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Original Article

Stable chloroplast transformation of immature scutella and inflorescences in wheat (*Triticum aestivum* L.)

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Chloroplast transformation in wheat was achieved by bombardment of scutella from immature embryos and immature inflorescences, respectively. A wheat chloroplast site-specific expression vector, pBAGNRK, was constructed by placing an expression cassette containing neomycin phosphotransferase II (*npt*II) and green fluorescent protein (*gfp*) as selection and reporter genes, respectively, in the intergenic spacer between *atpB* and *rbcL* of wheat chloroplast genome. Integration of *gfp* gene in the plastome was identified by polymerase chain reaction (PCR) analysis and Southern blotting using *gfp* gene as a probe. Expression of GFP protein was examined by western blot. Three positive transformants were obtained and the Southern blot of partial fragment of *atpB* and *rbcL* (targeting site) probes verified that one of them was homoplasmic. Stable expression of GFP fluorescence was confirmed by confocal microscopy in the leaf tissues from T_1 progeny seedlings. PCR analysis of *gfp* gene also confirmed the inheritance of transgene in the T_1 progeny. These results strengthen the feasibility of wheat chloroplast transformation and also give a novel method for the introduction of important agronomic traits in wheat through chloroplast transformation.

Keywords green fluorescent protein (*gfp*); site-specific integration; wheat

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Introduction

Chloroplast transformation offers several advantages over nuclear transformation. First, it improves gene containment due to the maternal inheritance of plastids, which incredibly reduces transgene transmission as compared with pollens [1,2]. Second, the gene expression position effect is eliminated because of the site-specific homologous recombination integration mechanism in the chloroplast genome [3]. Third, the co-expression of multiple genes is possible in the chloroplast as its translational machinery has the capacity to translate polycistronic transcripts [4–6]. And finally, there are nearly 100 chloroplasts in one leaf cell, each containing up to 100 plastome copies, and this multiploidy produces high expression levels of foreign genes [7,8]. Because of these remarkable advantages, the chloroplast transformation has been used for the production of pharmaceuticals in plants, modification of metabolic pathways, introduction of useful agronomic traits, etc. [9–11].

There have been a lot of efforts for chloroplast transformation in higher plants. First, successful chloroplast transformation was reported in *Chlamydomonas* [12]. Then chloroplast transformation has been extended to an increasing number of dicotyledonous plants, including important plant bioreactor for pharmaceutical proteins: tobacco [7,13–15]; the model plant species: *Arabidopsis thaliana* [16], petunia [17], and poplar tree [18]; species important in industrial economy: oilseed rape [19], *Lesquerella fendleri* [20], cotton [21], and sugar beet [22]; and edible crops: potato [23], tomato [24], carrot [25], soybean [26], lettuce [27,28], cauliflower [29], and cabbage [30]. Some preliminary studies have been made in chloroplast transformation of monocotyledonous species. Heteroplasmic and fertile transplastomic system in rice plant has been established [31]. Cell-type specific expression of fluorescent proteins was detected in the plastids of transgenic maize [32]. Also there is one report for transient expression of β-glucuronidase (*gus*) gene in the chloroplasts of wheat leaf and calli, but there is no report about stable integration events in wheat chloroplast transformation [33].

Wheat is an important cereal crop widely grown all over the world. Nuclear transformation in wheat has been

reported by many researchers [34–36]. The transformation and regeneration conditions were well established [37,38]. However, the stable transformation system for wheat chloroplasts has not been explored yet.

In the present paper, we reported the production of fertile, homoplasmic, chloroplast-transformed lines in wheat using a transformation vector that targeted the *atpB*/*rbcL* region of the wheat chloroplast genome. Stable expression of GFP fluorescence in the leaf tissues was confirmed by confocal microscopy from T_1 progeny seedlings. The successful transformation of chloroplasts offered the potential to introduce the functional genes for improving agronomic traits in wheat.

Materials and Methods

Plant materials

Triticum aestivum L. cv. Bobwhite, a model wheat cultivar often used in nuclear transformation was selected as an experimental material in present study. Wheat lines were sown in field in October and flowered at the beginning of the following April. The inflorescence was located above the final node of the tiller, and the immature inflorescence was usually at the right stage when there were three nodes and the space between the upper two was ~1 cm. After sterilization of the stem sections, the inflorescences were cut into transverse sections of ~1 mm or less and plated within the central area on medium in Petri dishes. Spikes were harvested 13–15 days post-anthesis and scutella were aseptically isolated from the immature embryos. Both immature inflorescences and scutella were used as the bombardment targets.

Construction of transformation plasmid

The chloroplast transformation vector, pBAGNRK, was constructed by placing the expression cassettes containing the green fluorescent protein gene (*gfp*) and the neomycin phosphotransferase II gene (*nptII*) in the intergenic spacer between the *atpB* and *rbcL* genes (which are located in the large single copy region of the wheat chloroplast genome, LSC). The chloroplast genome of *T. aestivum* (GenBank Accession No. AB042240) was used to design the primers. Chloroplast genome DNA was isolated to use as a template for polymerase chain reaction (PCR). A 1.5 kb fragment containing *rbcL* gene was amplified from wheat chloroplast DNA using the primers RBCLF (5′-CCG**CTCGAGAG**GGACTTATGTCACCA CAAACAGAAAC-3′) and RBCLR (5′-CGG**GGTACCGGATCC**CTCTTCTTTATCTTTAGTTTTATCTACTT-3′). The *Xho*I restriction site was added at the left end and *Bam*HI and *Kpn*I sites were introduced at the right end of the *rbcL* PCR product (restriction sites are shown in bold in the primer sequences). The PCR fragment was sequenced, digested with *Xho*I/*Kpn*I, and cloned into the pBluescript SK plus plasmid to obtain the pBRK intermediate plasmid. The 1.0 kb *atpB* gene fragment was amplified from the wheat chloroplast DNA using the primers ATPF (5′-GC**GAGCTCGGATCC**ACATTGCTAAAGCTCATGGGG-3′) ATPR (5′-ATAAGAAT**GCGGCCGC**TGCGATTTGTTCTCCTCTTCTAA-3′). The *Sac*I and *Bam*HI restriction sites were introduced at the left end and *Not*I site was added at the right end of the *atpB* PCR product (restriction sites are shown in bold in the primer sequences). The PCR fragment was sequenced, digested with *Sac*I/*Not*I, and cloned into vector pBRK to obtain the plasmid pBARK. The *gfp* gene sequence was amplified from the pAct1IsGFP-1 plasmid using primers GFPF (5′-CCC**AAGCTT**ATGGTGAGCAAGGGCGAGGAGCT GTT-3′) and GFPR (5′-ACGC**GTCGAC**CTTGTACAGC TCGTCCATGCCGAGAGT-3′), which added *Hin*dIII and *Sal*I restriction sites (shown in bold) at the left end and right end of the PCR product, respectively. The PCR product was sequenced and digested with *Hin*dIII/*Sal*I, and cloned into the p16ST plasmid, which was modified from pSB1 [22], containing the 16S ribosomal RNA promoter (*Prrn*), and the 3′ untraslated region (UTR) of the chloroplast *psb*A gene terminator to obtain the *gfp* gene expression cassette of *Prrn–gfp–TpsbA3′*. The *gfp* cassette was digested with *Not*I/*Pst*I and cloned into the pBARK plasmid to obtain pBAGRK plasmid. The *nptII* gene sequence was amplified from the pBI121 plasmid using the primers NPTF (5′-GCT**AAGCTT**ATGATTGAACAAGATGGA-3′) and NPTR (5′-GCG**GTCGAC**GAAGAACTCGTCAAGAAG-3′), which added *Hin*dIII and *Sal*I restriction sites (shown in bold) at the left end and right end of the PCR product, respectively. The *nptII* PCR fragment was sequenced and digested with *Hin*dIII/*Sal*I and cloned into the p16ST plasmid to obtain the *nptII* gene expression cassette of *Prrn–nptII–TpsbA3′*. The *nptII* cassette was digested with *Eco*RI/*Xho*I and cloned into the pBAGRK plasmid to obtain the final transformation plasmid pBAGNRK (**Fig. 1**).

Transformation and selection of transplastomic plants

Fifty to fifty-five scutella were circularly arranged in a circle of 2 cm diameter in 9 cm Petri dishes [Fig. 2(A)] and were bombarded with gold particles of 0.6 μm in diameter coated with pBAGNRK plasmid, using a PDS 1000/He particle gun (Bio-Rad, Richmond, USA). One milligram of gold particles (Bio-Rad) was washed in ethanol by sonication for three times and then twice with sterile water, and resuspended in 50 μl sterile water by sonication. Twenty microliters of 0.1 M spermidine and 50 μl of 2.5 M $CaCl_2$ solution were added into the particle suspension and mixed. Four and a half micrograms of plasmid pBAGNRK in 20 μl sterile water were added into the mixture. Then the mixture was distributed in seven microcarriers evenly for each shot. A pressure of 8.96×106 Pa and a vacuum of 28″ mercury

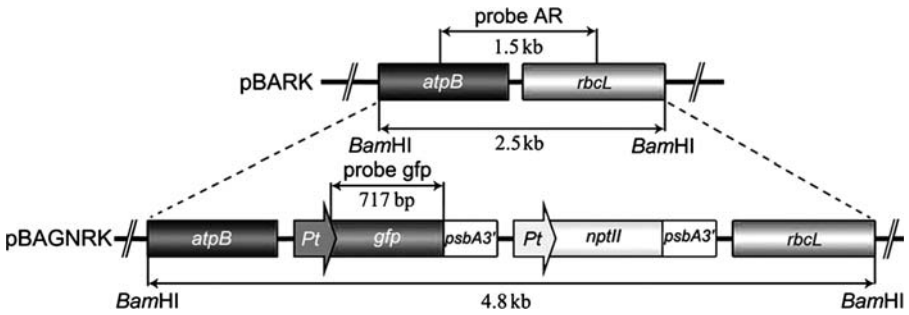

Figure 1 Map of the wheat chloroplast transformation vector Schematic map (not drawn to scale) of the atpB/rbcL region of the wheat chloroplast genome were used to construct the pBARK vector targeting the transgene cassette to this region. The Pt in pBAGNRK indicated the 16S ribosomal RNA promoter (Prrn) from tobacco chloroplast genome. PsbA3′ indicated the 3′ UTR of the psbA gene from tobacco chloroplast genome. Probe AR flanking the expression cassettes and probe gfp corresponding to the gfp gene used for Southern blot analysis were shown in pBARK and pBAGNRK; 1.5 kb and 717 bp showed the length of probe AR and probe gfp, respectively. The predicted hybridizing fragments (4.8 and 2.5 kb) were indicated when chloroplast DNA digested with *Bam*HI is probed with probe AR. Probe gfp detected a 4.8 kb fragment.

were applied, and the target distance was 9 cm. Thirty to forty sections of immature inflorescences were cut into 1 mm length, placed in one dish and were bombarded with plasmid-coated gold particles, following the same procedure as described above.

After bombardment, the scutella were cultured at 25°C in darkness for 3–4 weeks on callus induction medium (MSSP2), which contained the Murachige and Skoog (MS) salts and vitamins [39], supplemented with sucrose (90 g/l), agar (8 g/l), and picloram (2 mg/l). Then, the embryogenic calli were cultured on shoot regeneration medium (R_{ZD}) [40], which contained maltose (30 g/l), agar (8 g/l), Zeatin (5 mg/l), and 2,4-dichlorophenoxy acetic acid (2,4-D) (0.1 mg/l). Three or four weeks later, when the young shoots were transferred to the selective medium (R_{ZDS}), which contained R_{ZD} medium supplemented with 30, 40, and 50 mg/l of G418 in first, second, and third round selection cultures, respectively. The inflorescences were cultured on callus induction medium (L7-V) [40], supplemented with maltose (30 g/l), agar (8 g/l), and 2,4-D (2 mg/l). The regeneration and selection media were same as used in scutella culture. Scutella and immature inflorescences were recovered at 25°C in the 12 h/12 h light/dark during regeneration and selection phases. G418 was used as a selection agent, and concentration and selection procedure was adopted from the protocol developed by Sparks and Jones [41]. Juvenile plants that survived selection were transferred to soil and grown to maturity in greenhouse. The seeds were gathered from self-pollinated T_0 plants independently.

T_1 plant lines were obtained to detect GFP fluorescence and T_1 progeny PCR analysis. The seeds were germinated at 25°C in the 12 h light/12 h dark on MS medium supplemented with agar (8 g/l) and G418 (50 mg/l).

DNA analysis

Wheat chloroplast DNA was extracted from the leaf tissues of both the transformed and untransformed plants, using the Plant Chloroplast DNA Isolation Kit (Genmed Scientific Inc., Arlington, USA). The *gfp* and *nptII* genes were amplified using the primers GFPF/GFPR and NPTF/NPTR. When the transformants were confirmed for the presence of *gfp* and *nptII* genes, chloroplast DNA of the putative positive transformants was extracted to perform Southern blot. Fifteen micrograms of chloroplast DNA was completely digested by *Bam*HI and electrophoresed on a 0.8% agarose gel at 20 V for 20 h. The separated DNA was then transferred to Hybond-N^+ nylon membranes (Roche, Mannheim, Germany) using the method of Sambrook *et al.* [42]. These blots were hybridized with DIG-labeled probe gfp and probe AR (**Fig. 1**), which were generated by random prime label reaction. Labeling, hybridization, and chemiluminescence detection were carried out as described in the User's Guide for DIG High Prime DNA Labeling and Detection Starter Kit II (Roche). The membrane was auto-radiographed using X-ray film.

Western blot analysis

Total cell proteins were extracted from the leaves of the transformed and untransformed plants. The leaves were grinded in liquid nitrogen and homogenized in protein extraction buffer (0.1 M Tris–HCl, pH 8.0, 0.5 M NaCl, 5 mM dithiothreitol, 5 mM ethylene diamine tetraacetic acid, 0.2% Triton X-100, 2 mM phenylmethyl sulfonyl-fluoride, 1× proteinase inhibitor). After centrifugation at 4°C for 20 min at 24,000 *g*, the soluble protein extract was collected and electrophoresed on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel, then transferred to nitrocellulose membranes for immunoblotting. Monoclonal anti-GFP (Sigma, St. Louis, USA) was used as a primary antibody at a dilution of 1:500, and we used alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma) as a secondary antibody, at a dilution of 1:10,000. The SuperSignal West Pico chemiluminescent substrate

(Thermo Scientific, Waltham, USA) was used for detection.

Detection of GFP fluorescence

Leaf tissues of 10-day-old seedlings from T_1 progeny were used as slice samples for visualization. GFP fluorescence was monitored with Leica TCS SP5 confocal laser scanning microscopy. GFP fluorescence was collected in the range 510–532 nm and chlorophyll autofluorescence from 620 to 720 nm.

Results

Chloroplast expression plasmid

The wheat site-specific chloroplast integration vector construct (**Fig. 1**) targets the insertion of the two expression cassettes to the LSC region of chloroplast genome.

In the previous studies on chloroplast transformation, the insertion sites of foreign genes included the *rbcL–accD* [14,22,28], *rps7–ndhB* [19], *16S/trnI–trnA/23S* [21,31], *rrn16–rps12* [22], etc. However, there is no *accD* gene in wheat chloroplast genome and the site of the *atpB* gene and *rbcL* is similar to the *rbcL–accD*. The other gene pairs as described above are either too short or not adjacent to each other in the chloroplast genome [43,44]. Therefore, we chose *atpB–rbcL* intergenic region for insertion of foreign genes.

We chose two homologous genes, *atpB* and *rbcL*, both having length of 1.0–1.5 kb. Results of sequencing of PCR-amplified products of these two genes showed that they shared 100% homology to the corresponding sequences of wheat chloroplast DNA reported in the public databases. The synthetic *gfp* coding sequence with a threonine substituted for the serine in wild-type GFP at amino acid 65 (S65T) has been extensively used as a reporter gene [45–47]. The *nptII* gene encodes a neomycin phosphotransferase II, so that only cells with the *nptII* gene insertion in the chloroplast genome can be selected for resistance to Geneticin (G418) [48,49]. The selection gene *nptII* and reporter gene *gfp* were both driven by the strong plastid rRNA promoter, *Prrn*, which was isolated from tobacco chloroplast genome [14].

Regeneration of transplastomic plants

Chloroplast transformation was carried out by the bombardment of the scutella and immature inflorescences with the plasmid pBAGNRK. After bombardment, the total scutella and immature inflorescences were divided into groups each with 14 on the MSSP2 in a 9 cm Petri dish, and cultured at 25°C in the darkness for ~4 weeks to induce callus [**Fig. 2(B)**]. The callus induction was followed by differentiation on R_{ZD} medium at 25°C in the 12 h light/12 h darkness for 3–4 weeks [**Fig. 2(C)**]. The young shoots were subjected to three rounds of selection on R_{ZDS} medium [**Fig. 2(D)**]. Then, the well-developed green plantlets were cultivated in soil in greenhouse to maturity [**Fig. 2(E,F)**]. Following the biolistic procedure, we can obtain the putative transgenic plants in 5–7 months.

In six independent transformation bombardments, ~2500 scutella were used (on 40–55 individual bombarded

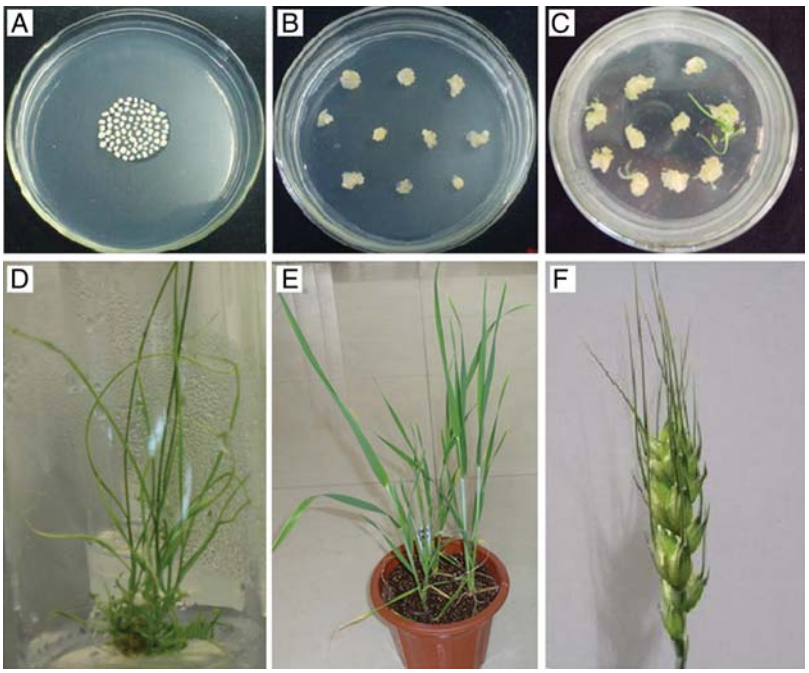

Figure 2 Regeneration of the transplastomic wheat plants (A) The scutella of donor wheat on target plate. (B) The callus induced from wheat scutella. (C) The cultures after 2–3 weeks on regeneration medium. (D) The cultures after three rounds on regeneration medium. (E) Plantlets cultured in the soil. (F) The spike of regenerated plant.

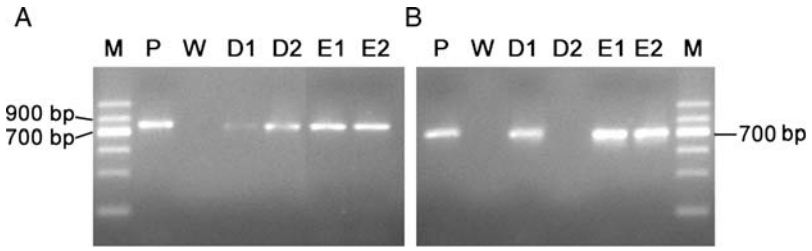

Figure 3 PCR analysis of transgene integration into wheat chloroplast genome PCR-amplified products from transplastomic plants were analyzed on a 1.0% agarose gel by electrophoresis. (A) Analysis of the *nptII* gene in transformed wheat plants using primers NPTF and NPTR yielded a 800 bp PCR fragment. M, DNA marker; P, plasmid pBAGNRK; W, wild-type plant; D1, D2, E1, and E2, transplastomic wheat plants. (B) Analysis of the *gfp* gene in transformed wheat plants using primers GFPF and GFPR showed ~717 bp PCR fragment. M, DNA marker; P, plasmid pBAGNRK; W, wild-type plant; D1, D2, E1, and E2, transplastomic wheat plants.

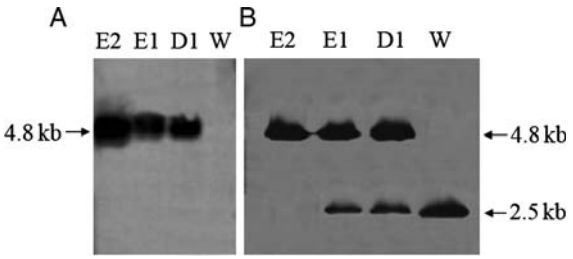

Figure 4 Southern blot analysis of three wheat transplastomic plants (A) The probe gfp was used in the hybridization, resulting in a 4.8 kb hybridization signal in D1, E1, E2 as expected, and the wild-type plant (W) has no signal. (B) The probe AR was used in the hybridization, resulting in a 2.5 kb hybridization signal from wild-type plants (W) and a 4.8 kb hybridization signal from transplastomic plants. The results indicated that the E2 is homoloplasmic and D1 and E1 are heteroplasmic.

dishes). Sixty-two independent plantlets survived after selection and were transplanted to soil and grown in greenhouse. In two independent transformation bombardments, ~600 immature inflorescences sections were bombarded and 10 plants were obtained after selection.

Analysis of transplastomic plants

The 72 putative transgenic plantlets were first tested via PCR for the presence of the *gfp* and *nptII* genes, using chloroplast DNA extracted from transformants as templates. One plant regenerated from immature inflorescences harbored both the *gfp* and *nptII* genes, and one only harbored *nptII* gene, and were named D1 and D2, respectively. Two plants obtained from immature embryos were found to have both *nptII* and *gfp* gene fragments [**Fig. 3(A,B)**], which were named as E1 and E2, respectively. Therefore, we used D1, E1, and E2 to analyze the integration of the *gfp* gene into the chloroplast genome in the following Southern blotting.

Southern blot analysis using the *gfp* probe detected a 4.8 kb fragment in the chloroplast DNA, digested with *Bam*HI [**Fig. 4(A)**]. In order to identify the integration of *gfp* and *nptII* gene expression cassettes in the chloroplast genome in the putative transformants, the probe AR was used in the Southern blot analysis of chloroplast DNA, again digested with *Bam*HI. There was only one 4.8 kb

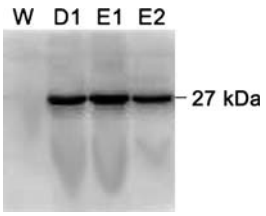

Figure 5 Western blot analysis of GFP expression in leaves Total soluble protein extracted from leaves was detected by immunoblotting. W, wild-type plants; D1, E1, and E2 indicate transplastomic plants respectively.

fragment in E2 sample which indicated homoplasmy. Whereas the D1 and E1 samples demonstrated heteroplasmy as there was another 2.5 kb fragment that only expected to appear in the wild-type wheat chloroplast DNA [**Fig. 4(B)**].

The expression level of *gfp* gene in transplastomic plants was examined by immunoblotting using total soluble protein extracted from the leaves and GFP monoclonal antibody. A 27 kDa GFP protein band was recognized in D1, E1, and E2. No GFP protein was detected in the wild-type control sample W (**Fig. 5**). The results demonstrate that the *gfp* gene was expressed after its integration into the chloroplast genome.

In order to examine the transgene expression and transmission in T_1 progeny, the seeds obtained from T_0 plants were genimated on MS medium with G418 selection. The leaves of 10-day-old green shoots were used for slice sample and were subjected to GFP fluorescence detection. Confocal laser scanning microscopic analysis confirmed the intense expression of GFP in mesophyll cells in leaves [**Fig. 6(A–C)**]. No GFP fluorescence was observed in the leaves from the wild-type wheat [**Fig. 6(D–F)**]. We also performed PCR analysis of the T_1 progeny. Primers GFPF/GFPR were used to detect the presence of *gfp* gene (**Fig. 7**). The GFP fluorescence and PCR results showed that the foreign gene was inherited by the progeny.

Discussion

In this study, we reported the stable transformation of wheat chloroplast using the biolistic technique, resulting in

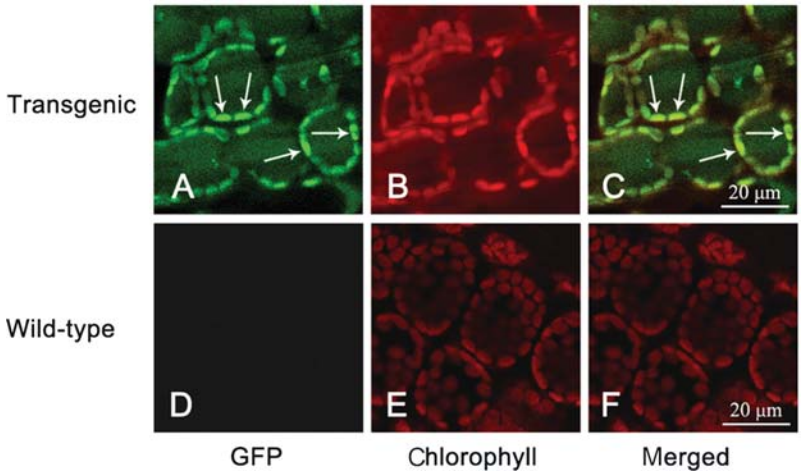

Figure 6 GFP fluorescence in chloroplasts of the wheat leaves Chloroplasts of mesophyll cells from transplastomic leaf show intense fluorescence in transgenic plants. The green GFP fluorescence (A) and the red chlorophyll autofluorescence (B) are shown separately and merged in (C). In panel A, the white arrows indicate chloroplasts. Whereas the wild-type wheat leaves showed only background level of autofluorescence (D–F).

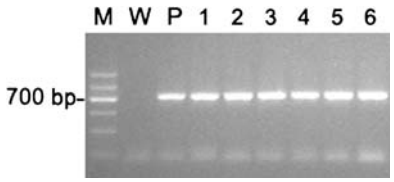

Figure 7 PCR analysis of chloroplast DNA extracted from leaves of T_1 progeny for the transgene *gfp* gene product (717 bp) was identified. M, DNA marker; P, plasmid pBAGNRK, positive control; W, wild-type plant; 1–6, transgenic lines.

one homoplasmic and two heteroplasmic transgenic plants. The fertile transplastomic wheat plants were obtained and the *gfp* gene was inherited by the T_1 progeny. Although the efficiency was low, this transformation system would be a novel tool for engineering the wheat plastome and creation of valuable biotechnological traits.

The vector used for wheat chloroplast transformation was constructed in our laboratory. GFP has been extensively used as a reporter marker for visualization of gene expression in both nuclear and chloroplast transformation in plants [46,47,50,51]. Till now, *gus* gene was only used in transient chloroplast transformation in anther derived albino wheat plants, wheat leaves, and regenerable calli derived from immature embryos [33]. GUS expression can be observed after 3 days of bombardment, but the tissues suffered cleavage of glucuronic acid from the substrate X-gluc to produce an insoluble indigo dye. Hence, *gfp* was chosen as reporter gene for its direct visualization of gene expression in individual cells without cell lysis and tissue distortion caused by fixation, staining, and sectioning [50]. Its subcellular localization and detection only needs a fluorescence microscope. Most cereal crops exhibit natural spectinomycin resistance, because of the presence of point mutations in their 16S rRNAs sequences [52,53]. In addition, the spectinomycin has been reported to hamper the transgenic callus regeneration in sugar beet chloroplast transformation [22]. Therefore, we could not consider *aadA* gene encoding aminoglycoside 3′-adenylyltransferase as the selection marker gene. The *nptII* gene has been widely used in the wheat nuclear transformation [36] and has also been applied in tobacco and cotton chloroplast transformation [21,48]. So, *nptII* gene was chosen as the selection gene in this study.

The target cell-type of explant tissues for bombardment is very important for successful regeneration and homoplasmy in transplastomic experiments. The leaf is the ideal tissue for expression of foreign genes in chloroplast in dicotyledons, such as tobacco [14], cotton [21], tomato [24], and potato [23]. Homoplasmic plants were successfully obtained from petioles in sugar beet through two additional rounds of selection [22]. Whereas there is no effective regeneration system for monocots' leaves and petioles yet, which is definitely hampering the development of chloroplast transformation in monocotyledonous cereal crops [31,33]. The calli from rice seed were bombarded for rice plastid transformation and resulted in heteroplasmic fertile plants [31]. In wheat, only two explant tissues, the scutella of immature embryos and immature inflorescences, were routinely used for nuclear transformation because of their easy *in vitro* regeneration and capability of obtaining fertile progeny [36]. There were no adult chloroplasts in the scutella of immature embryos; the protoplastids were spread in the scutella and could be bombarded. Therefore, the delivered foreign genes could be integrated into the genome of protoplastids along with the embryogenesis into adult chloroplasts [38].

In this study, the site-specific integration of *gfp* gene and the establishment of homoplasmic state were identified, in the transplastomic wheat. This is the first report about the

stable chloroplast transformation in wheat. Only one homoplasmic plant in T_0 generation was obtained from the scutella, and no homoplasmic but one heteroplasmic plant was regenerated from immature inflorescences. The transformation efficiency was estimated as 2 transplastomic plants per 42 bombarded plates of scutella and 1 transgenic line per 15 bombarded plates of immature inflorescences. It was comparable with that of sugar beet (1 transgenic line per 36 bombarded plates) and that of potato (3 transplastomic plants per 46 bombarded plates) [22,23]. But it was lower than that of tobacco (1–14 transgenic lines per bombarded plate) [1,14]. However, it remained difficult to estimate all reasons involved in the regeneration of a homoplasmic state. The bombardment parameters, the selection marker gene, selection pressure, and periods of selection all could be the possible essential factors. The homologous recombination between the two prrn regions and psbA3′ regions in the transformation vector might eliminate the *gfp* and *nptII* expression cassettes that had been integrated into the chloroplast genome, but there were very few relevant studies about this explanation. Alternatively, in order to achieve highly efficient regenerable positive plants, it needed to improve the selection pressure in the earlier stages of the targeted tissues. Or other more efficient monocot-specific selection genes should be introduced [31]. The explants for transformation might be developed further to the protoplasts or the calli induced from embryos or immature inflorescences. And highly efficient calli differentiation and shoot regeneration protocols would be useful to improve the transformation efficiency.

Chloroplast transformation in wheat was currently much more laborious and is less efficient than chloroplast transformation in tobacco and other plants. The procedure could be further optimized. Our present efforts aimed at making the chloroplast transformation system for wheat more efficient and introducing genes relevant to agronomical traits, such as tolerance to diseases and herbicides and resistance to salt and drought, into the chloroplast expression vector instead of *gfp* gene. This could be a novel approach for the development of molecular farming within chloroplasts in wheat.

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