NOTES

Integration and inheritance stability of foreign *Bt* toxin gene in the bivalent insect-resistant transgenic cotton plants

XIA Lanqin[1] & GUO Sandui[2]

1. Institute of Crop Breeding and Cultivation, Chinese Academy of Agriculture Science, Beijing 100081, China;
2. Biotechnology Research Center, Chinese Academy of Agriculture Science, Beijing 100081, China

Correspondence should be addressed to Guo Sandui (e-mail: gsdui@public.bta.net.cn)

Abstract **Genetic and expressional stability of *Bt* toxin gene is crucial for the breeding of insect-resistant transgenic cotton varieties and their commercialization. Genomic Southern blot analysis of R_3, R_4 and R_5 generations of bivalent transgenic insect-resistant cotton plants was done in order to determine the integration, the copy number and the inheritance stability of *Bt* toxin gene in the transgenic cotton plants. The results indicated that there was a 4.7 kb positive band in the Southern blot when the genomic DNA of the bivalent transgenic insect-resistant cotton plants and the positive control (the plasmid) were digested with *Hind*Ⅲ respectively. This result proved that the *Bt* toxin gene had been integrated into the genome of the cotton in full length. There is only one *Xho* Ⅰ restriction site in the *Bt* toxin gene. Southern blot analysis indicated that many copies of *Bt* toxin gene had been integrated into the genome of the cotton when the genomic DNA of transgenic plants was digested with *Xho* Ⅰ. Among them, there were four copies (about 17.7, 8, 5.5 and 4.7 kb in size) existing in all the tested plants of R_3, R_4 and R_5 generations. The preliminary conclusion was that there were more than four copies of *Bt* toxin gene integrated into the genome of the cotton, among them, more than one copy can express and inherit steadily. This result provides a scientific basis for the breeding of the bivalent insect-resistant transgenic cotton plants and its commercialization.**

Keywords: ***Bt* toxin gene, the bivalent insect-resistant transgenic cotton plants, integration, copy number, the genetic stability.**

The expression and genetic stability of *Bt* toxin gene is crucial for the breeding and the commercialization production of the bivalent insect-resistant transgenic cotton plants. Three elite varieties (GK95-1, GK-1 and GK-12) had passed the variety regionalization in the provinces of Shanxi, Anhui and Shandong, and renamed Jinmian 26, Guokangmian 1 and Guokangmian 12 respectively in 1998. The three varieties have been disseminated more than 18 million hm^2 in different areas of cotton belts, alleviating efficiently the great losses caused by cotton bollworm and the pollution caused by the chemical insecticide. At the same time, in order to delay the development of the insect resistance to the *Bt* toxin gene, since 1996, the *Bt* toxin gene and the *CpTI* gene that had different insecticidal mechanisms with the *Bt* toxin gene had been constructed into the same expression vector. We cooperated with the scientists of the Economic Crop Research Institute of Jiangsu Academy of Agriculture Science in transforming several cotton varieties such as Zhongmiansuo 19, Shiyuan 321, 3577 and 54 via the pollen tube pathway. And we obtained the bivalent insect-resistant transgenic cotton plants that could express the *Bt* toxin gene and the *CpTI* gene simultaneously[1, 2]. The bioassay and field evaluation indicated that they were highly resistant to the bollworm and nearly reached the homozygotic state in R_3 generation. Up to now, the genetic stability of the *Bt* toxin gene in the insect-resistant transgenic cotton plants had been studied only through the bioassay of the insecticidal activity of different generation materials[3, 4]. Many foreign scientists are in doubt about the successfully transformed insect-resistant cotton plants via the pollen tube pathway because there is no Southern hybridization evidence available at present, especially the transmission of the *Bt* toxin gene from one generation to the other. In this research, the integration, the copy number and the inheritance stability of foreign *Bt* toxin gene in the bivalent insect-resistant transgenic cotton plants were studied by Southern blot analysis in order to provide a theory basis for the breeding of the bivalent transgenic insect-resistant cotton plants and its commercial production.

1 Materials and methods

(ⅰ) Materials

(1) Bacterial strain and plasmid. The bacterial strain was *E. coli* (DH 5α). The plasmids were pG4AB and pGBI1214ABC deposited in our lab.

(2) Chemical reagents. The restriction endonucleases were products of Bio-lab. The isotope α-^{32}P-dATP was bought from Beijing Yahui Biological Ltd. The random primer DNA labeling kit was used for probe production. Other chemicals were from Hualüyuan Biological Co.

(3) The tested cotton materials. The plants of R_3, R_4 and R_5 generations of bivalent insect-resistant transgenic cotton (the receptor was *G. hirsutum*-Zhongmiansuo 19) were planted in our experiment field. And the non-transgenic cotton plants Zhongmiansuo 19 was as negative control.

(ⅱ) Method

(1) Probe preparation. The extraction, restriction endonuclease digestion and purification of the plasmid DNA were done according to Sambrook[5]. Probe was prepared according to the guide by the Promega Company of random primer DNA labeling system using the isotope α-^{32}P-dATP.

(2) Southern blot analysis. The extraction of cotton genomic DNA was done according to ref. [6]. 30 μg of genomic DNA were digested with restriction endonuclease for 16—20 h at 37℃. The total digestion volume was 500 μL, and 1 μg DNA was added with 5—10 units of enzyme. The electrophoresis, transfer, the pre-hybridization and hybridization were done according to Sambrook[5].

2 Results and analysis

(i) Southern blot analysis proves the integration of *Bt* toxin gene in the genome of cotton. The plasmid pGBI1214ABC (as the positive control, see fig. 1), the negative control (non-transgenic cotton plants) and the genomic DNA of the bivalent insect-resistant transgenic cotton plants were digested with *Hind* III respectively and probed with a 1.4 kb *Pst* I restriction fragment. Southern blot result proved that a 4.7 kb fragment appeared as a positive band in all the tested materials except the negative control. Therefore, it was proved at the molecular level that not only the *Bt* toxin gene had been integrated into the genome of the cotton, but also it existed in full length (as shown in fig. 2).

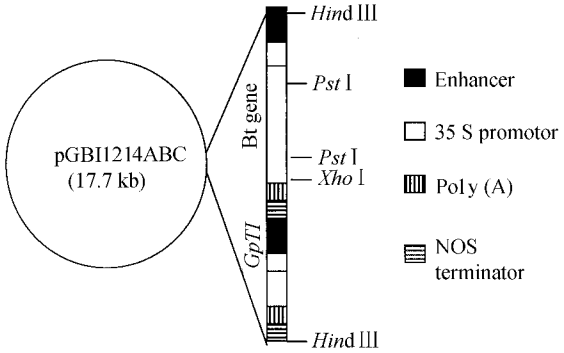

Fig. 1. The map of pGBI1214ABC

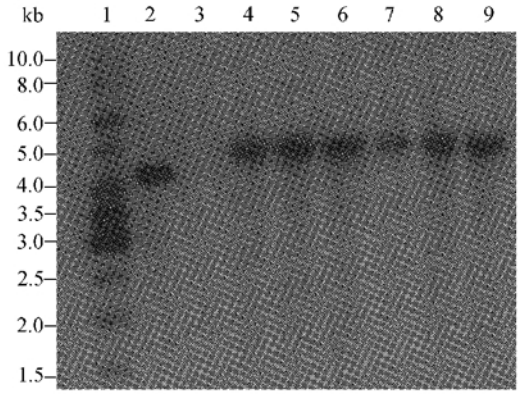

Fig. 2. Southern blot analysis of the genomic DNA of the transgenic cotton plants digested with *Hind* III. 1, 1 kb ladder; 2, CK+; 3, CK-; 4, 5, DNA of the R_3 generation materials of the bivalent transgenic insect-resistant cotton plants digested with *Hind* III; 6—9, DNA of the R_4 generation materials digested with *Hind* III.

(ii) Southern hybridization to determine the copy number of the *Bt* toxin gene and its genetic stability.

Xho I has one unique restriction site in the plasmid pGBI1214ABC, which is located at the outside of the probe DNA fragment. The copy number can be determined by Southern blot analysis after the genomic DNA of the transgenic cotton plants digested with *Xho* I. All the tested materials including the positive and negative control were digested at the same time. The digestion system was detailed in sec. 1(ii). The results (shown in fig. 3) were as expected, that is, the positive control had a positive band of 17.7 kb and no band appeared in the line of the negative control. But all the tested materials had four positive bands (about 17.7, 8, 5.5 and 4.7 kb), among them, the band of 17.7 kb was the most intensive, while the other bands were not concordant with each other. The preliminary conclusion was that more than four copies of *Bt* toxin gene had been integrated into the genome of the cotton. In order to testify the consistence of the restriction fragment of *Hind* III and *Xho* I, hybridization was done after the genomic DNA of transgenic cotton plants digested by *Hind* III and *Xho* I respectively. The result is shown in fig. 4, which further proved that the *Bt* toxin gene existed in full length in the genome of the cotton.

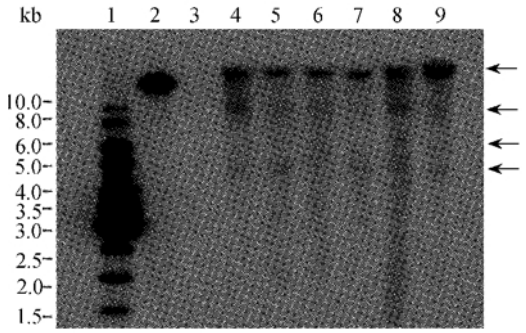

Fig. 3. Southern blot analysis of the genomic DNA of the bivalent transgenic cotton plants digested with *Xho* I. 1, 1 kb ladder; 2, CK+; 3, CK-; 4, 5, DNA of R_3 materials of the bivalent transgenic cotton plants digested with *Xho* I; 6—9, DNA of the R_4 generation materials of the bivalent transgenic cotton plants digested with *Xho* I.

In order to determine the inheritance stability of the *Bt* toxin gene, we extracted the genomic DNA of nine plants of R_5 generation at random. Digested with *Xho* I, hybridization was done to determine the copy number and the inheritance stability of the *Bt* toxin gene. The results are indicated in fig. 5. All the tested R_5 generation materials had positive bands that were about 17.7, 8, 5.5 and 4.7 kb. This result further proved the conclusion we had obtained from the R_3 and R_4 materials; that is, many copies of *Bt* toxin gene had been integrated into the genome of the cotton, among them four copies could be steadily inherited. In addition, a common band of 3.25 kb existed in all the nine tested materials. Compared with each other, the Southern blot patterns were similar among all the ma-

terials though there were some little differences. This indicated that gene recombination and gene amplification of the *Bt* toxin gene happened in the genome of the cotton and caused the variations of the Southern blot patterns among the different plants of the same progeny. But these did not impact the expression of *Bt* toxin gene in the progeny of the trangenic cotton plants.

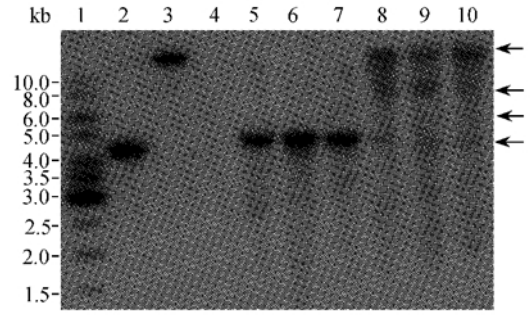

Fig. 4. Southern blot analysis of the R_3, R_4 generation materials of the bivalent insect-resistant transgenic cotton plants. 1, 1 kb ladder; 2, CK+(*Hin*d III); 3, CK+(*Xho* I); 4, CK-; 5,8, DNA of the R_3 generation materials digested with *Hin*d III and *Xho* I respectively; 6, 7, 9, 10, DNA of two R_4 generation plants digested with *Hin*d III and *Xho* I respectively.

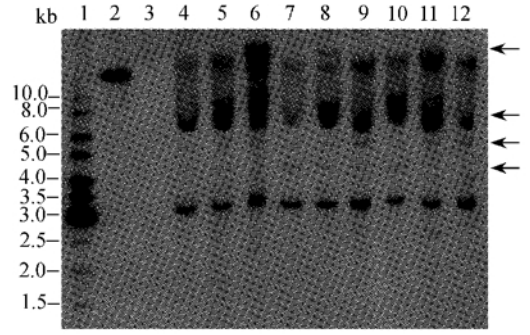

Fig. 5. Southern blot analysis of the R_5 generation materials of the bivalent transgenic cotton plants digested with *Xho* I . 1, 1 kb ladder; 2, CK+(*Xho* I); 3, CK-; 4—12, DNA of the R_5 generation materials digested with *Xho* I .

3 Discussion

Southern blot analysis of the genomic DNA digested with *Xho* I indicated that there were many positive bands that were different in size with the transformed plasmid DNA in addition to the same. Other researchers have observed this phenomenon too[7—9]. The above results indicates that there are many possible ways for the transformed plasmid DNA to exist on the receptor chromosome: (i) The transformed plasmid DNA concatamers arranged in head-to-tail repeats, then were integrated into chromosome at the same or different site. (ii) There was one full-length plasmid DNA flanked by two non-full-length plasmid at each side. (iii) The plasmid DNA recombination happened before or during the integration, while some restriction endonuclease sites might be methylated. On the other hand, recombination or deletion could happen in some DNA fragments. All these could lead to the positive bands in the hybridization which were more or less larger in size than the transformed plasmid DNA[7,9].

The Southern blot analysis indicated that although there were copy number differences among the R_3, R_4 and R_5 materials, four copies of *Bt* toxin gene that were about 17.7, 8, 5.5 and 4.7 kb in size were possessed by all the tested materials. This demonstrated that the four copies of *Bt* toxin gene could be steadily inherited. In addition, there appeared other different bands when compared with each other. This may be caused by two reasons: first, other copies of *Bt* toxin gene were not integrated at the same site with the above four copies, and they segregated during selfing; second, it was caused by rearrangement and translocation. This phenomenon has also been found in other transgenic plant studies. For instance, Matassi et al.[10] have found that in plants there exists the recombination repairing system which could identify prokaryotic plasmid DNA when he studied the transgenic rice plants. He thought that deletion and translocation happened often in the chromosome region that the foreign DNA integrated, and this would result in the rearrangement of the plant genomic DNA. Gou et al.[11] found that the rearrangement of the chromosome had caused changes in the morphology of the bivalent insect-resistant transgenic cotton plants. Although this was an individual phenomenon, it demonstrated that the mechanism of the rearrangement of chromosome existed in the genome of the cotton plants.

It had been proved by many researches that the inheritance modes of many transgenes were in accordance with Mendel's law of inheritance the expression of the transgene is dominant. No matter how many copies were arranged in tandemerization, if it was integrated at one site and its length was not longer than 1 cM, it would inherit as a single dominant gene. There are also some transformants which have two or more transgenes, inherited as two dependent or linked dominant genes. Zhang[4] studied the inheritance of insecticidal activity of the insect-resistant transgenic cotton plants in China, and proved that the *Bt* toxin gene can be steadily inherited in the progeny of the transgenic cotton plants, the insecticidal activity was concordant with each other in the following generations. The inheritance of target character in transgenic cotton transformed by the pollen tube pathway and agrobacterium-mediated transformation method was consistent with the Mendel's law of inheritance with the segregation ratio of 3 ∶ 1 or 15 ∶ 1[4].

The bioassay of the R_3, R_4 and R_5 generation materials indicated that the insecticidal activity could express and inherit steadily and reached homozygous state in R_3 generation. Therefore, it was postulated that the four copies of *Bt* toxin gene integrated at the same site or at different sites, only one copy could express. It was most possible that these four copies integrated at one site and inherited steadily because both the parents and their progenies possessed them[7,9,12].

There are many protocols developed for the transfer of foreign gene into the plant at present, such as agrobacterium-mediated, particlement microprojectile, PEG, pollen tube pathway and so on. *Rhizobium* agrobacterium-mediated transformation method is commonly used in the transformation of many dicotylodonous plants, but the transformed cells have to be cultured into plants and for many plant varieties which have low culture regeneration frequency it is difficult to obtain trangenic plants. In recent years, although the cotton culture techniques have been improved, the transformation is greatly impacted by the genotypes and other factors. Developed by Zhou et al.[13], the pollen tube pathway for the transfer of the foreign gene is easy to master and does not have the above difficulties. Thereafter, many reports demonstrated the transfer of foreign gene into plants by using this method, among them, the most successful was the transfer of the synthesized *GFMcryIA* gene into cotton by pollen tube pathway and the obtaining of the insect-resistant transgenic cotton plants[14]. But no evidence was found at molecular level, especially about the transmission of the *Bt* toxin gene into the progenies[15], so many foreign scientists doubted about the successful transformation of the insect-resistant transgenic cotton plants. This research has proved for the first time by Southern hybridization the integration and inheritance stability of the foreign *Bt* toxin gene transferred by pollen tube pathway in the trangenic cotton plants. This result fully illustrated the reliability and utility of the pollen tube pathway transfer system and set a scientific basis for the commercialization and variety breeding of the transgenic cotton plants.

In this research, we found that the foreign *Bt* toxin gene integrated on the chromosome of the cotton in many copies. It has been reported that the integration of many copies can result in gene silencing[12,16], but there were also researchers who did not agree with this opinion[17]. The results of our study indicate that although the *Bt* toxin gene exists in many copies, they can steadily be inherited and expressed, and no silencing has happened. This is consistent with the research of Hobbs[17]. It is proposed that the foreign *Bt* toxin gene may be integrated at the high expression site on the chromosome. Therefore, it is essential to analyze the characteristics of the integration site. That will be beneficial to the study of the transgenic plants.

Acknowledgements This work was supported by the National "863" High-Tech Program, the Special Foundation of the Ministry of Agriculture for "Developing Cotton Production" and the Chinese Foundation for Agriculture Science and Education.

References

1. Guo, S. D., Ni, W. C. et al., The application of the fused gene encoding the insecticidal protein and the expression vector (in Chinese), 1995, 12.28 Patent ZL 95119563.8,C12N.
2. Guo, S. D., Cui, H. Z., Xia, L. Q. et al., The study of the bivalent insect-resistant transgenic cotton plants, Scientia Agriculturia Sinica (in Chinese), 1999, 32(3): 1.
3. Tang, C. M., Sun, J., Zhu, X. F. et al., The genetic analysis of the insect-resistant activity of the three *Bt* transgenic cotton plants in China, Chinese Science Bulletin, 1999, 44 (19): 2064.
4. Zhang, T, Z., Tang, C. M., The utilization and dissemination of the *Bt* transgenic cotton plants and the management of the insect-resistance activity, Chinese Science Bulletin, 2000, 45(2): 119.
5. Sambrook, J., Fritsch, E. F., Maniatis, T., Molecular Cloning: A Laboratory Manual, New York: Cold Spring Harbor Laboratory Press, 1989.
6. Peterson, A. H., A rapid method for the extraction of cotton genomic DNA suitable for RFLP or PCR analysis, Plant Molecular Biology Reporter, 1993, 11: 122.
7. Peng, J., Wen, F., Lister, R. L. et al., Inheritance of gusA and neo genes in transgenic rice, Plant Mol. Biol., 1995, 27: 91.
8. Rathore, K. S., Chowdhury, V. K., Hodges, T. K., Use of bar as a selectable marker gene and for the production of herbicide resistant rice plants from protoplast, Plant Mol. Biol., 1993, 21: 871.
9. Cao, J., Duan, X., Mcelroy, D. et al., Regeneration of herbicide resistant transgenic rice plants following miroprojectile mediated transformation of suspension culture cells, Plant Cell. Rep., 1992, 11: 586.
10. Matassi, G., Montero, L. M., Salinas, J. et al., The isochore organization and the compositional distribution of homologous coding sequences in the nuclear genome of plants, Nucleic Acids Research, 1989, 17: 5273.
11. Guo, W. Z., Zhang, T. Z., Zhu, X. F. et al., Variation induced by DNA rearrangement in a transgenic *Bt+CpTI* cotton strain, Chinese Science Bulletin, 2001, 46(7): 559.
12. Finnegan, J., McElroy, D., Transgene inactivation: plants fight bank! Bio/Technology, 1994, 12: 883.
13. Zhou, G. Y., Wang, J., Gong, Z. et al., Molecular breeding of agriculture: a technique for introducing exogenouse DNA into plants after self-pollination, Scientia Agriculturia Sinica, 1988, 21: 1.
14. Ni, W. C., Zhang, Z. L., Guo, S D., The cultivation of the insect-resistant transgenic cotton plants, Scientia Agriculturia Sinica, 31(2): 8.
15. Langridge, P., Brettschneider, R., Lazzeri, P. et al., Transformation of cereals via Agrobacterium and the pollen pathway: a critical assessment, The Plant Journal, 1992, 2(4): 631.
16. Flavell, R. B., Inativation of gene expression in plants as consequence of specific sequence duplication, Proc. Natl. Acad., Sci. USA, 1994, 91: 3490.
17. Hobbs, S. L. A., Thomas, D. W., Delong, C. M. O., Transgene copy number can be positively or negatively associated with transgene expression, Plant Mol. Bio., 1993, 21: 17.

(Received March 12, 2001)