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RESEARCH PAPER

Low copy number gene transfer and stable expression in a commercial wheat cultivar via particle bombardment

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Abstract

Two groups of linear gene constructs (*gus* and *bar*, and *1Ax1* and *bar*) lacking vector backbone sequences were independently transferred into the elite wheat (*Triticum aestivum* L.) variety EM12, and genetically stable transgenic plants with low copy number transgene integration were recovered. Co-transformation experiments were carried out in parallel using either circular whole plasmid(s) or linear gene cassettes which were purified from the same plasmid by restrictive digestion, each cassette consisting of a promoter, an open reading frame, and a terminator. Six transgenic wheat lines transformed with *1Ax1* plus *bar* gene cassettes, five lines with *gus* plus *bar* gene cassettes, three lines with p1Ax1 plus pAHC20, and two lines with pAHC25 were regenerated with transformation frequencies of 0.6, 0.5, 0.3, and 0.2%, respectively. Southern blotting analysis showed that there were 1–4 hybridizing bands in transgenic lines carrying gene cassettes, of which most lines displayed single-copy transgene insertion. Expression analyses showed that 50.5% of the T1 lines carrying *gus* plus *bar* gene cassettes have the expression signals of two genes. SDS–PAGE analysis of the T_1 generation revealed that 71% of herbicide-resistant plants carrying *1Ax1* plus *bar* gene cassettes expressed the high molecular weight subunit 1Ax1 in the endosperm. Gene cassettes were transmitted and segregated in the subsequent generations, in simple Mendelian ratios. In addition, reverse tanscription–polymerase chain reaction (RT–PCR) results confirmed that *1Ax1* gene cassettes were expressed specifically in the endosperm of the transgenic wheat plant. It is proposed that gene transfer using multiple gene cassettes offers an efficient and rapid method to obtain the single-copy transgenic wheat.

Key words: Gene cassette, integration pattern, particle bombardment, vector backbone sequence, wheat.

Introduction

Genetic transformation provides a method for crop genetic manipulation in order to enhance its agronomic performance, resistance to biotic and abiotic stresses, yield, and end-use quality (Barcelo and Lazzeri, 1995; Rahman *et al.*, 2000; He *et al.*, 2001). Direct DNA delivery via particle bombardment is currently the standard technique used for such genetic transformation. However, this technique results in multiple copies of the introduced gene being integrated into the plant genome at a single locus, and this is associated with the silencing of transgenes in the subsequent progeny by a tendency to promote homologous rearrangement (Kohli *et al.*, 2003; Sparks and Jones, 2004). A low number of copies of a transgene in a plant chromosome has a much lower incidence of instability (Jones, 2005). For commercial success, it is essential to obtain transgenic lines with simple transgene integration patterns to make sure introduced traits are transmitted faithfully through successive generations in a predictable manner. Potential measures to achieve this aim generally include insulating the transgene with matrix attachment regions, avoiding repetition of the promoter or transgene sequences, and using a novel promoter. For effective implementation of these measures, one should start with low copy number transgene integration. However, the occurrence of plants containing low numbers of a transgene

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Abbreviations: 2,4-D, dichlorophenoxyacetic acid; DIG, digoxigenin; GUS, β-glucuronidase; HMW, high molecular weight; PCR, polymerase chain reaction; L-PPT, L-phosphinothricin; SDS–PAGE, sodium dodecylsulphate–polyacrylamide gel electrophoresis; X-Gluc, 5-bromo-4-chloro-3-indolyl β-D-glucuronide.

in a cereal is rare. To ensure that there is a low copy number of transgene in the plant genome, both the conventional approach and an 'agrolistic' method are expensive, labour-intensive, and complicated. Especially in wheat genetic manipulation, it is a challenging task to achieve transgenic lines by themselves, due to the low transformation frequency (Cheng *et al*., 2004). If the goal is also to obtain low copy number transgenic lines, then the task becomes an order of magnitude harder.

In routine plant transformation, the use of recombinant plasmids results, undesirably, in the integration of the bacterial vector backbone sequence into the host genome along with the attached exogenous genes (Kohli *et al*., 1999; Meza *et al*., 2002). The presence of the vector backbone sequence in the transplant serves no purpose in biolistic transfer procedures. Furthermore, the vector backbone sequences have a tendency to stimulate illegitimate recombination by providing AT-rich sequences as recombination hotspots during the formation of secondary structures (Muller *et al*., 1999). Additionally, it possibly produces new lengths of 'filler' DNA homologous to flanking plant genomic DNA, which will escape into the environment (Kohli *et al*., 1998; Pawlowski and Somers, 2000; Svitashev *et al*., 2002). Consequently, it has been suggested that the superfluous vector region should be eliminated before bombardment. Genetic transformation with transgene cassettes has been successfully applied to rice (*Oryza sativa*) and potato (*Solanum tuberosum*), and has eventually led to a higher transformation frequency and simple transgene integration pattern (Fu *et al*., 2000; Loc *et al*., 2002; Romano *et al*., 2003; Agrawal *et al*., 2005). This transformation technique has been suggested to generate a larger proportion of transgenic plants with simple integration patterns. In rice and potato, transgenes just included the reporter, and abiotic and herbicide resistance genes, but transfer of genes for improving agronomic characteristics of cereal plants has not been reported.

In this investigation, an initial attempt was made to produce transgenic wheat plants by introducing *gus* and *bar* gene cassettes into regenerable wheat tissue from which whole plants can be regenerated. For comparison, the source plasmid pAHC25 was used as the reference. Stable transgenic plantlets were regenerated with either *gus* and *bar* gene cassettes, or the pAHC25 plasmid. The results showed that low copy insertions of *gus* and *bar* genes in transgenic plants could be carried on with gene cassettes. Following this, the high molecular weight (HMW) glutenin subunit gene *1Ax1* was also introduced into wheat along with the *bar* gene using both gene cassettes and plasmids. Integration patterns and expression of *1Ax1* gene cassettes were also analysed in the subsequent progeny, and the endosperm specificity of the HMW subunit *1Ax1* gene was also identified in transgenic wheat plants recovered with *1Ax1* and *bar* gene cassettes.

Materials and methods

DNA constructs

Three different plasmids were used for bombardments: pAHC25, pHMW1Ax1, and pAHC20. Plasmid pAHC25 contains the selective *bar* gene (2.0 kb), which confers resistance to the herbicide BASTA [active ingredient L-phosphinothricin (L-PPT)/glufosinate ammonium], and the screenable *gus* gene (4.1 kb), encoding the β-glucuronidase (GUS) protein (Christensen and Quail, 1996). The minimal expression cassettes of *gus* and *bar* were released from pAHC25 by digestion with restriction enzymes *Hin*dIII and *Eco*RI, respectively. Plasmid pAHC20 included only the *bar* gene (Christensen and Quail, 1996). Plasmid pHMW1Ax1 included a 7.0 kb *Eco*RI genomic fragment including the complete coding sequence of the *Glu-A1-1a* (*1Ax1*) gene flanked by 2.2 and 2.1 kb of the 5′ and 3′ sequences (Halford *et al*., 1992). A linear gene cassette of *1Ax1* was excised from pHMW1Ax1 with *Eco*RI restrictive digestion. The *1Ax1* promoter was specific to wheat endosperm. The linear transgene constructs were delivered in the following combinations: *gus*+*bar*, pAHC25, *1Ax1*+*bar*, and pHMW1Ax1+pAHC20.

Plant material

EM12 is a commercial wheat cultivar from the centre of China, a hexaploid bread wheat species, with the genome constitution AABBDD. This wheat variety produces flour with poor processing properties, due to the absence of the HMW glutenin subunit 1Ax1. Experimental wheat lines were sown in the field in October and flowered at the beginning of the following April. Spikes were harvested 14–16 d post-anthesis.

Transformation procedure

The transformation procedure was performed based on the bombardment method developed by Barcelo and Lazzeri (1995), modified by Pastori *et al.* (2001) and Rasco-Gaunt *et al*. (2001), and fully described by Sparks and Jones (2004). Scutella were aseptically isolated from the immature embryos as the targets, which were to be bombarded.

For each bombardment, 30–50 scutella ~0.8–1.5 mm in diameter were placed in the centre of a plate of MS-based induction medium containing either 1 mg l^{-1} dichlorophenoxyacetic acid (2,4-D) or 2 mg l^{-1} picloram. Explants were cultured in darkness at 24 °C for 1 d prior to bombardment. Two extra plates of samples were used as controls, one bombarded with only gold particles and the other one not bombarded. Plasmids and gene cassette constructs were independently precipitated onto the gold particles (Bio-Rad, Richmond, CA, USA) to a total amount of 5 μg (plasmids) and 3.5 μg (gene cassettes) before bombardment. Bombardments were performed using a PDS 1000/He particle gun. After the bombardment, scutella were spread over the surface of the medium in groups of 15, and cultured at 24 °C in darkness for 3 weeks to induce embryogenesis. The callus induction stage was followed by regeneration of embryogenic calli in the light. Embryogenic calli were subsequently transferred to RZ regeneration medium containing 0.1 mg l^{-1} 2,4-D and 5 mg l^{-1} zeatin, and then cultured for 3 weeks prior to transfer onto hormone-free R medium containing 2 mg l^{-1} L-PPT. The regeneration stage was performed in four rounds of 3 weeks each, and the selection of putative transformants was done on the last three rounds of regeneration until control plantlets had been killed. Surviving plants were transferred to soil and grown to maturity under greenhouse conditions. The seeds were then collected from each independent T_0 progeny to produce the T_1 plant lines.

gus *and* bar *expression analysis*

The expression of the *gus* gene was examined histochemically using the substrate X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide) in different organs of transgenic plants. Leaf tissue was cut into small pieces, immersed in X-Gluc buffer and incubated overnight at 37 °C. Chlorophyll was extracted from the tissue by incubation in 70% ethanol followed by 100% ethanol.

The assay of *bar* gene expression activity was carried out by local application of 0.5% or 1.0% (v/v) BASTA solution, containing 0.1% (v/v) Tween-20, to mark regions of fully expanded leaves. Resistance to the herbicide solution was estimated 1–2 weeks after application by scoring the area of leaf necrosis.

PCR and RT–PCR analysis

Total genomic DNA was isolated from leaf material of the T_0 and T_1 regenerated plants using a cetyltriethylammonium bromide (CTAB) extraction method (Stacey and Isaac, 1994). Total RNA and mRNA of the T_0 generation were extracted from different tissues (root, leaf, inflorescence, embryo, and endosperm) using the Trizol reagent from Invitrogen. Polymerase chain reaction (PCR) was performed using 1 μl (50–100 ng μl^{-1}) of template DNA in a reaction mixture containing 10 mM TRIS–HCl pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% (v/v) Triton X-100, 200 μM of each dNTP, 0.3 μM of forward and reverse primers, 20–500 ng of DNA, and 0.66 U of *Taq* DNA polymerase (ABI). Reverse transcription–PCR (RT–PCR) was performed with the access RT–PCR system from Promega. PCR or RT–PCR analysis of the *gus* gene (primer pair: 5′-AGTGTACGTATCACCGTTTGTGTGAAC-3′, 5′-ATCGCCGCTTTGGACATACCATCCGTA-3′, annealing temperature 62 °C), the *bar* gene (primer pair: 5′-GTCTGCACCATCGTCAACC-3′, 5′-GAAGTCCAGCTGCCAGAAAC-3′, annealing temperature 57 °C), and the *1Ax1* gene (primer pair: 5′-GTGTGAGCGCGAGCTCCAGGAA-3′, 5′-CGGAGAAGTTGGGTAGACCCTGC-3′, annealing temperature 60 °C) was carried out and the products were detected in a 0.8% (w/v) agarose gel. The amplified fragment lengths were as follows: *gus*, 1047 bp; *bar*, 443 bp; and *1Ax1*, 450 bp. Thermocycling conditions of RT–PCR were as follows: 35 cycles, denaturation at 94 °C for 30 s, annealing for 30 s, and extension at 72 °C for 2 min.

Southern blot analysis

Around 15 μg of genomic DNA was completely digested with restriction enzymes that resulted in either a single recognition site in the gene cassette (single-cutter enzyme) or no recognition site in the gene cassette (non-cutter enzyme). The single cutters comprised *Sac*I (*gus*), *Sac*I (*1Ax1*), and *Bam*HI (*bar*), while the non-cutter enzymes comprised *Hin*dIII (*gus*), *Eco*RI (*bar*), and *Eco*RI (*1Ax1*). Plasmids digested with the same enzymes were used as a positive control and for the estimation of the copy number of the transgene inserted in the plant genome. The genomic DNA fragments were separated by electrophoresis on a 0.8% (w/v) agarose gel at 20 V for ~40 h and transferred by capillary blotting onto a positively charged nylon membrane (Roche Diagnostics GmbH, Lewes, East Sussex, UK) using the method of Sambrook *et al*. (1989). Blotting, hybridization, and chemiluminescent detection were carried out as described in the DIG System User Guide for Filter Hybridization (Roche, Welwyn Garden City, UK). Filters were hybridized with PCR-generated digoxigenin (DIG)-labelled probes produced using primers for the *gus* and *bar* genes. Hybridization signals were detected using a chemiluminescent detection system.

SDS–PAGE and gel scanning

Total proteins were extracted from single half grains or flour samples with 25 μl mg^{-1} of 62.5 mM TRIS–HCl buffer, pH 6.8, containing 2% (v/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, and 0.002% (v/v) bromophenol blue, and separated by SDS–PAGE using a TRIS-borate buffer system and 10% (w/v) acrylamide gels (Laemmli, 1970; Shewry *et al*., 1995).

Results

Regeneration of transgenic lines

The transgenic wheat plants were regenerated from the immature scutella transformed with multiple gene cassettes or plasmid(s) via particle bombardment. The mean regeneration frequency (percentage of cultures regenerating one or more shoots) in control (unbombarded) cultures was 51.2%. For explants undergoing bombardment, the corresponding value was 56.7%. In total, 993 scutella isolated from wheat EM12 were co-bombarded with the *ubiquitin–gus–nos* (Fig. 1a) and *ubiquitin–bar–nos* frames (Fig. 1b). The leaf genomic DNAs extracted from transgenic plants surviving after the selection for the *bar* gene were used for PCR analysis for the presence of transgenes. The results indicated that five independently regenerated plant lines had actually been integrated with the *gus* (Fig. 2A) and *bar* (Fig. 2B) gene cassettes. Another 1010 immature embryos were co-bombarded with *1Ax1* (Fig. 1c) and *bar* gene cassettes, and six independently transgenic lines carrying the *1Ax1* (Fig. 2C) and *bar* gene cassettes were identified.

When the whole plasmid pAHC25 (Fig. 1.1) was used in co-transformation, two independent transgenic wheat

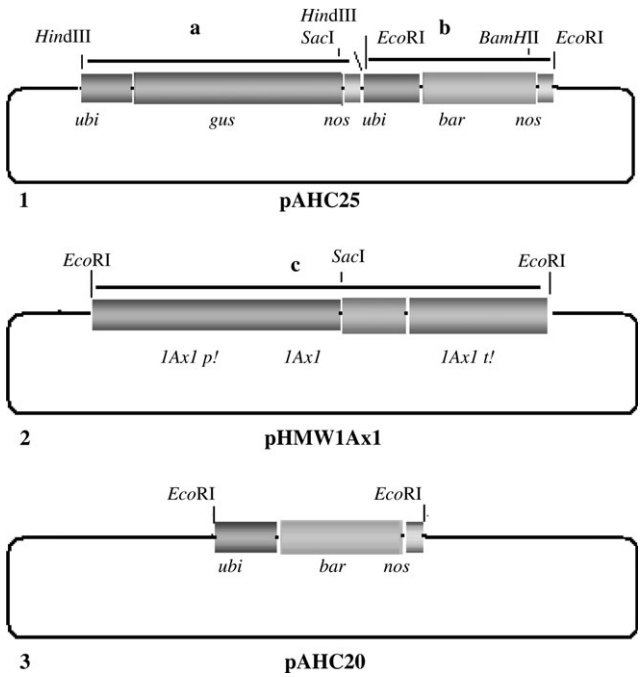

Fig. 1. Schematic maps of gene constructs used in co-transformation via particle bombardment. Circular recombinant plasmids: 1, pAHC25; 2, pHMW1Ax1; 3, pAHC20. Linear transgene cassettes: a, *gus*; b, *bar*; c, *1Ax1*. *ubi*, *ubiquitin* promoter; *nos*, *nos* terminator; *p!*, promoter; *t!*, terminator.

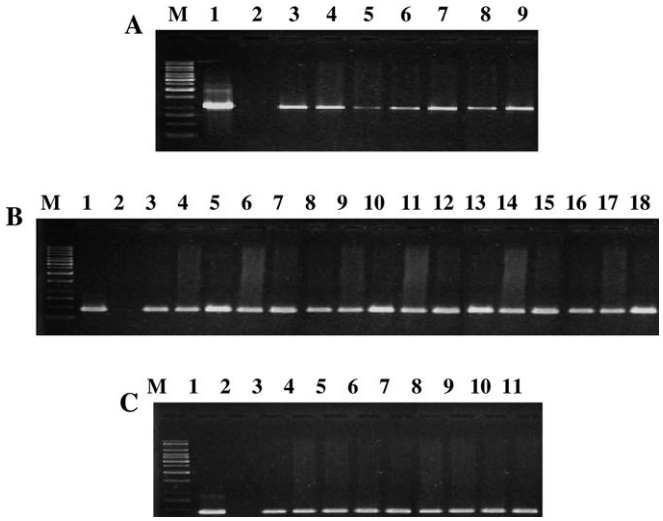

Fig. 2. PCR analysis of genomic DNA extracted from primary generations for the transgene. M, molecular weight marker; 1, pAHC25 or pHMW1Ax1; 2, water control. (A) *gus* PCR products (1056 bp); 3–9, transgenic lines B1-2-3, B1-3-4, B1-5-7, B2-4-3, B3-2-2, B6-4-3, and B6-5-1. (B) *bar* PCR products (443 bp); 3–18, transgenic lines B1-2-3, B1-3-4, B1-5-7, B2-4-3, B3-2-2, B6-4-3, B6-5-1, B12-2-1, B12-2-3, B12-4-5, B13-1-3, B13-2-5, B13-4-4, B16-2-3, B17-2-3, and B18-3-4. (C) *1Ax1* PCR products (450 bp); 3–11, transgenic lines B12-2-3, B12-4-5, B13-1-3, B13-2-5, B13-4-4, B16-2-3, B17-2-3, and B18-3-4.

plants recovered from 995 scutella were found to contain both *gus* and *bar* genes. Also three independent transgenic plants were regenerated from 998 scutella, which were transferred with the whole plasmids pHMW1Ax1 (Fig. 1.3) and pAHC20 (Fig. 1.2). Table 1 summarizes the results of PCR analysis of the primary transformants (T_0). Almost all the surviving wheat plants from the selection culture were demonstrated to be inserted with transgenes. An interesting observation was that the primary transgenic lines with either gene cassettes or whole plasmids all had a main shoot *in vitro* and on growth in soil produced only one or two tillers. However, T_1 plants showed relatively vigorous growth and produced multiple tillers (Fig. 3).

Integration of transgene cassettes

To investigate the patterns of integration of transgenes into the host wheat genome, further Southern blotting analysis of genomic DNA from the T_0 and T_1 regenerated plants was performed. It was previously reported that co-transformation with multiple gene cassettes predominantly resulted in the simple integration pattern in rice. Here it is proposed to identify the complexity of transgene integration pattern in wheat using two different gene cassettes. First, Southern blotting analysis of T_0 allowed the number of hybridizing bands to be estimated. All the individual T_0 lines carrying gene cassettes were studied after the digestion of genomic DNA, independently with a restriction enzyme that cut once and did not cut in the respective transgene cassettes. All the individual T_0 lines showed the simple integration

Table 1. *Integration and expression of transgenes in primary generations*

T_0 independent lines	Components of gene constructs	Integrated genes			Expressed genes		
		gus	*bar*	*1Ax1*	*gus*	*bar*	*1Ax1*
B1-2-3	*gus+bar*	+	+		+	+	
B1-3-4	*gus+bar*	+	+		+	+	
B1-5-7	*gus+bar*	+	+		+	+	
B2-4-3	*gus+bar*	+	+		Sterile	Sterile	
B3-2-2	*gus+bar*	+	+		Sterile	Sterile	
B6-4-3	pAHC25	+	+		–	+	
B6-5-1	pAHC25	+	+		+	+	
B12-2-1	*1Ax1+ bar*		+	+		+	+
B12-2-3	*1Ax1+ bar*		+	+		+	+
B12-4-5	*1Ax1 +bar*		+	+		+	+
B13-1-3	*1Ax1 +bar*		+	+		+	+
B13-2-5	*1Ax1 +bar*		+	+		+	+
B13-4-4	*1Ax1 +bar*		+	+		+	+
B16-2-3	p1Ax1+pAHC20		+	+		+	+
B17-2-3	p1Ax1+pAHC20		+	+		+	+
B18-3-4	p1Ax1+pAHC20		+	+		+	+

Fig. 3. Morphological comparison of T_0 and T_1 representative generations grown in soil. (A) T_0 plant line; (B) T_1 plant line.

pattern with 1–4 hybridizing bands per lane in Southern blots after the digestion of genomic DNA using the single cutters (Fig. 4), of which 13.6% showed a single hybridizing band. One band can be regarded as a single insertion, but may contain a number of copies present as concatenates or incomplete copies. Generally, comparison of the hybridizing intensity with the transgene bands revealed that the relative copy number of the independent transgenes had

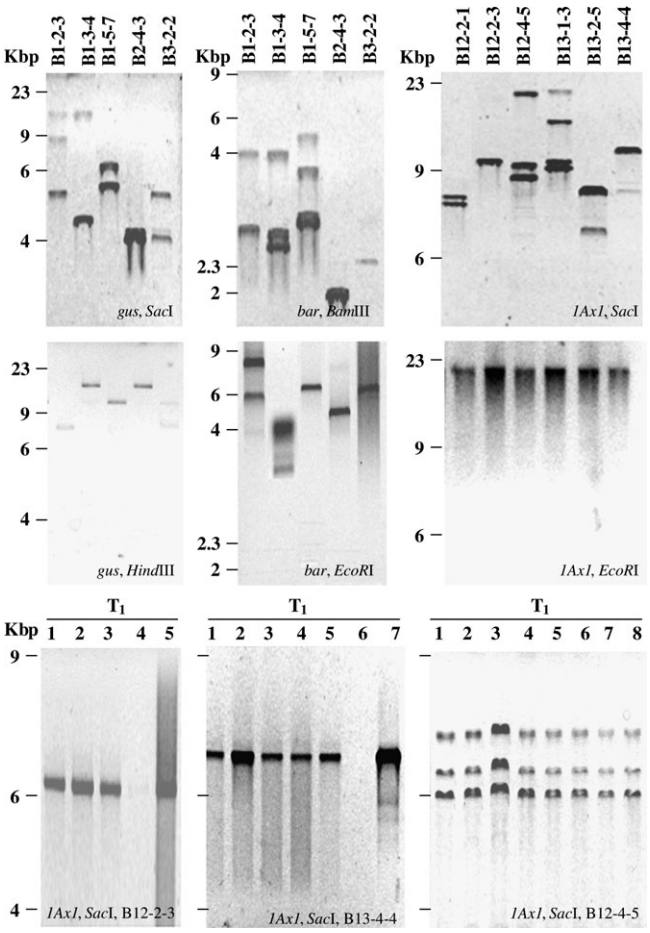

Fig. 4. Southern blot analysis of transgenic plants carrying gene cassettes, using leaf genomic DNA digested with the single cutters *Sac*I (*gus*, *1Ax1*) or *Bam*HI (*bar*), plus the non-cutter *Eco*RI (*bar*, *1Ax1*) or *Hin*dIII (*gus*). The lines named B1-2-3, B1-3-4, B1-5-7, B2-4-3, B3-2-2, B12-2-1, B12-2-3, B12-4-5, B13-1-3, B13-2-5, and B13-4-4 represent independent T_0 lines regenerated with gene cassettes. The numbers between T_1 and each blot correspond to different T_1 descendants of a T_0 line named B12-2-3, B13-4-4, or B13-2-5.

been inserted. However, Southern blotting analysis following the digestion of genomic DNA with restriction enzymes that do not cut in the relevant gene cassette could help to identify the single-copy transgene number. Southern blots of wheat lines which were integrated with a single-copy transgene are supposed to show the same number of bands using single-cutter enzyme as those using non-cutter enzyme. To distinguish the single-copy transgenic lines, the same T_0 lines as above were also assessed by Southern blotting analysis after the digestion using non-cutter enzyme. The results of Southern blots following the digestion using a single-cutter enzyme were compared with those using a non-cutter enzyme, indicating that 44.7% of the T_0 lines had the same number of hybridizing bands (Table 2). In the case of Southern hybridization with the labelled *1Ax1* probes after the digestion using non-cutter enzyme, the majority of the T_0 lines carrying the *1Ax1* and *bar* gene cassettes coincidently revealed a single hybridizing band. It is concluded that most of the integration events of gene cassettes involved the low copy number transgene insertions.

By the next generation, three T_1 plants were randomly chosen from each independent T_0 transformant for Southern blot analysis following digestion using a single cutter. Analysis of the individual T_1 progeny from each line allowed the patterns of inheritance of the HMW subunit and marker transgene to be determined. Table 2 summarizes the molecular analysis of T_1 segregants derived from each T_0 line and the corresponding T_0 line with gene cassettes. It was surprising to find that most of the T_1 lines carrying gene cassettes showed a single hybridizing band. In addition, each T_1 descendant of three T_0 lines named B12-2-3, B13-4-4, and B12-4-5, which were randomly chosen from the T_0 transgenic wheat lines carrying the *1Ax1* and *bar* gene cassettes, were further studied by Southern blotting analysis for the integration pattern of *1Ax1* gene cassettes (Fig. 4). Southern blotting of five T_1 progeny derived from the T_0 line named B12-2-3 indicated that the hybridizing band was absent in progeny 4, while the band was present in progeny 1, 2, 3, and 5. PCR analysis demonstrated that no *1Ax1* gene was inserted in progeny 4. The results of Southern blotting also showed that no *1Ax1* band was detected in one of seven T_1 descendants of the line B13-4-4, and this was confirmed by PCR analysis. It is suggested that lines B12-2-3 and B13-4-4 all contained a single-copy number HMW subunit *1Ax1* gene. Analysis of eight T_1 lines from line B12-4-5 showed no segregation of the *1Ax1* bands. It is concluded that the HMW subunit *1Ax1* gene cassette was inserted at a single locus in line B12-4-5. The data also indicated that the insertion of low copy number transgenes in plants occurred not only with the selective genes but also with the remarkable *gus* gene and HMW subunit *1Ax1* gene. As far as the plasmid transformation mentioned, the results of Southern blotting analysis revealed 6–10 hybridizing bands in most of the transgenic wheat plants (not shown).

Expression and inheritance of transgenes

Histochemical assays are routinely used to identify the expression of *gus* and *bar* genes. Table 1 shows the results of transgene expression in T_0 transgenic lines transformed with gene cassettes and plasmid(s). Subsequently, 97 T_1 transgenic lines carrying *gus* and *bar* gene cassettes, and 19 lines carrying *gus* and *bar* genes in a single plasmid were examined for the presence and expression of transgenes. Of the T_1 plants transformed with *gus* and *bar* gene cassettes, 50.5% were identified for the presence and expressions of two transgene cassettes. In the case of transformation using a single plasmid pAHC25, 26.3% transgenic wheat lines showed the presence and expression of *gus* and *bar* genes; 66.7% of T_1 transgenic plants carrying *gus* and *bar* gene cassettes showed the presence of the *gus* gene, but only 55.3% of those showed the expression of *gus* gene (Table 3).

The *gus* and *bar* transgenes were all under the control of the constitutive *ubiquitin* promoter from maize, a constitutive promoter which was demonstrated to have strong and stable expression in any tissues in several plant species. Here root, leaf, flower, inflorescence, and endosperm tissues were collected from transgenic plantlets, and all these tissues were found to produce the *gus* gene (Fig. 5B–E). A similarly strong signal of *gus* gene expression was observed in the tested tissues for transgenic plant lines carrying a gene cassette as those carrying the whole plasmid. It is clear that the exact expression of the *gus* gene was not influenced by removal of the vector backbone sequence. Leaves cut from each T_0 transgenic lines all showed resistance to herbicide solution, as shown in Fig. 5A.

Each of 85 T_1 progeny transformed with the *1Ax1* and *bar* gene in different gene cassettes was analysed for the presence and expression of transgenes by PCR and SDS–PAGE. Total protein fractions were separated by one-dimensional SDS–PAGE to resolve the HMW subunits from other seed proteins. Wild-type EM12 contains only the HMW glutenin subunits encoded by chromosome 1B and 1D, but no glutenin subunit encoded by chromosome 1A. Consequently, the additional HMW subunit encoded by the *1Ax1* gene cassette was obviously separated from the other endogenous subunits (Fig. 6). All of the transgenic lines were confirmed for the presences of *1Ax1* genes as indicated by the additional glutenin bands in the gel. Densitometric analysis of the gels showed that the total amount of HMW glutenin subunits as a mean percentage

Table 2. *Integration pattern of transgenes on Southern blot*

S, the number of hybridizing bands with labelled transgene probes in the Southern blot displaying genomic DNA following digestion by a single-cutter enzyme; N, the number of hybridizing bands with labelled transgene probes in the Southern blot displaying genomic DNA following digestion by a non-cutter enzyme; ND, not determined. A–C, the three plants were randomly selected from the T_1 lines.

T_0 independent lines	Hybridized bands of labelled *gus* or *1Ax1* probes				Hybridization of labelled *bar* probes			
	T_0 lines (S/N)	T_1 lines			T_0 lines (S/N)	T_1 lines		
		A	B	C		A	B	C
B1-2-3[a]	3/1	2	3	2	2/2	ND	2	1
B1-3-4[a]	2/1	2	2	2	3/2	3	1	1
B1-5-7[a]	2/1	2	ND	2	3/1	3	3	2
B2-4-3[a]	1/1	1	1	1	1/1	1	1	1
B3-2-2[a]	2/2	2	1	1	1/1	1	ND	1
B12-2-1[b]	2/1	2	2	1	2/1	2	2	2
B12-2-3[b]	1/1	1	1	ND	3/2	3	2	3
B12-4-5[b]	3/1	3	3	3	1/1	1	1	1
B13-1-3[b]	4/1	2	4	2	1/1	1	ND	1
B13-2-5[b]	3/1	3	2	1	2/2	1	2	2
B13-4-4[b]	2/1	1	2	1	3/2	3	3	3

[a] Produced following bombardment with *gus* and *bar* gene cassettes.
[b] Produced following bombardment with *1Ax1* and *bar* gene cassettes.

Table 3. *Expression and inheritance of transgene in T_1 progeny*

T_1 progeny are derived from the self-fertilization of the T_0 plants found to have the presence of different transgenes.

T_0 independent lines	Integration of gene confirmed by PCR (*gus* or *1Ax1*:*bar*)						Expresssion of gene examined by histochemical assay or SDS–PAGE (GUS or 1Ax1:BASTA)			
	+:+	+:–	–:+	–:–	χ^{2c}	*P*-value	+:+	+:–	–:+	–:–
B1-2-3[a]	16	5	5	1	0.325	>0.95	16	5	5	1
B1-3-4[a]	5	2	2	0	1.469	>0.25	5	2	2	0
B1-5-7[a]	10	3	3	1	3.183	>0.10	10	3	3	1
B2-4-3[a]	12	5	5	2	0.444	>0.75	12	5	5	2
B3-2-2[a]	6	3	3	1	0.538	>0.90	6	3	3	1
B12-2-1[b]	8	3	3	2	4.444	>0.05	6	3	3	4
B12-2-3[b]	5	2	2	0	1.469	>0.25	5	2	2	0
B12-4-5[b]	7	2	3	1	10.111	>0.005	6	2	1	3
B13-1-3[b]	4	2	4	1	0.152	>0.90	3	2	2	4
B13-2-5[b]	14	4	5	2	0.244	>0.5	10	2	8	5
B13-4-4[b]	5	2	3	1	0.798	>0.9	5	2	3	1

[a] Produced following bombardment with *gus* and *bar* gene cassettes.
[b] Produced following bombardment with *1Ax1* and *bar* gene cassettes.
[c] 9:3:3:1.

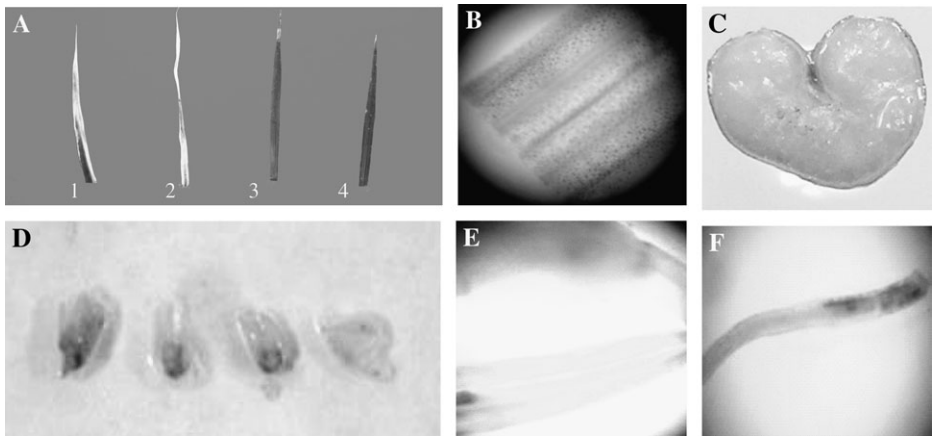

Fig. 5. (A) BASTA leaf painting assay: 1, 2, control leaves; 3, 4, BASTA resistance in the transgenic line B1-2-3 carrying *gus* and *bar* gene cassettes. (B–E) GUS expression in different tissues of the transgenic line B1-2-3: B, leaf; C, endosperm; D, inflorescence; E, flower; F, root.

of the total protein was increased from ~11% to 14% in transgenic lines, of which *1Ax1* transgene products accounted for ~2%.

It is essential that transgenes are stable and heritable over many generations for the successful application of transformation technology to the breeding programme of wheat and other plant species. From Table 3, it can be seen that all of the transgenic plants carrying gene cassettes were predominantly regenerated as simple integration patterns. The results of histochemical analysis and SDS–PAGE in transgenic plants confirmed the PCR and Southern blot data. The PCR-positive lines transformed with a single pAHC25 plasmid for the *gus* gene had no detectable GUS expression. In the transgenic lines co-transformed with pHMW1Ax1 and pAHC20, *1Ax1* and *bar* gene expression appeared to be correlated more closely with the PCR data.

Tissue-specific expression of the 1Ax1 *gene cassette*

In this investigation, transgenic wheat lines containing *1Ax1* and *bar* genes transferred in different gene cassettes were regenerated. The HMW glutenin subunit gene *1Ax1* cassette was under the control of the HMW glutenin subunit gene promoter, an endosperm-specific promoter. Twenty-one T_1 lines were randomly selected to determine the tissue-specific expression of the *1Ax1* gene promoter. Total RNA isolated from the seed, leaf, root, inflorescence, and embryo tissues was examined by RT–PCR analysis for the *1Ax1* transcripts in all the tested plants. The results showed that all 21 transgenic lines expressed the *1Ax1* gene only in the seeds. RT–PCR results of one line, a descendant of T_0 line B13-1-3, are shown in Fig. 7.

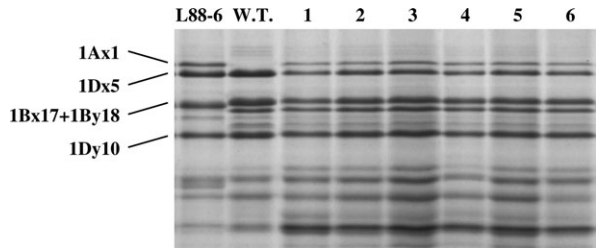

Fig. 6. SDS–PAGE analysis of endosperm proteins of T_1 seeds from six independent transgenic plants transformed with *1Ax1* and *bar* gene cassettes. L88-6, the positive standard wheat line containing subunits 1Ax1, 1Dx5, 1Bx17+1By18, and 1Dy10; L88-31, the negative standard wheat line containing subunits 1Bx17+1By18; W.T., wild type of EM12; 1, B12-2-1; 2, B12-2-3; 3, B12-4-5; 4, B13-1-3; 5, B13-2-5; 6, B13-4-4.

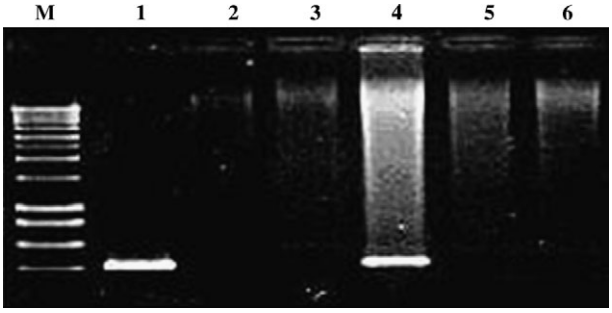

Fig. 7. RT–PCR analysis of RNA extracted from different tissues of one descendant of line B13-1-3. The *1Ax1* PCR product is a 450 bp fragment. M, molecular weight marker, 1, p1Ax1; 2, water control; 3, leaf; 4, endosperm; 5, root; 6, inflorescence.

Discussion

Transformation via particle bombardment is a favoured method in plant transformation, particularly in cereals. To address the transformation frequency problem encountered with wheat, genetic manipulation is necessary when applying the standard transformation procedure in elite wheat varieties. In the present study, when EM12 was first used as the experimental material, it was revealed that linear transgene cassettes lacking backbone sequence could be

integrated into the wheat genome as effectively as could circular plasmids, and a relatively little higher efficiency of transformation was also shown. This proves that removal of the vector backbone sequences had no significant influence on integration and inheritance of transgenes in the host genomic DNA. Due to the high transformation frequency of gene cassettes, it is suggested that the removal of the vector backbone sequence reduced the amount of concatemerization prior to transgene integration, which allowed genes to be integrated into plant genomes more efficiently than the whole plasmid. However, the frequency of EM12 transformation was relatively lower than that of the model variety. It was presumed that EM12 is sensitive to the agent L-PPT, or that an excessive concentration of the selection agent caused some successful transformants to escape from selection and the T_0 progeny to have one tiller. The selection procedure is critical for distinguishing the successful transformants among a large number of non-transformants. Increasing the concentration of the selection agent in the media eventually resulted in transgenic plants suffering from morphological, physiological, and fertility problems (He and Lazzeri, 1998). Therefore, there was a risk that transgenic plantlets would be killed in the selection stage. In the present study, primary transformants with either a gene cassette or whole plasmid(s) had few tillers, which shows that removal of the vector backbone had no effect on plant tillering. Furthermore, the T_1 generation recovered and grew back vigorously.

Instability of transgene expression in plants is often associated with multiple copies of transgenes being integrated at the same locus, as well as position effects due to random integration. Multicopy number integration may inhibit transgene expression and even lead to transgene silencing in transgenic plants. Moreover, large transgene loci can result in excision of the transgene locus and the undesirable loss of transgene expression in subsequent generations. It is reported that transgenes failed to be transmitted to the successive generations in wheat (Stoger *et al*., 1998) and tritordeum (Rooke *et al*., 1999). Cannell *et al*. (1998) even showed silencing or a gradual reduction in marker gene expression over three generations of transgenic wheat lines. A possible explanation for this is that very large transgenic loci may be meiotically unstable, which would account for the excision of the locus. Generally, when the single-cutter enzyme was used, the hybridizing bands in the Southern blot reflected the complexity of the transgene loci and copy number. In the present investigation, 6–10 hybridizing bands were predominantly observed in the transgenic plants transformed with whole plasmid(s), as reported before, reflecting the complex transgene integration patterns. A minimum of one band should be detected in any line, while other additional bands should be contributed by rearrangement. Transgenic plants were obtained using gene cassettes, and all of them showed 1–4 hybridizing bands. PCR and expression analyses also verified the simple integration and stable expression of gene cassettes in transgenic plants. Fu *et al*. (2000) found that 17 out of 22 of transgenic rice lines transformed with *bar* gene cassettes had only a single hybridizing band. They proposed that the removal of the vector backbone sequence decreased the high copy number integration events by limiting the amount of homologous recombination, and inhibited the effect of recombinogenic elements on the process of integration by offering fewer recombination hotspots. Zhao *et al* (2003) assumed that transgene recombination events happened during the course of integration of a transgene into the host genome. Agrawal *et al.* (2005) concluded that three possible elements, i.e. reducing the amount of concatemerization prior to transgene integration, limiting the occurrence of transgene rearrangements, and preventing homologous interactions between different transgenes during the integration events, worked together and therefore generated the simple and intact transgenic loci represented by simple hybridization patterns. However, it seemed more likely that the factors worked either independently or together to account for the simple transgene integration pattern and the high level of transgene expression. In these transformation experiments, the *bar* gene was used under the control of the cauliflower mosaic virus (CaMV) promoter 35S, in which there is a 19 bp palindromic sequence which could act as a recombination hot spot and lead to DNA rearrangement. In the present study, the selectable *bar* gene driven by the maize *ubiquitin* promoter was utilized, in which no hot spot has yet been identified. In this case, more simple copy number transgenic wheat lines were obtained with gene cassettes. Accordingly, it is possible to generate low copy number transgenic plants by eliminating the unnecessary exogenous sequence of the plasmid in bombardment-mediated transformation.

The commercial wheat variety EM12 was successfully engineered to biosynthesize the HMW glutenin subunit gene *1Ax1* carried on a gene cassette, and which resulted in an increase in the total amount of HMW subunit protein in the seeds of transgenic plants. Association between the number of expressed genes and quality has made the HMW subunits an attractive system for manipulation (Alvarez *et al*., 2000). It was shown here that the HMW glutenin subunit *1Ax1* gene cassettes were stably transmitted into progeny following Mendelian segregation ratios. The result also revealed that the *bar* gene was stable for three generations, showing Mendelian transmission ratios. It is clear that transformation with multiple gene cassettes was stable over three generations.

The HMW glutenin subunit gene *1Dx5* promoter has been widely used in wheat genetic manipulation. Much research supports the idea of endosperm-specific expression of HMW glutenin subunit gene promoters, except that Zhang (2001) observed expression of *gus* gene in roots, leaves, and pollen of transgenic barley, under the control of

the HMW glutenin subunit *1Dx5* gene promoter. This was presumably caused by genotype limitation. Generally, heterologous promoters do not maintain the activity found in the original species (Jones, 2005). Here the use of the *1Ax1* gene promoter isolated from wheat should make the expression of the foreign genes strong and stable in transgenic wheat plants.

This strategy will favour the frequency of single-copy insertions and efficient expression of the transgene in subsequent generations. Alhough *Agrobacterium*-mediated transformation was initially performed to obtain single-copy transgenic plants, the application to wheat and otherwise transformable plant species where DNA transformation is often far less efficient than that with model cultivars is mostly limited by the genotype of plant species (Jones, 2005). In the particle bombardment-mediated transformation, single-copy transgenic lines are desirable for a number of reasons, which permit simple structural documentation and potentially greater stability in gene structure and expression. The current approach of screening for single-copy transgenic plants is complicated, labour-intensive, and unpredictable (Srivastava, 1999). The strategy described here improved the frequency of single-copy transgenic lines and could avoid the generation of crosses for searching for introgression of the desired traits.

An important consideration with the current method is to remove directly the undesirable DNA fragment integrated into transgenic plants in the process of transformation. Recently, an increasing number of reports have revealed the presence of non-transgenic DNA in transgenic plants generated by either *Agrobacterium* or particle bombardment (Kohli *et al*., 2003). The interspersed non-transgenic DNA in the host genome is undesirable, and the conventional approach of deleting non-transgenic DNA in the host genome is more dependent on the speed and efficiency in testing the DNA sequence and the precise experimental design. The application of the transformation method described in the present study is a cost-saving step to obtain stable transgenic plants without the integration of non-transgenic DNA into the host genome. In conlusion, low copy number transgenic wheat was stably obtained with gene cassettes via particle bombardment at a relatively higher frequency. This technique improved the frequency of obtaining single-copy transgenic plants without vector backbone sequence via particle bombardment. It is proposed that the transformation procedure introduced in the current study will be a method routinely applicable in plant genetic manipulation.

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