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Transformation of Wheat Immature Embryos by Particle Bombardment

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Abstract

The objective of this study was to identify the major parameters controlling DNA delivery by particle bombardment to immature embryos of Chinese spring wheat (*Triticum aestivum* L.). Efficiency of DNA (*uid*A gene) delivery was assessed by transient GUS (β-glucuronidase) expression in bombarded tissues. Of the parameters analyzed, acceleration pressure, bombardment distance, chamber vacuum pressure, bombardment times, osmotic conditioning of culture had a remarkable influence on transient gene expression. A bombardment procedure suitable for Chinese spring wheat cultivars was developed which allowed high-efficiency DNA delivery combined with reduced damage to target tissues. The high efficiency made the system practical for wheat genetic transformation research and accelerating wheat breeding programs.

Key words: Genetics transformation; Particle bombardment; Transient expression; *Triticum aestivum* L.

Introduction

Wheat (*Triticum aestivum* L.) is one of the world's most abundant sources of energy and proteins for mankind. Although the wheat has been improved by classical breeding methods for a long time, the genetic engineering as an alternative breeding tool shows a great potential application value. In common wheat, particle bombardment developed by Klein et al. (1987) is a useful method for gene delivery to intact cells and currently the most widely used technique in wheat transformation. However, the first transgenic wheat plant was not obtained until 1992 (Vasil et al. 1992). Since then numerous laboratories have developed the capability of wheat transformation using biolistics (Vasil et al. 1993; Weeks et al. 1993; Becker et al. 1994; Nehra et al. 1994; Altpeter et al. 1996; Blechl and Anderson 1996; Barro et al. 1997; Chen et al. 1998b; Gabriela et al. 2001; Leonie et al. 2003). Within the past decade, much progress has been made in the development of genetic engineering technologies for the improvement of wheat. However, the production of transgenic fertile wheat plants has been hampered by factors such as genotype, particle bombardment parameters and the inherent complexity of the wheat system, the absence of reliable and efficient systems for delivering and expressing the gene of interest. Therefore, it is necessary to establish an efficient transformation system in wheat for routine application.

We have investigated the major factors controlling DNA delivery to immature embryos such as rupture disk pressure and bombardment distance, bombardment times, selection strategy of resistant calli and vacuum pressure. The objective of this study is to optimize the transformation procedure of particle bombardment which allowed high-efficiency DNA delivery combined with reduced damage to immature embryos of Chinese spring wheat cultivars.

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Materials and Methods

Plasmid construction

The plasmid pDM803 contains the *bar* gene under the control of *Ubi*-1 promoter as selectable marker, which confers resistance to the herbicide and the screenable *uid*A gene (under the control of the *Act*1 promoter), encoding for the enzyme β-glucuronidase. One enhancer was, respectively, inserted at the upstream of β-glucuronidase and bar to enhance their expression (Thompson et al. 1987).

Plant materials and particle bombardment

Three Chinese spring wheat (*Triticum aestivum* L.) genotypes of desirable agronomic and yield characters (i.e. Chuannong16, Y1496 and N711) were used in this study. Immature embryos, ranging from 0.5 mm to 1.5 mm and 12-14 days-post-anthesis, were isolated under aseptic conditions and placed with scutellum upwards (the embryo axis facing downward) on the induction medium (Table 1). Procedure for embryogenic callus induction and plant regeneration was summarized in Table 1. The pH of media was adjusted to 5.8 before being autoclaved for 15 min at 121℃ and 0.11 MPa. Media were gelled with 9 g/L agar. Cultures were maintained in the darkness at 25±1℃, with 50-70% relative air humidity, and transferred to fresh media every 15 days. Immature embryos were cultured 3-5 days on induction media. An osmotic treatment of the explants 4-6 hours prior to bombardment was performed according to Alpeter et al. (1996). Approximately 60 pre-cultured immature embryos were evenly distributed over a 2 cm diameter and the center of each 90 mm Petri dish containing the osmotic agent medium.

GUS Histochemical assay

Tissues were washed briefly in 100% ethanol and then in sterile distilled water, prior to incubating in X-gluc buffer containing 1mM X-gluc, 100 mM sodium phosphate buffer pH 7.0, 0.5 mM potassium ferricyanide, 0.5mM potassium ferrocyanide and 0.1% (v/v) Triton x-100, 20% (v/v) methanol (to eliminate the effect of endogenous GUS activity) after incubation overnight at 37℃. Chlorophyll was extracted from green tissues to allow visualization of GUS activity by washing tissues twice in 70% (v/v) ethanol, followed by several changes in 100% ethanol. Tissues were photo-

Table 1. Summary of medium composition

Medium type	Composition
Induction	Ms basal, 500 mg/L L-glutamine, 500 mg/l L-proline, 300 mg/L casein hydrolysate, 60 g/L sucrose, 100 mg/L myo-inostol, 2 mg/L 2,4-D, 0.5 mg/L Kinetic and 10 mg/L $AgNO_3$ added after autoclaving
Subculture	Same as induction medium, but 2×MS macro salt and 1 mg/L 2,4-D
High osmotic medium	Induction medium except $AgNO_3$, 0.2 M mannitol , 0.2 M sorbitol
Selection	Subculture medium, 5mg/L L-PPT
Differentiation	Induction medium except $AgNO_3$, 15 mg/L Mannitol, 1 mg/L Kinetic, 0.1 mg/L IAA, 05 mg/L TDZ, 1 mg/L Choline chloride, 3 mg/L L-PPT all these ingredients added after autoclaving, but solidified with 2.2g/L Phytagel
Regeneration	1/2MS, B_5, 1mg/L MET, 0.2 mg/L IAA, 5 mg/L L-PPT, 2% Sucrose, 2.2 g/L Phytagel

Note: A stock aqueous solution of silver nitrate (10 mg/L) was prepared and appropriate amounts filter sterilized into the media after autoclaving. These plates were stored in the dark immediately after the media solidified and the cultures were grown in the dark

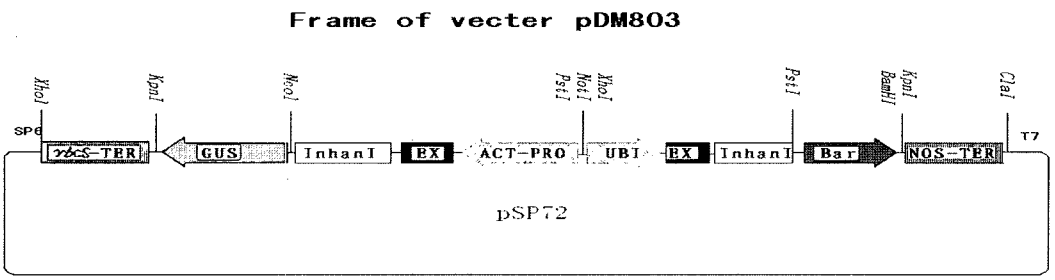

Figure 1. Plasmid structure of pDM803

graphed with a camera attached to an Olympus SZ-PT steromicroscope. DNA delivery was assessed by counting embryos and then counting the number of foci per embryo (Jefferson et al. 1987).

Leaf painting assay with Basta

For each plant tested, three approximately equal-sized, healthy-looking leaves from separate tillers were selected for leaf painting. Basta (at 0.25%) was applied with Tween-20 (0.1%), using a brush to paint the two surfaces of the distal half of the selected fully-expanded leaves. Tween-20 (0.1%) alone was used as a control.

PCR screening for putative transgenic plants

Genomic DNA was isolated from 100-200 mg leaf tissue by crushing in Cornell extraction buffer (100 mM Tris-HCl, pH 8.0, 500 mM NaCl, 50 mM EDTA, 0.84% SDS). After centrifugation to remove debris, DNA was precipitated with ethanol and the pellets were resuspended in sterile distilled water overnight at 4℃ without agitation. PCR was used to confirm the presence or absence of transgenes in the primary transformants. The primer sequences for *bar* gene was 5'-ACCATCGTCAACCACTACAT-3' and 5'-AGTCCAGCTGCCAGAAACCC-3' and about 440 bp gene fragments were expected.

Results

Sensitivity of embryogenic calli of Chuannong 16 to L-PPT

The sensitivity of embryogenic calli of Chuannong 16 to L-PPT was assessed. The results showed that L-PPT was effective as a selective agent at concentrations as low as 3 mg/L. However, some viable tissues were still detected in calli cultured on media containing up to 5 mg/L L-PPT. In this experiment, about 87.2% calli became brownish under the 4 mg/L L-PPT addition after 4 weeks (Figure 2). To reduce the number of calli escaping from selection, a concentration of 5 mg/L were used for the selection of stable transformants on the selection medium and regeneration medium, 3 mg/L on the differentiation medium, respectively. Healthy, growing sectors of calli were transferred every other week to fresh selection media. In 6 to 8 weeks, resistant callus lines were established. In various time after bombardment, green sectors, the precursors of shoots, originated from the callus were formed. At this stage, callus pieces were divided and the greening portions were transferred to the differentiation medium with 3 mg/L L-PPT.

Effect of bombardment parameters on transient expression

Ten combinations of pressure/distance were analyzed for optimizing DNA delivery into wheat immature embryos and cell viability in primary cultures. After bombarded, approximately one-fifth of the too small target materials stopped growing even took necrosis, while the remainders survived well. Gene transfer frequency was monitored by the transient expression of *GUS* gene (Figure 3). The effects of different pressure and distance on wheat immature embryos were determined by variance analysis and Least Significant Difference (LSD). It was observed that different combinations of pressure and distance had the significant effects on transient expression and the TEF% (Transient Expre-

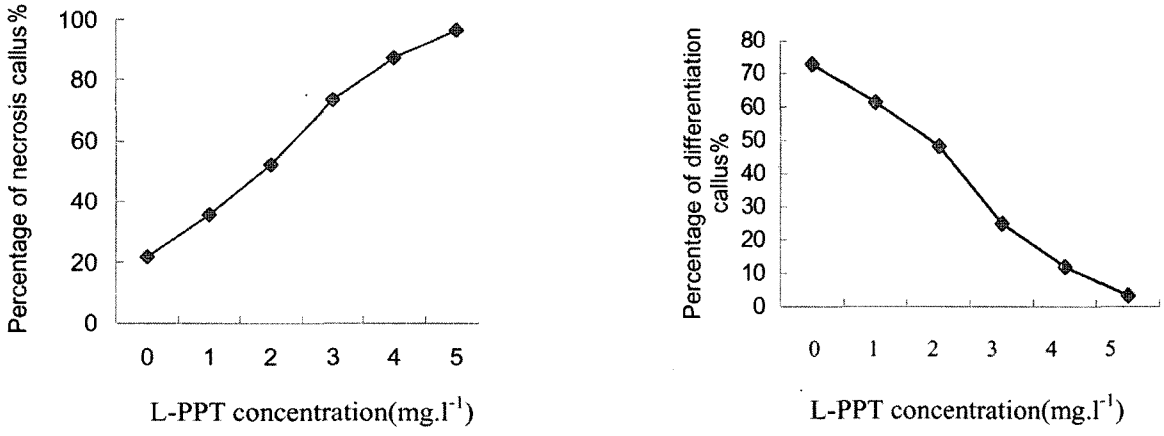

Figure 2. Variations of Chuannong16 calli resistant to L-PPT on selection (Left) and differentiation (Right) media

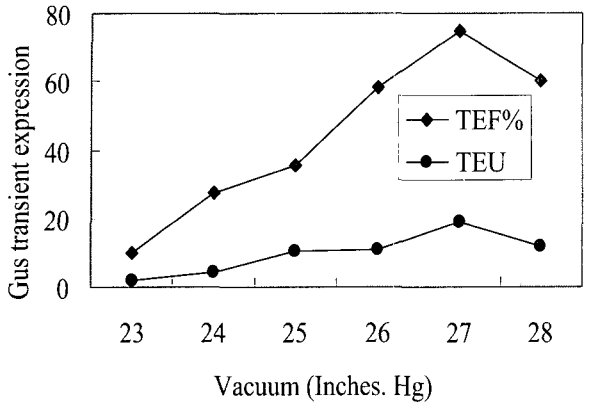

Figure 3. Effect of vacuum on transient expression. Transient expression frequency (TEF%) = embryo or callus numbers with blue spots/ total embryo numbers investigated. Transient expression unit (TEU) = average blue spot numbers per immature embryo or callus

ssion Frequency) ranged from 28.7% to 100%. Bombarding targets twice and using higher particle velocity (i.e. thicker rupture disks) generally increased the incidence of transient expression. The highest number of GUS positive foci per embryo (44.4) and the highest TEF% (100%) was obtained from bombarded tissue once with a 1100 psi×9 cm and once with a 900 psi×6 cm. Otherwise with 9 cm bombardment distance under the same pressure showed significantly higher TEF% and TEU (Transient Expression Unit) (Table2).

However, *GUS* transient expression results increased with the vacuum pressure increasing from 23 to 27 inches.Hg and transient expression efficiency was decreased when it was 28 inches. Hg (Figure4). Under vacuum 27 inches. Hg, TEF% and TEU were 75% and 18.7 respectively at 1100 psi and 9 cm bombardment distance.

According to the transient expression results (Table 2 and Figure 4), Chuannong16 and N711 immature embryos were bombarded with three bombardment pressures and distances groups under 27 inches. Hg, in order to further substantiate optimal bombardment parameters. By LSD (at 95% level) analysis, significant genotype difference was observed in resistant calli and transformed plantlets (Table 3). These results also indicated that Chuannong16 was better response than N711 in regeneration ability. Otherwise, we found that 1100 psi×9 cm showed better shoot regeneration than other combinations.

Selection and regeneration

Bombarded immature embryos, cultured for 18 hours on high osmotic media, were transferred onto the media with 5mg/L L-PPT directly or after transition culture for 5-7 days. Pale yellowish, friable, compact resistant calli grew normally after 6 week selection. Some slowly growing non-resistant calli turned into brown or milk white, soft and died quickly because of strong restrain from L-PPT (Figure 5A). In subsequent experiments, shoot production from resistant calli was allowed to proceed on regeneration media with the presence of 3 mg/L selection agent (Figure 5B). However, some resistant calli only developed into green sector with numerous roots without shoots (Figure 5C). The transformation efficiency between direct selection and transition

Table 2. *Effects of bombardment distance and ruptured disk pressure on transient expression of wheat embryos*

Times/plate	Rupture disk pressure (psi) × Bombardment distance (cm)	Transient expression frequency (TEF%)	Transient expression unit (TEU)
1	900×6	32.2^{g}	11.3^{e}
1	900×9	40^{f}	6.9^{f}
1	900×12	28.7^{g}	5.8^{f}
1	1100×6	55.6^{e}	22.3^{cd}
1	1100×9	75.4^{bc}	17.8^{d}
1	1100×12	69.2^{cd}	10.5^{ef}
2	1100×9 900×6	100^{a}	44.4^{a}
2	900×9 900×6	78.8^{b}	18.9^{d}
2	1100×9 1100×9	64.5^{d}	26.7^{c}
2	1100×6 900×6	45.8^{f}	35.3^{b}

*Values sharing the same letter in each column are not significantly different based on the LSD (α =0.05) test.
Transient expression frequency (TEF%) = embryo or callus numbers with blue spots/ total embryo numbers investigated
Transient expression unit (TEU) = average blue spot numbers per immature embryo or callus

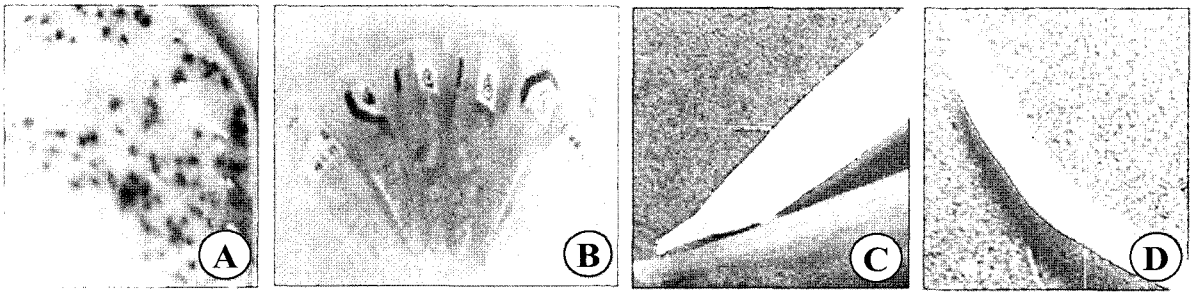

Figure 4. Transient expression of *GUS* gene. Note: Histochemical GUS assay for expression of the *Uid*A gene in transformed calli (A, B). Evidence of stable transformation in developing leave GUS (C); assays of root tip samples from transgenic wheat plant (D).

culture of 5-7 days was compared (Table 4). Our data confirmed that transition culture after bombardment improved cell recovery ability and enhanced the percentage of resistant- calli and final plantlets. Different varieties showed difference of tolerance to late selection on L-PPT. The resistant- calli and regeneration rates were statistically significant at the 95% level in all varieties except N711, but particularly marked in Chuannong16, which showed higher regeneration rate, even in the direct selection approach. The regeneration percentage in the three varieties tested ranged from 10.5% to 54.9% in the case of transition culture. Sometimes, more than one shoot was derived from a single callus. Average1-2 shoots per resistant callus of N711 and Y1496, however, 3 shoots per resistant callus of Chuannong16 showed stronger regeneration ability (Figure 5 D). After 4 weeks, some of the regenerated plants retained a green color, grew quickly, and developed strong roots; some grew slowly and produced short roots, others became yellowish and necrotic. Only well-rooted plants were transplanted to the garden pots (Figure 5E, F). 7 days after painting, Basta resistance was determined according to the percentage of necrosis suffered over the area painted with the herbicide solution. A plant was scored as herbicide-resistant if the necrosis area was less than 10%, and herbicide-sensitive if the necrotic area was greater than 80%. Plants having 10-80% necrosis were scored as partially resistant (Figure 5G).

Identification of putative transgenic plants

After selection for 6-8 weeks on media containing 5 mg/L L-PPT a total of 429 resistant colonies were recovered from bombardments. Fifty-nine herbicide-resistant and partially resistant plants from 148 Chuannong16 colonies were screened for the presence or absence. As a result, 6 plants show expected fragment (Figure 6).

Discussion

A milestone in wheat transformation was made when Vasil et al. (1992) obtained several independent transgenic wheat lines by particle bombardment. Subsequently DNA transfer via particle bombardment is the most widely used method for wheat transformation. The stable expression of transgenes has now been achieved by several groups (Vasil et al. 1993; Weeks et al. 1993; Becker et al. 1994; Nehra et al. 1994; Zhou et al. 1995; Altpeter et al. 1996; Ortiz et al. 1996; Barro et al. 1997). However, widespread application of the technology is still limited by relatively low and erratic stable transformation efficiencies and by the general

Table 3. Resistance analysis of wheat immature embryos bombarded at various parameters

Genotype	Rupture pressure (psi) × Bombardment distance	No. bombarded embryos	Callus resistant to PPT		Plant resistant to PPT	
			No.	Frequency	No.	Frequency
Chuannong16	1100×9	430	232	53.95[a]	33	21.2[a]
	1100×12	280	95	33.92[c]	11	15.2[b]
	900×6	359	174	48.47[b]	15	13.5[c]
N711	1100×9	408	197	48.28[b]	9	8.4[d]
	1100×12	204	51	25.00[d]	7	7.6[d]
	900×6	582	134	23.02[e]	2	0.8[e]

*Values sharing the same letter in each column are not significantly different based on the LSD (α=0.05) test.

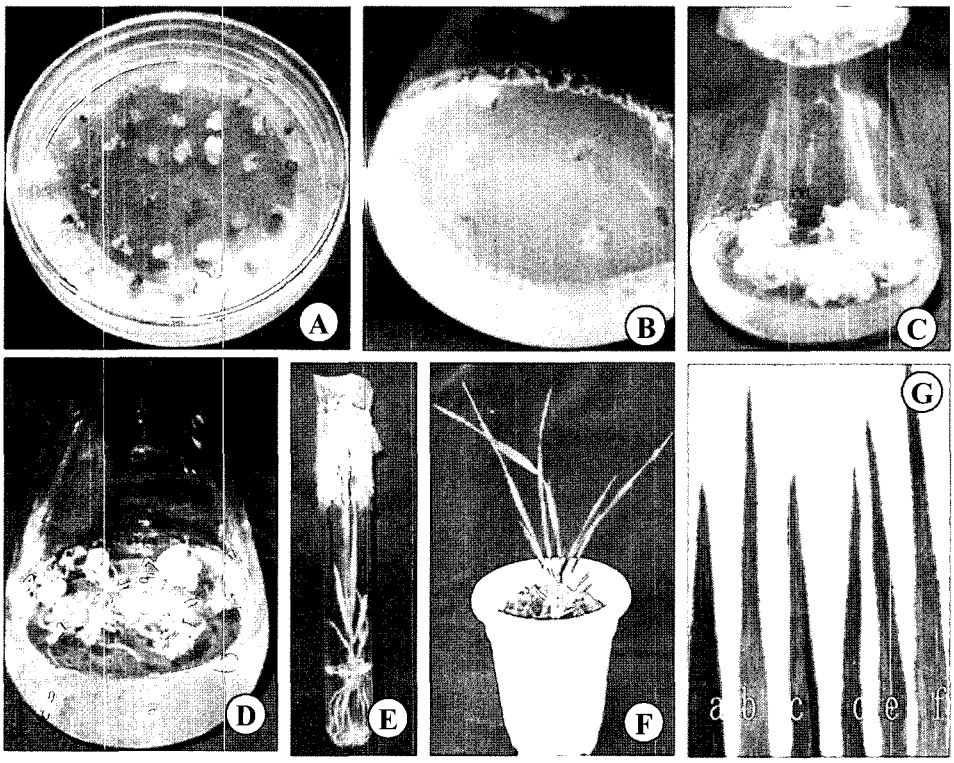

Figure 5. Transformation, selection and regeneration of wheat calli derived from immature embryos. A-B, Calli on the selection medium; C-D, shoots regenerated from the transformed calli on selection medium; E, regenerated plantlets rooted on the media; F, transgenic plant transferred to soil; G, Expression of *bar* was confirmed by leaf-painting. Basta (0.25%) was applied with Tween-20 surfactant (0.1%) to the terminal 5-8 cm of selected leaves, leaves from transformed plants (a-c), typical leaves from non-transformed plants (d-f).

Table 4. The effect of transition culture on transformation efficiency

Genotype		No. of bombarded immature embryos	Frequency of resistant calli	Regeneration frequency (%)
Chuannong16	①	121	56.1^{b}	47.6^{b}
	②	336	83.6^{a}	54.9^{a}
N711	①	90	25.7^{d}	12.1^{e}
	②	216	26.9^{d}	10.5^{e}
Y1496	①	80	47.5^{c}	20.3^{d}
	②	101	56.4^{b}	32.8^{c}

Note: ① directly selected without transient culture; ② selected after 7-day transient culture
Values sharing the same letter in each column are not significantly different based on the LSD (α =0.05)test.

use of tissue culture-responsive but agronomically less desirable "model" genotypes such as 'Bobwhite' and 'Florida'.

Wheat transformation frequency using particle bombardment is often around 1% and usually performed in model cultivars (Weeks et al. 1993; Becker et al. 1994; Blechl and Anderson 1996; Nehra et al. 1996; Stoger et al. 1998). Increased frequencies of individual transformation experiment were reported (Harvey et al. 1999; Zhang et al. 2000; Rasco-Gaunt et al. 2001). However most attempts to increase transformation efficiency have obtained in model genotypes but not reproducible. Moreover, the use of elite varieties in wheat transformation is a difficult task due to, among many factors, variety-specific differences in the formation of embryogenic calli and plant regeneration (Viertel et al. 1998; Iser et al. 1999). Elite varieties, however, have a wide range of response to tissue culture and efficiency of callus induction and regeneration seem to be genotype-dependent (Felfoldi and Purnhauser 1992; Fennell et al. 1996; Viertel et al. 1998). This work confirms the relatively high embryogenic and regeneration capacity observed in Chuannong16 which was a new wheat cultivar with high

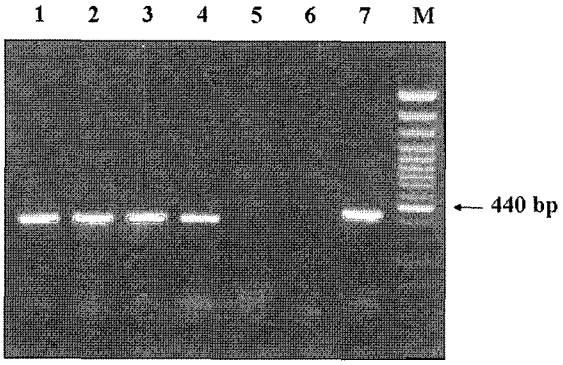

Figure 6. 1-4. transgenic plants; 5, water control; 6, non-bombarded plants; 7, pDM803; M, molecular weight marker.

yield, good stability and extensive adaptability (Zheng et al. 2002), under bombarded conditions. The high embryogenic and regeneration capacity indicated that the stress provoked by the bombardment itself does not affect the good response of these varieties to tissue culture.

The development of reliable transformation protocols for recalcitrant species depends on the ability to deliver the intact DNA molecules into the nuclear genome of reproducible cells and to recover fertile adult plants from tissue culture. The choice of starting material (explants) including embryo size and physiological condition had been proved to be crucial in successful wheat transformation via particle bombardment (Gabriela et al. 2001). Present study indicated that gray white and sub-transparent immature embryos, 0.5-1.5 mm diameter, 12-14 days post anthesis were optimal explants. They recovered from mechanical injury quickly and produced pale yellowish and intense embryogenic calli. However, the data presented here concur with recent results obtained by Rasco-Gaunt et al. (2001) who demonstrated that, embryos in 0.5-1.5 mm showed better embryogenesis and shoot regeneration than those smaller or larger than that size group. Moreover, it was demonstrated that in this study the transition culture treatment after bombardment was superior to direct selection in the transformation frequency. Our conclusion was that transition culture promoted transformation efficiency and eliminated partial pseudo positive clones.

In wheat, although some parameters have been examined by different workers (Perl et al. 1992; Becker et al. 1994; Alpeter et al. 1996), there was still a need for coherent study on the conditions influencing DNA delivery and transient expression, particularly in elite varieties. The objective of this work was to develop a particle bombardment transformation system for Chinese spring wheat elite varieties using immature embryos. Therefore, we investigated a number of bombardment parameters with the aim of not only optimizing DNA delivery conditions but also identifying conditions, which minimized the damage to the target tissues. Apart from the obvious effect on depth of penetration, particle acceleration and distance also had an important influence on the distribution of particles (Liu et al. 2002). At low pressures expression events were evenly distributed and at a relatively lower density, thus reducing bombardment shock and tissue injury. At high pressures, a small area of the tissues was very strongly targeted and thus likely to be damaged, while tissues at the periphery showed very few expression events (Rasco-Gaunt. et al. 1999). Twice bombardments were useful mainly when primary delivery was not efficient, but they increased the damage to target tissues (Lonsdale et al. 1990) and thus were undesirable for stable transformation. It has been demonstrated that the best gene expression was achieved by using 1100 psi$\times$9 cm in single bombardment and 1100 psi$\times$9 cm+900 psi$\times$6 cm in two bombardments, respectively.

The results of these analyses allowed the development of an optimized bombardment procedure for Sichuan wheat new varieties. The following parameters were optimized from the standard procedure: 0.6 mm gold particle, 1100 psi $\times$9 cm acceleration pressure and bombardment distance, 5-7 days transition culture of bombarded tissues. Chamber vacuum pressure was also modified to 27 inches. Hg to enhance the DNA delivery efficiency.

A number of laboratories have developed wheat transformation system using the bar gene as the selectable marker and L-PPT as the selectable agent (Lonsdale et al. 1998a; Barro et al. 1998; Harvey Alison et al. 1999; Zhao et al. 2002; Leonie et al. 2003). Different receptors selected by various groups showed diverse levels of tolerance on L-PPT. At the outset of this investigation, the dose of L-PPT in selection media after particle bombardment for donor wheat to endure was unknown. Initially, Chuannong16 calli were sacrificed at 4 weeks inoculation in order to confirm for selection dosage. In subsequent experiments, embryogenic calli with green spots were transferred onto differentiation media with L-PPT. Selection was carried out with 0-5mg/L L-PPT for two to three rounds of 2 weeks each (Fig. 2). To eliminate escapers and gain optimal efficacy of L-PPT selection, we employed 5 mg/L L-PPT selection culture 3 weeks then transferred the resistant calli to differentiation media with 3 mg/L L-PPT. Such studies constitute an important starting point for long term wheat breeding programs involving genetic engineering.

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