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RESEARCH ARTICLE

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Establishment of transgenic acceptor and transformation of *barnase* gene by particle gun in maize inbred line 18-599 (white)

Abstract The efficient acceptors for maize transgenic engineering are currently insufficient in China. Seed production by male sterility is the best method for advancing the authenticity of maize hybrid. Maize inbred line 18-599 (white) is an antivirus high-quality maize inbred line in China, which has been used for lots of maize hybrid cultivars. The establishment of high efficiency transgenic acceptors is necessary for advancing the transgenic efficiency in maize transformation work. In this study, the efficient transgenic acceptors were optimized and established. 18-599 (white) was studied in state, types of culture mediums, times of callus regeneration and concentration of the screening reagent, Basta. The results showed that N6-4 medium was the best in 8 types of mediums for the immature embryo of 18-599 (white), 1.6 mm length was the feasible length of immature embryos for tissue culture in establishing the transgenic acceptor system, and it was within 5 times for suitable callus subculture. With the optimized transgenic acceptors, *barnase* gene was translated successfully into 18-599 (white) by a particle gun using *bar* as a marker gene. Basta was used as the screening reagent, its lethal concentration was 8 mg·L^{-1} and its working concentration for screening was 6, 8 and 6 mg·L^{-1} in 3 turns for callus regeneration, respectively. In this work, a transgenic plant with male sterility was obtained through molecule detection and observation in the field. The result has an important significance for the creation of new male sterility inbred lines in maize in the future.

Keywords maize, inbred line, transgenic acceptor, particle gun, molecular detection

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1 Introduction

Genetic engineering is currently an effective breeding method for improving characteristics amendment and the germplasm in maize. Transformation must depend on a transgenic acceptor with high efficiency. The previous work chose suspended cells (Wang et al., 1995a), immature embryos (Wang et al., 1995b; Li et al., 1990) and plumules (Li et al., 2001) in the establishment of transgenic acceptors, and on some maize materials, studied the transmissibility and variation of inductivity in the ability and components of culture mediums. As a result, the immature embryo culture was an effective method (Pan et al., 2003; Fu et al., 2005; Zhang et al., 2000). 18-599 (white) is an antivirus maize inbred line, which has high quality and high combining ability. Many hybrids came from it, obtained examination and approval and planted in large areas in southwest China. 18-599 (white) is a promising maize inbred line. It is important to study the state of immature embryos and the types of culture mediums, which could be used in many new hybridizing combinations, times for cloning of callus and concentrations of dosage. However, reports on the optimization of the transgenic acceptor on 18-599 (white) have not been found yet in China.

Male sterility is an inheritance phenomenon, where a plant is incapable of producing either normal anther or pollen or male gamete during the sexual propagation stage. Researchers are trying to make use of this in hybrid strain reproduction. At present, the primary quality problem is the low purity of maize hybrid strain in China, which arose from the traditional mode of maize

hybrid strain reproduction. Emasculating the female inbred line artificially has been done in the traditional mode of hybrid strain reproduction, which may result in the untimely or incomplete emasculation, and lead to a decline of purity of maize hybrid strain (Sun et al., 2003), because of the female inbred line's self-crossing. The problem of low purity of the maize hybrid strain need to be solved to establish a new female inbred line with male sterility characteristics by genetic engineering technology in maize hybrid strain reproduction in China. *RNase* gene and *barnase* gene, in the expression controlled by a special promoter in the anther, can cause heteroplasia of fabric carpet veneer and pollen can neither be produced nor have vitality. *Barnase/barstar* genes were transformed into tobacco and rape, and the transgenic male sterile line and restorer line were successfully obtained for the first time in the world (Mariani, 1990; Mariani et al., 1992; Sun et al., 2007). Subsequently, studies of *barnase* gene controlling the sterility have developed rapidly in recent years, and the genes expressed differentially in anther have been cloned from many plants, such as rice (Ling et al., 1998; Lu et al., 2000; Zou et al., 1994; Zhan et al., 1996), rape (Zhou et al., 1997) and tomato (Li et al., 1997), and the transgenic male sterile lines were successfully gained. Particle bombardment (Klein, 1987) has a unique predominance in improving genetic characteristics and effectively breaking misogamy between species. Wang et al. (1995b) translated the *Bt* gene into maize suspended cells by particle bombardment and obtained the transgenic maize. Zhang et al. (2000) also obtained it using the same method later. All the above studies showed a fine background by genetically engineering the male sterility line.

2 Materials and methods

2.1 Materials

The immature embryos of the maize inbred line 18-599 (white) were selected to establish a transgenic acceptor system. The ears were selected 7–20 days after pollinating by shucking out the bract of each layer and thereafter sterilizing, with 1/3 length of the grain top cut out and the immature embryos sought out. These were placed on abductive mediums by inoculating 15 immature embryos in each utensil. The immature embryos with scutellum upward were cultivated in abduction culture medium under 27°C in the dark, 4 weeks each time.

The immature embryos at the length of 1.0, 1.2, 1.6, 2.2, 2.6 and 3.0 mm were calculated after 20 days on N6-4 culture mediums. The growth rates of the immature embryos were surveyed every 2 days, and the net increased growth and expansion multiple were calculated to determine the best length of immature embryos.

2.2 Methods

2.2.1 Abduction medium

The germfree immature embryos selected from ears were inoculated on the abduction culture mediums of N6-1, N6-2, N6-3 and N6-4 or MS-1, MS-2, MS-3 and MS-4 (Table 1). Every type of the 8 culture mediums with 100 $mg \cdot L^{-1}$ inositol, 500 $mg \cdot L^{-1}$ casein hydrolysate, 30 $g \cdot L^{-1}$ sucrose, 7 $g \cdot L^{-1}$ agar and a normal quantity of other elements at pH 5.8 was used. Based on N6, the abduction culture mediums were at normal concentrations of B5 drop element I, N6 drop element and N6 organic, with the 4 types of culture mediums all at normal concentrations of MS drop element and MS organic based on MS. 20 immature embryos were inoculated in each utensil with 3 replications. The number, the length and the rate of callus for immature embryo were record. The most feasible from all the above medium types was selected finally as the most available culture medium.

Table 1 Some components adjusted in N6 and MS culture mediums

medium	component adjusted		
	L-Pro/g	2,4-D/mL	NAA/mL
N6-1/MS-1	1.38	4	0
N6-2/MS-2	1.38	6	0
N6-3/MS-3	1.38	8	0
N6-4/MS-4	0.69	4	1

Note: 2,4-D and NAA represent that the concentrations of stock solution are 0.5 and 0.2 $mg \cdot mL^{-1}$ respectively. Values in Table 1 are based on 1 L of medium.

2.2.2 Clone medium and differentiation medium

Key components of the clone medium and the filtration medium were N6 (major element) + B5 (drop element) + N6 (organic) + Fe^{2+} + CH + L-Pro + 2,4-D + sucrose + agar at pH 5.8, with Basta added to the filtration medium. Key components of differentiation medium were N6 (major element) + B5 (tiny element) + N6 (organic) + Fe^{2+} + CH + L-Pro + sucrose + agar at pH 5.8.

2.2.3 Critical concentration of selective reagent Basta

The callus cultured was chosen for the study of selective reagent Basta, whose concentration gradients were 0, 2, 5, 8, 11 and 14 $mg \cdot L^{-1}$. There were 20 calluses in one utensil, with 3 utensils used as 3 replications. Growth status and death rate of calluses were recorded. Ultimately, the lethal concentration of selective reagent Basta was ascertained. The rate of callus regeneration, at the percentage of the callus number between twice, was

used for judging the clone capability of calluses. A callus, on which a new callus could unceasingly grow, was regarded as having the regeneration ability, while the callus, which was aquiform or brown, was determined to be dead in this study.

2.2.4 Objective gene

Barnase gene was given by Prof. Fang Rong-xiang from Chinese Academy of Sciences. *bar* gene was regarded as a marker gene with herbicide resistance while establishing the expressive vector. The construction of the plamid pBBN is as follows (Fig. 1).

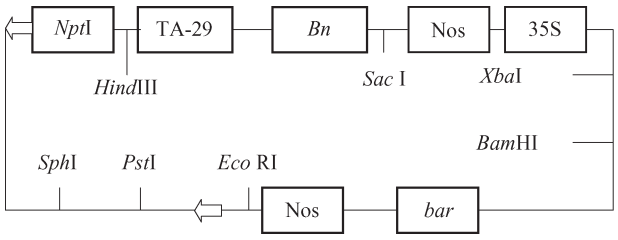

Fig. 1 Structure of T-DNA region of plant expression

2.2.5 Preparation of tiny bomb

Five microlitre solution of pBBN plasmid was immersed in 50 μL of the suspended solution of gold powder (diameter = 1.0 μm). Then 50 μL 2.5 mol·L^{-1} $CaCl_2$ solution and 20 μL 0.1 mol·L^{-1} solution of spermidine were added slowly under a swirl condition. The solution was vibrated continuously for 3 min, and then centrifuged at 70000 × g for 5 s after 10 min. After removing the supernatant, 250 μL of ethanol was added into the precipitate, vibrated continuously, and centrifuged at 70000 × g for 5 s. Then the supernatant was discarded again. Finally, 60 μL of ethanol was added into the precipitate, accompanied by adequate shaking.

2.2.6 Particle gun

The calluses of Type II of 2–4 μm in diameter were selected and pretreated in the culture medium with a high infiltration potential energy. Then the selected calluses were transformed by popping in a 3 cm diameter, with a cannon-shot of 900 mm, and at a vacuum pressure in the sample room (Wan et al., 1995). The bombed calluses were then placed in a filtration medium for about 5–7 days of recovery and cultivation.

2.2.7 PCR analysis of T_0 transgenic plants

The total DNA of the anthers was extracted using the 2 × CTAB method (Murray and Thompson, 1980). PCR amplifications were carried out using anther DNA, control DNA (plasmid NDA) and Negative DNA (non-transgenic maize DNA). PCR analysis of 18-599 (white)-*barnase*-*bar*-transgenic plants in T_0 and the non-transgenic plants was done. The objects of detection were *barnase* gene and *bar* gene. *Taq* enzyme was obtained from TaKaRa Company.

The primer sequences for the PCR analysis of the *barnase* gene were PN1: 5′-ATGGCACAGGTTATCAACAC-3′ and PN2: 5′-TTATCTGATTTTTGTAAAGG-3′, with a concentration at 250 ng·μL^{-1}. The primer sequences for the PCR analysis of the *bar* gene were P1: 5′–CTGACGTAAGGGATGACGC–3′ and P2: 5′–CATCGCAAGACCGGCAAC–3′, with a concentration at 250 ng·μL^{-1}. The PCR reaction system was 20.0 μL including 10 × buffer 2.0 μL, 25 mmol·L^{-1} $MgCl_2$ 1.0 μL, 2.5 mmol·L^{-1} dNTP 1.2 μL, P1 0.2 μL, P2 0.2 μL, Taq enzyme (5 unit·μL^{-1}) 0.2 μL, DNA 1.0 μL as a template, and ddH_2O 14.2 μL. The condition for PCR reaction of *barnase* gene and *bar* gene was as follows: at 94°C for 5 min; at 94°C for 40 s, 52°C for 40 s, 72°C for 1 min, in 30 cycles; 72°C for 10 min. PCR amplification products were separated with 0.8% agarose gel, and 0.5% TBE electrophoresis buffer, dyed by ethidium bromide (EB), and developed using a Bio-Rad gel imaging system.

2.2.8 PCR-Southern Blotting on T_0 transgenic plants

With the production of PCR of transgenic plants, it was assayed with a reagent box from Roche Company and DIG-labeled by a probe of random primer.

2.2.9 Fertility analysis of pollen from transgenic maize

The fertility, size and development status of the fresh anthers from transgenic maize were observed. The pollen coloration was examined by 0.1% I_2-KI solution.

3 Results

3.1 Establishment of transgenic acceptor with immature embryos

3.1.1 Selection of inductive mediums

After 20 days of inoculation, of the four kinds of N6 culture mediums, the immature embryos on the N6-4 had the maximum of net increase and expanding coefficient, and their increase rate per day was close to that on the other three mediums (Fig. 2). Therefore, the N6-4 was the best in the 4 types of N6 mediums, with the N6-3 being next. The MS-1 was the best in the 4 types of MS mediums (Fig. 3).

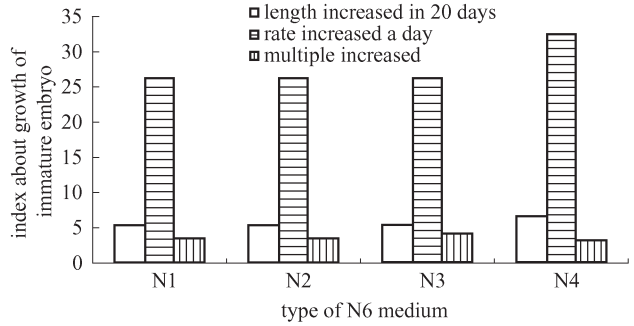

Fig. 2 Growth of immature embryos on N6 mediums

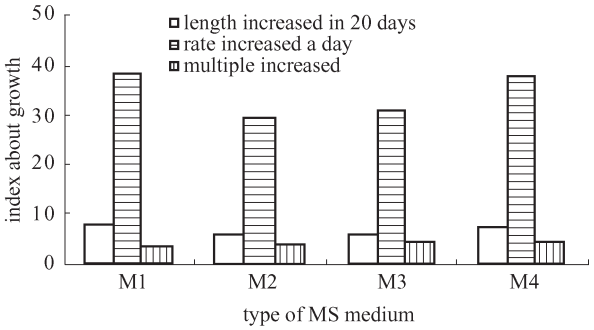

Fig. 3 Growth of immature embryos on MS mediums

Through the comparison of callus growth between inductive mediums N6 and MS (Table 2), the average rate of embryo callus on the N6 medium was 41.0%, which was twice as high as that on the MS medium. Therefore, when the transformation acceptor system was constructed using 18-599 (white), much more embryo calluses could be gained on the N6 medium.

Table 2 Comparison of callus generation between inductive mediums N6 and MS

medium		No. of calluses cultured	rate of embryoid calluses/%
N6	N6-1	42	40.0
	N6-2	44	42.0
	N6-3	36	34.0
	N6-4	51	48.0
	average		41.0
MS	MS-1	26	25.0
	MS-2	3	2.9
	MS-3	13	12.0
	MS-4	20	19.0
	average		21.3

Note: 105 immature embryos were inoculated.

3.1.2 The feasible length of immature embryos for tissue culture

The length of immature embryos, ranging from 1.6–2.2 mm, had a bigger net increase in 20 days, thereunto, the immature embryos of 1.6 mm had the biggest net increase (Fig. 4). These results showed that the most feasible length for the immature embryos was 1.6 mm when the N6-4 medium was used to construct the transformation acceptor system. In addition, the 1.6 mm long immature embryo was easily stripped off the maize ears.

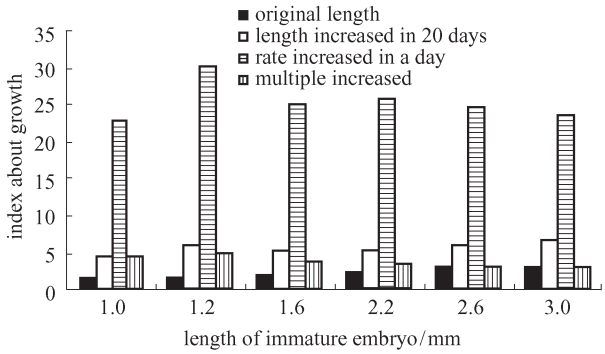

Fig. 4 Growth of immature embryos of different lengths on N4 medium

3.1.3 Times of regeneration culture for calluses

The regeneration rate of calluses could be over 96% if the times for the regeneration culture of immature embryos did not exceed 5, and the surviving rate of plantlets in the field was over 35% (Table 3). The regeneration rate of calluses and the surviving rate of plantlets all were higher, but lower if the times exceeded 7. Therefore, the times of callus generation under five was important for enhancing the survival rate of transgenic plantlets, the result could ensure the amount of transformed immature embryos and the transformed plantlets.

Table 3 Effect of clone times on callus regeneration and plantlets survival

Clone times	No. of callus	percentage of callus regeneration /%	percentage of induced plantlets /%	percentage of plantlets in the field /%
1	120	99	31	38
3	120	97	29	36
5	121	96	30	35
7	118	78	17	6
9	120	47	9	0

3.2 The concentration of the selective reagent Basta

Table 4 shows that, 8 $mg \cdot L^{-1}$ Basta with herbicide resistance as the selective reagent could be the lethal concentration. Considering the cell damage in calluses during the transformation, the concentrations of Basta were at 6, 8 and 6 $mg \cdot L^{-1}$ in three tests, respectively. In the first round of tests, 6 $mg \cdot L^{-1}$Basta was used to

recover the transformed calluses, 8 mg·L^{-1}Basta was used to eliminate the un-transgenic calluses in the second round test, and in the third round test, 6 mg·L^{-1} Basta was used to reduce the damage of Basta and the disadvantage of callus differentiation.

Table 4 Effect of Basta concentration on callus regeneration

Basta concentration/mg·L^{-1}	percentage of brown callus/%	growth of calluses
0	0	normal
2	17	a few calluses died
5	48	a half calluses died
8	100	all of calluses died
11	100	all of calluses died
14	100	all of calluses died

3.3 Validation of transgenic plants

3.3.1 Molecular detection of transgenic plants

PCR method was used on the transformed plants in the field. In only 1 of the 23 transgenic plants, the specific band at 341 bp could be amplified, which was the same as that of the positive control but did not occur in the negative control (Fig. 5 C).

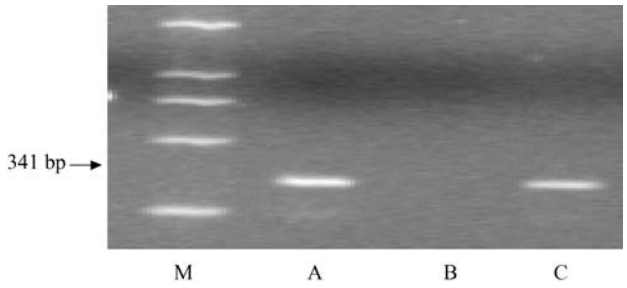

Fig. 5 PCR detection of *barnase*-transgenic maize
Note: M, A, B and C represent DL2000 marker, pBBN-*barnase* CK, Negative control and Positive plants, respectively.

The 23 transgenic plants were detected for *bar* gene by PCR amplification. The result showed that 3 of the 23 transgenic plants had the specific amplified band at 750 bp and were positive (Fig. 6 D,E,G). Among the 3 plants, one plant was also positive by the *barnase* gene detection.

The 23 transgenic plants were examined by PCR-Southern blotting. The result showed that 1 (Fig. 7 C) of the 23 transgenic maize was positive, which the *barnase* gene had been transformed into the genome of 18-599 (white).

3.3.2 Observation of anthers of the male anthotaxy about transgenic plants in the field

The transgenic plants with *barnase* gene were transplanted in the field; the anthers of the male anthotaxy

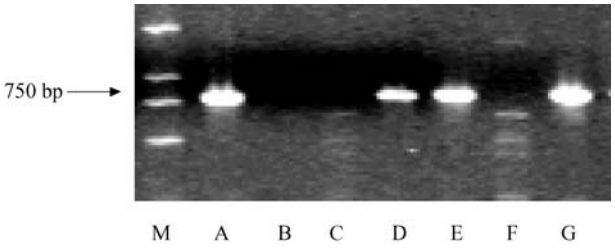

Fig. 6 PCR detection of *bar* of transgenic maize
Note: M represents DL2000 marker; A represents pBBN CK-*bar*; B represents negative control;
C and F represent non-transgenic plants; D, E and G represent positive plants, respectively.

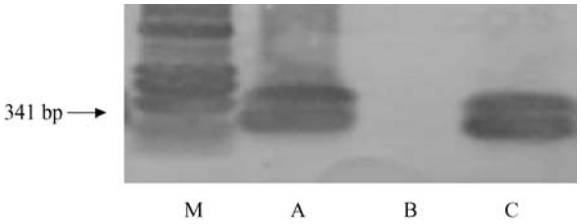

Fig. 7 PCR-Southern blotting assay of *barnase*-transgenic maize
Note: M represents DL2 000 Marker; A represents pBBNCK-*barnase*; B represents negative plant; C represents transgenic plant.

were observed carefully for its fertility. As a result, the anthers were empty without the pollen, the inner membrane of the anther was thinner than that of the normal and pale green, its length was about 1/2–1/3 of the normal, so the male anthotaxy was sterile. The dyed inner membrane of the anther by I_2-KI reagent did not turn blue, so there was no pollen in the anther.

All the above results showed that the obtained *barnase* transformed plant was sterile. The characters of male anthotaxy on transformed and untransformed plants are shown in Fig. 8.

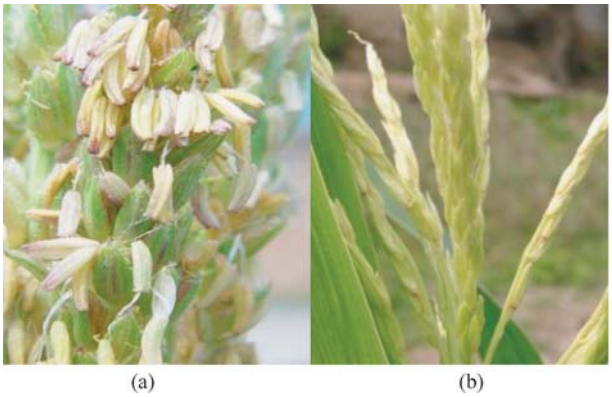

Fig. 8 Comparison of male ears between transgenic plant and normal plant
Note: (a) represents Tassal of non-transgenic plant; (b) represents Tassal of transgenic plant with *barnase* gene.

4 Discussion

The seed production of hybrid cultivars with male sterility can change the existing artificial castration mode, reduce the production cost, and radically improve the seed purity in maize. The transgenic plants with male sterility were obtained by genetic engineering in this work, which offered an important reference to the seed production mode of hybrid cultivars by male sterility in maize.

Immature embryos are currently the best material for transgenic acceptors in maize, with a high rate of induced plantlets and obvious growth predominance. It is actually difficult to establish an efficient transgenic acceptor as many techniques are needed in the process. It is found that the primary factors affecting the construction of the acceptor system are the genotype, the immature state, the inductive medium, the regeneration medium and the times of regeneration. In this study, the least amount of immature embryos could be speculated through the rate of induced plantlets for self-line 18-599 (white) to improve the work efficiency and gain the transgenic plants. Generally, the amount of immature embryos should be enlarged to 2–3 times of the least amount to reduce the venture of not gaining the transgenic plants, probably because the deficient immature embryos are used in the work.

The younger the immature embryos, the more easily the embryoid calluses would be differentiated, but the more slowly the calluses may grow, with more difficulties in gaining the immature embryos. If the embryos are bigger, the rate of their embryoid calluses and the type II embryoid calluses will be much lower, the calluses may generate buds and roots more easily, with a lower rate of transformed plants. The 1.6 mm length for immature embryos may be appropriate for differentiating embryoid calluses and seedlings.

The contents of growth hormones and the nutrition component all may change markedly during the regeneration culture. When the contents change to a threshold value, the calluses may be brown because of decompensation, so the regeneration (clone) of calluses must be timely.

5 Conclusions

The inbred line 18-599 (white) belongs to the genotype with a high ability of cloning and inducement. In this study, the calluses were induced twice, cloned 4 times, and selected 3 times. 2813 masses were gained from the initial 675 immature embryos, thereunto 1274 masses of embryoid calluses were transformed by particle bombardment. 322 tube seedlings were gained, 208 seedlings passed excision and 23 seedlings survived finally in the field. There was one seedling tallied with the goal characteristics of male sterility caused by *barnase* gene through molecule check. The rate of genuine transformation of immature embryos was only 1.48% in this study.

The length of immature embryos can affect the rates of embryoid calluses and differentiation of seedlings. The 1.6 mm length for immature embryos may be appropriate for both generating calluses and differentiating seedlings. The components and the types of culture mediums should be ensured from genotypes of self-line in maize. The suitable times of clone for embryoid calluses should be within 5 times.

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