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Trehalose Synthase Gene Transfer Mediated by *Agrobacterium tumefaciens* Enhances Resistance to Osmotic Stress in Sugarcane

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ABSTRACT

Trehalose synthase gene (*TSase*) from *Grifola frondosa* was transferred into sugarcane (*Saccharum officinarum* L.) using *Agrobacterium*- mediated method to improve sugarcane drought-tolerance. The results indicated that embryogenic callus of sugarcane was sensitive to *A. tumefaciens* EHA105 strain in the transformation system employed. The high frequency of PPT-resistant plants were obtained from transformated with 3 weeks callus after incubation, which reached 4.5% on average. The transgenic plants was confirmed by PCR and southern blot analysis. Some transgenic plants showed multiple phenotypic alterations and some plants demonstrated improvement tolerance to osmotic stress.

Key words: *Agrobacterium tumefaciens*, transformation, trehalose synthase gene, sugarcane

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is an important industrial crop in China. Because of upland growing area, poor water capacity soil and seasonal dry climate, drought has become more and more serious threat to production of sugarcane. Moreover, sugarcane is an asexual propagated polyploidy crop with a relatively complex genetic background. However, using traditional breeding techniques to improve drought tolerance is a formidable task. In contrast, genetic engineering technique is playing a more powerful role in plant improvement.

Trehalose is widely distributed in resurrection plants in desert such as *Myrothamnus flabellifolius*, yeast, spore and fruiting body of fungi, etc., which serves as a protectant against dehydration (Drennam *et al.*, 1993; Machenzie *et al.*, 1998). Trehalose-6-phosphate synthase genes (*TPS1*) from yeast or *Escherichia coli* are transformed into tobacco and potato, and the accumulation of trehalose is detected in the transgenic plants. Production of trehalose can improve plant tolerance to drought, but often lead to stunted growth, lancet-shaped leaves and short, thick roots in some plants (Holmstrom *et al.*, 1996; Goddijin *et. al.*, 2000; Zhao *et al.*, 2000). The synthesis of trehalose in basidiomycete, *Grifola frondosa* was catalyzed by trehalose synthase (TSase) (Saito *et al.*, 1998) and the Tsase gene had been transferred into sugarcane via microprojectile bombardment. This gene could improve drought resistance in sugarcane (Drennan, *et al.*, 1993; Mackenzie *et al.*, 1988; Holmstrom *et al.*, 1996; Goddijn *et al.*, 1997; Romero *et al.*, 1997; Yeo *et al.*, 2000; Zhao, 2000; Saito *et al.*, 1998; Zhang, 2000)

Unlike dicotyledonous plants, the initial development of *Agrobacterium*- mediated transformation systems for graminaceous plants, which were originally outside the host range of *A. tumefaciens*, was not very efficient. The first description of successful transformation of gene into graminaceous plant is on rice by Chan *et al.*, (1993) subsequently were on maize and wheat. The transformation in sugarcane was reported in 1998 (Arencibia *et al.*, 1998). The transgenic sugarcane plants were recovered from co-cultivation of suspension culture calli , meristematic explants and embryogenic calli [with *A. tumefaciens* respectively. In our study, an efficient method of transformation by co-cultivation of embryogenic calli with *A. tumefaciens* was described. Many *TSase* transgenic plants were obtained, and the integration, morphological changes and improvement of osmotic stress tolerance of the transgenic plants were also demonstrated (Chan *et al.*, 1993; Ishida *et al.*, 1996; Cheng *et al.*, 1997; Arencibia *et al.*, 1998., Enriquez-Obregon *et al.*, 1998; Elliot *et al.*, 1998).

MATERIALS AND METHODS

Plasmids and *Agrobacterium* strain

The trehalose synthase gene (*TSase*), cloned from *Grifola frondosa* by RT-PCR, (Zhang *et al;* 2000) was digested with EcoRI and treated with Klenow for filling in at 3' end and

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cleaved with BamHI at 5' end from pUC29, and inserted into pBBB to create the plant expression vector pBBBT. The resultant T-DNA region contained *TSase* and *bar* (a selective marker gene conferring resistance to phosphinothricin, PPT) genes. The *TSase* was controlled by a 2 copies CaMV35S promoter and the *bar* gene was controlled under a CaMV35S promoter (Fig. 1). pBBBT was introduced into *A. tumefaciens* EHA105 (a 'super-virulent' strain harboring pTiBo542) by triparental mating (Fu et al., 1994).

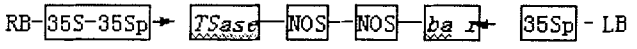

Fig.1: Diagram of T-DNA region of plant expression vector pBBBT

Plant materials

Meristematic explants of commercial variety sugarcane were taken from field-grown plants, and incubated in M1 medium (MS+1mg/L 2,4-D) to induce calli and embryonic calli. The calli were then transferred to M2 medium (MS+1mg/L 6-BA+0.5mg/L KT) to induce shoot regeneration. The shoots were rooted in M3 medium (1/2MS+1mg/L IAA). In order to determine the suitable concentration of PPT (Sigma) for selection, a series of pre-experiments on calli and shoots were carried out, with some calli and some shoots in M2 medium containing PPT concentrations gradients, respectively. The gradient concentrations of PPT were 0.00 mg/L, 0.50 mg/L, 0.75 mg/L, 1.00 mg/L, 1.25 mg/L and 1.50 mg/L. In order to determine the PPT sensibility to rooting, the shoots were incubated in the same gradient of PPT except M3 medium, instead. Another experiment was conducted to determine the competence of sugarcane cells by transforming starting materials at different ages, which were confined within 1week, 2, 3, 4 and 7 weeks after incubation, and investigating the frequency of resistance produced in plants.

Preparation of *A. tumefaciens* suspension

EHA105 harboring pBBBT was grown at 28°C in YEP medium supplemented with 50 mg/L kanamycin (Sigma), 25 mg/L rifampicin (Sigma) and 25 mg/L streptomycin (Gibco-BRL). When the cell density reached OD_{600} = 0.6, the cells were collected and re-suspended in the same volume of MR medium. A liquid MS medium reduced the content of macro-elements to 1/5 of its original and supplemented amount with 150 μmol/L acetosyringone (AS, Sigma), 10 mmol/L fructose, 10 μmol/L glucose, pH 5.3. The bacterial cells were cultured for 2 h to induce the expression of *vir* genes.

Transformation and selection

Before initiating the infection by *A. tumefaciens*, the calli were transferred onto fresh M1 medium for 4d, and collected on filter paper. A brief dried treatment under flow in super clean bench was made for 30 to 60 min, till the calli dried and begin shrinking. The dried calli were infected by immersing in the *A. tumefaciens* suspension and agitated at 100 rpm on a rotary shaker for 30 min, and then blotted dry. The infected calli were divided into pieces of 0.3–0.5cm in diameter, placed onto M1 medium containing 100–μmol/L AS, and co-cultured at 23°C in dark for 3–4d. After co-cultivation, the materials were rinsed thoroughly with sterile water, dried on filter paper, and then incubated onto M2 medium, which supplemented with 500mg/L carbenicillin (Sigma) and 0.75 mg/L PPT, and cultured at 26°C under illumination at 1500 lux with 14 h/d for selection. The selective medium was replaced once in 3–4 weeks. Proliferated calli were excised with scalpel and sub-cultured on medium of the same composition. Regenerated shoots were transferred into the same selective medium till the shoots grew to 6–8 cm in height, and then transferred into rooting selective medium, which was M3 medium containing PPT 0.5 mg/L and cefoxitin (Gibco-BRL) 300 mg/L. The flow chart was illustrated in Fig.2. Evidence of *A. tumefaciens* contamination was confirmed by incubating old leaves of PPT-resistant plant on antibiotics-free YEP solid medium and culturing for weeks.

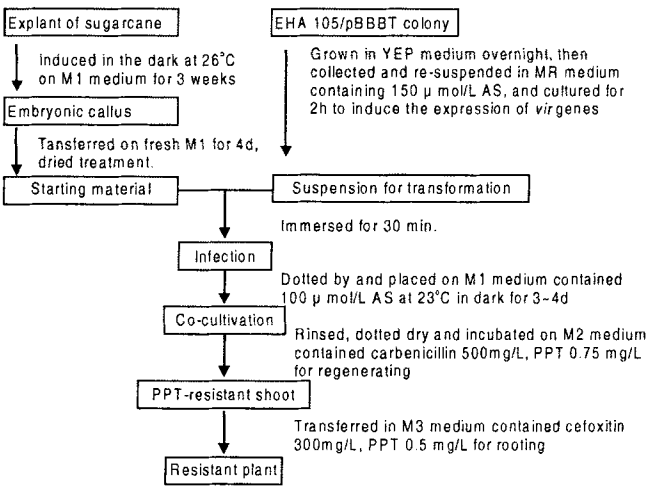

Fig.2: Protocol of transformation of sugarcane mediated with *A. tumefaciens*

PCR and southern-blot analysis

Total DNA was extracted from leaf tissues of sugarcane plant using SDS method. PCR was carried out using the DNA from PPT-resistant plants and control plant. The primers were designed based on the ends sequence of *TSase*. The reactions were done with annealing temperature of 58°. Southern-blot analysis was performed using total genomic DNA from PCR positive plants and control plant. The DNA was dotted onto NC hybond membrane (LKB). The fragment of *TSase*, digested with BamHI/EcoRIfrom pUC29, was DIG labeled and used as probe. The procedure of labeling and hybridizing followed the manufacturer's protocol of DIG labeling and detection kit (Boehringer).

Osmotic stress tolerance of the transgenic plant

In some plants, Southern-blot positive lines were transferred in M2 medium for propagation. The propagation shoots and control shoots were transferred into MS medium in presence of PEG_{8000} 17.4% (w/v) and cultured at the same conditions as selection.

RESULTS

Construction of pBBBT and its introduction into *A. tumefaciens*

The putative resultant plasmid was 11 kb long and there was a Sac 1 site located at 1469bp of *TSase*. pBBBT was identified by digestion with BamHI/SacI (Fig.3). The correct in-frame fusion was further verified by DNA sequencing. *TSase* harbored in EHA105 was identified by hybridization *in situ* hybridization of bacterial colony with labeled *TSase* probe, and all colonies showed positive.

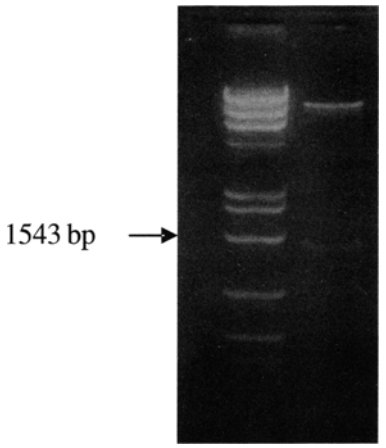

Fig.3: Identification of pBBBT digested with BamHI/SacI

Establishment of sugarcane transformation system mediated by *A. tumefaciens*

The competence of the accepter cell was the key factor of the transformation, and the somatic embryo could behave similarly to sexual embryo for T-DNA transfer. Calli produced from the cut of the explants after incubating on M1 medium in 3d. The embryonic calli, with the characteristic of white-yellow color, lustrous, dense and grain-like tissues, projected from the surface of the yellow, smooth and watery homogeneous calluses after being cultured for 3 weeks (Fig.4). Embryoids developed from embryonic calli after being transferred on M2 medium and shoots appeared in another 3 weeks. The results of the pre-experiments of transformation with different starting materials ages indicated that transformed calli of 3 weeks resulted in the highest production frequency of PPT-resistant plants, which defined as number of resistant plants per number of co-cultivated calli. It reached 4.5% on average. Co-cultivated materials from 1 week age starting materials turned brown in a few days, and then died after selection; while those from 7 weeks age starting materials continuously multiplicated for a few months and never regenerated. The recovered frequency of starting materials at 2w and 4w age were 3.7% and 2.8%, respectively (Fig.5). The results of the pre-experiments of PPT concentration on sugarcane materials indicated that 0.75 mg/L PPT was suitable concentration for calli and shoots selection. They turned brown and died in a few weeks under the concentration. For rooting, shoots were not able to root in 0.5 mg/L PPT. This concentration was used for rooting selections.

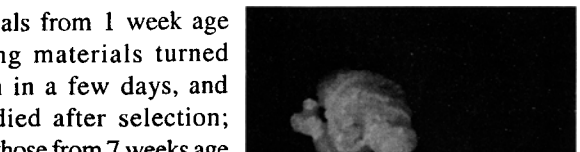

Fig.4: Embryonic callus projected from the surface of callus of starting material

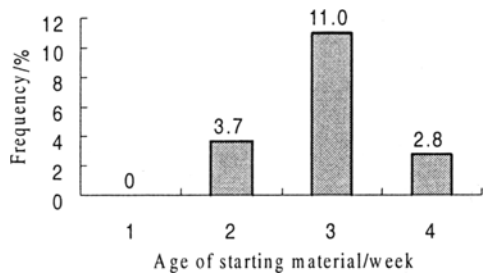

Fig.5: Frequency of PPT-resistant plant recovered from different ages of starting material

Development of resistant plant

1026 pieces of resistant calli produced from 1247 pieces of co-cultivated calli after PPT resistance selection for a few months, the resistant calli produced 536 shoots. After long time selection, 93 shoots survived and were transferred into rooting medium. Fifty six shoots could root against PPT pressure. The frequency ranged from 2.8% to 11.0%, with the average value of 4.5%. *A. tumefaciens* contamination was suppressed for months inhibited by carbenicillin.

Observation of morphological changes in resistant plants

Most of resistant plants showed no obvious morphological changes except slow growth, while 12 of them exhibited multiple phenotypic alterations, including yellowish fine leaves and vertical growth; zigzag-shaped leaves or roots; short, thick, rigid and stunting roots; and retarded growth and difficult rooting. The plants with morphological changes did not recover normal state even in absence of PPT for long time *in vitro* (Fig. 6).

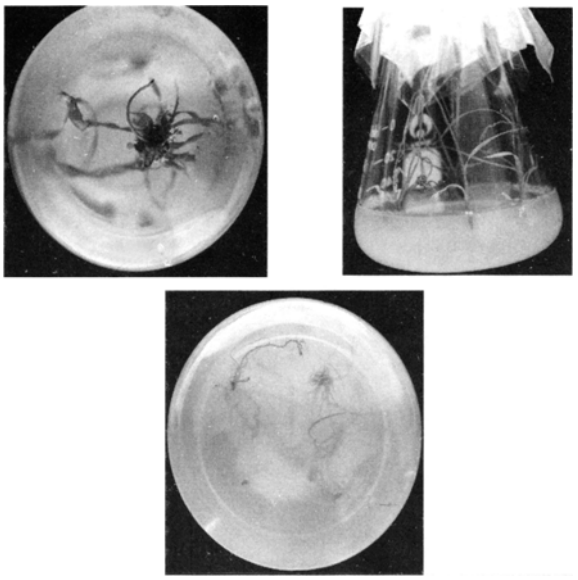

Fig.6: Morphological alterations of leaves and roots of transgenic plants

Integration of TSase in the genome of transgenic sugarcane plants

An expected, a 2.2 kb fragment was amplified from some DNA template extracted from resistant plants and was not amplified from that of control plant (Fig.7). DNA from 3 PCR

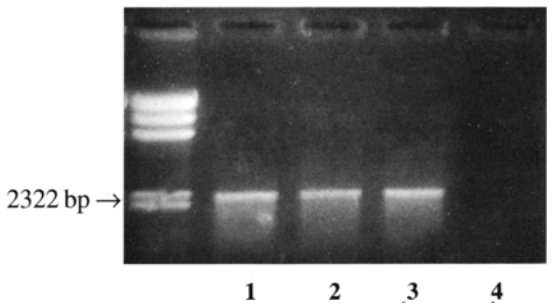

Fig.7: PCR analysis for detection of TSase in the transgenic sugarcane plants 1–3 DNA extract from transgenic plants; 4 DNA extract from non-transformation plant.

positive sugarcane and pBBBT sample were hybridized with *TSase* probe and all showed positive reaction while DNA from non-transformation plants showed negative (Fig.8). The results indicated that *TSase* had been integrated into the genome of sugarcane and the transgene maintained intact. DNA from some normal plants and morphological changes plants were detected and showed positive by PCR and dlot-Southern analyses. Twenty-four plants or lines were detected and seven of them showed positive. About 30% of the PPT-resistant plants were detected positive by PCR (Table.1), two lines out of which demonstrated little morphological change and was easier to propagate. The PPT-resistant plants, which recovered rapid growth after absence of PPT, were not detected positive

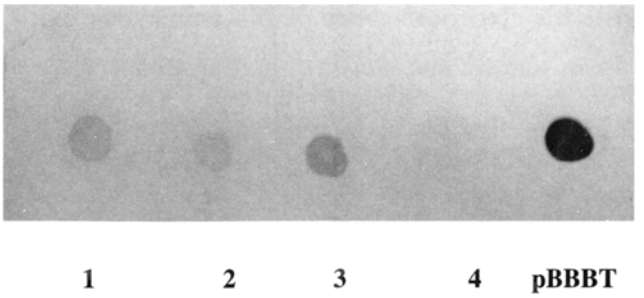

Fig.8: Dot-southern analysis for detection of TSase in the transgenic sugarcane plants 1–3 DNA extract from transgenic plants; 4 DNA extract from non-transformation plant.

Table 1: Detection results of PCR and dot-Southern for PPT-resistant plants

Detection Times	PPT-resistant plants	PCR positive plants	Dot-Southem positive plants	PCR positive lines/PPP-resistant lines
1	3	1		Undetected
2	8	3		7:24
3	14	4		Undetected

by PCR. This results indicated that the selective pressure of PPT was too low to work well, and too many plants escaped from the selection.

Improvement of osmotic stress tolerance of the transgenic plant

Under the osmotic stress of PEG (8000 MW), the non-transformed plants began turning yellow at the third day and wilted and dried extending from old leaves to young leaves in 7d (Fig.9). It showed severe damage, while all transgenic plants kept green and began turning yellow at the seventh day (Fig.9). The results indicated that sugarcane transformed with *TSase* could improve its osmotic stress tolerance.

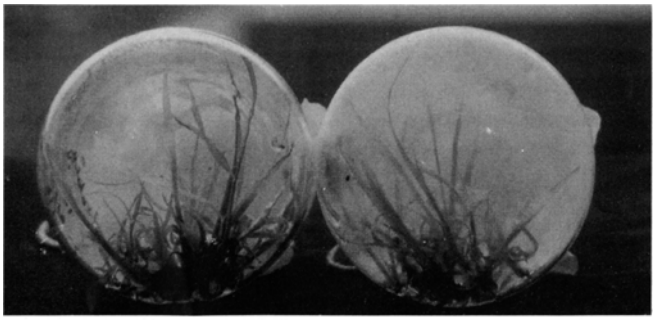

Fig. 9: Transgenic sugarcane integrated with *TSase* improved osmotic stress tolerance.
A Non-transgenic control plants; B TSase transgenic sugarcane plants.

DISCUSSION

The types and physiological states of receptor cells are critical to success of transformation of graminaceous plants mediated by *A. tumefaciens*. The first successes of transformation of rice , maize and wheat with *A. tumefaciens* were using immature embryos as starting materials. Uses of other tissues such as embryonic callus were tried and had succeeded subsequently (Hiei *et al.*, 1994). Because of difficulty in flowering and pollen infertility, the obtainment of immature embryos of sugarcane is fruitless in many areas. The successful starting materials for sugarcane transformation by *Agrobacterium*-mediated had been callus suspension cultures (Arencibia *et al.*, 1998), meristematic explants and embryogenic calli (Enriquez-Obregon *et al.*,1998) but the procedures of preparation reported were complex and tedious. In this study, the developmental processes of callus starting from explant and embryogenic callus starting from homogeneous callus had been investigated and some pre-experiments of transformation on different ages of callus had been conducted. It was found that the callus at the stage of a great deal grain-like embryogenic calluses projection emerging was most suitable for transformation by infecting with *A. tumefaciens* (Fig.4). It facilitated the transfer and integration of the T-DNA, because the synthesis of DNA and cell division are required for incorporation of foreign DNA into a host genome(Binns *et al.*, 1988). In order to facilitate the cell division and uniformity, the callus was subcultured on fresh medium for 4d. Co-cultivation of dry calli with *A. tumefaciens* suspension produced a rehydration of the plant cells. This process could facilitate the adhesion of *A. tumefaciens* to the cell wall, likewise, the entrance of *A. tumefaciens* into the intercellular spaces of the callus .

A. tumefaciens-plant cell recognition and attachment, sensing of plant signals by *A. tumefaciens* and activation of *A. tumefaciens vir* genes after the transduction of plant sensed signal molecules, were the early and essential steps of the transformation process. Wounded dicotyledonous cell exuded phenolic compounds, which activated *vir* genes. Graminaceous plants appeared not to produce these compounds, or the levels are insufficient to serve signals if they do. Addition of AS was indispensable to or greatly enhanced the expression of *vir* genes (Hiei *et al.*, 1994). The bacterium had been cultivated in MR medium, which contained 150 μmol/L AS, for 2h to provide sufficient level of signals and induce expression of *vir* genes before infection. During the 3–4d co-cultivation, 100μmol/L ass had been supplemented to the medium for expressing sufficient productions of *vir* genes. The other factors such as addition of sugars, an acidic pH and low temperature during co-cultivation were favorable to infection(Cangelos *et al.*, 1990; Turk *et al.*, 1991; Banta *et al.*, 1998). In the MR medium, we supplemented with 10mmol/L fructose, 10mmol/L glucose and pH5.3. The temperature of co-cultivation was 23°C.

The established transformation system was efficient on sugarcane. The frequency could reach 4.5% on average. In addition to its efficiency, this system expressed easy manipulation, short-time preparation and well repetition. Calli at 3 weeks age was used on transformation. It eliminated long tedious culture procedures and probable problem with difficult regeneration and somatic variation(Arencibia *et al.*, 1998). We also noted that some starting materials, which had been sub-cultured for a long time, were difficult in regeneration after transformation, while, starting materials younger than 2 weeks, which was apt to turn brown and produce low frequency after infection, were unsuitable for transformation. Continuous selection just after co-cultivation, uses of PPT as selective agent and EHA105 strain as transformation mediator could also have contributed to the efficiency.

Trehalose did not accumulate in higher plants naturally except for resurrection plants (Growe *et al.*, 1992). And limited amounts of trehalose accumulated in transgenic plants could increase in drought tolerance of the plants, but often accompanied pleiotropic phenotypes . In view of the low amounts of trehalose present in plants, it seems unlikely that trehalose has an important function in osmotic stress protection in plants. If so, it may activate the sugar-sensing mechanism and lead to accumulation of alternate osmolytes Goddijn *et al.* (1997). It is suggested that trehalose or related metabolites might have a function as regulators of plant growth and development. The phenotypes of transgenic sugarcane were various, some changed on leaves or on roots mainly and the others on the whole plants, which tempts us to speculate on the different profile of growth and development regulation. The increase in tolerance of osmotic stress of transgenic sugarcane implied the improvement of its drought tolerance. That could lead to commercial potential for production if the limit of growth reduced to a degree of acceptable.

In conclusion, our studies demonstrate that embryogenic callus of sugarcane is sensitive to *A. tumefaciens* EHA105 and the genetic transformation system is efficient. The established transformation system is practical and easier to operate than those reported before. Transformed with trehalose synthase gene from *Grifola frondosa*, some plants of transgenic sugarcane show multiple phenotypic alterations and increase in tolerance of osmotic stress.

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