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Full Length Research Paper

Transformation of trehalose synthase gene (*TPS* Gene) into corn inbred line and identification of drought tolerance

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Trehalose synthase gene (*TPS* gene) was transferred into maize inbred line Zheng58 with the improved pollen tube path method. At the three to five leaf stage, selection with spray Basta (0.7%) solution was conducted, and was then followed by polymerase chain reaction (PCR) and PCR-southern detection. The results showed that the new gene was transferred into the plants and integrated into the plant chromosomes. The initial drought-tolerance test in farmland confirmed that the drought resistance of transgenic plants was higher than that of non-transgenic plants. The results show that the new maize drought resistance germplasm can be gained by means of pollen tube path which is a feasible, effective and new way for maize drought resistance breeding.

Key words: Maize, trehalose synthase gene (*TPS* Gene), drought resistance.

INTRODUCTION

With water resources increasingly diminishing, drought has become the upmost abiotic factor that influences corn production. China's water-deficient area accounts for 52% of its whole territory, and the drought inundated region covers an area of about 9 million hm^2, which directly results in the reduction of the grain output of 10 billion kg. So drought has become the biggest challenge that China's agriculture production faces (Li, 1990; Chen and Zou, 2007). In the study of breeding drought-resistant material, using plant transgenic technique, Kishor et al. (1995) imported the P5CS gene into tobacco; as such, transgenic plants increased the proline content by 10 to 18 times, which provided an indirect proof that the drought resistance of transgenic plants was improved (Kishor et al., 1995). Yang et al. (2009) used CBF4 Arabidopsis transcriptional factor to transform the immature embryos and embryogenic calli of maize elite inbred line, by particle bombardment, into transgenic plants. Moreover, it proved that the drought resistance of transgenic plants has been improved to a certain extent (Yang et al., 2009). Although it is the most economical and efficient way to improve the maize's drought resistance capability by means of breed improvement, drought resistance capability is mostly dominated by micro genes. So, it is practically very difficult to improve heredity; as such, no improvement can be seen in this way. Therefore, using transgenic technology to transfer salt tolerance gene into maize inbred line is of great significance in breaking the reproductive isolation between species and creating new drought resistance germplasm. Many microbes can survive for a long time in drought, high temperature, high salt and other severe conditions, due to the high concentration trehalose content in their cells. Research shows that trehalose has special function of preserving the vitality of organisms and protecting the cell membrane and protein structure effectively, so as to make organisms remain intracellularly moist in extreme cases, such as dehydration in high temperature and frozenization in low temperature. This, in

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Abbreviations: TPS, Trehalose synthase; **PCR,** polymerase chain reaction; **PPT,** phosphinothricin.

turn, can prevent cells from losing their nutrients and avoid damages caused by dehydration (Eleutherio et al., 1993; Yang and Yang, 2000; Growe, 1996; Zhang, 1998; Zhang et al., 2007). In this study, we used the improved pollen tube channel method to transfer TPS into maize inbred line (Zheng58), taking advantage of its drought resistance capability to obtain genetically modified maize plants with better drought resistance capability. It will provide the material for cultivating new drought-resistant maize breed.

MATERIALS AND METHODS

Plant materials

Stable maize inbred line Zheng58 (receiver), preserved by Arid Farming Research Center, Shanxi Academy of Agricultural Sciences, was used for this study; donator plasmid for conversion was p3UbiTPS while the target gene was TPS (GenBank Registry Number X68496); botany for screening marks bar gene, all initiated by Ubiquitin gene.

Besides, agrobacterium strain EHA101 was provided by Professor Lin Zhongping from Peking University.

Deoxyribonucleic acid (DNA) importing

First of all, maize inbred line Zheng58 that grew vigorously in Sanya, Hainan was selected in years 2009 and 2010, after which self pollinating was done on it at 14:00 to 16:00 on a sunny day. After pollinating for 16 to 20 h, that is, from 10:00 to 12:00 in the next morning, the leaves were cut off fast in the middle of the pollination style with a scalpel and then dropped into the imported liquid (15 to 20 ul) at incision with a dropper at three different times. Finally, the container containing the liquid was covered and the solution was allowed to pollinate itself in order to obtain T_0 materials.

Field experimenting

In March 2010, T_0 materials collected in Sanya Hainan were sown, and then T_1 materials were obtained through selection. In July, the selected T_1 materials were planted in Beiying, Taiyuan. In December 2010, T_2 materials that were screened in Sanya, Hainan were planted. All materials were planted monoseedingly by rows at random, with row length of 3 m and line space of 30 × 80 cm. All screened plants were strictly bagged for self pollinating and were managed in the field, which was in Sanya city. However, Sanya city in Hainan province is located in the tropics and it belongs to monsoon tropical climate zones. Its annual average temperature is 23.8°C, its average annual rainfall is abundant, and it has a sunny day for more than 300 days.

Progeny plants screening

Progeny materials were selected at the three to five leaf stage by means of Basta (0.7%) solution (the solution was purchased from the Bio Basic Inc Company). After ten days, the number of survivals was investigated.

The survival seedings can be preliminarily identified as phosphinothricin (PPT) herbicides seedlings with resistibility. At the six to seven leaf stage, DNA was extracted for molecule detection and the survival plants were marked.

DNA extraction

200 mg of the samples were collected from every generation at the 5 leaf stage, after which they were frozen rapidly in liquid nitrogen, and then conserved under -40°C. Subsequently, the total DNA was extracted in accordance with Wang Guan-lin's Method (Wang and Fang, 2005). Plasmid DNA was extracted using alkaline decomposition method (Sambrook et al., 1992) and was purified with PCR fragment recovery kit.

PCR detection

PCR amplification sequence of *PTS* gene was designed by Primer V5 and synthesized by Shanghai ShengGong Biological Engineering Technology Company, Limited. The upstream primer sequence was 5'-GGATCAGGTGAGGAAGGACTTGC-3' and the downstream primer sequence was 5'-TCT TCC ACA TCT CCA CGA CTT GG-3'. Fragment length was amplified by 805 bp in the system of 20 µl. Afterwards, plant DNA (150 ng) was extracted with the following amplification conditions: 94°C pre denaturation for 4 min, 94°C denaturation for 45 s, 55°C annealing for 45 s, 72°C extension for 60 s, and 30 cycles of 72°C for 10 min. PCR products were kept in 1% agarose gel electrophoresis for separation, after which they were observed and photographed with SYN imaging system.<../ Program Files/Youdao/Dict/resultui/queryresult.html> Southern bolt detection. 15 µg DNA of transgenic plants, 15 µg DNA of the compared plants, and DNA of the plasmid were taken from the samples in order to confect the enzyme cutting system. Restriction enzymes *HindIII* was used to cut short-term buffer Buffer0 $^{+}$ (Shanghai Shenggong) in the reaction system (50 µl), after which restriction enzymes (15) were added to each one. After slight offcentering, the enzyme was cut in 37°C water-bath overnight. The enzyme cut products were separated electrophoretically in 1% agarose gel, and then DNA denaturation was conducted on them before they were transferred to the nylon membrane. Later they were fixed and then allowed to hybridize, after which the DIG DNA Labeling and Detection Kit, and the CSPD Immunofluorescence stain box bought from Roche were used to analyze them. The TPS gene hybridization probe followed the kit instructions laid down for preparation of the samples, and after hybridization and staining, the samples were autoradiographed with X-ray film.

Identification of the drought resistance of transgenic plants

After treating transgenic maize T_2 with drought resistance artificially for six days, the free proline content of leaves was determined by using sulfonylureas base salicylic acid method. Similarly, after treating transgenic maize T_2 with drought resistance artificially for six days, the chlorophyll content was determined by the method of Peng and Liu, (1992). Samples were taken from the middle of the maize leaves, after which they were weighed accurately and then cut into pieces, before placing them in 15 m test tube. The sample's solution was extracted using acetone and anhydrous ethanol (2:1) mixture ion. 10 ml of mixture ion was added to each sample, and extraction was done for 24 h in an absolutely dark environment. Afterwards, the extract solution was taken and the OD value was read with 721 spectrophotometer at 663 and 645 nm wavelength, before chlorophyll a and b content (Ca and Cb) was calculated by the following formula:

$$C_a = \frac{(12.7OD_{663} - 2.69OD_{645})}{1000W}$$

$$C_b = \frac{22.69OD_{645} - 4.68OD_{663}}{1000W}$$

Figure 1. PPT resistance seedlings.

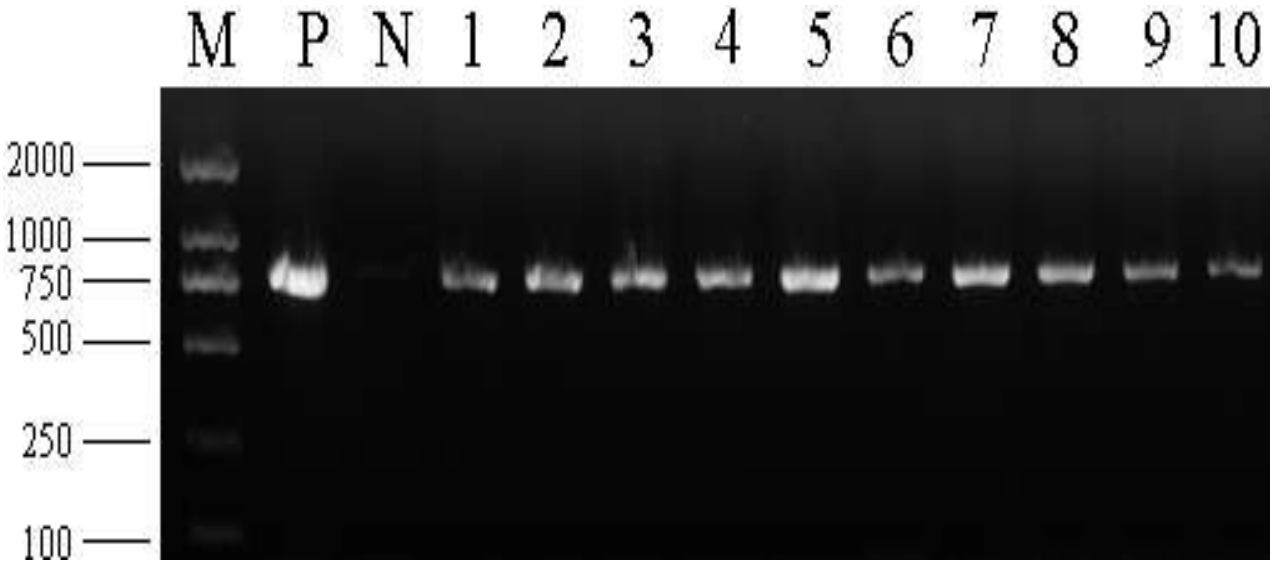

Figure 2. Electrophoresis results of some materials by PCR detection. M, *Trans2KTM* DNA marker; P, positive control; , negative control; lanes 1-10, transgenic plants.

RESULTS

Attaining the transferred plants

Using the pollen tube channel method and by means of preliminary screening, we attained seven assumed transferred spikelets with 1520 grains, and the harvested seeds were the T_0 materials. At the 3 to 5 leaf stage, we obtained eight PPT resistant seedlings by means of Basta (0.7%) solution (Figure 1), after which we then used the blade molecular to detect molecules at the 6 leaf stage. Besides, T_1 generations were plants selected from T_0 with the feature of PPT resistance. However, the result of molecule detection was positive; as such, the same principle was used for others.

Result of PCR detection

The result of the PCR detection of transferred plants, negative control plants and the extraction DNA of plasmid (Figure 2) show that the positive PCR amplification rate of T_1 generation was only about 43%, while that of T_2 generation was up to 75 to 85% and that of T_3 was up to 96%. All these indicate that transgenic materials are purifying, while genetic segregation decreases generation by generation. The electrophoretic separation bands of the transferred plants extension were consistent with

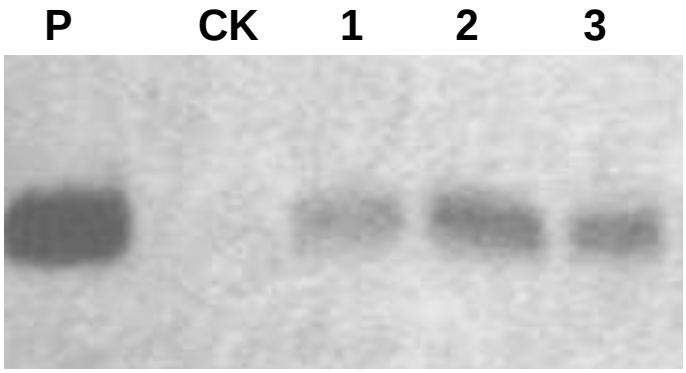

Figure 3. Southern blot hybridization analyses of T3 transgenic plants. P, plasmid; CK, negative plant; 1, 2 and 3, transgenic plants.

those of the positive control plants, and all PCR negative control results were negative. All these prove that the PCR amplification method was correct and its results are reliable.

Southern blot hybridization analysis

The results of the southern blot hybridization analysis also prove that target gene has been integrated into the genome of transferred plants, and the target gene can be inherited and expressed stably in transferred plants. This can be verified from the result of drought resistance indexes analysis that follows. Figure 3 shows the southern blot hybrid analysis result of some T_3 generation transferred plants. The hybrid strip proves that target genes are integrated into plant genomes by a single copy.

Identification of drought resistance in transgenic plant

The content of free proline in transgenic plants

Proline is an important intracellular osmolyte, and the content of proline in plants is proportional to the plants' drought resistance capacity. Proline can accumulate greatly when the plant suffers water stress. In order to identify the drought resistance capability of transgenic lines in this study, we planted T_2 seeds harvested from well-growing T_1 transgenic lines and were positively tested by molecular detection in greenhouse pots. Then, we screened ten positive plants by PCR, after which we measured the content of proline of these ten positive plants under drought treatment. As can be seen from Table 1, with the exception of No. 4, all the other plants showed higher proline content than the control plants, with No. 6 having the highest proline content, reaching 196.20 μg / g, which was about 2.5 times higher than that of the control plants. However, other transgenic plants had 20% higher proline content when compared with that of the control plants. The results indirectly show that the transgenic plants have better drought resistance capacity than the non-transgenic plants.

The chlorophyll content in transgenic plants

In addition to proline content, the chlorophyll content of the ten screened positive transgenic plants were also detected. Chlorophyll is the main photosynthetic pigment of plant leaves and an important indicator in plant physiology research. From Table 1, we can see under drought treatment that the chlorophyll content of some transgenic plants was far higher than that of the control plants. Moreover, plant No. 6, with a content of 2.330 mg/g, was twice higher than that of the control plants. This result also clearly portrays that the drought resistance capability of some transgenic plants is better than that of non-transgenic plants.

DISCUSSION

Using transgenic technology to create new plant germplasm can break the boundary between species; thus, to realize gene transference between species, the biological traits of existing species should be improved upon and then new species should be bred. At present, the pollen tube channel method created by China's scholars has been widely used in cotton, soybeans, maize, etc. For example, Qi et al. (1998) reported that exogenous DNA was imported into maize inbred line by means of pollen tube channel method to establish mature import technology in the exact manner, while Wang et al. (2002) imported Bt gene into Jilin backbone inbred line. In this study, drought resistance gene, *TPS*, was imported into maize inbred line by means of improved pollen tube channel method, and by spraying Basta (0.7%) solution for generations to screen and detect molecule, before

Table 1. Measurement results of proline and chlorophyll content in plants.

Number of plant	Proline content		Chlorophyll content	
	μg/g	Compared to CK (%)	mg/g	Compared to CK (%)
CK	82.92	—	1.052	—
1	115.27	38.90	1.419	34.9
2	144.47	74.25	1.691	60.8
3	107.55	29.72	1.342	27.7
4	77.81	-0.07	1.136	8.0
5	133.19	60.64	1.528	45.3
6	196.20	136.65	2.330	122.1
7	104.10	25.57	1.266	21.1
8	116.18	40.13	1.382	31.5
9	120.09	44.84	1.582	50.5
10	123.28	48.70	1.437	36.6

acquiring T_3 transgenic plants which have higher resistance.

The electrophoresis band of the transgenic plant's amplification product was consistent with the positive control zone, where all the negative control zones show signs of negative PCR. Using such a method to import exogenous DNA can help us to attain new drought resistance maize resources. Therefore, it is a feasible and effective method to cultivate seeds with drought resistance. Genetic transformation of trehalose synthase gene in plants dates back to Holmstorm's report (Holmstrom et al., 1994, 1996). Yeo et al. (2002) transformed potatoes with TPS1 of yeast driven by promoter CaMV35S. However, the transferred plants grew badly including dwarfing, yellowing, willow-leaf shaping, unusual root development, deformity and so on; but after they were transplanted, they got right soon and drought resistance capacity was improved obviously (Yeo et al., 2000). Garg et al. (2002) mixed Otsa of coli and Otsa gene, and then imported them into rice. After doing a series of experiments on T_5 generation, they obtained results which show that transgenic rice can grow better in severe conditions than ordinary rice, and it also has features such as: cold resistance, drought resistance, salt resistance, etc.

Nevertheless, the transgenic plants have higher photosynthetic efficiency under non-stress conditions. More so, Zhao et al. (2000) cloned the trehalose-6-phosphate gene (TPS) from *Saccharomyces cerevisiae* Hansen, and it was observed that the transgenic tobacco with TPS could express drought tolerance under drought stress. Jia et al. (2007) imported TPS genes into ryegrass, and the PCR analysis showed that TPS gene had already been integrated into ryegrass. The identification of drought resistance shows that cold resistance and water retaining capacity of transgenic synthase was improved under the condition of drought stress, and their electrolyte leakage rate was distinctively lower than that of the control plants. In this laboratory, we imported *TPS* genes into maize inbred line "Zheng-58", and found that the drought resistance capacity of the attained transgenic plants was strengthened. This proves that the importation of TPS genes cannot only stabilize inheritance, but can also improve the drought resistance capacity of transgenic maize. PCR and Southern analysis showed that TPS gene had been integrated into the maize, although the results of drought-tolerance investigation in farmland confirmed that the drought-tolerance of transgenic plants was higher than that of non-transformed plants. In conclusion, developing drought-tolerance breeding by plant gene engineering technique was a practicable path.

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