGGAAGTTGTGGAATT, and GGAGAAGTTTACATGCTAA of *BmEP80*.

Each siRNA of the candidate genes was diluted to 10 and 20 ng/μl, respectively. The three siRNAs of each gene were mixed in a 1:1:1 ratio. Pupae were divided into two groups. One group was injected with 100 ng siRNA mixture on day-2 and day-5 of the pupa stage, whereas the other group was injected with 200 ng siRNA mixture on the same day. The same dosage of EGFP siRNAs was injected into another group of pupae as a control. The eggs laid by the tested moths were incubated at 25 °C and 60% relative humidity. The relative humidity and temperature were controlled by artificial climate incubators (SPX-250, China).

2.7. Extraction of ovary proteins and two-dimensional gel electrophoresis (2-DE)

2-DE was used to analyze the differential expression of ovary proteins between the WT and *l-e*^{m} mutants. The proteins were extracted from each 2 g to 5 g ovary from the 6th day of the pupal stage to the 1st day of the moth stage. The protein concentrations were determined using a Bradford kit (Sangon). 2-DE was performed according to the manufacturer's recommendations (GE Healthcare). The 2-DE gels were stained by silver staining and scanned using an HP Scanjet G2410 (Hewlett Packard). The images were analyzed using ImageMaster™ 2D Platinum 6.0 software (GE Healthcare).

3. Results

3.1. Mapping of the *l-e*^{m} mutation

Polymorphic markers of each linkage group and twenty BC_1F progenies were used to determine the linkage group on which the *l-e*^{m} gene is located. Given that chromatid exchanges do not occur in female silkworms, if a polymorphic marker is linked with the *l-e*^{m} gene, the banding patterns of the WT and *l-e*^{m} mutants of the BC_1F progenies are thus consistent with the F_1 individual and mutation parent (P_2), respectively. The results of marker S1012 indicated that 10 BC_1F progenies that laid normal eggs exhibited a banding pattern similar to that of F_1, whereas 10 BC_1F progenies that laid dead eggs exhibited a banding pattern similar to that of P_2 (Fig. 1A). Thus, the *l-e*^{m} gene is located on the 10th linkage group, and marker S1012 is linked to the *l-e*^{m} gene.

Based on the linkage analysis, we roughly mapped the *l-e*^{m} gene using three polymorphic markers (S1005, S1012 and S1014) and 36 F_2 female individuals that laid dead eggs. Three-point mapping using MapMaker 3.0 narrowed the linked region to that upstream of