BIOLOGIA PLANTARUM 51 (4): 746-748, 2007

BRIEF COMMUNICATION

Transgenic tobacco plants containing *Bt* and *GNA* genes

H.Y. ZHANG*,**[1], X.Z. LIU*, L. WEI***, L.Y. ZHOU*** and Y.M. YANG*

*Southwest Forestry College, White Dragon Temple, Kunming, Yunnan Province-650224, P.R. China**
*South China University of Tropic Agriculture, Dangzhao, Hainan-571737, P.R.China***
*Institute of Microbiology, Chinese Academy of Science, Beijing-100080, P.R. China****

Abstract

A new plant expression vector (pBSbtCry1Ac-GNA) containing two insect resistant genes, a synthetic chimeric gene *SbtCry1Ac* encoding the insecticidal protein CrylAc and a gene *GNA* encoding snowdrop lectin (*Galanthus nivalis* agglutinin) was constructed. Transgenic tobacco plants containing these two genes were obtained through *Agrobacterium*-mediated transformation of tobacco leaf discs. Results from PCR detection and genomic DNA Southern blot analysis indicated that both *SbtCrylAc* gene and *GNA* gene were integrated into the genome of these plants. Results of Western blot analysis indicated that these two proteins were expressed in the analyzed plants. Bioassays of *Myzus persicae* and *Helicoverpa assulta* on detached leaves of transformed tobacco plants were carried out. The average aphid inhibition rate of these plants tested at 12 d post-infestation was 71.9 %. The average *H. assulta* mortality of these plants tested at 6 d post-infestation was up to 89.8 %. The kanamycin resistance of the T_1 progeny of these transgenic plants was analyzed and a typical 3:1 segregation was observed.

Additional key words: agglutinin, insect-resistant, *SbtcrylAc*.

Infest with insects is one of the main reasons of reducing crop production. The use of plant-transformation methods to introduce resistance genes into plant genomes may have an important impact on yield. Reproducible and efficient methods were successfully established in rice (Sawahel 2003), tobacco (Velazhahan and Muthukrishnan 2003), *Triticum aestivum* (Mitić *et al*. 2004), *etc*. This study was aimed at construction of a new plant expression vector (pBSbtCry1Ac-GNA) containing two insect resistant genes, a synthetic chimeric gene *SbtCry1Ac* encoding the insecticidal protein CrylAc and a gene *GNA* encoding snowdrop lectin (*Galanthus nivalis* agglutinin). Transgenic tobacco plants expressing these two genes were obtained through *Agrobacterium*-mediated transformation of tobacco leaf discs.

Escherichia coli DH5α, *Agrobacterium tumefaciens* LBA4404 (containing helper plasmid pAL4404), pGNA34 (Zhou *et al*. 1998), pD12 (Tian *et al*. 2000), pBin513 (Zhang and Liu 2004), and pSbtCry1Ac were preserved or constructed in our laboratory. The chimeric *SbtCry1Ac* gene was isolated from pSbtCry1Ac as a *Bam*HI and *Xho*I fragment and inserted into the *Bam*HI and *Sal*I sites of the binary vector pBin513 (Zhang and Liu 2004) to form the plant expression vector pBSbtCry1Ac. The *GNA* gene was isolated from pGNA34 as a *Bam*HI and *Xho*I fragment and inserted in *Bam*HI～*Sal*I sites of pD12 (Tian *et al*. 2000) to form recombinant plasmid pDGNA. The *GNA* gene expression cassette was isolated as a *Hin*dIII fragment and inserted into *Hin*dIII site of pBSbtCry1Ac to form the plant expression vector pBSbtCry1Ac-GNA (Fig. 1).

This expression vector was transformed into *Agrobacterium tumefaciens* as described by Li *et al* (1994). Transformation of *Nicotiana tabacum* L. cv. Yun Yan 79 was carried out by *Agrobacterium*-mediated method (Horsch *et al*. 1985).

Received 15 October 2004, *accepted* 10 September 2006.
Abbreviations: Bt - *Bacillus thurigiensis*; *GNA* - *Galanthus nivalis* agglutinin; MS - Murashige and Skoog; PCR - polymerase chain reaction; SDS-PAGE - sodium dodecyl sulphate-polyacrylamide gel electrophoresis.
Acknowledgements: The authors thank Ms. M.L. Xu (South China Tobacco Breeding Research Center) for providing seeds used in this study. We also thanked Dr. C.Z. He (Laboratory of Plant Biotechnology, Institute of Microbiology, Chinese Academy of Sciences) for his critical review of the manuscript. The study was supported by grants from National Basic Research Program ("973" Plan) of China (Grant No. 2003 CB 415102).
[1] Author for correspondence; fax: (+81) 0871 3862178, e-mail: hanyaoz@163.com

Tobacco DNA used for PCR detection was isolated according to Li *et al*. (1994). Gene specific primers for *cry1Ac* amplification are $P_1^{(+)}$ (5'ATC TAT GCA GAG TCT TTC AGA) and $P_2^{(-)}$ (5'GAG GTT ATC CAA GGA GGT). A fragment of 1370 bp should be amplified by using these two primers. For the amplification of *GNA* gene, the primers are $P_3^{(+)}$ (5′ > CTCTCTCTACCGGG GAATTTCG < 3′)and $P_4^{(-)}$ (5′ > CATCGCAAGACCGG CTTC < 3′). A fragment of 500 bp should be amplified by using these two primers.

*Bam*HI ~*Xho*I fragment of *SbtCry1Ac* gene or *Bam*HI ~*Xho*I fragment of *GNA* gene were ^{32}P-labeled and used as probes in Southern blot analysis.

Fresh tobacco leaf samples (100 mg) were ground to powder in liquid nitrogen and then suspended in 0.1 cm^3 of 2× sample buffer. The suspension was used for SDS-PAGE and Western blot analysis following the procedure as described by Zhou *et al.* (1998). Proteins were separated by 10 % sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the proteins were transferred to a polyvinylidene difluoride membrane (*Amresco*, USA). The rabbit anti-*Bt* or anti-GNA antiserum was diluted for 1:5000 (v/v) as primary antibody, and the second antibody was alkaline phosphatase-conjugated goat anti-rabbit IgG (1:3000, v/v).

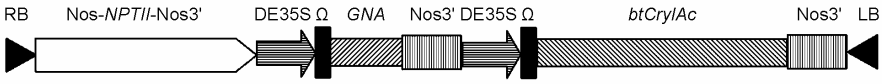

Fig. 1. Gene structure of plant expression vectors.

Insect bioassay of transgenic tobacco plant was carried out as described by Gou *et al*. (2004) and by Li *et al.* (1994). Fully expanded young leaves were detached from the top of transgenic tobacco plants (or control plants), then divided into several pieces and placed into test tubes of 2 cm in diameter. To each tube, one *Helicoverpa assulta* larvae of the second instar were transferred. The tubes were sealed with a cotton agglomerate, and placed in the growth chamber at 26 °C and 90 % relative humidity. To each plant, 10 to 30 insects were tested. After 6 d, the extent of leaf consumed, insect mortality, the mass of survived larvae and the number of excrements of the survived larvaes were recorded.

Segregation of kanamycin resistance in T_1 progeny of transgenic plants was checked according to Li *et al.* 1994). The sterilized seeds were planted on MS media containing 300 mg dm^{-3} kanamycin and grew in the growth chamber at 25 °C. Green seedlings (kanamycin resistant) and white seedlings (kanamycin sensitive) were counted for χ^2-test of segregation of kanamycin resistance.

Tobacco leaf tissues were co-cultivated with *Agrobacterium tumefaciens* LBA4404 carrying the binary vector pBSbtCry1Ac-GNA. On the MS shoot-induction medium containing 30 mg dm^{-3} kanamycin, the induction rate of shoots was 10 - 40 %. The number of shoots produced on each leaf explant varied from 1 to 5. Kanamycin resistant shoots were rooted on MS root-induction medium containing 50 mg dm^{-3} kanamycin. About 70 - 80 % of the shoots produced roots and regenerated into a complete plantlets. With this system, a total of about 200 independently transformed plants were obtained from co-cultured transformation of leaf tissues with *Agrobacterium tumefaciens* LBA4404 containing pBSbtCry1Ac-GNA. The results of PCR analysis revealed that more than 80 % of the regenerated plants could produce the expected gene-specific PCR product, implying that they were possibly transgenic.

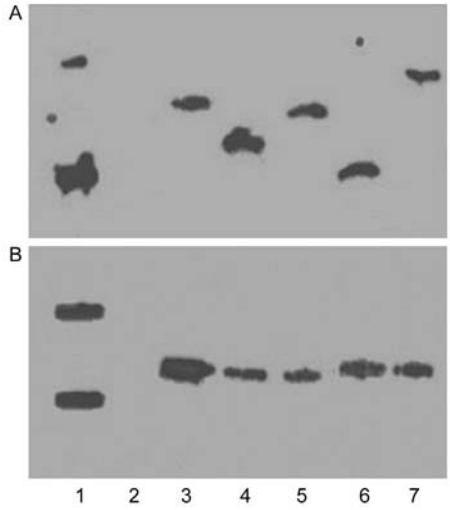

Fig. 2. Southern blot hybridization patterns of pBSbtCryAc-GNA transformed tobacco plants.

Table 1. Kanamycin (Km) resistance of the T_1 progeny of some transgenic plants. In all T_1 plants 3:1 segregation was observed.

Transgenic plant	Number of Km resistant plants	Number of T_1 plants	χ^2	*P*
3	168	229	0.32751	>0.70
8	173	231	0.00144	>0.99
14	183	261	3.32184	>0.10
15	159	204	0.94117	>0.50
18	181	253	1.61396	>0.30
CK	0	241	-	-

PCR positive plants were further selected by Southern blot hybridization to confirm their transgenic nature. Fig. 2 shows the results of the transgenic plant DNA hybridized with ^{32}P-dCTP labeled *SbtCry1Ac* or *GNA* gene probes, respectively. It was demonstrated that the plants analyzed were transgenic for both genes.

The patterns of hybridization with *Bt* and *GNA* gene probe suggested that the insect-resistant genes had been inserted into the genome of 5 selected plants as a single copy. The different size of the hybridization bands for different plants (Fig. 2) reflected the different integration sites of *Bt* gene and *GNA* gene in plant genome.

Western blot analysis of proteins from the transgenic plants was performed using Cry1Ac antiserum and GNA antiserum, respectively. The results of the Western blot immuno-detection indicated that the proteins of the transgenic plants specifically reacted with antiserum of Cry1Ac and GNA, respectively, suggesting that these two proteins were expressed in the analyzed plants.

Bioassays of *Myzus persicae* and *Helicoverpa assulta* on detached leaves of transformed tobacco plants were carried out. The transgenic plants could strongly inhibit the *Myzus persicae* population development. The average inhibition rate of plants tested at 12 d post-infestation was 71.9 %. The results shown that *Helicoverpa assulta* resistance of transgenic tobacco plants was significantly higher than that of non-transformed tobacco plants based on the leaf damage rate, the amount of excrements of tested insects and the average mass of survived larvae. The average mortality of plants tested at 6 d post-infestation was up to 89.8 %. The kanamycin resistance of the T_1 progeny of these transgenic plants was analyzed and a typical 3:1 segregation was observed (Table 1). This also confirmed the conclusion of a single copy insertion as revealed by Southern blot analysis.

References

Guo, H.N., Jia, Y.T., Zhou, Y.G., Zhang, Z.S., Ouyang, Q., Jiang, Y., Tian, Y.C.: Effects of transgenic tobacco plants expressing *ACA* gene from *Amaranthus caudatus* on the population development of *Myzus persicae*. - Acta bot. sin. **46**: 1100-1105, 2004.

Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eichholtz, D., Rogers, S.G., Fraley, R.T.: A simple and general method for transferring genes into plants. - Science **227**: 1229-1231, 1985.

Li, T.Y., Tian, Y.C., Qing, X.F., Mang, K.Q., Li, W.G., He, Y.G., Shen, L.: [Transgenic tobacco plants with efficient insect resistance.] - Sci. China (Ser. B) **37**: 276-282, 1994. [In Chinese.]

Mitić, N., Nikolić, R., Ninković, S., Miljuš-Djukić, J., Nešković, M.: *Agrobacterium*-mediated transformation and plant regeneration of *Triticum aestivum* L. - Biol. Plant. **48**: 179-184, 2004.

Sawahel, W.: Improved performance of transgenic glycinebetaine-accumulating rice plants under drought stress. - Biol. Plant. **47**: 39-44, 2003/2004.

Tian, Y.C., Zheng, J.B., Yu, H.M., Liang, H.Y., Li, C.Q., Wang, J.M.: Studies of transgenic hybrid poplar 741 carrying two insect-resistant genes. - Sci. Silvae sin. **42**: 263-268, 2000.

Velazhahan, R., Muthukrishnan, S.: Transgenic tobacco plants constitutively overexpressing a rice thaumatin-like protein (PR-5) show enhanced resistance to *Alternaria alternata.* - Biol. Plant. **47**: 347-354, 2003/2004.

Zhang, H.Y., Liu, X.Z.: [Construction of expression vector of wood-boring pest resistant gene transferred into *Populus yunnanensis*.] - J. Southwest Forest. College **24**: 12-14, 2004. [In Chinese.]

Zhou, Y., Tian, Y.C., Wu, B., Mang, K.Q.: [Inhibition effect of transgenic tobacco plants expressing snowdrop lection in the population of *Myzus persicae*.] - Chin. J. Biotechnol. **14**: 13-19, 1998. [In Chinese.]