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Construction and long term preservation of clonal transgenic silkworms using a parthenogenetic strain

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ABSTRACT

For the functional analysis of insect genes as well as for the production of recombinant proteins for biomedical use, clonal transgenic silkworms are very useful. We examined if they could be produced in the parthenogenetic strain that had been maintained for more than 40 years as a female line in which embryogenesis is induced with nearly 100% efficiency by a heat shock treatment of unfertilized eggs. All individuals have identical female genotype. Silkworm transgenesis requires injection of the DNA constructs into the non-diapausing eggs at the preblastodermal stage of embryogenesis. Since our parthenogenetic silkworms produce diapausing eggs, diapause programing was eliminated by incubating ovaries of the parthenogenetic strain in standard male larvae. Chorionated eggs were dissected from the implants, activated by the heat shock treatment and injected with the transgene construct. Several transgenic individuals occurred in the daughter generation. Southern blotting analysis of two randomly chosen transgenic lines VTG1 and VTG14 revealed multiple transgene insertions. Insertions found in the parental females were transferred to the next generation without any changes in their sites and copy numbers, suggesting that transgenic silkworms can be maintained as clonal strains with homozygous transgenes. Cryopreservation was developed for the storage of precious genotypes. As shown for the VTG1 and VTG14 lines, larval ovaries can be stored in DMSO at the temperature of liquid nitrogen, transferred to Grace's medium during defrosting, and then implanted into larvae of either sex of the standard silkworm strains C146 and w1-pnd. Chorionated eggs, which developed in the implants, were dissected and activated by the heat shock to obtain females (nearly 100% efficiency) or by a cold shock to induce development to both sexes in 4% of the eggs. It was then possible to establish bisexual lines homozygous for the transgene.

1. Introduction

Transgenic silkworms have been used as a post genomic tool for the analysis of gene function in lepidopteran insects. For example, the function of JH esterase, the impacts of mutated genes, the promoters of silk genes, and the resistance to insect viruses and to *Bacillus thuringiensis* have been examined (Tan et al., 2005; Sakudoh et al., 2007; Quan et al., 2007; Sezutsu et al., 2009; Tatematsu et al., 2010; Ito et al., 2008; Atsumi et al., 2012). The silkworm has also been used as a bioreactor for the production of recombinant proteins; several vectors have been developed and shown to mediate production of mammalian proteins such as interferon, human albumin, mouse antibody, human collagen, etc. (Tomita, 2011; Tatematsu et al., 2012). Since the quality and the amount of products ordained for pharmaceutical purposes must strictly be controlled, the genotypes of transgenic silkworms must be stabilized and the chosen strains must sustain long time storage and propagation without changing any character. The medicines based on recombinant protein production in the transgenic silkworm cannot be approved

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without establishment of reliable rearing and long term preservation techniques of the genetically stable silkworms. Ordinary silkworms are propagated by standard mating whereby each fertilized female lays 300–600 eggs that survive only one year. With current rearing methods, transgenic silkworm strains must be propagated by mating at least once per year. Since the development of gametes is associated with the recombination of chromosomes, the propagation and long term preservation of the transgenic silkworms without genetic changes is very difficult. In addition, the current method of silkworm transgenesis uses a transposon vector that provides random transgene insertion with virtually no control of the level of transgene expression that is subjected to strong position effect. Transgene copy number and the genetic background of the strain also affect the expression level of introduced genes (Uchino et al., 2007, 2008). To circumvent all these problems, we need targeted transgene insertion, fast cloning for homozygous transgenes and genotype stability, and long term preservation of chosen genotypes without the need of continuous silkworm rearing. The achievement of the second and third of these requirements is described in this paper.

Use of a parthenogenetic strain is the crucial innovation of our study. Two types of the thermally induced parthenogenesis have recently been compared (Zabelina et al., 2015). The "ameiotic" parthenogenesis can be induced by a heat shock treatment of unfertilized eggs followed by rapid temperature decrease (Astaurov, 1940). This treatment suppresses the first meiotic division and induces egg development without the male pronucleus. Two female pronuclei of oocyte I remain diploid: one of them is aborted, while the other one becomes nucleus of the oocyte II and initiates cleavage. Since no crossing over of chromosomes occurs in female gametogenesis (Sturtevant, 1915), the pronuclei are genetically identical with the genotype of the parent female, including the ZW chromosomes determining the female sex. Male sex is determined by the ZZ configuration; the factor specifying the femaleness is located on the W chromosome (Hasimoto, 1933; Kiuchi et al., 2014). Exclusively clonal female silkworms develop from the unfertilized eggs in the thermally induced parthenogenesis. However, the propagation of most silkworm strains by means of thermal parthenogenesis is limited because development is activated only in a portion of the unfertilized eggs, their hatchability is very low in most strains, and the mortality of hatchlings is high. Astaurov (1973) created special silkworm strains that are adapted to thermal parthenogenesis and can be maintained as isogenic lineages for long periods of time. The strains were constructed by repeated selection of females with high hatching and survival rates in their parthenogenetic progeny. Some parthenogenetic strains with more than 90% hatchability of the treated eggs have been maintained for more than 40 years exclusively by thermal parthenogenesis. The use and possible benefits of parthenogenesis in the cloning of the transgenic silkworms were suggested by Grenier et al. (2004) but no one has reported successful production and maintenance of transgenic parthenogenetic silkworms.

In this paper we describe the procedure of transgenesis in a parthenogenetic strain. Southern blot analysis of the transgenic silkworms revealed that the genes inserted into the genome were faithfully transferred to the next generation without changing the positions and copy numbers, proving that these silkworms were real clones of their parents. We also demonstrate that the ovaries of the clonal transgenic strains can be preserved in liquid nitrogen and retain capacity to produce viable eggs after defrosting and implantation into a larva of either sex. Furthermore, we show that a cryo treatment of unfertilized eggs yields males that can be mated with clonal females to obtain progeny homozygous for the transgene in both sexes.

2. Materials and methods

2.1. Silkworm strains

The parthenogenetic strain P14 was used to produce transgenic silkworms. The strain was established in 2010 by the selection of females obtained by crossing the parthenogenetic strain P29 of Astaurov (1973) with the bivoltine strain PVN from the Italian silkworm collection. The P14 strain has almost 100% effectual parthenogenetic reproduction (Klymenko et al., 2013) and gives diapausing eggs. The strain possesses the chocolate (*ch*) gene in heterozygous state (ch^+ causes dark brown, and ch^- light brown body color of hatchlings). Male larvae of the non-parthenogenetic strain Soviet 5 (Strunnikov and Gulamova, 1969) were used in our study as the recipients of the P14 ovary implants to obtain non-diapausing parthenogenetic eggs. The eggs of Soviet 5 are either white (males) or black (females). The strains C146 and w1-pnd were used as the recipients of ovaries chilled to the temperature of liquid nitrogen. The strains P14 and Soviet 5 were reared in the Faculty of Genetics and Cytology, Kharkiv National University of Y.N. Karazin (Kharkiv, Ukraine). The strains C146w1 and w1-pnd were maintained at the Transgenic Silkworm Research Unit, Genetically Modified Organism Research Center, at the National Institute of Agrobiological Sciences (Tsukuba, Ibaraki, Japan). The silkworms were reared on artificial diet (Nihon Nosan, Yokohama, Japan) at 25 °C.

2.2. The induction of parthenogenesis and elimination of diapause

To obtain clonal silkworms by ameiotic parthenogenesis, the unfertilized eggs dissected from the moths were thermoactivated by exposure to 46 °C for 18 min followed by immediate cooling to 15 °C for three days (Klymenko, 2001). To break the diapause, the eggs were either soaked in HCl solution of specific gravity 1.110 for 1 h at 25 °C or kept for weeks at low temperature. Meiotic parthenogenesis was induced by exposing unfertilized eggs to −11 °C for 30 min (Strunnikov, 1987).

2.3. The construction of transgenic silkworms

The non-diapausing eggs of the P14 strain were obtained as described by Zabelina and Klymenko, 2008. Ovaries were dissected from the P14 larvae on the 2nd day of the 4th instar and transplanted into the Soviet 5 male larvae of the same age. Transplantation was performed according to Spiridonova et al. (1987) and the hosts were grown until the adult stage. Chorionated eggs were then dissected from the ovary implants and activated by the heat shock treatment. After 12 h at 15 °C, plasmid DNA was injected into the eggs by the method reported elsewhere (Tamura et al., 2000, 2007). The injected eggs were incubated at 15 °C for 3 days and then at 25 °C until hatching. The vector pBac[3xP3DsRedafm] (Fig. 1a, Horn et al., 2002) and the helper plasmid (Tamura et al., 2000) DNAs were prepared with the HiSpeed Plasmid Midi Kit (Qiagen, Hilden, Germany). DNA preparations were repeatedly washed with 70% ethanol, dried in vacuum and dissolved in the injection buffer (5 mM KCl/0.5 mM phosphate buffer at pH 7.0). Hatched larvae were reared until the adult stage when the unfertilized eggs were dissected, thermoactivated, incubated at 15 °C for 3 days and then transferred to the rearing temperature 25 °C. At 3–4 days before hatching, the eggs were screened under fluorescent microscope equipped with the DsRed filter.

2.4. Southern blotting

Genomic DNAs of the 2nd (G2) and the 3rd (G3) generations of transgenic silkworms were purified from the posterior silk gland