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RESEARCH ARTICLE

Fusion insect-resistant gene mediated by matrix attachment region (MAR) sequence in transgenic sugarcane

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Abstract With the embryogenic calli of sugarcane variety ROC25 as the acceptors, a genetic transformation system mediated by *Agrobacterium tumefaciens* was established and the fusion insect-resistant gene (constructed by *Amaranthus viridis* L. agglutinin gene and soybean Kunitz tripsin inhibitor, AVAc-SKTI) transformed plants were acquired. The plant expression vector was constructed with the fused gene which was flanked on both sides of the MARs. The plant vectors were transformed into the embryogenic calli with the plant expression vector without MAR control. In the genetic transformation of sugarcane, the vector ligated with MARs in the same direction on both sides of the fused gene could increase the number of transgenic lines, which was 55.56% higher than that of the control. Moreover, the number of transgenic plants regenerated from each resistant callus increased by 26.43%. The AVAc-SKTI fusion insect-resistant gene transformed plants would improve the resistance of sugarcane plants to the attacks of borer (*Diatraea saccharalis*) and aphid (*Ceratovacuna lanigera* Zehnther).

Keywords Sugarcane, matrix attachment region, fusion insect-resistant gene (AVA+SKTI), *Agrobacterium tumefaciens*

Introduction

Sugarcane (*Saccharum* hybrids) is an important sugar crop in southern China. However, sugarcane crop is often attacked by a variety of insect-pests at various growth stages, of which *Diatraea saccharalis* and *Ceratovacuna lanigera* Zehnther cause considerable losses in cane yield and sucrose content in cane. Because of a relatively complex genetic background of sugarcane, traditional breeding technique to improve insect resistance is a formidable task. In contrast, genetic engineering is playing an important role in sugarcane improvement. *Amaranthus caudatus* agglutinin (ACA) and *Amaranthus hypochondriacus* agglutinin (AHA) transformed tobacco plants have shown to have higher resistance to *Myzus persicae* than the GNA transgenic plants (Guo *et al*., 2004; Rinderie *et al*., 1990; Zhou *et al.*, 2001). Soybean Kunitz tripsin inhibitor (SKTI) is specific for serine proteases, and it has extensive resistance to inhibit the larva development with little accumulating tolerance (Song *et al*., 1993; Kim *et al*., 1985; Koide *et al*., 1973; Gatehouse *et al*., 1993). MARs (matrix attachment regions) can stabilize and enhance gene expression in transgenic plants (Allen *et al*., 1993, 1996, 2000; Breyne *et al*., 1992; Han *et al*., 1997; Lee *et al*., 1998), therefore, it will be useful to investigate MARs effects on fusion gene expression in transgenic sugarcane. In the present study, the fusion insect-resistant gene (AVAc-SKTI) mediated by MARs was introduced into embryogenic calli of sugarcane by *A.tumefaciens* and transgenic plants were obtained.

Materials and Methods

Plasmids and *Agrobacterium* strain

Plasmids of pC13-2MAR and pC13 were preserved in our laboratory. AVA gene from *Amaranthus viridus* L. and SKTI gene were connected by the linker with six amino acids. The fusing gene was inserted into pC13-2MAR and pC13 to create

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the plant expression vectors of pC13-2MAS and pC13-AS. Fusion gene was flanked by pea MARs arranged in direct repeat in plasmid pC13-2MAS. The T-DNA regions of the two vectors contained fusion gene and Hyg gene as a selective marker. The fusion gene was controlled by an Ubi promoter and the Hyg gene was controlled under a CaM35S promoter (Fig.1). The pC13-2MAS and pC13-AS were transferred into *A.tumefaciens* EHA105 by frozen-melting method, respectively.

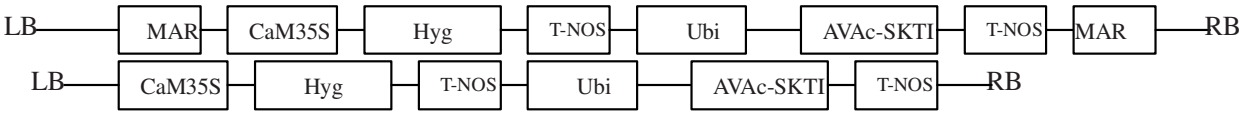

Fig. 1. Diagram of T-DNA region of plant expression vectors of pC13-2MAS and pC13-AS.

Plant materials

Young leaves of sugarcane (*Saccharum officinarum* L.) variety ROC25 were cut into small pieces and cultured in M1 medium (MS + 3.0 mg/L 2,4-D) to induce embryonic calli. The calli then were transferred onto M2 medium (MS+2.0mg/L 6-BA +0.1mg/L NAA) to induce shoot regeneration. The developed shoots were transferred to M3 medium (1/2MS +4.0mg/L NAA +50g/L sucrose + 8g/L agar) for rooting. To determine the suitable Hyg concentration for selection, the calli and shoots were subsequently transferred to the selection medium containing varying levels of Hyg, that is, 0, 10.0, 20.0, 30.0, 40.0 and 50.0mg/L, respectively. The media were changed several times by transferring the cultures into a fresh medium in every two weeks. Three repeats were conducted and the media without Hyg were used as the controls.

Preparation of *A. tumefaciens* suspension

A. tumefaciens strains EHA105 harboring pC13-2MAS and pC13-AS, respectively, were grown in Luria Broth medium supplemented with 25mg/L streptomycin and 25mg/L rifampicin at 28 ^{0}C till OD_{600} value of the bacterial suspension reached to 0.6. The bacteria were collected and re-suspended in hormone-free half-strength MS liquid medium supplemented with 10mmol/L fructose, 10mmol/L glucose and 150mmol/L AS, pH5.3. Later, the cells were cultured for 2 h to induce the expression of *vir* gene and the suspension was used directly to infect calli of sugarcane.

Transformation and Regeneration of transgenic sugarcane plants

Before infected by *A. tumefaciens*, the embryonic calli were transferred onto fresh M1 medium for 4 days. The cultures of approximate 2mm^2 were treated by flow in super clean bench for 30 to 60min till to dry and begin shrinking. The dried calli were immersed in the bacterial suspension containing 150μmol/L AS for 45 min, and then dried with the filter paper. Then the infected calli were co-cultured onto hormone-free M1 medium containing 100μmol/L AS at 23^{0}C in dark for 4 days. After the co-cultivation, the calli were placed on selection medium (M1 medium containing 500mg/L carbenicillin and 50mg/L Hyg) and cultured in dark for three weeks at 26^{0}C to induce the new resistant calli. The proliferated calli were selected for 2 times on the same selective medium as described above and transferred onto M1 medium for 4 days. Then, the resistant calli were cultured at 26^{0}C onto M2 medium supplemented with 500mg/L carbenicillin (Cb) and 50mg/L Hyg under a 14h light/12h dark photoperiod (50μmol/m^2.s light intensity provided by white fluorescent tubes) until shoots developed. The selective medium was replaced once every two weeks. The regenerated green health shoots of 3~4cm high were divided into single plant and transferred to the hormone-free MS medium containing 300mg/L Cb for growing stronger. When the shoots grew to 6-9cm high, they were transferred for rooting induction onto M3 medium which was supplemented with 300mg/L Cb and 30mg/L Hyg. The well-rooted plants were eventually planted to soil in pots in greenhouse. *A. tumefaciens* contamination was suppressed for months inhibited by carbenicillin.

PCR and Southern blotting

The genomic DNA were extracted from the leaf of Hyg-resistant and control plants, respectively, by the method of Yao *et al*. (2005). PCR analysis for the detection of the fusion gene of AVAc-SKTI was carried out by using the forward primer A1 (5'-GGAAGATCTACCATGGCGGGATTACCA GTG-3') and the reverse primer A2 (3'-GAAAGAGCGTCA CTCACTCTAGAC-5'). The expected product size was about 1600bp. PCR reactions were performed in a thermal cycle programmed for 36 cycles of 95^{0}C for 1min, 50^{0}C for 1min and 72^{0}C for 2min. The amplified DNA fragments were resolved on 0.8% agarose gel and visualized under UV transillumination. Southern blotting was performed using the total genomic DNA from PCR positive and control plants, respectively. The fragment of AVA gene was DIG labeled and used as probe. The labeling and hybridization were done by following the manufacturer's protocol of DIG labeling and detection kit (Roche).

Results and Discussion

Selection concentration of Hyg

The embryonic calli on the selective medium supplemented with 50mg/L Hyg, were turned brown and died in a few weeks. And the shoots were not able to root in the M3 medium containing 30mg/L Hyg. Therefore, 50mg/L Hyg was the suitable concentration for regeneration of embryonic calli and shoots, and 30mg/L Hyg for rooting.

Development of transgenic resistant plants

The embryonic calli infected were co-cultured with *A. tumefaciens* onto M1 medium supplemented with 100μmol/L AS in dark for 4 days at 23^{0}C, and then transferred to selective medium. After 3 to 4 weeks, non-transformed calli became brown and died gradually. However, the resistant calli, with the characters of white-yellow color, lustrous, dense and grain-like tissues were appeared on the surface of transformed embryonic calli. The resistant calli were separated from homogeneous calli and cultured on the M1 selective medium again. After selection for 2 times, the embryoids were developed. When the survived calli were transferred on M2 medium containing 50mg/L Hyg, shoots appeared in another 3 weeks. The single shoot (2-3cm in height) was separated from bud cluster and cultured on hormone-free MS medium for 2 weeks and later transferred onto the M2 selective medium. The shoots of 6-8cm high were planted in M3 medium with 30mg/L Hyg for rooting. After 3-4 weeks, the roots of regeneration shoot were gradually developed and shoots grew stronger. Most resistant plants showed no obvious morphological changes except slow growth as compared with the control (Figs. 2 and 3).

Fig. 2. The transformed plants of sugarcane; 1 - Transformed plants with MAR; 2 - Transformed plants without MAR.

Fig. 3. The roots of transformed plants with MAR.

Southern blotting

As expected, a 1600bp fragment of fusion gene was amplified from some DNA template extracted from the plants transformed with pc13-2MARAVA and pc13-AVA and was not amplified from that of the control (Fig. 4). Southern blotting analysis was performed with the PCR positive plants. DNA from four PCR positive sugarcane were hybridized with AVA probe and all showed positive reaction while DNA from non-transformation plants showed negative (Fig. 5). The results indicated that the fusion gene had been integrated into the sugarcane genome. A total of 98 PCR positive plants were acquired, which included 65 transformed plants with MAR and 33 transformed plants without MAR. The 65 plants with MAR derived from 14 transgenic sugarcane lines and the Hyg-resistant plants regenerated from each resistant calli were 4.64 on an average. Thirty three transgenic plants without MAR were derived from 7 transgenic lines. The Hyg-resistant plants regenerated from each resistant calli were 3.67 on average. The results demonstrated that the number of transgenic lines with MAR was 57.12% higher than those of the control without MAR and the regenerated plants per number of resistant calli also increased by 26.43%.

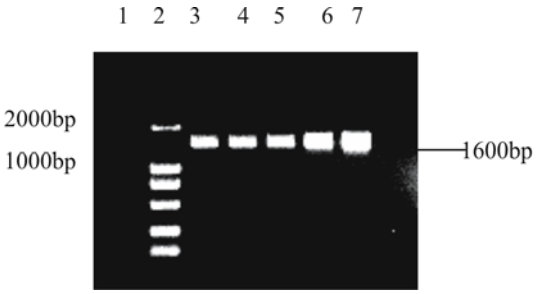

Fig. 4. PCR of transgenic plants; Lane1, non-transformed plants; Lane2, marker DL-2000; Lane3-4, transgenic plants with MAR; Lane5-6, transgenic plants without MAR.

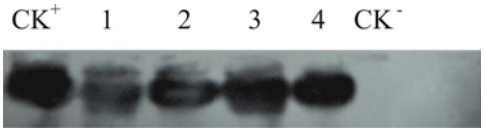

Fig. 5. Southern blotting of DNA from transgenic plants; Lane1-2, transgenic plants with MAR; Lane3-4, transgenic plants without MAR; CK-, wild type plants as negative control; CK+, pC13-2MAS PCR products.

Conclusions

A genetic transformation system was set up using mediated by *Agrobacterium tumefaciens* was set up embryogenic calli of sugarcane variety ROC25 and AVAc-SKTI transformed plants were developed in the present study. The results also indicated that the transformation mediated with MARs could increase the transgenic plants or lines generated from each callus and enhance the transformation efficiency. Hyg not only affected the normal growth of calli, but also inhibited strongly the regeneration of shoots. The different Hyg-resistance of the two calli (the calli infected by EHA105 harboring pc13-2MAS and by EHA105 harboring pc13-AS, respectively) resulted in obvious difference in the number of transformed

plants mediated with MAR and without MAR. At the same time, the results indirectly reflected the Hyg expression level difference of the two calli. The AVAc-SKTI fusion gene resistance to insects and gene expression level among different transgenic lines mediated with MARs and without MARs, together with untransformed sugarcane controls, are under investigation.

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