Int. J. Indust. Entomol. Vol. 7, No. 1, 2003, pp. 11~14

International Journal of *Industrial Entomology*

Screening of RAPD Markers for Fluoride Resistance in *Bombyx mori* L.

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(Received 14 April 2003; Accepted 8 June 2003)

NF733xin, the near allele line was obtained by means of crossing and backcrossing the silkworm race T6, which contained fluoride resistance major gene, to race 733xin, which was highly susceptible to fluoride toxicity. Two hundred RAPD random primers were used in the RAPD analysis of these 3 strains. Two molecular markers, OPB-08850 and OPB-10917, were obtained. OPB-10917 was used to detect the backcross generations. It was found that all the fluoride resistant individuals in each backcross generation had the same special band. These results proved that this marker was reliable.

Key words: *Bombyx mori*, Near allele line, Fluoride resistance, RAPD marker

Introduction

Silkworm (*Bombyx mori* L.) is one of the important economic insects. Silk fabric has long been welcome all over the world. Silk industry plays an important role in China, India and other developing countries. Among many factors that drive silk industry, the breeding and commercialization of high yield and quality silkworm varieties has contributed greatly to the stabilization and strengthening of this industry. However, as industrialization advanced in these countries, air pollution had become more and more serious. One of the pollutants poisonous to silkworms is fluoride. It has greatly reduced the productivity of the commercial silkworm races in the past decade. Therefore, how to breed commercial silkworm varieties with high resistance to fluoride effectively and efficiently has become one of the most important tasks in front of the sericultural researchers.

Natural silkworm races vary greatly in their resistance to fluoride (Lin *et al.*, 1996; Zhao *et al.*, 1996). In the past decades, sericultural researchers have taken the advantage of this property to breed commercial silkworm varieties that had resistance to fluoride. However, the traditional breeding work involves great time and labor input. Moreover, its selection accuracy and efficiency is quite low. In recent years, RAPD analysis has been widely used to screen the molecular markers for near allele lines so as to establish the molecular marker-assisted breeding protocols (Demeke *et al.*, 1996; Hart *et al.*, 1995).

Lin *et al.* (1997) discovered the major dominant gene that controls silkowrms resistance to fluoride. We used the fluoride resistant race T6 which contained the major gene and the highly susceptible race 733xin to establish the near allele line, NF733xin. Molecular markers of fluoride resistance were screened out with RAPD amplification. The markers were further used to examine individuals of each backcross generation so as to identify if they are reliable.

Materials and Methods

Establishment of the near allele line

T6 (containing the major gene of resistance to fluoride, Chinese strain, bivoltinism) and 733xin (highly susceptible to fluoride, Chinese strain, bivoltinism) were maintained by single pair matings. The near allele line NF733xin was established by crossing 733xin females to T6 males followed by using 733xin as cyclical backcross parent for 6 continuous generations. The progeny of each generation was fed with mulberry leaves treated with 200 mg/l sodium fluoride solution. The males that had resistance to fluoride were mated to 733xin females till the 6th

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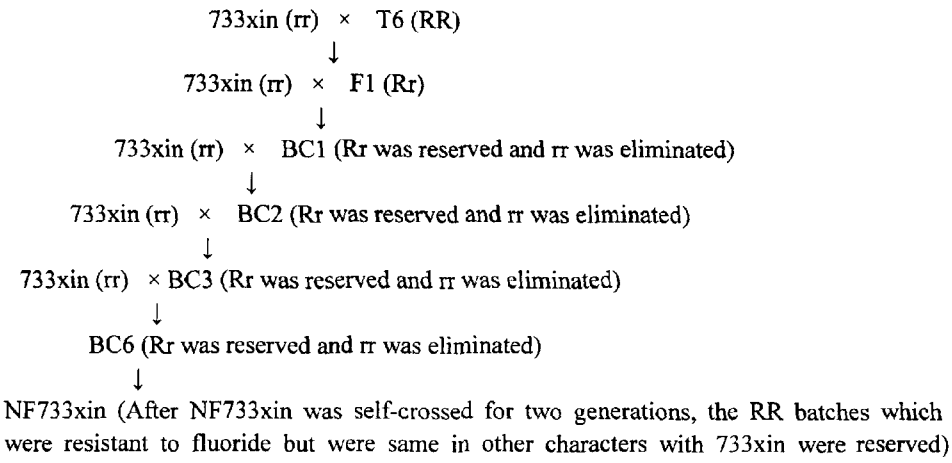

Fig. 1. Preparation of the near allele line resistant to fluoride.

generation (BC6). Then the individuals of BC6 were self-crossed for 2 generations. NF733xin was the progeny of the moths in which the resistance had no separation (*i.e.*, all of its individuals had resistance to fluoride) (Fig. 1).

Preparation of materials

The larvae of T6, 733xin and the near allele line, NF733xin were raised under identical conditions. Those of BC1, BC2, BC3, BC4 and BC5 were raised to test the validity of the molecular markers. Larvae of the 3rd instar till the 3rd day of the 5th instar were fed with mulberry leaves treated with 200 mg/l sodium fluoride solution. The silk glands of 50 surviving larvae of each line were extracted and blended to make their genetic background as consistent as possible.

DNA extraction and purification

Silk glands of 50 larvae were previously frozen in liquid nitrogen and were ground into powder. The powder was divided into 3 samples. From each sample 0.5 g powder was taken to dissolve in 0.5 ml extraction buffer, incubated with proteinase K (final concentration was 100 μg/ml in the mixture) at 56°C for 5 hrs followed by extraction with phenol:chloroform (1:1) and phenol:chloroform:isopentanol (25:24:1), respectively. The DNA solution was precipitated with 0.2 times (V/V) 10 M ammonium acetate and 2 times (V/V) pure ethanol. The pellet was washed with 70% ethanol twice and then dissolved in TE buffer (pH 8.0). The dissolved DNA was incubated with RNase (50 μg/ml) at 37°C for 2 hrs. Then it was extracted with phenol:chloroform (1:1), phenol:chloroform:isopentanol (25:24:1), precipitated with 0.2 times (V/V) 10 M ammonium acetate and 2 times (V/V) pure ethanol, and washed with 70% ethanol twice, and dissolved in TE buffer (pH 8.0) again. DNA concentration and purity was examined with a spectrometer and the sample was stored at −20°C for further analysis.

PCR amplification

PCR amplification was referred to the protocol of Williams (Williams *et al.*, 1990) with some modifications. Total reaction volume was 25 μl which contained 10 × Taq polymerase buffer 2.5 μl, 200 μM dNTPs, 2 mM Mg^{2+}, 0.5 μl 10-mer primer, 10−20 ng template DNA, and 0.2 μl Taq polymerase. Each PCR amplification consisted of 35 reaction cycles plus a final extension reaction at 72°C for 7 minutes. Each reaction cycle was performed as follows: 94°C 30 sec, 40°C 60 sec, 72°C 180 sec. PCR products were analyzed with electrophoresis on 1.2% agarose gels under 5 V/cm for 2 hrs. The gels were stained with ethidium bromide and were consequently photographed and analyzed by using GelDoc-2000 gel image analysing system (Bio-Rad).

Results

RAPD amplification

Two hundreds arbitrary 10-mer primers were used in the RAPD analysis of T6, 733xin and NF733xin. Among 200 primers used, 2 of them, OPB-08 (5GTCCACACGG-3) and OPB-10 (5-CTGCTGGGTG-3), showed polymorphic amplification products. The result of OPB-08 primer was shown in Fig. 2. The figure showed that: (1) A special band of about 850 bp was found in T6 and NF733xin but not in 733xin, indicating that this band was from the resistant parent T6 and was probably linked to the fluoride resistance gene. (2) A band of < 850 bp was found only in NF733xin. It was absent in its two parents, T6 and 733xin. This implied that it was a recombination type. (3) A special band of 2,322 bp was seen in the 733xin but not in NF733xin, showing that it had probably been substituted by the donor resistant parent.

The amplification result of OPB-10 primer was shown in

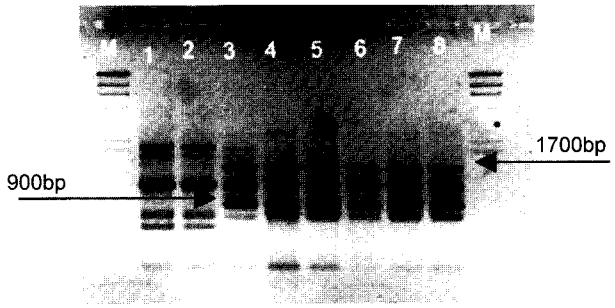

Fig. 2. Amplification results of primer OPB-08 of T6, 733xin and NF733xin. M, molecular marker (λ *Hind*III); Lanes 1 and 2, 733xin; Lane 3, T6; Lanes 4 to 8, NF733xin.

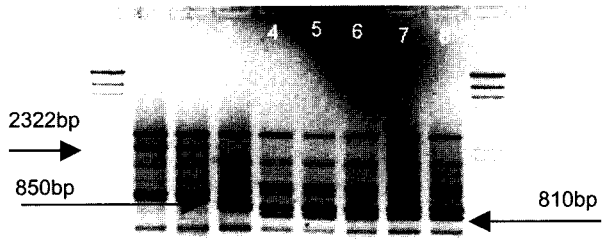

Fig. 3. Amplification results of primer OPB-10 of T6, 733xin and NF733xin. M, molecular marker (λ *Hind*III); Lanes 1 and 2, 733xin; Lane 3, T6; Lanes 4 to 8, NF733xin.

Fig. 3. Its arrangement was similar to that of Fig. 2. There was a special band of 900 bp in T6 and NF733xin but not in 733xin, showing that it was from the resistant parent T6. This band was about the same size to that from primer OPB-08. In accordance with the primer amplification nomenclature, these two special bands were named OPB-08850 and OPB-10900, respectively. Another band of 1,700 bp was found in the resistant parent. It was also found in the near allele line. It was probably related to the fluoride resistance.

Verification of the molecular marker

The BC1, BC2, BC3, BC4, BC5 and the near allele line NF733xin were all raised in the spring of 1999. All larvae were fed with mulberry leaves treated with 200 mg/l sodium fluoride solution. DNA of the surviving individuals was amplified with primer OPB-10. The special OPB-10900 band could be found in each backcross generation (Fig. 4). This result proved that the molecular marker OPB-10900 was reliable.

Discussion

Resistance to fluoride is a very important economic character in *Bombyx mori* L. It is controlled by one major

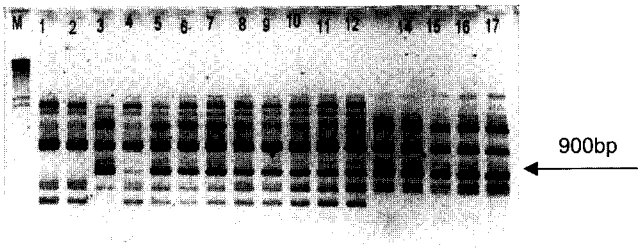

Fig. 4. Amplification results of primer OPB-10 of backcross generations. M, molecular marker; Lanes 1 and 2, 733xin; Lane 3, T6; Lanes 4 and 5, BC1; Lanes 6 and 7, BC2; Lanes 8 and 9, BC3; Lanes 10 and 11, BC4; Lanes 13 to 17, NF733xin.

dominant gene. This had been proved by classical heredity theory recently. Our study used one susceptible race and one resistant race as parents to prepare the near allele line. The near allele line was obtained after 6 generations of backcrossing and 2 generations of self-crossing. Theoretically, the characters of the near allele line should be the same as the susceptible race 733xin except that it carries the major dominant gene of resistance to fluoride. Fig. 2 and Fig. 3 indicate that the near allele line has not carried all of the DNA sequences of the backcross parent. However it did contain the sequence linked to the fluoride resistance gene. A comprehensive analysis to the special bands from the amplification products of primer OPB-10 and OPB-08 reveals that OPB-10900 band is more relevant to the character of fluoride resistance, and is linked to the major dominant gene of fluoride resistance. Reliability of the molecular marker has been demonstrated by using it to examine the backcross generations. The special band had been sequenced. The sequence is now being used to design special primers of 20–25 bp by using SCAR (Sequence characteristic amplified region) technique. Upon completion of the special primers, a molecular marker-assisted selection system for silkworm fluoride resistance can be established.

Acknowledgments

This paper was supported by the Jiangsu Agricultural High-Tech Research Fund (No. BG2001317).

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