ISSN 1672-9145 **Acta Biochimica et Biophysica Sinica** 2007, **39**(5): 377–383 **CN** 31-1940/Q

Inheritance and Expression of Copies of Transgenes *1Dx5* and *1Ax1* in Elite Wheat (*Triticum aestivum* L.) Varieties Transferred from Transgenic Wheat through Conventional Crossing

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Abstract To study the inheritance and expression of multiple copies of transgenes from transgenic wheat lines, three crosses between transgenic wheat lines B72-8-11b and B102-1-2 and Chinese elite wheat varieties Chuan89-107 and Emai18 were carried out. Chuan89-107×B72-8-11b, Chuan89-107×B102-1-2 and Emai18×B72-8-11b, and F_1 plants were selfed or backcrossed to obtain different generation populations. Protein analysis in grains of F_1 and F_2 and backcross progenies of BC_1F_1, BC_1F_2, BC_1F_3, BC_2F_1, BC_2F_2 and BC_2F_3 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that the transgenes *1Dx5* and *1Ax1* were expressed and segregated in the target wheat according to Mendelian laws. A range of *1Dx5* expression levels were observed in the progenies of Chuan89-107×B72-8-11b and Emai18×B72-8-11b, but the expression levels of *1Ax1* in progenies of Chuan89-107×B102-1-2 rarely changed. It suggested that the two foreign genes had different mechanisms of expression in the cross progeny, even though they were produced in the same way and the foreign *1Dx5* gene of 5–10 copies had the more complicated expression mechanism than the *1Ax1* gene of 4–5 copies.

Key words cross; elite wheat; *1Dx5* gene; *1Ax1* gene; inheritance; expression

Wheat is the most important crop in the world in terms of its geographical distribution, area under cultivation and total yield. Ninety-five percent of cultivated wheat is of the hexaploid type used for the preparation of bread and other baked products. The types and quantities of high molecular weight glutenin subunits (HMW-GS) of wheat have a direct influence on the elasticity and strength of dough, which determine the bread-making quality [1–3].

Two HMW-GS genes are present on each of the homologous group one chromosomes of wheat, encoding an x-type and a y-type subunit [4]. Cultivars of hexaploid bread wheat containing three, four or five individual subunits (1Dx, 1Dy, 1Bx and 1Ax and/or 1By subunits) exist, but cultivars rarely contain six subunits because one or more of the six HMW-GS genes may be silenced. These differences in HMW subunit composition result in both quantitative and qualitative effects on bread-making performance in bread wheat [5,6]. Therefore, attempts to improve grain quality have focused on manipulating the amount and composition of the HMW-GS, especially 1Dx5 and 1Ax1 subunits which are known to be associated with good bread-making quality [4,7].

In China, most elite wheat varieties are not bread-making, and there is increasing demand for high quality dough for a range of food products. Good bread-making quality depends on the presence of protein subunit combinations such as 1Dx5+1Dyl0, 1Ax1, 1Ax2* and 1Bxl7+1Byl8 [8], but these are scarce in Chinese wheat. Only 30% of the varieties contain 1Dx5+1Dyl0 subunits compared to a higher percentage in the varieties in other countries [9].

Transgenic wheat line B72-8-11b contains 1Dx5, 1Bx17 and 1By18 subunits and line B102-1-2 contains 1Ax1, 1Bx17 and 1By18 subunits [10]. In these two wheat lines, the subunits they contain confer good wheat bread-making

Received: December 25, 2006 Accepted: February 25, 2007

This work was supported by a grant from the Major State Basic Research Development Program of China (No. 2002CB111302)

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DOI: 10.1111/j.1745-7270.2007.00284.x

quality and they can offer multiple copies of the foreign gene. However, these two wheat lines lack other agricultural assets, for instance, they can not adapt well to the climate and environment in China and are not good in yield. The two elite varieties have good agricultural qualities such as high yield, resistance to some diseases, early ripening, and adaptation to the environment in Hubei province, but they might also have bread-making quality if they expressed the *1Dx5* or *1Ax1* gene. Crossing and backcrossing can combine the high HMW-GS in B72-8-11b and B102-1-2, with the assets of the elite varieties.

In the present study, crosses and backcrosses were made between transgenic lines and elite cultivars, Chuan89-107 and Emai18. Inheritance and expression of transgenes were analyzed with the aim of increasing the proportions of the HMW subunits and expression levels of foreign genes in Chinese elite wheat and improving its bread-making performance.

Materials and Methods

Plant materials

Transgenic lines B72-8-11b and B102-1-2 were used [10]. They were in the L88-31 background [11] and were produced by co-bombardment with the plasmid pAHC25 and either the p1Ax1 plasmid [5] or the p1Dx5 plasmid [12] containing the HMW subunit *1Ax1* and *1Dx5* genes, respectively, under the control of their own endosperm-specific promoters. B72-8-11b contains approximately 5–10 copies of the *1Dx5* gene and B102-1-2 contains approximately 4–5 copies of the *1Ax1* gene [10,13,14]. The two elite varieties are Chuan89-107 and Emai18, both with high yield and stress resistance.

All the wheat lines in this study were planted at the end of October or the beginning of November and were harvested the next May. The HMW-GS compositions of the materials used in this study are listed in **Table 1**.

Crossing and backcrossing

Three crossing and backcrossing combinations were carried out between Chuan89-107, Emai18 and B72-8-11b, B102-1-2: Chuan89-107×B72-8-11b, Chuan89-107×B102-1-2 and Emai18×B72-8-11b. Transgenic wheat lines were used as male parents and the other two lines were used as female parents and recurrent parents. Seeds of cross progenies F_1, F_2 and backcross progenies BC_1F_1, BC_1F_2, BC_1F_3, BC_2F_1, BC_2F_2 and BC_2F_3 were obtained from plants expressing the foreign gene in the previous generation.

Table 1 High molecular weight glutenin subunit (HMW-GS) composition of parents of crosses

Wheat line	HMW-GS composition		
	1A	1B	1D
B72-8-11b	null	17+18	5*
B102-1-2	1*	17+18	null
Chuan89-107	null	7+9	2+12
Emai18	1	14+15	2+12

1* and 5* represent *1Ax1* and *1Dx5* genes, respectively, but it does not mean that the *1Ax1* gene was exclusively on the A genome, or that the *1Dx5* gene was exclusively on the D genome of wheat chromosomes.

Analysis of transgene integration

For polymerase chain reaction (PCR) and Southern analysis, total genomic DNA was isolated from leaf tissue of wheat using the hexadecyltrimethylammonium bromide (CTAB) method [15]. PCR of the *1Ax1* and *1Dx5* genes was carried out with 50–200 ng of genomic DNA in a reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 μM of each dNTP, 0.3 μM of each primer, and 0.66 U *Taq* DNA polymerase (TaKaRa, Dalian, China). The conditions for PCR of the *1Ax1* gene were one cycle of denaturation at 94 ºC for 5 min, followed by 30 cycles of 94 ºC for 30 s, annealing at 60 ºC for 30 s, extension at 72 ºC for 1 min, and a final extension at 72 ºC for 10 min. The primers for the *1Ax1* gene were 5′-GTGTGAGCGCGAGCTCCA-GGAA-3′and 5′-CGGAGAAGTTGGGTAGTACCCTGC-3′. There was one difference in the *1Dx5* reactions, in that the extension phase was 72 ºC for 2.5 min in the cycles. The primers for the *1Dx5* gene were 5′-GCCT-AGCAACCTTCACAATC-3′ and 5′-GAAACCTGCT-GCGGACAAG-3′. Products of PCR amplification were analyzed by electrophoresis in 1.0% (*W*/*V*) agarose gels. Integration of foreign HMW subunit genes was examined by PCR and Southern blot analysis of digested genomic DNA. Genomic DNA was digested with *Eco*RI, which cut the plasmid pHMW1Ax1 into two parts, one of which was 7 kb long and includes the *1Ax1* gene, and the other was the 2.7 kb of plasmid pUC8. *Eco*RI also cut the plasmid pHMW1Dx5 into two fragments of 8.7 kb and 2.7 kb. DNA was then separated by electrophoresis in 0.8% (*W*/*V*) agarose gel and transferred by capillary blotting to positively charged nylon membrane (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Filters were hybridized with PCR-generated digoxigenin-labeled probes produced using a PCR digoxigenin probe synthesis kit (Roche Diagnostics).

Analysis of transgene inheritance and expression

Seeds from each generation of the crossed and backcrossed wheat lines were germinated and grown in the field. Foreign gene segregation and inheritance were investigated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The distal, endosperm-containing parts of individual seeds were analyzed by SDS-PAGE for expression of the foreign HMW subunit genes. The corresponding proximal parts containing the embryo were then germinated, allowing transgene presence to be correlated with expression.

Total proteins of seeds were extracted from single half-grains in 25 μl/mg extraction solution of 62.5 mM Tris-HCl buffer, pH 6.8, containing 2% (*W*/*V*) SDS, 10% (*V*/*V*) glycerol and 0.02% (*W*/*V*) bromphenol blue, and separated by SDS-PAGE using a Tris-borate buffer system and 10% (*W*/*V*) polyacrylamide gels with approximately 0.09% ammonium persulfate (Sigma, St. Louis, USA) and 0.0008% tetramethylethylenediamine (TEMED) [16]. Acrylamide concentration in the stacking gel was 2%. The electrophoresis conditions were 130 V in the concentrating gel and 240 V in the separating gel. When the electrophoresis was finished, the gel was stained with a solution of 40% methanol, 10% trichloroacetic acid and 0.1% Coomassie Brilliant Blue R250.

Results

Analysis of crossing validity

To avoid false positives from crossing, SDS-PAGE was used to detect the expression of foreign genes of the positive seeds from the F_1 generation. The results showed that the whole HMW-GS was correctly integrated and expressed (**Fig. 1**). Three combinations of crosses were also analyzed for their foreign HMW-GS genes by PCR (**Fig. 2**). In the F_1 generation, foreign genes could be amplified in all individual plants. The *1Ax1* gene was more

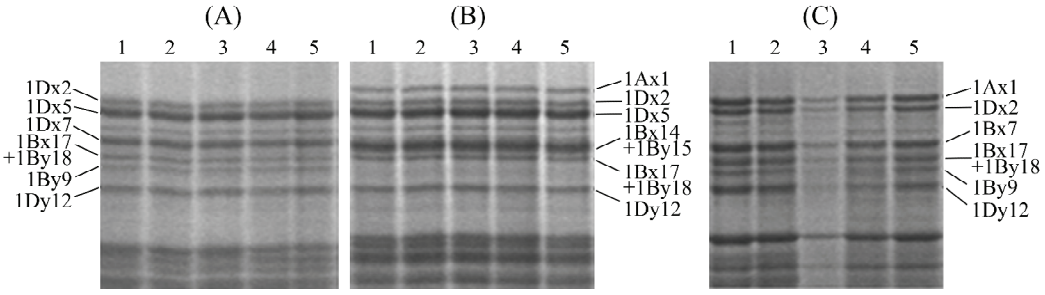

Fig. 1 Uniformity of composition and expression of F_1 wheat high molecular weight glutenin subunits

(A) Chuan89-107×B72-8-11b progenies. (B) Emai18B72-8-11b progenies. (C) Chuan89-107×B102-1-2 progenies.

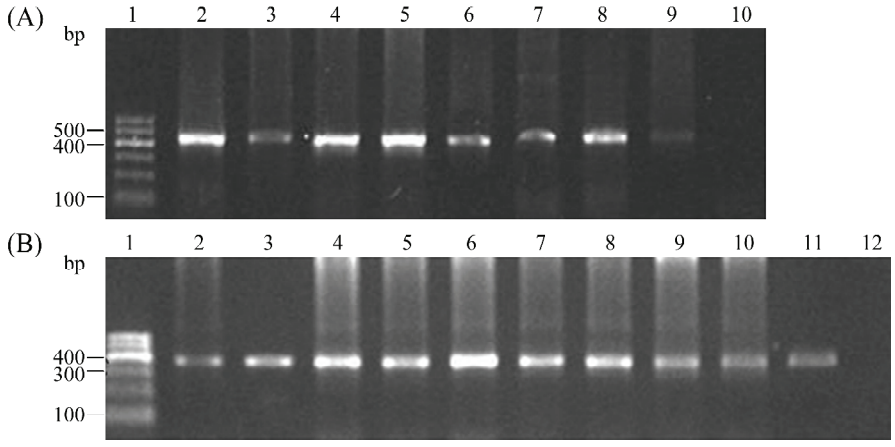

Fig. 2 Polymerase chain reaction analysis of F_1 for *1Ax1* and *1Dx5* genes

(A) 1, 600 bp marker; 2, pHMW1Ax1; 3–9, F_1 progenies of Chuan89-107×B102-1-2; 10, null. (B) 1, 600 bp marker; 2, pHMW1Dx5; 3–7, F_1 progenies of Chuan89-107×B72-8-11b; 8–11, F_1 progenies of Emai18×B72-8-11b; 12, null.

than 400 bp, and the *1Dx5* gene was between 300 and 400 bp as shown on the agarose gel. These results showed that the crosses were valid and the seeds could be used for breeding and backcrossing.

Analysis of foreign gene integration

PCR analysis was also carried out in other generations. In the backcross progenies, foreign genes were segregated and absent from some plants of F_2, BC_1F_2 and BC_2F_2. Southern blot was carried out and the results showed that there were still multiple copies of foreign genes in the offspring of the cross and backcross (data not shown).

HMW-GS expression and segregation

As shown in **Fig. 3**, the composition of HMW-GS was determined in the seeds of crossed and backcrossed prog-

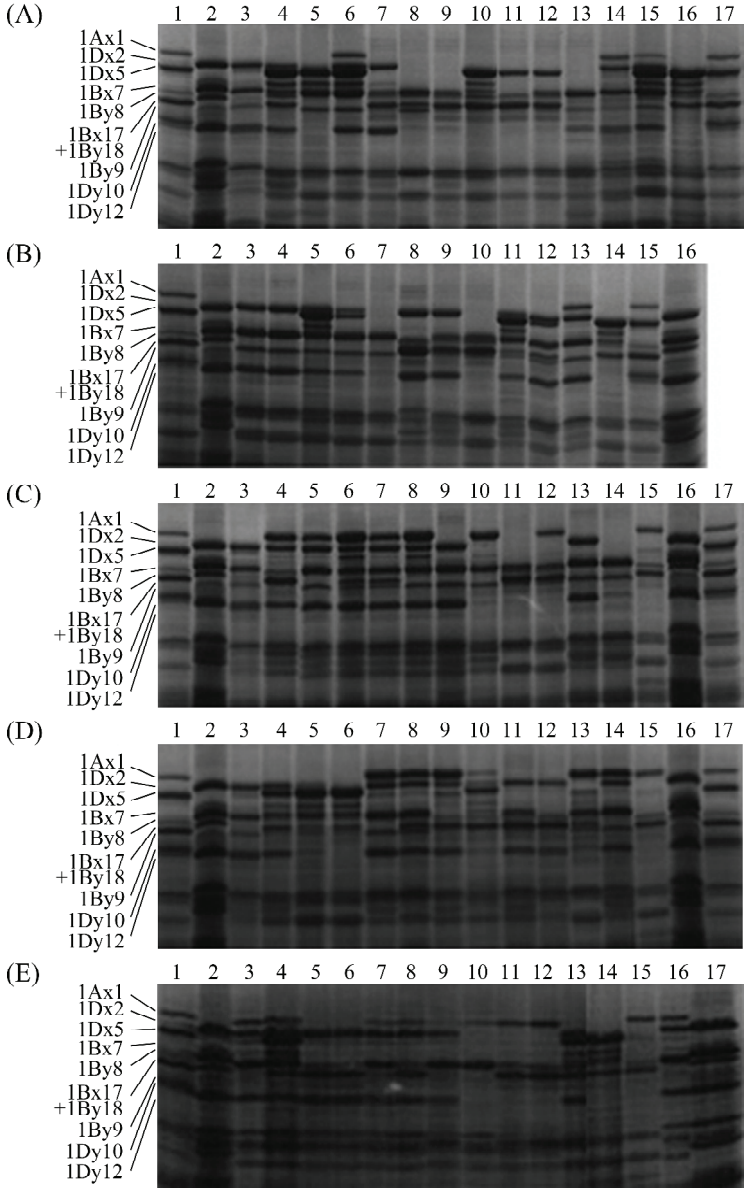

Fig. 3 Segregation of foreign genes in BC_1F_2 progenies

(A) 1, L88-6; 2, CS; 3, Chuan89-107; 4–15, Chuan89-107×B72-8-11b progenies; 16, B72-8-11b; 17, L88-6. In this part, we can see that the expression levels of multiple copies of foreign *1Dx5* gene in lane 4, 5, 6, 10 and 15 were higher than the expression level of the single copy of endogenous *1Dx5* gene in L88-6 in lane 1. (B) 1, L88-6; 2, CS; 3, Chuan89-107; 4-13, Chuan89-107×B72-8-11b progenies; 14, B72-8-11b; 15, L88-6; 16, CS. (A) and (B) also showed the extra subunit whose molecular weight was similar to that of *1Ax1* [lanes 6, 14 and 15 in (A) and lanes 13 and 15 in (B)]. (C) 1, L88-6; 2, CS; 3, Chuan89-107; 4–14, Chuan89-107×B102-1-2 progenies; 15, B102-1-2; 16, CS; 17, L88-6. (D) 1, L88-6; 2, CS; 3, Chuan89-107; 4–15, Chuan89-107×B102-1-2 progenies; 16, CS; 17, L88-6. (E) 1, L88-6; 2, CS; 3, Emai18; 4–15: Emai18×B72-8-11b progenies; 16, B72-8-11b; 17, L88-6. Only BC_1F_2 progenies are shown.

enies F_2, BC_1F_2, BC_1F_3, BC_2F_1, BC_2F_2 and BC_2F_3. HMW-GS segregation was observed in the backcrossed progenies, and there were many genotypes. There were some new HMW subunits which did not exist in the parent wheat lines [**Figs. 3(A)** and **4(B)**].

Foreign HMW-GS expression

Foreign genes in transgenic wheat B72-8-11b and B102-1-2 were transferred by particle bombardment and the copy numbers of foreign genes were 5–10 for *1Dx5* and 4–5 for *1Ax1* [13,17]. Leonie [17] *et al.* found that 4–5 copies of *1Ax1* genes had been inserted at two loci in B102-1-2. Theoretically, when occurring at two loci, these *1Ax1* genes would segregate in the progenies of crosses and backcrosses, and the segregation ratios in F_2 and BC_1F_1 were calculated, as shown in **Table 2**. Obviously, the observed ratios are close to the predictions of segregation ratios of two loci, but not for one locus.

Despite the possibility that the number of *1Ax1* genes varied, the expression levels of *1Ax1* were rarely changed [**Fig. 4(A)**].

In the chromosomes of wheat line B72-8-11b, 5–10 copies of foreign genes could be integrated at one locus or several loci. When considering only the foreign gene *1Dx5*, the theoretical segregation ratio of BC_2F_1 would be 1:1, whereas the segregation ratios in F_2, BC_1F_2 and BC_2F_2 generations would be 3:1 if the copies of the foreign gene were linked to form a single locus. A χ^2-test was carried out to test whether the foreign genes were inserted in one locus or two loci. The results were consistent with the predictions of one locus (**Table 2**). Results show that the multi-copy foreign genes in the transgenic wheat were inserted at one locus and they were inherited by the next generation according to Mendelian patterns.

However, the expression levels of the *1Dx5* gene were clearly different among the different progenies [**Fig. 4(B, C)**]. The expression of the *1Dx5* gene was at high levels in some plants but at low levels in others. Generally, the expression level of transgenes was higher than that of the endogenous *1Dx5* gene in L88-6, which was the isogenic wheat line of L88-31 containing one copy of the*1Dx5* gene [**Fig. 3(A)**].

Discussion

Although particle bombardment has become the most popular method for the transformation of wheat, the disadvantages such as high copy number, unstable heritability and expression of the foreign gene were difficult to

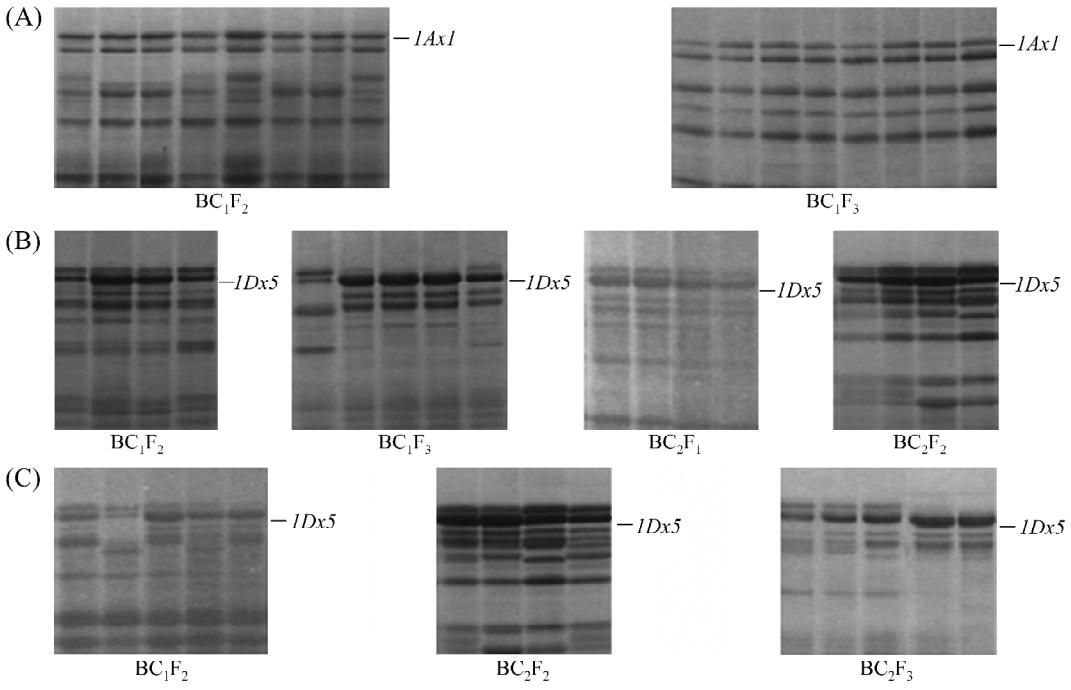

Fig. 4 Expression levels of foreign *1Ax1* and *1Dx5* genes in different progenies

(A) Chuan89-107×B102-1-2 progenies: BC_1F_2 and BC_1F_3. (B) Chuan89-107×B72-8-11b progenies: BC_1F_2, BC_1F_3, BC_2F_1 and BC_2F_2. (C) Emai18×B72-8-11b progenies: BC_1F_2, BC_2F_2 and BC_2F_3. In each generation, there are different expression levels of the *1Dx5* gene.

Table 2 χ^2-Test of expression and segregation of the foreign genes in cross progeny of wheat lines compared with Mendelian predictions

Foreign gene	Generation	Crossing	No. of individual plants		Segregation ratio of foreign gene in single locus		Segregation ratio of foreign gene in two loci	
			+	–	Expected ratio (+:–)	χ^2	Expected ratio (+:–)	χ^2
1Dx5	F_2	Chuan89-107×B72-8-11b	36	12	3:1	0.0278	15:1	20.3556
		Emai18×B72-8-11b	19	5	3:1	0.0556	15:1	6.4000
	BC_1F_2	Chuan89-107×B72-8-11b	549	197	3:1	0.7194	39:9	28.2132
		Emai18×B72-8-11b	86	25	3:1	0.2432	39:9	0.8041
	BC_2F_2	Chuan89-107×B72-8-11b	124	40	3:1	0.0081	311:73	2.7434
		Emai18×B72-8-11b	15	8	3:1	0.7101	311:73	2.7623
1Ax1	F_2	Chuan89-107×B102-1-2	235	21	3:1	37.6302	15:1	1.3500
	BC_1F_1	Chuan89-107×B102-1-2	197	70	1:1	59.4607	3:1	0.1511

The chic2 was calculated with the equation of the χ^2-test with Yates' correction for continuity because df=1:chi-squared=sigma [(|observed-expected|–1/2)²]/Expected. $\chi^2_{0.05,1}$=3.84. +, foreign gene was expressed; –, foreign gene was not expressed.

resolve. Embryos of many wheat lines have low embryogenic capacity, and the transformation frequency by particle bombardment is also very low (<1%). Therefore, it is not easy to obtain transgenic plants for these wheat lines in this way. Crossing and backcrossing between transgenic wheat and elite wheat can avoid these problems. It is easier to obtain lines with good desirable characters for many elite varieties using transgenic technology with conventional breeding than through the transgenic method of bombardment. There are many reports about the inheritance and stability of transgenes in donor wheat lines, and some research showed that once integration of foreign genes occurred, foreign DNA would be retained through meiosis and maintained in the progenies [9,14,18–20]. However, there are few reports about inheritance and expression of transgenes in elite wheat varieties. Only when foreign genes can be inherited stably by their progeny can this breeding mode be applied. The analysis of the inheritance and expression of copies of foreign genes in elite wheat became the goal of the present study.

In this work, crosses and backcrosses were used to study the inheritance and expression of multiple copies of the foreign genes *1Dx5* and *1Ax1* in the elite wheat lines whose genotypes are different from the foreign gene donors. The results showed that the multiple copies of foreign *1Dx5* and *1Ax1* genes could be inserted into the chromosomes of elite wheat lines during crossing and backcrossing, and these two genes were inherited by their progenies. The HMW-GS genes in these progenies segregated normally and there were various genotypes, some of which included the foreign genes, but the others did not.

If the foreign genes are inserted at a single locus, they will be linked and inherited together. In the course of inheritance, multiple copies of the same gene unite together and are inherited as a whole. Alternatively, the foreign gene insertion locus could be multiple and the foreign gene copy numbers are not always the same in each insertion locus. These foreign genes are dispersed randomly. It has been reported frequently that multiple foreign genes have been inserted in one locus during transformation by particle bombardment [18,21,22] and, in our trial, the foreign multiple copies of *1Dx5* gene were inserted in one locus and inherited as a whole.

Although the functional foreign *1Dx5* gene was inserted at a single locus, transformation by particle bombardment frequently results in foreign genes with high copy numbers. Some, but not all, of these foreign genes may be silenced because of gene rearrangement or co-suppression. Silenced genes are not expressed and therefore can not be observed by SDS-PGE. Non-silenced foreign genes showed various expression levels in the cross progenies of Chuan89-107×B72-8-11b and Emai18×B72-8-11b. *1Dx5* genes (5–10 copies) were inserted in one locus in the transgenic parent B72-8-11b. The expression levels of *1Dx5* in the cross progenies were sometimes lower than in the cross parent B72-8-11b and we can speculate that a proportion of multiple copies of a gene are silenced. Why the *1Dx5* gene was overexpressed stably in B72-8-11b but its expression level varied in the cross progenies is not clear.

Integration of *1Dx5* gene also led to the expression of

an extra subunit whose molecular weight was similar to that of *1Ax1*. Similar observations have been reported in transgenic wheat line B73-6-1 [23]. These might be caused by the insertion of foreign genes, with the genes silenced in the parents but activated in the cross progenies. However, the absence of some HMW-GS was the result of gene segregation in the cross progenies.

Four to five copies of the *1Ax1* gene have been observed as inserted into two loci in the chromosome of B102-1-2 [10,13,14], and we also found the *1Ax1* gene at two loci in the cross progeny. However, in contrast to *1Dx5* in cross progenies, the *1Ax1* gene in progenies of Chuan89-107×B102-1-2 expressed as an endogenetic gene. Its expression level was hardly changed and was approximately the same as that in the parent B102-1-2.

Our results indicated that the foreign genes could be inherited stably by their cross progenies. It was also suggested that the two foreign genes had different mechanisms of expression in the cross progeny, even though they were produced in the same way. The foreign *1Dx5* gene of 5–10 copies had the more complicated expression mechanism in our observation than the *1Ax1* gene of 4–5 copies.

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Edited by
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