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Optimization of a biolistic transformation system for transfer of antifreeze gene *KN2* and the *bar* herbicide resistance gene in common wheat

L. Cai[1], D.F. Sun[1] and G.L. Sun[1,2]

[1]College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, China
[2]Department of Biology, Saint Mary's University, Halifax, Nova Scotia, Canada

Corresponding authors: D.F. Sun / G.L. Sun
E-mail: sundongfa1@mail.hzau.edu.cn / genlou.sun@smu.ca

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ABSTRACT. We studied the effects of different media for callus induction and differentiation, and pre-culture period of immature wheat embryo culture on biolistic transformation efficiency for including antifreeze gene *KN2* and *bar* conferring resistance to the herbicide bialaphos. The percentage of plantlets generated from induction and differentiation media without Cu^{2+} was lower than those cultured on differentiation media with Cu^{2+} (71.15%) or induction media with Cu^{2+} (68.45%) and both induction and differentiation media with Cu^{2+} (52.17%). The combinations of Nor medium for callus induction and Cu^{2+} medium for regeneration, and Cu^{2+} medium for induction and R medium for regeneration were superior for biolistic transformation. The calli induced on Cu^{2+} medium and pre-cultured for 4 d before biolistic transformation, and cultured on R medium after biolistic transformation produced the highest percentage (65%) of transgenic plantlets with the *KN2* gene. Overall, about 50% plantlets regenerated from calli pre-

cultured 4d before bombardment carried the *KN2* gene; 44.7% of the plantlets carried the *bar* gene, which was higher than for any other treatment, followed by pre-culture 1d with 31.43% transformation rate for the *KN2* gene and 20% transformation rate for the *bar* gene.

Key words: Common wheat; Cold-resistance; Biolistic transformation; Antarctic fish superoxide dismutase gene

INTRODUCTION

Wheat (*Triticum aestivum* L.) is one of the staple food crops grown worldwide. Genetic engineering is widely used for improving agronomic traits, for instance, resistance or tolerance to biotic and abiotic stresses in wheat. Genetic transformation enables the introduction of novel genes directly into locally adapted cultivars to create new varieties (Jones et al., 2005). The first key requirement for a successful transformation system is a highly regenerable target tissue. Immature embryos has been the most widely used target and a range of culture medium components have been evaluated to enhance regeneration (Barro et al., 1999; Schulze, 2007; Sikandar et al., 2007). Among the DNA delivery methods, biolistic and *Agrobacterium*-mediated methods have played important roles in wheat transformation (Tao et al., 2011). The *Agrobacterium*-mediated method is an efficient system for dicotyledons, but is much less efficient for monocotyledons such as wheat, rice and corn, since monocotyledons have natural resistance to *Agrobacterium* infection (Ziolkowski, 2007). Currently, the biolistic system is widely used for DNA delivery in wheat (He and Lazzeri, 1998; Pellegrineschi et al., 2002; Hardwood, 2012). However, efficient transformation systems are usually developed for a single responsive genotype and are not transferrable to alternative genotypes, and challenges remain for the transformation of particular wheat cultivars (Hardwood, 2012).

Low temperature (cold and frost) has a significant negative impact on wheat productivity, and has become one of the serious abiotic stresses in wheat production in China in recent years. Chilling or freezing temperatures can lead to dysfunction and increased production of activated oxygen species such as superoxide, hydrogen peroxide and hydroxyl radicals (McKersie et al., 1993; Kuk et al., 2003). These reactive oxygen species can cause peroxidation of membrane lipids (Mead, 1976) and DNA strand breaks (Brawn and Fridovich 1981). Plants' first reaction against oxyradical-mediated injuries is the action of superoxide dismutases (SODs), a group of metalloenzymes that protect cells from superoxide radicals by catalyzing the dismutation of superoxide radicals to oxygen and hydrogen peroxide (Bowler et al., 1992). SODs are considered the first and most important line of antioxidant enzyme defense systems by removing the superoxide anion (Zelko et al., 2002), and has been proposed to be of importance in stress tolerance such as antifreeze activity. McKersie et al. (1993, 1996, 1999) demonstrated that transgenic alfalfa expressing high levels of SOD displayed significantly high tolerance to freezing stress. Similar results have been obtained in transgenic tobacco (Gupta et al., 1993) and tomato (Wang et al., 2005).

In the present study, Antarctic fish superoxide dismutase gene, *KN2*, was selected for the genetic transformation of *T. aestivum*, with the objective of developing transgenic plants with increased tolerance to freezing stress. The effect of different media for callus induction, differentiation and pre-culture before biolistic transformation of wheat immature embryo cultures on transformation efficiency of the antifreeze gene *KN2* and *bar* gene, which confers

resistance to the herbicide bialaphos, were studied. This protocol developed here could be used for developing transgenic plants, integrated with other agronomically important genes.

MATERIAL AND METHODS

Plant materials and culture conditions

Wheat variety Longchun 23 (L23) was used in this study. The immature embryos from L23 were collected from seeds in approximately 15 days after anthesis. The seeds were immersed in 70% ethanol for 1 min, rinsed with sterile water once, and then immersed in 0.1% $HgCl_2$ for 10 min, followed by 4 rinses in sterile water and drying on filter paper. The immature embryos were aseptically excised from caryopses, placed with the scutellum upwards on 4 induction media (Nor, Cu^{2+}, Ag^{+} and N) (Table 1), and inoculated for 1-12 days in the dark at 25°C. The cultured tissues were grown on osmotic medium for 4-6 h before bombardment.

Table 1. Media used in this study.

Media	Compositions
Induction medium (Nor)	MS (Murashige and Skoog medium) + 500 mg/L Casein Hydrolysates + 100 mg/L glutamine + 100 mg/L proline + 10 mg/L Vitamin B_1 + 40 g/L maltose + 2 mg/L 2,4-D + 8 g/L agar, pH 5.8
Induction medium (Cu^{2+})	Nor medium +1.25 mg/L $CuSO_4$, pH 5.8
Induction medium (N^{+})	Nor medium with 5006 mg/L NH_4NO_3 - KNO_3
Induction medium (Ag^{+})	Nor medium + 2.5 mg/L $AgNO_3$, pH 5.8
Osmotic medium	Nor medium + 0.2 M mannitol + 0.2 M sorbitol
Differentiation medium (R)	MS + 500 mgL Casein Hydrolysates + 100 mg/L glutamine + 100 mg/L proline + 10 mg/L Vitamin B_1 + 40 g/L maltose + 5 mg/L kinetin + 8 g/L agar + 3 mg/L bialaphos, pH 5.8
Differentiation medium (Cu^{2+})	R medium + 0.25 mg/L $CuSO_4$, pH 5.8
Regeneration medium	½ MS basic medium + 500 mg/L Casein Hydrolysates + 100 mg/L glutamine + 100 mg/L proline + 10 mg/L Vitamin B_1 + 30 g/L maltose + 8 g/L agar, pH 5.8
Root strengthening medium	1/2MS basic medium + 30 g/L maltose + 0.2 mg/L naphthalene-acetic acid + 0.5 mg/L paclobrtrazol + 8 g/L agar, pH 5.8

Plasmid vector, plasmid transformation and plasmid DNA isolation

Plasmids pUBI::*bar* and pUBI::*KN2*, which contain the *bar* and *KN2* gene, respectively, were kindly provided by the Genetics and Development Institute, Chinese Academy of Science. Both genes are under the control of the ubiquitin promoter and *nos* 3' terminator. Ampicillin was used as selectable marker (Figure 1).

For biolistic transformation, the plasmids were amplified in *Escherichia coli* DH5α strain, transformed by routine heat-shock procedure. Plasmid DNA was isolated using the Tian Pure Mini Plasmid DNA isolation kit (Tiangen Biochemical Co., Beijing, China).

Preparation and coating of gold particles with plasmid DNA

One milliliter 96% ethanol was added to 20 mg gold microcarrier particles, and the mixture was shaken for 2 min and centrifuged for 1 min at 13,000 rpm. The supernatant was removed and 1 ml 96% ethanol with gold microcarrier particles was added, and the above step repeated 3 times. Washing using 1 ml sterile water was repeated 3 times, and after the final wash, particles were suspended in 1 ml sterile water and kept at -20°C until use.

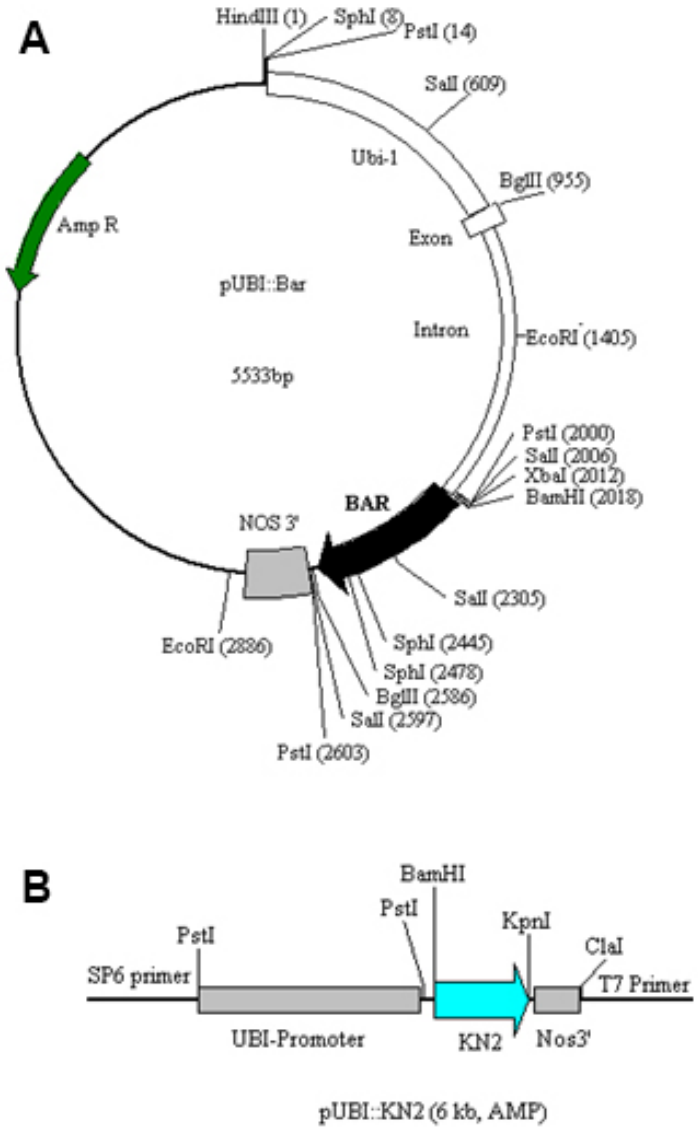

Figure 1. Vector maps of pUBI::*bar* (**A**) and pUBI::*KN2* (**B**).

Each 5 μg pBUI::*bar* and pUBI::*KN2* plasmid DNA were separately added to each tube containing 35 μL prepared gold microcarrier particles and mixed. Next, 50 μL 2.5 M $CaCl_2$ and 20 μL 0.1 M spermidine were added, vortexed, and kept at room temperature for 10 min. Particles were then pelleted by centrifugation for 1 min and the supernatant

was discarded. Particles were washed once with 140 μL 70% ethanol and once with 48 μL absolute ethanol.

Particle bombardment, differentiation, and regeneration of transformants

Immature embryo-derived calli were bombarded 5-6 times at 1100 psi helium pressure, at a target distance of 6 cm and under a vacuum of 28 mmHg with appropriate plasmid DNA coated microprojectiles by employing the Biolistic PDS-1000/He particle delivering system (Bio-Rad, USA). Bombardments with *bar* and *KN2* were conducted on the same calli. Following bombardment, the calli were cultured on osmotic medium for 18 h, transferred to induction medium for 15 days (see Table 1), and then transferred to two differentiation media (R, Cu^{2+}) for 2 months at 25°C, with a 16/8 h light/dark cycle. The healthy explants were subcultured on the differentiation media every 15 days. The regenerated plantlets were grown on regeneration medium for 30 days and then transferred to root strengthening medium. The rooted plantlets were vernalized at 4°C for 1 week and then transferred to pots to be grown until maturity at 25°C.

Genomic DNA isolation and PCR analysis

Total genomic DNA was isolated from wheat leaves at the three-leaf stage using the CTAB method (Stein et al., 2001). PCR analysis of genomic DNA was carried out using 12-20 ng wheat DNA, 1.5 μM $MgCl_2$, 0.2 μM of each primer, 0.2 μM dNTPs, 0.04 U *Taq* polymerase in a 25-μL reaction volume. The forward and reverse primers used for amplification of the *bar* gene were 5'-CGGTCTGCACCATCGTAACCACT-3' and 5'-GAAACCCACGTCATGCCAGTTCCC-3' (Zhou et al., 2008). The primers used for amplification of the *KN2* gene were 5'-TGCATCAGTGCAGGCCCTCAC-3' and 5'-CCTTTTCCCAGGTCGTCGGCC-3', which were designed by us using the Primer 3 software. The PCR protocols were as follows: for the *bar* gene, an initial denaturation at 95°C for 3 min, 35-40 cycles of 94°C for 30 s, 62°C for 45 s, 72°C for 1 min, and final extension at 72°C for 5 min; for the *KN2* gene, an initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 30 s, 66°C for 45 s, 72°C for 1 min, and final extension at 72°C for 5 min. The PCR products were run on a 1.5% agarose gel in 1X TAE along with a size marker.

RESULTS

Effect of different induction media

The effect of induction medium supplemented with Cu^{2+}, Ag^{+} or N^{+} on callus induction from L23 was determined. The frequencies of induced calli on different media ranged from 95.28 to 97.33% (Table 2). Different induction media showed no effect on the frequency of induced callus, but showed an effect on the differentiation frequencies of calli in sequential culture on differentiation media. The differentiation frequency of calli was increased from 38.36% on the normal medium (Nor) to 42.99% on the medium with Cu^{2+}. The media supplement with either N^{+} or Ag^{+} decreased the differentiation frequency of calli. In addition, the number of induced buds from the calli cultured on the medium with Cu^{2+} was higher than that

on the other three media. The calli induced on the Cu^{2+} medium showed quick regeneration of plantlet when cultured on regeneration medium, and the regeneration rate (64.95%) was higher for the calli induced on Cu^{2+} medium compared to the other media.

Table 2. Effect of callus induction media on L23 immature embryos culture and differentiation*.

Media	No. of immature embryos cultured	No. of calli induced	% of induced calli	No. of calli differentiated	% of calli differentiated**	Plantlets obtained	% of Plantlets obtained
Nor	150	146	97.33%	56	38.36%	70	47.95%
Cu^{2+}	220	214	97.28%	92	42.99%	139	64.95%
N^{+}	178	170	95.28%	50	29.41%	43	25.29%
Ag^{+}	126	122	96.82%	25	20.49%	13	10.66%

*The percentage (%) of induced calli was calculated before biolistically transformation, the % of calli differentiated rate was calculated after biolistically transformation. **The callus differentiation data is a mean from two differentiation media (Cu^{2+} and R).

$CuSO_4$ effects

The calli induced with induction media were transferred to the differentiation media R and Cu^{2+}. More shoots were produced from the calli cultured on differentiation media with $CuSO_4$ (Cu^{2+} medium) than on differentiation media without $CuSO_4$ (R medium) (Figure 2). A high percentage of calli induced on Nor medium subcultured on the differentiation medium supplemented with Cu^{2+} generated plantlets (71.15%). The calli induced on the medium with Cu^{2+}, subcultured on the differentiation medium without Cu^{2+} showed a 68.45% regeneration rate, and those subcultured on the differentiation medium Cu^{2+} showed a 52.17 % regeneration rate. In general, the percentage of plantlets regenerated using both the induction and differentiation media without Cu^{2+} was much lower compared to culture on either differentiation medium with Cu^{2+} (71.15%) or induction medium with Cu^{2+} (68.45%) and both induction and differentiation media with Cu^{2+} (52.17%) (Table 3).

Effect of culture period before transformation on L23 differentiation and genetic transformation

Prior to bombardment, the immature embryos were cultured on induction media for 12, 10, 7, 4 and 1 days to test transformation efficiency. After transformation, the tissues were cultured for 15 days and then transferred to the differentiation medium R and Cu^{2+}. The effects of pre-culture period and induction and differentiation media on biolistic transformation are presented in Table 4. The results showed that the calli induced on Cu^{2+} medium and pre-cultured for 4 days before biolistic transformation, and cultured on R medium after biolistic transformation produced the highest percentage (65%) of transgenic plantlets with the *KN2* gene (Table 4). PCR amplification of genomic DNA from explants using primers specific for the *bar* and *KN2* genes were used to identify the presence of transgenes in the transformants (Figure 3). The overall results showed that 50% of plantlets regenerated from calli pre-cultured 4 days before bombardment carried the *KN2* gene and 44.7% of plantlets carried the *bar* gene, which was higher than with any other pre-culture treatments, followed by pre-cultured 1 day with a 31.43% transformation rate for *KN2* gene and 20% for *bar* gene (Table 5).

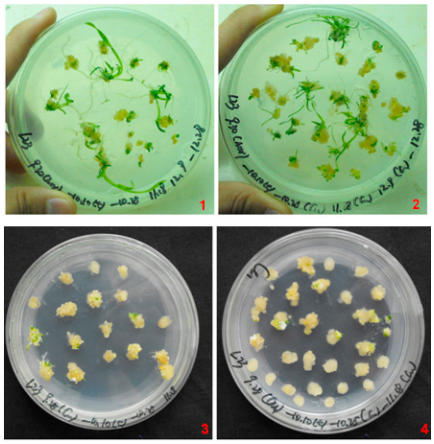

Figure 2. Callus induced on Nor induction media from immature embryos of L23 was transferred to differentiation media R (Figure 2.1) and Cu^{2+} (Figure 2.2). Figure 2.3 and 4 showed that callus induced on Cu^{2+} induction media from immature embryos of L23 was transferred to differentiation media R (Figure 2.3) and Cu^{2+} (Figure 2.4).

Table 3. Effects of $CuSO_4$ on differentiation of L23 callus induced from four induction media*.

Differentiation media	No. of calli biolistically transformed	No. of calli regenerated	% of regeneration	No. of calli biolistically transformed	No. of calli regenerated	% of regeneration
	Nor			Cu^{2+}		
Cu^{2+}	52	37	71.15%	46	24	52.17%
R	94	33	35.11%	168	115	68.45%
	N+			Ag^{+}		
Cu^{2+}	68	16	23.53%	36	0	0
R	102	27	26.47%	86	13	15.12%

*The calli induced on four induction media, after biolistically transformation, the the biolistically transformed calli were cultured on differentiation media Cu^{2+} and R.

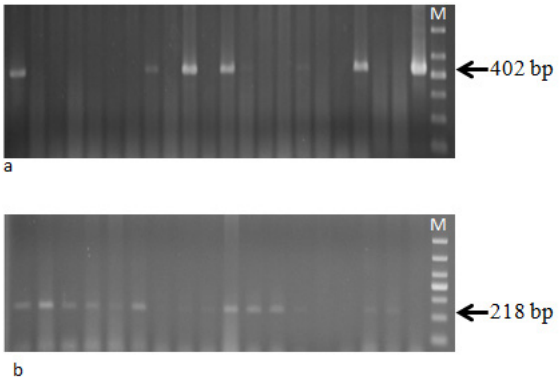

Figure 3. Examples of PCR analysis of transgenic plantlets. *Lane M* = molecular marker, 100 bp; **a.** PCR analysis of the *bar* gene, expected size of the amplified *bar* gene is 402 bp; **b.** PCR analysis of the *KN2* gene, expected size of the amplified *KN2* gene is 218 bp.

Table 4. Effects of pre-culture period, induction media, differentiation media on biolistic transformation of L23 immature embryos.

Induction media			Nor		Cu^{2+}		N^{+}		Ag^{+}	
Differentiation media			R	+ Cu^{2+}	R	+ Cu^{2+}	R	+ Cu^{2+}	R	+ Cu^{2+}
12 days	No. of calli transformed		29	-	28	28	32	-	-	-
bar gene	No. of positive plantlets		4	-	12	0	4	-	-	-
	Rate		13.79%	-	42.86%	0	12.50%	-	-	-
KN2 gene	No. of positive plantlets		1	-	14	0	3	-	-	-
	Rate		3.45%	-	50.00%	0	9.38%	-	-	-
bar + *KN2* gene										
No. of positive plantlets	1		-	8	0	2	-	-	-	
	Rate		3.45%	-	28.57%	0	6.25%	-	-	-
10 days	No. of calli transformed		25	29	32	-	27	-	26	-
bar gene	No. of positive plantlets		6	5	13	-	0	-	3	-
	Rate		24.00%	17.24%	40.63%	-	0	-	11.54%	-
KN2 gene										
	No. of positive plantlets		3	9	6	-	0	-	4	-
	Rate		12.00%	31.03%	46.15%	-	0	-	15.38%	-
bar + *KN2* gene	No. of positive plantlets		1	3	3	-	0	-	3	-
	Rate		4.00%	10.34%	23.08%	-	0	-	11.54%	-
7 days	No. of calli transformed		40	23	54	-	45	68	25	36
bar gene	No. of positive plantlets		1	2	9	-	6	3	0	0
	Rate		2.50%	8.70%	16.67%	-	13.33%	4.41%	0	0
KN2 gene	No. of positive plantlets		1	9	12	-	5	1	0	0
	Rate		2.50%	39.13%	22.22%	-	11.11%	1.47%	0	0
bar + *KN2* gene	No. of positive plantlets		0	0	3	-	2	0	0	0
	Rate		0	0	5.56%	-	4.44%	0	0	0
4 days	No. of calli transformed		-	-	20	18	-	-	35	-
bar gene	No. of positive plantlets		-	-	6	9	-	-	2	-
KN2 gene	Rate		-	-	30.00%	50.00%	-	-	5.71%	-
	No. of positive plantlets		-	-	13	6	-	-	0	-
	Rate		-	-	65.00%	33.33%	-	-	0	-
bar + *KN2* gene	No. of positive plantlets		-	-	6	1	-	-	0	-
	Rate		-	-	30.00%	5.56%	-	-	0	-
1 day	No. of calli transformed		-	-	35	-	-	-	-	-
bar gene	No. of positive plantlets		-	-	7	-	-	-	-	-
	Rate		-	-	20.00%	-	-	-	-	-
KN2 gene	No. of positive plantlets		-	-	11	-	-	-	-	-
	Rate		-	-	31.43%	-	-	-	-	-
bar + *KN2* gene	No. of positive plantlets		-	-	4	-	-	-	-	-
	Rate		-	-	11.43%	-	-	-	-	-

(-) data not available.

Table 5. Effects of culture period before transformation on L23 differentiation and genetic transformation.

Pre-culture time before bombardment	No. of regenerated plantlets	No. of plantlet with *bar* gene	Transformation rate of *bar* gene	No. of plantlet with *KN2* gene	Transformation rate of *KN2* gene
12 days	50	20	17.54%	18	15.79%
10 days	56	27	19.42%	22	15.83%
7 days	96	21	7.22%	28	9.72%
4 days	38	17	44.74%	19	50.00%
1 day	18	7	20.00%	11	31.43%

DISCUSSION

Cu^{2+} is a cofactor of many enzymes, such as polyphenol oxidase and ascorbic acid oxidase in metabolic pathways, and an essential element for plant growth and development.

Previous studies have suggested that Cu enzymes could play an important role in plant tissue culture (Purnhauser, 1991; Purnhauser and Gyulai, 1993). Purnhauser (1991) examined the effects of six copper levels on regeneration from callus cultures in hexaploid wheat and found that regeneration rates were eight times higher on medium containing 10 μM $CuSO_4$ than on the original MS copper level (0.1 μM). Similar results were also found for wheat anther cultures and triticale immature embryo-derived callus (Purnhauser and Gyulai, 1993). Ghaemi et al. (1994) tested the effects of different copper levels on the production of embryoids from anthers of tetraploid wheat (*T. turgidunt* L.), and found that the addition of 40 μM $CuSO_4$ to the medium significantly increased embryoid production in three of the four genotypes tested. Chen et al. (2004) showed a positive correlation between the rate of wheat callus regeneration and concentration of Cu^{2+} in the culture medium, and suggested that the optimal concentration of Cu^{2+} for wheat tissue culture is 5 μM. In rice, Yang et al. (1999a,b) showed that an increased level of copper in the culture medium dramatically improves the proliferation and regeneration rate of calli. Yu et al. (2008) observed that a greater number of plantlets regenerated on medium containing 2 mg/L (~8 μM) $CuSO_4$ survived, which may have resulted from an increased number of well-developed roots. In general, our results here correspond well with previous studies, suggesting that the micronutrient copper in the induction medium (1.25 mg/L, ~5 μM) can increase the differentiation rate and regeneration rate of wheat calli.

The effect of silver nitrate on somatic embryogenesis and plant regeneration in several plant species has been investigated, for example, *Brassica* ssp (Kuvshinov et al., 1999), rice (Adkins et al., 1993), and barley (Castillo et al., 1998). In wheat, the effects of silver nitrate on somatic embryogenesis and plant regeneration have also been studied. Purnhauser et al. (1987) first reported that 10 mg/L $AgNO_3$ effectively promoted wheat shoot regeneration from calli derived from immature embryos. Chen et al. (2006) suggested that the induction medium supplemented with 0.1 mg/L ABA (abscisic acid) and 2.5 or 5.0 mg/L $AgNO_3$ enhanced induction of calli and differentiation of wheat calli. Yu et al. (2008) optimized the culture system for mature embryo-based callus induction and plant regeneration for elite wheat cultivars grown in China, and found that the addition of 10 mg/L $AgNO_3$ in regeneration medium promoted plant regeneration. Fernandez et al. (1999) found that the addition of 1 mg/L $AgNO_3$ to the medium improved the induction of somatic embryogenesis in durum wheat. In our study, the lowest regeneration rate (15.12%) was found for the calli induced from medium with $AgNO_3$ used for biolistic transformation. Our results disagreed with previous findings, and suggested that $AgNO_3$ decreased the plantlet regeneration and transformation rate. In previous studies, the healthy or undamaged calli were cultured on the medium supplemented with $AgNO_3$. In this study, bombardment was used to introduce foreign genes into host cells. Microcarriers used for biolistic transformation usually damage cells by perforating them and allowing cytoplasm to escape (Greer et al., 2009). The calli induced on $AgNO_3$ medium may contain Ag^+, which may inhibit cell differentiation and growth. It is also possible that $AgNO_3$ interacts with gold microcarrier particles to inhibit the induction and differentiation of calli.

Nitrogen is one of the major macronutrients required for plant growth and development. Mordhorst and Lörz (1993) claimed that nitrogen content strongly affected the development of barley microspores, and that optimal nitrogen content was between 20 and 35 mM. Nitrogen effects are highly dependent on both total amount of nitrogen and proportion of NH_4^+ and NO_3^-, and affect a wide range of *in vitro* responses, including callus growth, shoot and root organogenesis, embryogenesis, and shoot multiplication (George and Klerk, 2008). A

strong positive correlation was found between the increase in the number of regenerated embryos and the increase in the number of regenerated plants in Superb cultivar exposed to high concentrations of nitrogen (Greer et al., 2009). It was found that increasing nitrogen content 3- and 6-fold resulted in respective 8.4- and 13.4-fold increases in the regeneration of plantlets from the bombarded scutella (Greer et al., 2009). However, in our study, the high concentration of nitrogen in induction medium did not show an increase in regeneration of plantlets. No strong connection between content of nitrogen and somatic embryogenesis yield in wheat was reported by Menke-Milczarek and Zimny (2001). Grimes and Hodges (1990) found the level of nitrogen did not significantly affect the number of regenerated rice plants. It was suggested that each species, cultivar and even tissue has its own unique preference for different salt concentration (He et al., 1989; Maës et al., 1996). Greer et al. (2009) also found that the nitrogen content used was ideal for regenerating the cultivar Superb, but was suboptimal for regenerating other cultivars. Thus, the effect of nitrogen on the induction and regeneration of calli is cultivar dependent.

The condition of the host is an important factor determining the success rate of genetic transformation. Using explants at an optimal developmental stage for genetic transformation can improve the transformation efficiency. The duration of immature embryo culture before bombardment is one of the important factors. It has been reported that transformation can be dependent on the pre-culture period (Gless et al., 1998). A study on the transformation of immature maize embryos found that transformation frequency was greatly increased when the scutella were pre-cultured for 2-6 days (Brettschneider et al., 1997). With triticale scutellum tissue, a clear reduction in tissue damage was achieved when the explants were pre-cultured for 2-7 days (Zimny et al., 1995). Takumi and Shimada (1996) showed that embryos cultured for less than 4 day before bombardment failed to produce transformed plants, and transgenic wheat plants were obtained from the immature embryos pre-cultured 5 days or more prior to bombardment. However, a culture of leaf segments of longer than 2 days prior to bombardment led to a 33% decrease in transient expression events in oats (Gless et al., 1998). Our results showed that wheat immature embryos pre-cultured for 4 days are more suitable for genetic transformation than the embryos pre-cultured more than 4 days.

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