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Full Length Article

Development of Plant Expression Vector with Taq DNA Polymerase Gene to Yield Heat-tolerant Maize Lines

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Abstract

Thermus aquaticus (*Taq*) DNA polymerase has been extensively used in DNA amplification procedures, However, the application of *Taq* DNA polymerase gene in plant has not yet been reported. In this study, we have focused on using *Taq* DNA polymerase gene to develop heat-tolerant maize transgenic lines, which would aid in controlling crop loss due to heat stress and potentially increase the economic value. Firstly, an expression vector with *Taq* DNA polymerase gene was constructed and introduced into the maize ZhongKai inbred lines, Zhong sweet No. 1 and Zhong glutinous No. 2 by pollen-mediated transformation to obtain the transgenic maize plants. Then, the harvested T1 generation seeds were confirmed to be positive transformants by PCR and Southern blotting. The screening of the T2 generation by exposure to heat stress conditions in the field combined with physiological indexes resulted in nine heat-tolerant transgenic maize lines. The expression vector with *Taq* DNA polymerase gene, developed in the present study, will certainly expand more avenues for heat-tolerant genetic transformation in plants. © 2017 Friends Science Publishers

Keywords: Maize; *Taq* DNA polymerase gene; Pollen-mediated transformation; Heat-tolerance

Introduction

The heat shock response is commonly believed to be dominated by multiple genes. When plants are stimulated by heat, the heat shock factors may specifically combine with the binding sites, thereby activating the expression of the genes that encode heat shock proteins (HSP) and promote the accumulation of these proteins *in vivo*. This phenomenon leads to an improvement of heat tolerance in plants (Morimoto, 1998; Hartl and Hayer-Hartl, 2002; Andrade *et al*., 2013). On the contrary, two different studies have shown that under heat stress, the species that can produce new proteins harbor genes with poor heat tolerance (Stout and Al-Niemi, 2002; Mishra and Grover, 2016), have shown that under heat stress, the species that can produce new proteins harbor genes with poor heat tolerance. Their results demonstrated that most of the proteins synthetized at room temperature, can also be synthetized under heat stress, since it is a response to high temperature in species with poor heat tolerance. These findings were found to be consistent with previous reports (Freiden *et al.*, 1992; Wahid *et al.,* 2007), which also suggested that the phenomenon of heat tolerance is not limited to HSP alone.

Taq DNA polymerase is isolated from the thermophilic bacteria, *Thermophilus aquaticus*, and has a stable natural structure. It can synthesize arbitrary sequence of DNA under high temperature *in vitro* and presents fidelity to its replicated templates. It is a key ingredient of polymerase chain reaction (PCR) and at present, its application is confined to this technique. Interestingly, Yang *et al.* (1995) established a "PCR-like system" without template and primers, and proved that Taq DNA polymerase can mediate spontaneous enzymatic synthesis of DNA. Meanwhile, a higher dose of enzyme, higher reaction temperature, and prolonged heat preservation time were considered favorable for the synthesis of small DNA molecules. These observations further corroborated the findings of Schachman (Schachman *et al*., 1960). For the amplified DNA sequences of sizes less than 194 bp and of the range, 603 bp-4.3 kb, the corresponding protein molecular weights obtained were smaller than 8.4 KD and between 20.4 KD and 120 KD, respectively (Yang *et al.*, 1995). The sizes of these proteins were found to be similar to the sizes of the HSP detected in the Yang *et al.* (1995) experiment.

Currently, the transgenic methods commonly used for most of the plants include the *Agrobacterium tumefaciens* mediated technique and particle bombardment. These two methods require tedious and lengthy tissue culture procedures and are carried out by applying the callus as a receptor, which may in turn induce somatic variations and is genotype dependent (Frame *et al.*, 2011; Omer *et al.*, 2013). Although bombardment is not restricted by hosts, it is likely to cause damage and mutations. *A. tumefaciens* has a natural transformation mechanism to develop a single copy, but

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maize is not a natural host for *Agrobacterium* (Jackson *et al.*, 2013). Various improvement methods, such as adding Acetosyringone (Sheikholeslam and Weeks, 1987), screening strains, and utilizing binary vector (Miller *et al*., 2002; Vega *et al.*, 2008; Deeba *et al.*, 2014) have been adopted in the infection process; however, the transformation ratio still remains low. Among the reproductive system transformation methods, the first proposed pollen-tube pathway (Zhou, 1983) showed a great success in cotton (Yang *et al.*, 2007). Further, pollen-mediated transformation was proposed based on the blooming characteristics of maize; therefore, the operation was observed to be more suitable for the transformation of plants, especially for maize, which produced large amounts of pollen (Eapen, 2011).

The question that whether Taq DNA polymerase gene can be directly applied to promote normal DNA replication in plant cells under heat stress, still needs to be answered. The present study was therefore undertaken in order to investigate the phenomenon of spontaneous synthesis of DNA in plants *in vivo* under heat stress conditions. The maize transformation experiment was done by producing an expression vector with Taq DNA polymerase gene using pCAMBIA1380 as the binary vector. This vector system was then transformed into maize inbred lines using the pollen-mediated method. The use of molecular techniques and exposure to high temperature conditions in the field further facilitated to screen and obtain the heat-tolerant maize lines.

Materials and Methods

Plants and other Experimental Materials

Zhong sweet No. 1 and Zhong glutinous No. 2 inbred lines was used for the experiments in this study. All maize plants were cultivated in field and bagged during flowering period. *Escherichia coli* strain DH5α was used for the cloning. The pCAMBIA1380 was used as the binary vector for pollen-mediated transformation.

Establishment of Binary Expression Vector

Based on the 2.5 kb gene sequence information of Taq DNA polymerase gene (Di *et al*., 2012), a pair of primers (*Taq*1: 5′-GGCCGAATTCATGCTGCCCCTCTTTGAGCCC-3′) and *Taq*2: 5′-GGCCGAATTCTCACTCCTTGGCGGAGAGCC-3′) were designed, with *Eco*RI located at each end of the gene. PCR was performed in a 25μL reaction containing 1μL of plasmid containing Taq DNA polymerase gene as the template, 18.5 μL of ddH_2O, 2.5 μL of 10×PCR buffer, 0.5 μL of dNTP mix (10 mM each), 1 μL of primer *Taq*1 (10 μM), 1 μL of primer *Taq*2 (10 μM), and 0.5 μL of DNA polymerase (2.5 U/μL; Takara, Dalian, China). PCR was performed using the following cycling profile: 94°C for 5 min, 36 cycles at 94°C for 1 min, 56°C for 1 min, 72°C for 4 min, and then 72°C for 10 min. The PCR amplicons about 2.5kb DNA fragment (Fig. 1B) were sequenced by Dingguo Biotechnology Co. (Beijing, China). Preparation of competent *E. coli* cells, as well as the insertion of PCR amplicons into the pUC19 vector, were carried out based on the protocols reported by Li and Liu (Li and Liu, 2004). The recombinant plasmids was firstly screened by Blue-white screening (Fig. 1C) method (Stout and Al-Niemi, 2002) and further confirmed by PCR (Fig. 1D). Then, the gene fragment was recycled (Fig. 1E) and inserted into pCAMBIA1380 binary vector to yield the expression vector pCAMBIA1380-*Taq*.

Maize Transformation by Pollen-mediated Method

Approximately 0.1 g of fresh pollen was collected at 8–9 AM and stored in 5 mL of 10% sucrose solution. Subsequently, the pollen grains were mixed with 50 μg plasmid DNA followed by being treated twice using ultrasonication (200 W, 5 seconds each time and at an interval of 3 sec). Then, the precipitated pollen and the remaining solution were transferred to a petri dish for artificial pollination. In this experiment, 20 ears with filament length of 3–5 cm were selected. The pollen solution following ultrasonic treatment was applied to the filaments using a brush for artificial pollination. The ear bags were hitched and labeled to record data on burliness.

PCR and Southern Blotting Detection of Transformants

Seedling leaf DNA extraction of T1 and T2 generation of transformants was performed with improved SDS alkaline lysis (Wang *et al*., 2007). The PCR amplification of T1 and T2 generation was carried out in a 25 μL mixture with an annealing temperature of 56°C. For Southern blotting of T1 generation of transformants, 20 μg of DNA were digested with *Eco*RI to hybridization with probes. The probes from the plasmids was labeled using the PCR digoxin probe labelling kit, and the random primers method was used to label the molecular weight marker. The Chemiluminescent detection (CSPD) method was used for color development (Liang *et al.*, 2016).

Screening of Maize for Heat Tolerance

T2 generation of transformants was sown on March 25, that was 15 days later than the normal spring seeding, and the study was conducted at the experimental plots of Zhongkai University of Agricultural and Technology, China between 2010~2013. The growth robustness, extent of damage and pollen quantity were observed and divided into three grades: (i) Bad, no anther spited, no pollen, short, with yellow-green blades; (ii) Moderate, anther spited but thin and shriveled, small amount pollen, weak blades; (iii) Normal, lots of pollen, dark green upright blade

(Chen *et al.*, 2010).

The number of kernels was recorded after the maize ears were harvested (Kaur and Saxena, 2008). Furthermore, the leaves were sampled at the plant flowering stage to determine the physiological parameters. Of which, the superoxide dismutase (SOD) activity was measured by nitro blue tetrazolium (NBT) photo reduction, and the malondialdehyde (MDA) concentration was determined by thiobarbituric acid spectrophotometric method (Li *et al*., 2002).

Results

Acquisition of Taq DNA Transgenic Plants

To obtain the transgenic plants of Taq DNA polymerase gene, we firstly amplified the target gene from plasmid containing *Taq* DNA polymerase gene (Fig. 1B), which was confirmed by sequencing. Subsequently, the gene fragment amplified above was connected to plasmid vector pUC19 and then inserted into pCAMBIA1380. The results of EcoRI digestion identification indicated that the expression vector, pCAMBIA1380-*Taq* was successfully established (Fig. 1A). The transformed maize ears using pollen-mediated method were harvested (Fig. 2).

Confirmation of Taq DNA Transgenic Lines

To investigate positive transformed maize, a PCR analysis of T1 generation was performed. The results showed that a total of 18 plants were successfully amplified to obtain the expected band (Fig. 3A). To confirm the PCR results and further determine the precise copy of the transferred gene, Southern blotting of the T1 generation was conducted (Fig. 3B). The results indicated that the target gene was integrated into the genome of the transformed plants, and all of positive transformants were one copy insertion. Totally, six positive T1 lines from 18 PCR positive plants were obtained.

Screening of Maize for Heat-tolerance in the Field

To investigate the growth of the plant and pollen quantity under high temperature stress, we postponed sowing the T2 generation of transgenic plants and make the flowering period delayed to mid-June, when the average temperature was 33°C, 0.5–1°C higher than normal sowing time; and the filling period was in late June, the average temperature was 33.2°C, 0.5–2°C higher than normal sowing time, which resulted in a moderate damage on maize. Consistent with PCR detection results (Table 1), some of T2 generation plants were short, with yellow blades and weak growth vigor, and not tassel and no pollen, which was similar to the control. However, some of these had relatively strong growth vigor, with dark green and upright blades, and much more pollen that could be pollinated and fertilized (Fig. 4).

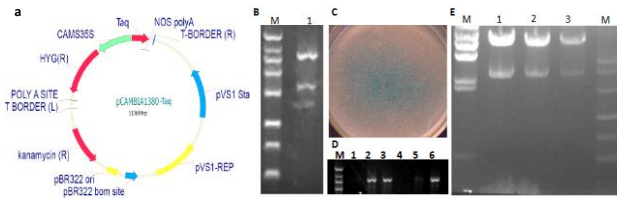

Fig. 1: Establishment of binary expression vector
A. Structure of binary vector pCAMBIA1380-Taq; B. PCR amplification of the *Taq* DNA polymerase gene; C. Blue-white screening; D. PCR identification of recombinant plasmids; E. Digestion identification of pCAMBIA1380-Taq

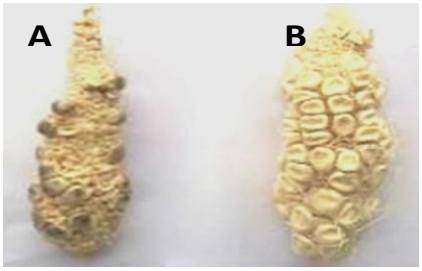

Fig. 2: Ears of transgenic plants
A: Zhong glutinous No.1, B: Zhong sweet No. 2

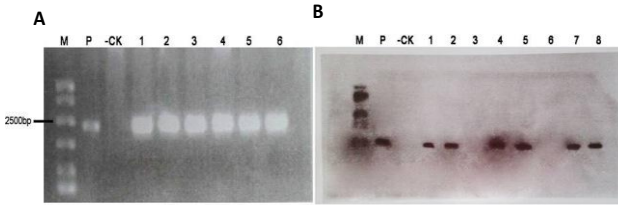

Fig. 3: Detection of T1 generation transgenic plants
A. PCR detection; B. Southern hybridization. M: Marker, P: Plasmid, -CK: Negative control, 1-8: Transgenic plants

Fig. 4: High temperature screening of T2 generation in field

Analysis of Heat Tolerance Index

To confirm the heat-tolerant screening, heat tolerance index such as SOD activity and MDA concentration of the transgenic plants were conducted (Table 1) on June 15 (during the flowering period), when the maximum

Table 1: PCR positive rate and heat-tolerant screening of $T_{2\text{-}4}$ line of T_2 generation plants

Identity of T2 - 4 line	PCR （+，-）	Blossom SOD (U/g)*	MDA concentration (nmol/g)*	Growth	Pollination	Kernel number per panicle (n)	Resistance line
1	-	143.22fg	46.78a	Bad	Few	8	
2	+	175.03d	17.09c	Moderate	Moderate	12	
3	+	226.00bc	15.25d	Normal	Many	19	
4	-	135.17g	36.40b	Bad	Few	6	
5	+	175.17d	17.26d	Moderate	Moderate	16	
6	+	164.52e	17.01d	Moderate	Moderate	17	
7	+	168.83e	15.19d	Moderate	Moderate	11	
8	+	176.02d	22.92bc	Excellent	Moderate	16	
9	+	165.77e	16.49c	Moderate	Moderate	12	
10	-	129.78h	37.15b	Bad	Few	8	
11	-	110.65c	37.29b	Bad	Few	5	
12	+	187.54d	14.45e	Bad	Few	6	
13	+	219.64c	15.16de	Excellent	Moderate	18	
14	-	106.18i	69.00a	Moderate	Moderate	15	
15	+	246.99a	16.17d	Excellent	Many	26	R
16	+	242.77ab	16.80d	Excellent	Many	22	
17	-	135.69g	30.81bc	Bad	Few	10	
18	+	226.43b	19.78c	Moderate	Moderate	15	
19	-	143.73f	32.44b	Bad	Moderate	14	
20	+	218.68c	18.70c	Moderate	Moderate	15	
21	+	235.57b	18.00cd	Excellent	Many	18	
22	+	226.94bc	17.99cd	Excellent	Many	23	
23	+	235.93b	15.78cd	Excellent	Many	19	
24	-	15.78cd	41.48b	Bad	Few	11	
25	+	286.35a	14.98e	Excellent	Many	31	R
26	-	179.96d	26.04c	Bad	Few	13	
27	+	248.87a	12.38e	Excellent	Many	27	R
28	+	238.14b	14.45d	Excellent	Many	25	
29	+	237.66b	10.60f	Excellent	Many	26	
30	+	265.11a	8.77g	Excellent	Many	41	R
31	-	150.00f	49.69a	Bad	Few	18	
CK1	-	149.25f	33.65b	Bad	Few	17	
CK2	-	157.19f	30.14bc	Bad	Few	15	
CK3	-	183.44d	29.87c	Bad	Few	15	

*Duncan's new multiple range method was applied for significance test. a-i: indicate a 0.05 level of significance

temperature was particularly as high as 34°C. Under this condition, the SOD activity reflected the heat capacity of the plant itself, and MDA concentration reflected the degree of heat damage. In table 1, high SOD activity but low MDA content were appeared in PCR-positive plants and the number of spike grain has a positive correlation with the SOD activity. It was illustrated that Taq DNA polymerase gene is beneficial to form the normal antioxidant enzyme, decrease the stress, enhance growth vigor, finally increase the kernel per spike.

Discussion

Currently, researches about maize heat tolerance were seldom carried out than drought or chill. Also, a complete system for identification of maize heat tolerance is lacked, especially reliable and effective criterion. The existing studies were ether only on seedling stage, or under artificial high temperature condition, which cannot be linked with flowering or seed setting (Hussain *et al*., 2006). The paper studied the experiment under the real field condition by postponing the sowing date and imposing high temperature during maize flowering period to trigger heat stress. The formation of plant resistance is often closely related to the activity of enhanced antioxidant enzyme system, in which SOD and MDA were usually taken as heat tolerance physiology index (Bowler, and Fluhr, 2000; Vranova *et al*., 2002). Also, growth vigor, pollen quantity and blade color were used as direct field index, while kernel number per spike was used as indirect parameter. Combined with positive PCR, above indexes can comprehensively screen transgenic heat tolerance lines. This system has a direct production application value.

Taq DNA polymerase shows the potential to maintain its activity at 94°C, and the ability to catalyze DNA synthesis in a relatively wide temperature range. Thus, it forms a good heat-resistant gene resource in the field of plant genetic engineering. Now, a few heat-stable genes have been used in the development of expression vectors. For instance, heat shock protein gene, HSP (Dietrich *et al.*, 1991), phytase gene, phyA (Li and Liu, 2004) and thermo-tolerance genes, TTOs (Ko *et al*., 2007). The expression vector with Taq DNA polymerase gene, developed in the present study, will certainly expand more avenues for heat-tolerant genetic transformation in plants. Particularly, in southern China, where asynchronous silking is associated

with hot and humid weather conditions as well as abnormal temperature fluctuations during summer, nurturing new heat-tolerant species will have far-reaching significance and broad developmental prospects.

Conclusion

In this study, we developed heat-tolerant maize transgenic lines using Taq DNA polymerase gene, which will benefit to controlling crop loss due to heat stress and increase the economic value. SOD activity, MDA concentration, PCR-positive plants and growth vigor, as well as the number of spike grain, all should be used as heat tolerance index to form an ideal heat-tolerant screening system.

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