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 T1 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 transcription–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...
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 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 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 selectable 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 HindIII and EcoRI, respectively. Plasmid pAHc20 included only the bar gene (Christensen and Quail, 1996). Plasmid pHMW1Ax1 included a 7.0 kb EcoRI genomic fragment including the complete coding sequence of the Glu-Al-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 EcoRI 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 T0 progeny to produce the T1 plant lines.
gus and bar expression analysis

The expression of the gus gene was examined histochemically using the substrate 5-Bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc) 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 leaf 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) 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′-AGTGTACGATTACCCGCGACATCATCAGCTGCGAAGACGAAAC-3′, annealing temperature 62 °C) and the bar gene (primer pair: 5′-GTGTGACATCTGCACTGCTACCC-3′, 5′-GAATGGTCGTCGCGGAACAC-3′, annealing temperature 57 °C) and the 1Ax1 gene (primer pair: 5′-GGTTGAGCCGCCGCTCCGAGAAGAAATC-3′, 5′-CGGGAAGTTGGGTTAGACCCCTGC-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 non-cutter enzyme. The single cutters comprised SacI (gus), SacI (1Ax1), and BamHI (bar), while the non-cutter enzymes comprised HindIII (gus), EcoRI (bar), and EcoRI (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 pAHHC25 (Fig. 1.1) was used in co-transformation, two independent transgenic wheat lines were co-transformation, two independent transgenic wheat
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 (T0). 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, T1 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 T0 and T1 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 T0 allowed the number of hybridizing bands to be estimated. All the individual T0 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 T0 lines showed the simple integration 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...
It is concluded that most of the integration events of gene cassettes involved the low copy number transgene insertions.

By the next generation, three T1 plants were randomly chosen from each independent T0 transformant for Southern blot analysis following digestion using a single cutter. Analysis of the individual T1 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 T1 segregants derived from each T0 line and the corresponding T0 line with gene cassettes. It was surprising to find that most of the T1 lines carrying gene cassettes showed a single hybridizing band. In addition, each T1 descendant of three T0 lines named B12-2-3, B13-4-4, and B13-2-5, which were randomly chosen from the T0 transgenic wheat lines carrying the IAx1 and bar gene cassettes, were further studied by Southern blotting analysis for the integration pattern of IAx1 gene cassettes (Fig. 4). Southern blotting of five T1 progeny derived from the T0 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 IAx1 gene was inserted in progeny 4. The results of Southern blotting also showed that no IAx1 band was detected in one of seven T1 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 IAx1 gene. Analysis of eight T1 lines from line B12-4-5 showed no segregation of the IAx1 bands. It is concluded that the HMW subunit IAx1 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 IAx1 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 T0 transgenic lines transformed with gene cassettes and plasmid(s). Subsequently, 97 T1 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 T1 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 T1 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 T0 transgenic lines all showed resistance to herbicide solution, as shown in Fig. 5A.

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

<table>
<thead>
<tr>
<th>T0 independent lines</th>
<th>Hybridized bands of labelled <em>gus</em> or <em>1Ax1</em> probes</th>
<th>Hybridized bands of labelled <em>bar</em> probes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T0 lines (S/N)</td>
<td>T1 lines</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>B1-2-3*</td>
<td>3/1</td>
<td>2</td>
</tr>
<tr>
<td>B1-3-4*</td>
<td>2/1</td>
<td>2</td>
</tr>
<tr>
<td>B1-5-7*</td>
<td>2/1</td>
<td>1</td>
</tr>
<tr>
<td>B2-4-3*</td>
<td>1/1</td>
<td>1</td>
</tr>
<tr>
<td>B3-2-2*</td>
<td>2/2</td>
<td>2</td>
</tr>
<tr>
<td>B12-2-1*</td>
<td>2/1</td>
<td>2</td>
</tr>
<tr>
<td>B12-2-3*</td>
<td>2/1</td>
<td>1</td>
</tr>
<tr>
<td>B12-4-5*</td>
<td>3/1</td>
<td>3</td>
</tr>
<tr>
<td>B13-1-3*</td>
<td>4/1</td>
<td>2</td>
</tr>
<tr>
<td>B13-2-5*</td>
<td>3/1</td>
<td>3</td>
</tr>
<tr>
<td>B13-4-4*</td>
<td>2/1</td>
<td>2</td>
</tr>
</tbody>
</table>

* Produced following bombardment with *gus* and *bar* gene cassettes.

### Table 3. Expression and inheritance of transgene in T1 progeny

<table>
<thead>
<tr>
<th>T0 independent lines</th>
<th>Integration of gene confirmed by PCR (<em>gus</em> or <em>1Ax1:bar</em>)</th>
<th>Expression of gene examined by histochemical assay or SDS–PAGE (<em>GUS</em> or <em>1Ax1:BASTA</em>)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>χ²  P-value</td>
<td>+: =: =: =: =: =:</td>
</tr>
<tr>
<td></td>
<td>+: +: =: =:</td>
<td>χ²  P-value</td>
</tr>
<tr>
<td>B1-2-3*</td>
<td>16 5 5 1 0.325 &lt;0.05</td>
<td>16 5 5 1 0.325 &lt;0.05</td>
</tr>
<tr>
<td>B1-3-4*</td>
<td>5 2 2 0 1.469 &gt;0.25</td>
<td>5 2 2 0 1.469 &gt;0.25</td>
</tr>
<tr>
<td>B1-5-7*</td>
<td>10 3 3 1 3.183 &gt;0.10</td>
<td>10 3 3 1 3.183 &gt;0.10</td>
</tr>
<tr>
<td>B2-4-3*</td>
<td>12 5 5 2 0.444 &gt;0.75</td>
<td>12 5 5 2 0.444 &gt;0.75</td>
</tr>
<tr>
<td>B3-2-2*</td>
<td>6 3 3 1 0.538 &gt;0.90</td>
<td>6 3 3 1 0.538 &gt;0.90</td>
</tr>
<tr>
<td>B12-2-1*</td>
<td>8 3 3 2 0.444 &gt;0.05</td>
<td>8 3 3 2 0.444 &gt;0.05</td>
</tr>
<tr>
<td>B12-2-3*</td>
<td>5 2 2 0 1.469 &gt;0.25</td>
<td>5 2 2 0 1.469 &gt;0.25</td>
</tr>
<tr>
<td>B12-4-5*</td>
<td>7 2 3 1 10.111 &lt;0.005</td>
<td>6 2 1 1 10.111 &lt;0.005</td>
</tr>
<tr>
<td>B13-1-3*</td>
<td>4 2 4 1 0.152 &gt;0.90</td>
<td>3 2 1 4 0.152 &gt;0.90</td>
</tr>
<tr>
<td>B13-2-5*</td>
<td>14 4 5 2 0.244 &gt;0.5</td>
<td>10 2 8 5 0.244 &gt;0.5</td>
</tr>
<tr>
<td>B13-4-4*</td>
<td>5 2 3 1 0.798 &gt;0.9</td>
<td>5 2 3 1 0.798 &gt;0.9</td>
</tr>
</tbody>
</table>

* Produced following bombardment with *gus* and *bar* gene cassettes.

b Produced following bombardment with *1Ax1* and *bar* gene cassettes.

c 9:3:3:1.
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 T1 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 T0 line B13-1-3, are shown in Fig. 7.

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 T0 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 T1 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 subunit 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. Although 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 crossovers for searching into 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 conclusion, 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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