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Zoological Research

Screening and Cloning of RAPD Marker of Fluoride Tolerance Gene in Silkworm, *Bombyx mori*

XU Qing-gang, CHEN Ke-ping*, YAO Qin, LIU Xiao-yong
(*Institute of Life Sciences, Jiangsu University, Zhenjiang, Jiangsu* 212013, *China*)

Abstract: In this study, silkworm strain T6, tolerant to fluoride, and silkworm strain 733xin, highly sensitive to fluoride, were used to construct the near-isogenic lines. 300 random primers were used in RAPD amplification to DNAs of these lines. A molecular marker named S207 was found linked to the fluoride tolerance gene. Examination to F_2 segregated individuals of the above lines verified that this molecular marker was reliable. Subsequently, the molecular marker was cloned into a T vector (pUCm-T) for sequencing. Comparing with sequences available in the GenBank showed that this molecular marker was novel. We plan to convert it into a SCAR marker to facilitate establishment of a molecular marker assisted breeding system.

Key words: *Bombyx mori*; Near-isogenic lines; Fluoride tolerance; RAPD marker

家蚕耐氟基因 RAPD 分子标记的筛选及其克隆

徐庆刚，陈克平*，姚 勤，刘晓勇
（江苏大学 生命科学研究院，江苏 镇江 212013）

摘要：以家蚕耐氟品种 T6 和高敏感品种 733 新为材料，并构建其近等基因系，采用 300 个随机引物进行 RAPD 扩增，获得了与家蚕耐氟性有关的一个分子标记 S207，并在 F_2 代分离个体中得到验证，证明了此分子标记的可靠性，进而将此标记克隆进 T 载体 pUCm-T 中，完成了测序，分析发现此序列是新的未有报道的序列。计划下一步将此 RAPD 标记转化成 SCAR 标记，建立分子标记辅助育种技术体系。

关键词：家蚕；近等基因系；耐氟性；RAPD 标记
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Fluoride contained in the industrial waste gas can pollute mulberry leaves. The polluted leaves impede silkworm growth and even kill them. In 1970s, Japan's silkworm rearing suffered great loss caused by soot (Fujii & Hayashi, 1972). From 1980s, a number of industrialized areas of China also suffered from fluoride toxicity in silkworm rearing (Shen & He, 1997). Studies (Liu, 1981; Feng et al, 1999) have showed that, to the 3rd instar silkworms, fluoride content for 30 ppm per gram dry leaves is the safe threshold value. If the content exceeds 30 ppm, the silkworms will be poisoned (Liu, 1981). But different varieties of silkworm have varied tolerance to fluoride (Lin et al, 1996). Lin et al (1997) discovered that there were highly fluoride-tolerant strains among the national silkworm germplasm resources for the first time. The forthcoming hereditary experiments indicated that the fluoride tolerance trait is controlled by a

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* Corresponding author (通讯作者), E-mail: kpchen@ujs.edu.cn, Tel: 0511-8791923
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major dominant gene (Lin et al, 1997). After being transferred into the sensitive strain through hybridization, the major gene made the sensitive strain resistant to fluoride, verifying existence of the major gene for fluoride tolerance (Lin et al, 1997).

Silkworm hybridization has long been adopted to breed silkworm varieties for practical uses and has been very successful in the past. However, in the traditional breeding method, the direction of the gene flow is handled by phenotype, its accuracy is unsatisfactory, the breeding cycle is long, and the efficiency is relatively low. With the advent of the molecular markers, it is hopeful to break the bottleneck that hampers the animal and plant breeding, and to realize the monitoring of the gene flow at DNA level (Fang et al, 2001). At present, there are a lot of reports on molecular marker assisted breeding in plants (Chen & Xia, 2002). The molecular marker researches on silkworm also have achieved significant progress because of its unique biological characteristics and rich heredity knowledge (Chen et al, 2003). The traditional breeding of fluoride-tolerant silkworms has the disadvantage of poor selection efficiency and needs high labor input. So we used fluoride tolerance strain T6 and highly sensitive strain 733xin to cross, then backcross for 6 generations to construct near-isogenic lines. Randomly amplified polymorphic DNA (RAPD) was employed as a tool to screen molecular markers linked to the fluoride resistance gene in silkworm. A specific DNA band was proved to be linked to the fluoride resistance gene. We cloned and sequenced the DNA segment, established the foundation for setting up a molecular marker assisted silkworm breeding system.

1 Materials and Methods

1.1 Silkworm strains

The silkworm strains are maintained by the Institute of Life Sciences of Jiangsu University. We employed fluoride tolerance strain T6 as the male parent and highly sensitive strain 733xin as the female parent to obtain the F_1 progeny. Then, 733xin was used as recurrent parent to backcross the F_1 progeny for 6 generations (Yao & Chen, 2002). For each generation fluoride was administrated to the larvae and the individuals with strong resistance were selected for the subsequent backcrosses with 733xin till the near-isogenic lines were obtained. Meanwhile, F_2 generation was established and employed to verify the molecular marker. The *E. coli* strain of DH5α is maintained by the Institute of Life Sciences of Jiangsu University.

1.2 Preparation of DNA

The fluoride resistant parent T6 and the near-isogenic lines were fluoridated from 3rd instar to 5th instar with 320 ppm NaF, and silk glands were picked up from the normal individuals fed by fluoride. The susceptible parent 733xin were fluoridated from 3rd instar to 5th instar with 40 ppm NaF, and silk glands were picked up from the sensitive individuals separately. The F_2 progeny were fluoridated from 3rd instar to 5th instar with 320 and 40 ppm NaF, and then picked up from the normal individuals and sensitive individuals separately. Genomic DNA was extracted from the silk glands by the method of Chen et al (2001).

1.3 RAPD-PCR and specific segment verifying

T6, 733xin and the near-isogenic lines were amplified with 300 primers. If any specific primer was obtained, we used it to amplify the F_2 progeny to verify the linkage to fluoride tolerance gene.

PCR was performed in a total reaction mixture of 25 μL consisting of 2.5 μL 10 × PCR Buffer, 1.5 mmol Mg^{2+}, 33 ng of template DNA, 0.5 μmol primers, 1 U of Taq polymerase (Takara), and dNTPs (Takara) at 0.20 mmol each. The amplification was carried out with a 2 min denaturation at 94 ℃, followed by 40 cycles of 30 s denaturation at 94 ℃, 1 min hybridization at 40 ℃, and 90 s elongation at 72 ℃, then followed a 10 min elongation at 72 ℃, 4 ℃ soak finally. Electrophoresis was carried out at 3 – 5 V/cm in the 1.2% agarose gel in 1 × TAE buffer, dyed by EB.

1.4 Identification of the recombinant plasmid

we cloned the specific DNA segment that reclamation from the PCR production into T vector of pUCm-T (Songon). The methods of cut by restriction endonuclease and amplified by the molecular marker were employed to identify of the recombinant plasmid.

2 Results and Analysis

2.1 Analysis of the marker screening result

We employed 300 random primers in this experiment. A molecular marker S207 (GGCAGGCTGT) that linked with the fluoride tolerance gene was obtained. It amplified a 930 bp specific DNA segment. We amplified the separated individuals of the F_2 progeny and compared with the amplified results of T6, 733xin and the near-isogenic lines. The strain T6, the near-isogenic lines, and the fluoride-resistance individuals of F_2 progeny have a 930 bp specific DNA segment. But there is no this specific DNA segment in 733xin and the fluoride-susceptible individuals of F_2 progeny. It is verified that the specific DNA segment come from resistant parent T6 and link to resistance gene (Fig.1).

2.2 Cloning of the specific DNA segment and identification of the recombinant plasmid

The recombinant plasmid was cut by restriction endonuclease of *Pst* Ⅰ, which has two cut sites on the T vector. The objective DNA was just cut off from the recombinant plasmid. A same DNA segment was obtained from the amplified recombinant plasmid by primer S207. All these show the objective DNA had being cloned into the recombinant plasmid (see Fig.2).

2.3 Sequencing and analysis

The recombinant plasmid was sequenced by GENE Co. Ltd. The Target DNA segment is 938 bp (including the primers' sequences).

The sequence was analyzed by DNAstar version 5.01. We searched the sequence in GenBank, but did not find similar DNA sequence. The sequence that we got is a new one.

3 Discussions

Resistant to fluoride is a very important economic property of silkworm. It is already verified that fluoride tolerance property of silkworm is controlled by a major dominant gene by classical heredity theory at present. In theory, the near-isogenic lines have the same hereditary materials as the recurrent parent 733xin after 5 – 6 generation backcross except the resistance gene (Yao & Chen, 2002).

There are RAPD polymorphism between the near-isogenic lines and 733xin. It shows that the hereditary materials of donor parent T6 are not replaced all by

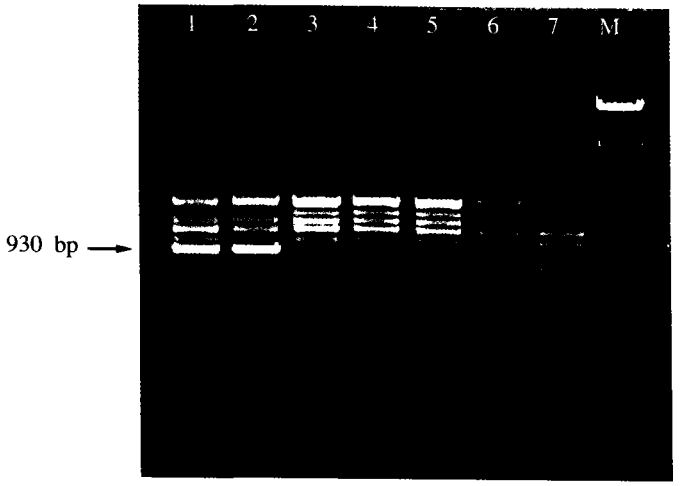

Fig.1 Validation of the specific DNA band

Lane 1: Resistant parent; Lane 2: Near-isogenic lines; Lane 3: Susceptible parent; Lane 4 – 7: Susceptible individuals of F_2; M: Marker (λDNA/*Hand*Ⅲ + *Eco*RⅠ).

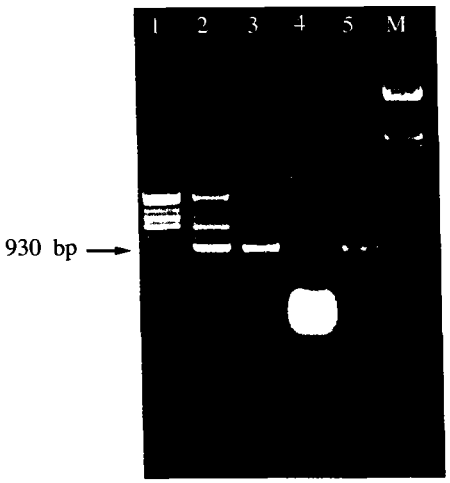

Fig.2 Identification of the recombinant plasmid
Lane 1: Susceptible parent; Lane 2: Resistant parent; Lane 3: Amplifying of the recombinant plasmid; Lane 4: Result of the recombinant plasmid cut by *Pst* Ⅰ; Lane 5: Target DNA fragment; M: Marker (λDNA/*Hand*Ⅲ + *Eco*RⅠ)

733xin, but the near-isogenic lines has got the resistance gene and the DNA segment has linked to it.

At present the research work on molecular marker of silkworm fluoride tolerance has been did only by Chen et al (2001). Two molecular markers linked to gene have been obtained (Chen et al, 2001). But the RAPD linkage maps of silkworm are not dense enough. The RAPD linkage map constructed by Li Bin is not accord to the classic genetic linkage map because of the molecular markers' insufficiency (Li et al, 2000). Recent progress in genome biology relies on detailed linkage maps, but it is often difficult to construct such maps for organisms worked on by not enough molecular markers. So more molecular markers are needed to be screened, then we can make an accuracy orientation of the alleles and cloning them. This paper had already sequenced the specific DNA segment. The result of the sequence analysis showed that it is a new sequence. The next step of the plan is to transforms this RAPD marker into SCAR (sequence characterized amplified region) marker, so as to set up a auxiliary breeding technology system of molecular marker.

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