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花粉介导法获得玉米转基因植株

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摘要: 利用花粉作为外源基因的载体进行遗传转化在玉米 (*Zea mays* L.) 上获得了成功。以玉米自交系太 9101、综 31 等为受体,以 pCL Ⅱ-RG-1 质粒为供体,在玉米开花期,用花粉与质粒 DNA 混合并附加超声波处理,然后辅以人工授粉的方法将外源基因导入到受体中。DNA 斑点杂交和 PCR 扩增以及 PCR-Southern blot 杂交检测结果证明,几丁质酶基因确已导入玉米自交系中。所得结果表明:玉米花粉可以介导外源基因的转化。利用花粉作为载体介导外源基因转化,避免了传统的基因枪法和土壤杆菌法转化所要求的组织培养技术,转化方法简单,易操作,具有很强的实用性。

关键词: 玉米; 花粉介导法; 基因转化
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Transgenic Maize Plants Obtained by Pollen-mediated Transformation

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Abstract: The genetic transformation was achieved by pollen-mediated approach on maize (*Zea mays* L.) inbred lines Tai 9101 and Zong 31. Plasmid DNA of pCL Ⅱ-RG-1 was mixed with fresh pollen of maize inbreds in sucrose solution. The pollens were treated by ultrasonication and collected, pollinated on silks of maize ears. Transformants were confirmed by dot blot hybridization, PCR amplification and PCR-Southern blot hybridization. The pollen-mediated transformation approach could circumvent the tedious tissue culture procedures like in particle bombardment and *Agrobacterium* infection, etc. This approach is simple, easy to operate, and could be widely used in practice.

Key words: maize; pollen mediated; genetic transformation

Maize is an important cereal crop. Genetically, transformed maize plants could be obtained by various approaches, such as ovary injection[1], ultrasonication treatment[2], particle bombardment[3,4], *Agrobacterium* infection[5], and electroporation[6], etc. Plant tissue culture procedures are prerequisites for most of the approaches above except for the ovary injection. Calli or even protoplasts as receiptors are needed in these approaches. Mutations are often induced during the process of callus induction and plant regeneration, resulting in causing the transformed plants to be difficult to transplant from test tube to field (greenhouse) due to easy death of plantlets and production of aberrant sterile plants. Significantly these approaches are limited in application.

Chitinase hydrolyses chitin existing in hyphae of fungi, and prevents fungi from infecting plants and propagating inside plant tissues. Hence, attempts were made to transform the chitinase genes into tobacco and potato, etc., and highly resistant plants to *Trichdermy harzianum* were obtained[7].

Most of maize diseases are fungal diseases, such as, leaf blight (*Setosphaeria turcica*), leaf spot (*Cochliobolus heterostrophus*), smuts (*Ustilago maydis* and *Sphacelotheca reiliana*). Introducing the chitinase gene into maize inbred lines may result in selection of plant lines resistant to above diseases. The goals of this study are to develop fungal disease-resistant maize lines as well as the pollen-mediated transformation method.

1 Materials and Methods

1.1 Materials

Maize inbred lines, Tai 9101 and Zong 31 were

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1.2 Methods

1.2.1 Preparation of plasmid DNA The plasmid pGL Ⅱ-RC-1 contains the chitinase gene and hygromycin gene for resistant-selection. The chitinase gene fragment is 1.1 kb (Fig. 1). The plasmid DNA was prepared with alkaline lysis[8] and purified with PCR Fragment Recovery Kit (TaKaRa Biotechnology (Dalian) Co., Ltd).

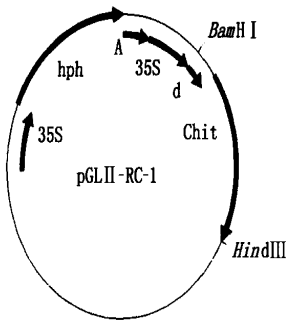

Fig. 1. Physical map of plasmid pGL Ⅱ-RC-1.
35S, CaMV 35S promoter; A, poly (A); Chit, chitinase coding region; d, adaptor; hph, hygromycine resistant gene.

1.2.2 Transformation method Seeds of maize inbreds were sown in the early May, and plants flowered in the early and mid-July. Maize ears were bagged before silking. About 0.3 g of fresh pollens were collected in the morning, and mixed with about 10 μg of the plasmid DNA in 20 mL of solution with 5 % sucrose (The ratio of the plasmid DNA to the pollens (W/W) was 1∶30 000). The solution was treated with ultrasonication before and after adding the plasmid DNA. By using JY92-Ⅱ Ultrasonicator from Ningbo Xinzi Scientific Instrument Institute the parameters for sonication treatment were: sonic intensity of 300 W, treatments for 8 times each for 5 s and 10 s interval. Then, the treated pollens were pollinated on clipped maize silks.

1.2.3 DNA dot blot hybridization T_0 seeds were sown in experimental plots next spring. Young leaves were collected from plants with 5 - 6 leaves. Total plant DNA was extracted according to Pich and Schubert[9], and purified using the instruction of PCR Fragment Recovery Kit. The plasmid DNA was digested with *Bam*H Ⅰ and *Hin*d Ⅲ, and the chitinase gene fragment of 1.1 kb was recovered by electrophoresis on low melting point agarose. The recovered DNA fragment was labeled with the DIG DNA Labeling and Detection Kit from Boehringer Mannheim. Ten μg total plant DNA grom each plant were dripped on nylon membrane, which was then baked at 80 ℃ for 2 h. DNA dot blot hybridization was carried using labeled chitinase gene fragment as probe at 68 ℃, and the membrane was probed with NBT and X-phosphate.

1.2.4 PCR amplification Two pairs of primers were designed based on the sequence of the chitinase gene and synthesized by Sangon Biotechnology Company of Shanghai. The primers, numbered as 8341/8342 and 49040/49041 sequenced as follows. 8341: 5′-GCGGTGGTGGCCATGGCGGT-3′; 8342: 5′-GGGCCTCTGGTTGTAGCAAT-3′; 49040: 5′-GCTCCACCTCCGATTACTGC-3′; 49041: 5′-GCGTTGCCGTTGTTCTCCTC-3′. The fragment sizes between the two pair primers were 923 bp and 376 bp, respectively. The PCR conditions were as following: program 1: 94 ℃, 5 min; 94 ℃, 0.5 min; 50 ℃, 0.5 min; 72 ℃, 1.5 min; 30 cycles; 72 ℃, 10 min. Program 2: 94 ℃, 4 min; 94 ℃, 0.75 min; 45 ℃, 0.75 min; 72 ℃, 1.75 min; 30 cycles; 72 ℃, 10 min. PCR amplification was completed using TaKaDa Taq™ Kit and PTC-200 Thermal Cycler.

1.2.5 PCR-Southern blot hybridization Following the method of Wang and Fang[10], the 376 bp fragment of the chitinase gene as probe was labeled with DIG-dUTP using PCR DIG Probe Synthesis Kit. PCR products of total DNA as temperate isolated from transformed plants were fractionated by electrophoresis in 1.2 % agarose gel, and transferred onto nylon membrane, hybridized with DIG labeled probe, detected with CSPD (disodium 3-(4-methoxyspiro {1, 2-dioxetane-3, 2′-(5′-chloro) tricyclo [3.3.1.1^{3,7}] decan}-4-y1) phenyl phosphate) florescence staining and exposed to X-ray film.

2 Experiment Results

2.1 The medium for pollen mediated transformation

2.1.1 Sucrose solution If pollens are mixed with DNA solution directly, they will loss their vitality because they are swollen by water in solution. Therefore, the solution with a suitable solute has to be selected. We prepared the solution with certain amount of sucrose which could maintain normal turgidity of the pollens and facilitate them to be germinated. But the optimum sucrose concentration varies with the pollens from different plant species[11].

A test for selecting suitable sucrose concentration was conducted. Seeds from maize inbred lines were sown in spring. Plants grew normally and silked and bloomed from middle to late July. At full blooming stage the fresh pollens were taken at around 10∶00 AM and suspended into the solution (pH 7.0) with sucrose of 5 %, 10 %, 15 % and 20 %, respectively. The pollens in the solution were stored at 4 ℃ for about 5 h and then, observed under an optical microscope. The result was listed in

Table 1. It could be seen from Table 1 that the sucrose solution could, to certain extent, protect the pollens from damage due to water exchange between the pollens and the solution. The solution with 5 % sucrose could best reserve the pollens, integrity. With the increase of sucrose concentration percentage of the damaged pollen grains was increased. 68.4 % of the pollen grains were damaged when the sucrose concentration was up to 20 %. Therefore, 5 % sucrose solution was used for pollen mediated transformation in the present study.

Table 1 Pollen grain changes in various concentrated sucrose solution (var. E28)

Sucrose concentration (%)	No. pollen grains	No. of pollen grains with plasmolysis	Percentage of total	No. of pollen grains with broken wall	Percentage of total	Percentage of damaged pollen grains
5	82	2	2.4	0	0	2.4
10	59	20	33.9	7	11	45.8
15	47	15	37.9	8	17.0	54.9
20	57	6	10.5	33	57.9	68.4
0		Part		More		About 95

2.1.2 Ultrasonication treatment Xu *et al* ① succeeded in introducing foreign fluorescent molecules and genes into mammal cells by ultrasonication treatment. They considered that transient vacuolization induced by ultrasonication was an important mechanism for foreign molecules (including DNA) to enter cells. In present study, we introduced the plasmid DNA into the pollen grains with transiently released high energy and vacuolization by ultrasonication. The pollens could, then, be used for transformation by pollination. Because the intensity of sonication may affect the vitality of pollens, we studied the extent of the damaged maize pollens with various sonic intensities. The results were listed in Table 2.

Table 2 Pollen grain changes after ultrasonication treatment (var. Huangzaosi)

Sucrose concentration	Sonication strength (W)	No. of treated pollen grains	No. of pollen grains with plasmolysis	Percentage of total	No. of pollen grains with broken wall	Percentage of total	Percentage of damaged pollen grains
5 %	200	101	32	31.7	8	7.9	39.6
	300	52	20	38.5	5	9.6	48.1
	400	71	30	42.3	6	8.5	50.8
	0	43	13	30.2	3	7.0	37.2

All of the sonication treatments were 8 time treatments with 5 s working time and 10 s working interval.

It is indicated from Table 2 that ultrasonication treatment could cause some of the pollen grains to be damaged. The damaged extent was related to sonication intensity. As the intensity was increased from 200 W to 400 W, the percentage of the damaged pollen grains increased from 39.6 % to 50.8 %. We considered that certain degree of the sonic intensity was needed to facilitate the plasmid DNA entering pollens, so 300 W was used in present study.

The physical force produced by ultrasonication not only can hurt the pollen grains, but also shear the plasmid DNA. We examined the DNA by electrophoresis after sonication treatment and found that the parts of the plasmid DNA was kept intact.

2.2 Identification of transformed plants

Maize ears from inbred lines were pollinated as described in **1.2.2**. Thirty-four of the maize ears were treated and six ones had seeds. Totally, 32 seeds were obtained and sown in experimental plots. Young leaves were taken when maize plants had 5 - 6 leaves. Total DNA was extracted from the leaves and used for molecular assay.

2.2.1 DNA dot blot hybridization The total DNA from 13 transformed T_1 plants and non-transformed ones as controls were assayed by dot blot hybridization, 6 out of the 13 plants were positive, and the controls were negative.

2.2.2 PCR amplification analysis The primers 8341/8342 was used for PCR amplification of the positive plants (Tai 9101-3, Tai 9101-11, Tai 9101-18, Zong 31-4, Zong 31-2 and Zong 31-1) identified in DNA dot blot hybridization in the condition of program 1. The results were shown in Fig. 2. The primers 49040/49041 was

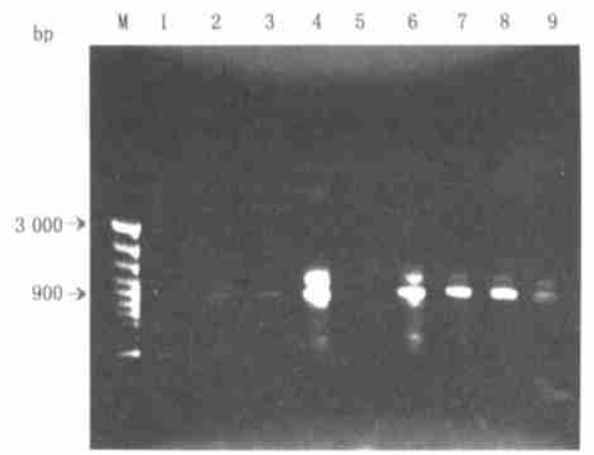

Fig. 2. PCR analysis of total DNA from transformed T_1 plants and CK (1.2 % agarose gel electrophoresis).
M, molecular marker; Lane 1, Zong 31 plant (CK); Lanes 2 - 4, transformed plants: 2, Zong 31-2, 3, Zong 31-4, 4, Zong 31-1; Lane 5, Tai 9101 (CK); Lanes 6 - 8, Tai 9101 transformed plants: 6, Tai 9101-11, 7, Tai 9101-18, 8, Tai 9101-3; 9, pCL Ⅱ-RG-1 plasmid.

① Xu N(许宁), Zou X(邹翔), Chen B-Q(陈伯权), Zhao N-M(赵南明). A possible theoritical model for ultrasonication induced transformation cell membrane perforation caused by "vacuolation effect". Proceedings of the 5th national symposium on Bio-Membrane. Haikou, China, 1993. 178. (in Chinese)

used for PCR of T_2 plants in the condition of program 2. Nineteen out of 24 T_2 plants examined were positive and the results were shown in Fig. 3.

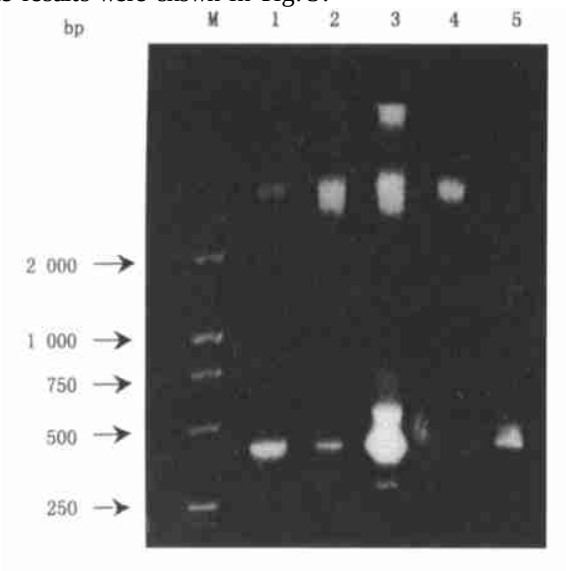

Fig. 3. PCR analysis of total DNA from transformed T_2 plants and CK (1.2 % agarose gel electrophoresis).
M, molecular marker; Lanes 1 - 3, descendant of Tai 9101 transformed plants, Tai 9101-3-5, Tai 9101-19-6, Tai 9101-3-4, respectively; Lane 4, Tai 9101 (CK); Lane 5, pCLTRG-1 plasmid.

2.2.3 PCR-Southern blot hybridization The above detected positive T_2 plants were assayed again with PCR-Southern blot hybridization. Eleven of the 15 tested plants were positive (Fig. 4). The results implied that the chitinase gene was introduced into maize inbred lines.

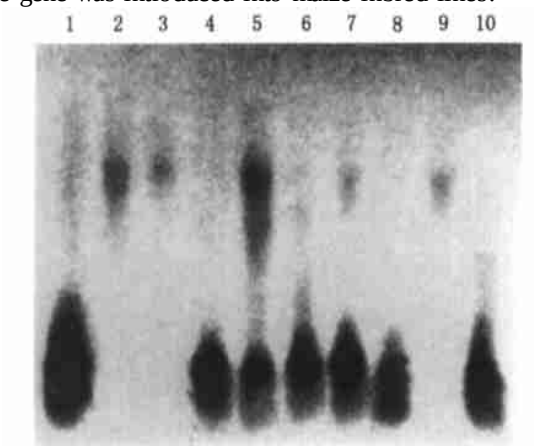

Fig. 4. PCR-Southern blot hybridization of total DNA from transformed T_2 plants and CK.
1, plasmid; 2, Zong 31(CK); 3, Zong 31-1-3; 4, Zong 31-3-8; 5, Zong 31-1-6; 6, Zong 31-4-2; 7, Zong 31-1-2; 8, Zong 31-4-2; 9, Tai 9101(CK); 10, Tai 9101-3-5.

2.2.4 Seedlings resistant to hygromycin The seeds from transgenic and non-transgenic maize plants were germinated in 25 mg/L (for Zong 31) or 15 mg/L (for Tai 9101) hygromycin solution in dark. The transgenic plant seeds could germinate, generate normal roots and shoots under the selection pressure, but the control seeds were germinated slowly, and the root development was obviously inhibited, and the plants died at last (Fig. 5.).

Fig. 5. Germination of seeds from transformed and CK maize plants in the solution with 15 mg/L hygromycin.
Left, Tai 9101(CK); Right, Tai 9101-3.

3 Discussion

It was reported that generative cells were used as vectors for genetic transformation in animals[12]. There has not been any similar successful results done in plants till now, though some attempts were made by Sanford *et al*[13], Ohta[14], Booy *et al*[15], and Li *et al*[16]. We have here reported an easy-applicating genetic transformation approach using plant pollen as a vector.

Booy *et al*[15] reported that the digestion of plasmid DNA by nuclease was a major hurdle for using pollen as a genetic transformation vector. They attempted to wash off nuclease or to inhibit the activity of the enzyme with EDTA. They found that the former could only eliminate partial enzyme and the latter inhibited the germination of pollens completely. We conceived that the ultrasonication treatment at certain intensity before adding plasmid DNA might destroy the activity of nuclease, meanwhile, not severely harm the germination activity of the pollens. We adopted the ultrasonication treatment at the intensity of 300 W before adding plasmid DNA to destroy the nuclease on the pollen surface.

Dong *et al*[17] obtained transformants of maize and millet with the frequencies of 0.05 % - 0.21 % and 0.18 %, respectively by pollinating with bombarded pollens. We assumed that it was difficult for the bombarded pollens to compete with the normal pollens due to the pollens wounded by hitting of microparticles. Therefore, the transformation frequency would be low even though some transformants could be got. Further more, the pollen might be splashed up by bombardment. In our approach,

the transformed and untransformed pollens have the vitality at the same level, thus, the transformation frequency would be relatively higher.

Using pollen-mediated transformation approach, we could avoid tedious tissue culture procedures adopted in *Agrobacterium* and bombardment approaches. Thus, it is easy to apply this approch in practice and ready to integrate it into conventional breeding program. Facilities used for this approach is relatively simple and cost less.

The study on enhancing seed setting of the transformants and their progenies resistant to maize head smut (*Sphacelotheca reiliana*) and leaf spot (*Cochliobolus heterostrophus*) are undergoing.

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