

## Optimization of Regeneration System of Tissue Culture and Transformation of *1Dx5* Gene without Markers in Wheat

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**Abstract:** To improve bread making quality of flour and produce transgenic plants free of selectable markers, Immature embryo scutella from Xindong No. 26 were used as explants for establishing an efficient and stable wheat regeneration system, then the linear expressing box of *1Dx5* without selectable markers was transformed into the immature embryo of Xin Dong No. 26 via particle bombardment. The result showed that MS medium containing 2, 4-D 1.5 mg/L and Dicamba 0.5 mg/L for callus induction, R medium containing ZT 1 mg/L and 2,4-D 0.01 mg/L for callus differentiation were the best efficiency, the differentiation frequency of callus was 90%; Transformed plants were screened by PCR, three transgenic plants were detected among 1000 transformed plants, only yielding the transformation rate of 0.3%. The compositions of HWM-GS were analyzed by SDS-PAGE. The HMW-GS gene *1Dx5* was expressed in some seeds of transgenic plants. Our studies lay the foundations for obtaining marker-free transformants using the linear gene via particle bombardment.

**Keywords:** *1Dx5*, particle bombardment, regeneration, selectable markers, wheat

### INTRODUCTION

Through the biological technology and genetic engineering method to improve wheat agronomic characters had been widely attentioned (Lazzeri *et al.*, 1997) and become conventional breeding auxiliary mean (Birch, 1997). Using this technology, the researchers could transfer the valuable genes into the local varieties of wheat so that produced transgenic wheat varieties which had production value (Patnaik and Khurana, 2001). It was well known that land salinization and lower wheat processing quality always restricted the Xinjiang's grain production. In order to make the rapid development of wheat industry in arid areas, it was urgent to breed new traits of salt-tolerance wheat which improved processing quality. Research showed that HMW-GS had molecular weight for 90~147 kd and contained disulfide bond, which played a decisive role in bread elasticity (Ng and Bushuk, 1988) and baking quality (Shewry *et al.*, 1989). 1Ax1, 1Dx5, 1Dy10 were considered the high quality subunit genes (Shewry *et al.*, 1992). Therefore, the wheat which had these subunit genes and can be expressed had a broad application prospects in the production. In order to use the high quality 1Dx5 gene of HMW-GS in wheat, the author had separated, purified and cloned the subunit gene from high quality variety of Xinjiang (Xiao-Yu, 2011) and transformed it into common wheat planted widely to improve their processing quality. At present,

mediated genetic transformation with Micro projectile bombardment had been matured (He *et al.*, 1998). But it was need to establish an effective tissue culture system (Fennell *et al.*, 1996). Along with the establishment of plant tissue culture technology, wheat genetic transformation research had made a great progress. But significant differences between the different genotypes of wheat and the low regeneration frequency still seriously restricted the wheat genetic engineering research development.

So, in this study, immature embryo scutella of Xindong No. 26 was taken as research materials for establishing an efficient regeneration system. With the methods of particle bombardment to transform the 1Dx5 gene into salt-tolerant variety would lay a foundation for wheat processing quality improvement.

### MATERIALS AND METHODS

The study was conducted in Research Room of Molecular Biology and Informatics, Xinjiang Normal University from 2009 to 2012. The wheat receptor material was salt-tolerant wheat of Xindong No. 26 in Xinjiang and positive control was L88-6. They were all sowed in Xinjiang Normal University experimental field.

Plasmid pMon 530 and E coli strain DH5 $\alpha$  were saved in laboratory.

Callus induction medium, contained MS salt, sugar 30 g/L, Agar 8 g/L and its pH was adjusted at 5.8. Differentiation medium, contained R (Qin and He, 2001) which except KT.

In induced phase, immature embryo scutella callus were cultured in dark and temperature was maintained at (25±1) °C. When cultures were shifted to differentiation phase, temperature was maintained at (25±1) °C with 16 h light (about 2000 lux) cycle in every 24 h.

**Methods:** Took ears of wheat of flowering 15 days after pollination. Picked off the immature seed and sterilized by washing with 1% HgCl<sub>2</sub> for 12 min followed by 3-5 washes with sterilized distilled water. Took the immature embryo carefully, picked off the plantule, turned up the scutella and inoculated it on the induction medium. After 30 days, these induced callus were inoculated to the differentiation mediums according to the groups. Counted callus induction rate after 30 days on the induction medium and counted differentiation rate in 30 days after transferred to the differentiation medium. The specific statistical analysis method referred to Liu (2005). In addition, average rate of emergence (%) =  $\frac{\text{The number of seedling differentiation}}{\text{The number of inoculation of explants}} \times 100\%$ .

Ten to fourteen days after plant flowering, stripped out of the seeds, disinfected the surface, got rid of the immature embryo and took the scutella as the test material. Osmotic treatment of immature embryo scutella was done for 4 h with 0.4 M mannitol after it two days preculture. Bombardments were carried out with particle bombardment (PDS 1000/He, Bio-Rad) under a vacuum of 27 inches of Hg and the conversion process refers to improvement methods of Rasco *et al.* (1999). The variables included rupture disc pressures (1,100 psi), distance from rupture disc to macrocarrier (6 cm), type of micro carrier (gold), size of micro carrier (1.0 µm diameter of gold particles), number of bombardments per Petri plate (1), precipitating agent of ackage the bullets (0.1 M Spermidine and 2.5 M

CaCl<sub>2</sub>). After bombardment, scutella were kept on medium supplemented with 0.4 M mannitol for 16 h then transferred to induction medium without mannitol. The Experiment designs two controls: gold bombardment (without exogenous gene) and no bombardment.

Total DNA was extracted from the regenerative plants and positive transgenic plants were selected by PCR with *IDX5* specific primers (F: 5'-GCCTAGCAACCTTCACAATC-3' and R: 5'-GAAACCTGCTGCGGACAAG-3'). About 450 bp amplified fragment was obtained.

The PCR reaction conducted as followed program: An initial denaturation at 9°C for 5 min, followed by 35 cycles of 94°C for 30s, 6 for 30s and 72°C for 90s and a final extension step at 72°C for 7 min. The a °C amplified products were separated on 1% agarose gel and photographed using Gel Documentation System.

Harvested seed of positive plant separately, chipped half off spring seeds without germ and picked with study to crush by sample clamp. As mentioned, to extract its high molecular weight glutenin subunit for SDS-PAGE analysis (Zhu *et al.*, 2008).

## RESULTS AND DISCUSSION

Immature embryo scutella were cultured on ten kinds of callus induction medium (had different concentration of 2, 4-D and Dicamba). Then, transferred the callus to three kinds of differentiation medium with 2, 4-D 0.01 M and gradient concentration of ZT.

The study found that a green point appeared in the callus after it transferred onto differentiation medium 2~3 days. On the 30<sup>th</sup> day, most of green point differentiated into plants. Green plantlet differentiation rate There were no significant differences on green plantlet differentiation rate between the two mediums supplemented 2, 4-D 0.01 M and ZT on the concentration gradient (Table 1). To the rooting rate, 1M ZT (89.04%) was significantly higher than 3 M ZT

Table 1: Effect of ZT, 2, 4-D on callus differentiation/%

Radio of auxins/mg/L		Number of transplanted immature inflorescences	Frequency of calli rooted	Frequency of all plants
ZT	2, 4-D			
1	0.01	258	89.04±2.8a	72.69±6.6a
3	0.01	231	80.68±3.5a	55.15±4.7b
5	0.01	235	66.43±4.1b	61.09±5.8ab

The same letter within the same line (column) means difference not significant with Duncan's multiple range test at 0.05 level

Table 2: Effects of induction media containing different ratio of 2, 4-D/Dicamba on the callus differentiation/%

Radio of auxins/mg/L		Number of transplanted immature inflorescences	Frequency of calli rooted	Frequency of all plants
2, 4-D	Dicamba			
0.5	1.5	111	84.67±6.1a	94.28±12.7ab
1.5	0.5	97	84.87±5.1a	100±11.5a
1	1	89	75.37±4.7a	78.21±10.4ab
2	2	90	82.54±5.0a	67.76±7.3b

The same letter within the same line (column) means difference not significant with Duncan's multiple range test at 0.05 level

(80.68%) and 5 M ZT (66.43%). The experiment result showed that 1 M ZT was beneficial for root differentiation. Furthermore, seedling which differentiate in 1M ZT would growth better than others and is more feasible to transplant.

This experiment results also showed that combination of 2, 4-D and Dicamba had profound effect on differentiation frequency of callus tissue. Different combination ratios of 2, 4-D and Dicamba had significant effects. On the combination of 0.5 M 2, 4-D and 1.5 M dicamba, callus induction and green plant regeneration rate were higher than any other combination. The green plantlet frequency and the average rate of seedling emergence were 79.33 and 100%, respectively (Table 2). The result showed that the combination of 0.5 M 2, 4-D and 1.5 M Dicamba was more advantageous to the differentiation of induced callus.

Base on the best induction medium (0.5 M 2, 4-D and 1.5 M Dicamba), R medium added 1 M ZT and 0.01 M 2, 4-D, the best differentiation rate was gotten. The green plant regeneration rate was 90% (Fig. 1). The regeneration plant was transplanted after the seedling formation. The survival rate was above 95% and these plants can be matured to seed.

Immature embryo was the earliest explants used in the wheat genetic transformation. Many studies suggested that, the induction and plant regeneration ability of immature embryo callus was stronger than the young spike and mature embryo (Lü *et al.*, 2007). Based on immature embryo scutella for transformation receptor, not only the false positive bringed by embryo could be reduced, but also its induced callus had the best plant regeneration frequency compared to other explants materials (Qin and He, 2001). For all the gramineous plants, the regeneration ability of immature embryo scutella was better. Immature embryo scutella were transformed by particle bombardment had achieved success (Birch, 1997).

It was generally acknowledged that 2, 4-D was an essential plant hormone in callus induction (Zhou *et al.*, 2000). Two, 4-D to callus relied on the genotype of wheat (Umer *et al.*, 2009). Some studies found that the induction effect of this research result indicated that the callus tissue differentiation rate was influenced obviously by 2, 4-D combined with Dicamba than each of them in solo. The result was the approximate homology with the result of research to Bryl genotype by Przetakiewicz *et al.* (2003). The reason may be that the combination of Dicamba and 2, 4-D is beneficial to the formation of embryonic callus. Nbaors *et al.* (1983) was also reported that the 2, 4-D or 2, 45-T used separately would promote the formation of non-embryonic callus. Embryonic callus had regeneration characteristics and could be regenerated plantlet by embryogenesis or organogenesis. Non-embryonic callus only could be proliferation, but not regeneration.

Based on the regeneration system, linear expression box of HMW gene *IDx5* without selection



Fig. 1: Plant regenerated on the differentiation medium for 30 days

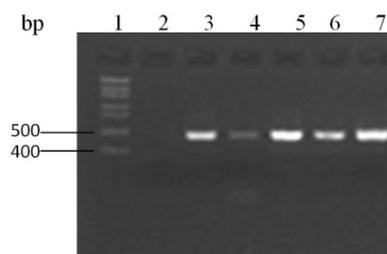


Fig. 2: Selection of transgenic wheat plants with PCR  
1: Marker; 2: Xin Dong NO. 26 donor plants for negative control; 3: 1Dx5 for positive control; 4: L88-6 genotype for positive control; 5-7: Different transgenic plants

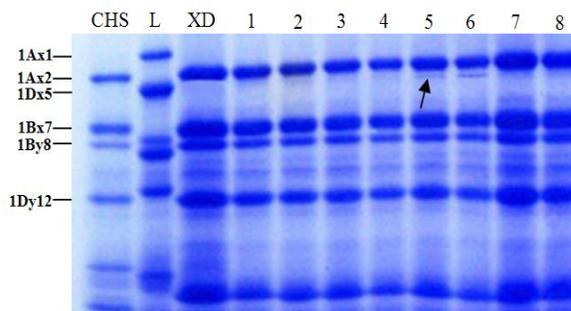


Fig. 3: SDS-Page of total protein from partial T1 transgenic seeds  
CHS: China Spring; L: L88-6 genotype for positive control; XD: Xin Dong NO. 26 donor plants for negative control; 1-8: Endosperm specific expression of 1Dx5 subunit gene in different transgenic plants

markers was bombardmented into wheat immature embryo scutella and regeneration plants were obtained Transgenic plants were selected from all the regeneration plant by PCR with *IDx5* specific primer. About 450bp target fragment can be amplified. PCR results showed that transformation rate of *IDx5* without selection markers was 0.3% and only three strains can detect clear specific banding (Fig. 2).

To determine if the transgene was expressed in transformed plants, the total proteins of seeds of transgenic plants progeny were analyzed by SDS-PAGE. It was showed that *IDx5* would be expressed in seeds of transgenic offspring to some extent (Fig. 3).

This research transformed the linear expression frame without selection markers into wheat immature embryo scutella by particle bombardment. Results showed that *IDx5* had expressed in several grains of transformed plants. It confirmed previous reports (Uzé *et al.*, 1999) that of the minimum expression box of purpose gene only contained promoter, coding sequence and terminator can be used in wheat particle bombardment operation successfully. Although this transformation system had lower transforming rate compared with Rooke *et al.* (1999) (1.4%) and Gadaleta *et al.* (2008) (1.5%), it can improve the safety of transgenic plants. However, UZE co-transformed the wheat gene by the linear segment GUS and bar and the results show that transforming rate of linear fragment was higher than complete plasmid. The different results may be that there no use resistance selection pressure in particle bombardment transforming process. Mullins *et al.* (1990) etc., also thought that transformed cell would be at a disadvantage competition state with no-transformed cell without selection pressure during genetic transformation. So this transformation system should be optimized to improve transformation frequency.

### CONCLUSION

This research established a high efficiency and stability wheat plant regeneration system with the immature embryo scutella as the explants. On that basis, non-selection markers linear *IDx5* genes was transformed into the immature embryo callus of wheat of Xindong No. 26 in xinjiang and transgenic plants were obtained which *IDx5* expressed in the offspring seeds. All of that laid a foundation for obtaining the safe new transgenic variety of wheat, which also had salt-tolerant characteristic and high processing quality.

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