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LETTER TO THE EDITOR

Biolistic Genetic Transformation of a Wide Range of Chinese Elite Wheat (*Triticum aestivum* L.) Varieties

Wheat (*Triticum aestivum* L.) is a major staple food crop worldwide. It is economically important because it can be grown in a wide range of climates and geographic regions, and it has made an enormous contribution to the increase in global food production over the past four decades (Dixon et al., 2009). Wheat is produced on more than 18% of the arable land in the world, and is the most cultivated crop after maize and rice (FAOSTAT data, 2014). Despite its global strategic significance, progress in genomic and genetic engineering research on wheat has lagged behind that on other major crops due to the difficulty of culturing tissues, and the complexity of its hexaploid genome. The first successful wheat transformation was achieved by particle bombardment (Vasil et al., 1992). Since then additional transgenic wheat plants have been obtained by various transformation methods (Harwood, 2011). Microprojectile bombardment is considered to be a promising method, since it is robust, versatile and relatively efficient in terms of gene delivery.

The prototype of gene transfer by particle bombardment is the transfer of GUS gene using tungsten projectiles (Krysiak et al., 1999), and this system has been gradually optimized (Li et al., 2012). However, for the purpose of transformation, wheat tissues are generally limited to immature wheat embryos or embryogenic calluses initiated from immature embryos, which can only be harvested from wheat plants growing in the field of greenhouse for a very short period. Very few reports have been published using mature embryos and embryogenic calluses initiated from mature embryos as explants (Li et al., 2012). The majority of studies have been confined to a few responsive varieties including "Bobwhite" (Zhou et al., 1995; Altpeter et al., 1996). Moreover, it may take a long time to transfer an interesting gene from model wheat to agronomically-acceptable germplasms by conventional breeding. We describe here the application of a highly efficient biolistic wheat transformation system to a diverse set of current Chinese elite wheat varieties. The system was optimized by screening for responsive tissue culture genotypes, and adjusting various factors influencing transformation efficiency.

We compared callus induction, regeneration capacity and stable transformation in immature embryos (IEs) and mature embryos (MEs) of 18 Chinese wheat varieties (Table S1). The average rate of induction of calluses from immature embryos, was 73.5 ± 3.9%, substantially higher than the 16.4 ± 1.1% from mature embryos (Table S1) ($P < 0.01$). For "Xiaoyan39", "Xiaoyan41", "Longchun23", "Kenong199", "Yangmai19", "Xiaoyan60" and "Zhengmai366", the callus induction efficiency from IEs could exceed 95%. Similarly, the average rate of plant regeneration from each immature embryo-derived callus, was 14.5 ± 1.3%, compared with 6.8 ± 0.6% from each mature embryo-derived callus.

About 600 IE/ME calluses (ten replicates of 60 calluses on average) and 250−600 IEs (five replicates of 50−120 IEs on average) from each variety were bombarded with plasmid pDM803 (Table 1 and Fig. 1A) (Gao et al., 2008). Three weeks after bombardment, all the calluses were transferred to regeneration medium, M1G supplemented with 5 mg/L PPT (Fig. 1B and C). Fully-recovered PPT-resistant shoots were transferred to rooting containers (rooting medium supplemented with the same concentration of selective agent as in the regeneration medium) for an additional 10−14 days (Fig. 1D). Putative transgenic plants were subsequently transferred to soil in the greenhouse (Fig. 1E). For winter wheat varieties, transgenic plantlets were vernalized for 2−3 weeks in containers before being transferred to soil and then grew to maturity.

We selected 10 of the 18 Chinese elite varieties and compared their stable transformation efficiencies when immature embryos, and calluses derived from immature and mature embryos were used as target explants bombarded with pDM803 (Table 1). As indicated in Table 1, IEs had the highest transformation efficiency (5.15 ± 0.62%) followed by IE calluses (2.26 ± 0.44%) and ME calluses (1.89 ± 0.22%). No significant differences were observed in stable transformation efficiencies between IE calluses and ME calluses ($P > 0.05$). For both "Baofeng104" and "Zhengmai366" , there was, exceptionally, no significant difference in transformation efficiency between any of the three explant sources.

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Table 1
Comparison of the stable transformation efficiencies of 10 Chinese varieties using immature embryos and calluses from immature and mature embryos as bombarded targets

Variety	IE		IE callus		ME callus	
	Number of transgenic lines/Number of IE bombarded	Mean* ± SE	Number of transgenic lines/Number of IE callus bombarded	Mean* ± SE	Number of transgenic lines/Number of ME callus bombarded	Mean* ± SE
Yangmai19	32/242	13.22a ± 1.10	4/284	1.41cd ± 0.21	NA	NA
Kenong199	70/608	11.51a ± 1.19	14/230	6.09b ± 1.35	10/332	3.01c ± 1.12
Longchun23	38/508	7.48b ± 0.67	49/1553	3.16c ± 0.35	23/812	2.83c ± 0.24
Xiaoyan60	19/442	4.30c ± 1.05	6/417	1.44cd ± 0.07	2/173	1.16cd ± 0.12
Xiaoyan39	10/241	4.15c ± 0.55	8/397	2.02c ± 0.40	6/383	1.57cd ± 0.06
Jing411	6/238	2.52c ± 0.00	5/333	1.50cd ± 0.10	NA	NA
Yangmai18	16/646	2.48c ± 0.46	5/226	2.21c ± 0.32	11/680	1.62cd ± 0.20
Xinong2611	10/442	2.26c ± 0.40	6/372	1.61cd ± 0.03	6/396	1.52cd ± 0.20
Baofeng104	5/372	1.34cd ± 0.30	6/593	1.01cd ± 0.21	4/408	0.98d ± 0.26
Zhengmai366	4/339	1.18cd ± 0.00	6/428	1.40cd ± 0.00	3/251	1.19cd ± 0.21
Mean ± SE	210/4078	5.15 ± 0.62	109/4833	2.26 ± 0.44	65/3435	1.89 ± 0.22

IE, immature embryos; ME, mature embryos; NA, not applicable. 80−120 scutella or 50−70 calluses were bombarded per plates. *Means followed by the same letter are not significantly different at the 5% level of significance.

Significant effects of genotype on stable transformation efficiencies were also detected ($P < 0.01$). IEs, IE calluses and ME calluses of "Kenong199" in each case regenerated the most herbicide (Basta) resistant plants. For "Yangmai19" and "Jing411", we obtained hardly any herbicide resistant plants from ME calluses.

In this experiment, significant genotypic effects on IE transformation efficiency ($P < 0.05$) were detected. The efficiencies ranged from 1.18 ± 0.00% to 13.22 ± 1.10%, with an overall efficiency of 5.15 ± 0.62%. The transformation efficiencies of cvs. "Yangmai19" and "Kenong199" exceeded 10%, with the highest efficiency (13.22%) obtained for "Yangmai19". The transformation efficiencies obtained in this study were comparable to, if not better than, the highest efficiencies previously reported for biolistic transformation of wheat (Zhang et al., 2000; Rasco-Gaunt et al., 2001).

One of the advantages of particle bombardment for engineering crop species is that vectors are not necessarily required for bombardment. Fu et al. (2000) devised a clean DNA strategy in which all the vector sequences were removed prior to particle loading. We therefore compared the effect of circular and linear plasmids on stable transformation efficiencies. Transgenic wheat plants were regenerated from immature embryos of cv. "Kenong199" transformed with either circular intact plasmid pAHC20 (Christensen and Quail, 1996) or the linear gene cassette pL-Bar (Materials and Methods). In total, 43 PPT-resistant plants were recovered from 420 IEs in the pAHC20 transformation, compared with 26 healthy plants from 906 IEs in the pL-Bar transformation. In our hands, the overall transformation efficiency with the intact plasmid (10.2 ± 0.9%) was significantly higher than that obtained with the linear cassette (2.9 ± 0.4%). In contrast, Yao et al. (2007) found that the stable transformation frequency of wheat was 1.1% with gene cassettes, compared with 0.4% using intact plasmids. In rice, on the other hand, Fu et al. (2000) and Breitler et al. (2002) found that microprojectile bombardment-mediated transformation using gene cassettes gave similar transformation efficiencies to using whole plasmid DNA. We assume that these variations in relative efficiencies are caused by different gold coating processes and other bombardment parameters that affect the efficiency of transformation.

To detect the presence of the *gus* gene, GUS transient activity was measured 3 days after bombarding immature embryos and calluses with pDM803 (Fig. 1F and G). GUS activity was also detected in segments of leaves sampled from PPT resistant plants (Fig. 1H). This stable GUS expression confirmed that the *gus* gene had integrated into the wheat genome.

Stable integration of the *bar* gene into the genome of putatively transformed plants was further analyzed by DNA gel blot hybridization. Genomic DNA from ten randomly selected putative transgenic T_0 plants with pAHC20 was digested with *Sph* I, which cuts once inside plasmid pAHC20 generating an internal 2.44-kb fragment containing the *bar* gene. DNA gel blot analysis confirmed the presence of the transgene and the independent nature of all the tested plants except for the product of event C5-05, in which *bar* was not detected (Fig. 1I). About half of the tested events yielded the expected 2.44-kb fragment in addition to other bands. Events C5-01 and C5-07 produced only one band, while the remaining events involved multiple integrations suggesting that 1 to 14 copies of the *bar* gene had integrated into the wheat genome. As in many other species, the biolistic transformation approach often results in an insertion of multiple transgene copies and

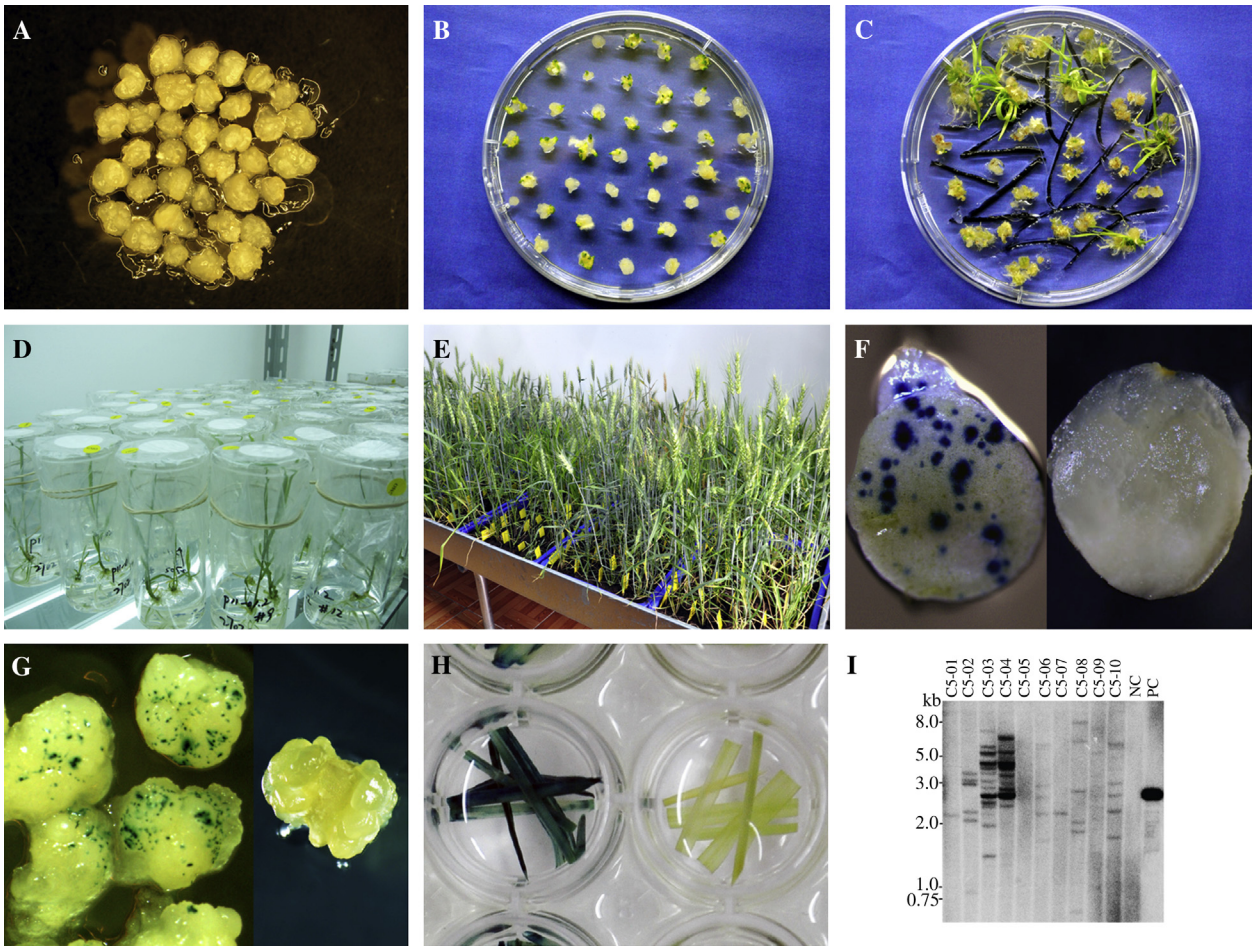

Fig. 1. Production of transgenic wheat plants (cv. "Kenong199") by microparticle bombardment.

A: Embryogenetic calluses of mature embryos on osmotic medium prior to bombardment. **B:** Shoots regenerated from calluses after 2 weeks on regeneration medium. **C:** Growth of shoots and roots after 4 weeks on regeneration medium. **D:** Plants surviving on rooting medium after two rounds of selection. **E:** Putative transgenic plants transferred into soil and grew in an artificial climate room. **F:** Immature embryos stained histochemically for GUS activity 24 h after bombardment with pDM803 (left) and without pDM803 as control (right). **G:** Wheat callus tissue cultured for 28 days from immature embryos exhibiting transient GUS activity 24 h after bombardment with pDM803 (left) and without pDM803 as control (right). **H:** Segments of leaves from a transgenic event showing stable GUS expression (left) and non-transformed plants as control (right). **I:** DNA gel blot analysis of pAHC20-transformed wheat plants. Genomic DNA was digested with *Sph* I and hybridized with a ^{32}P-labeled 562 bp *bar* probe. Lanes 1−10, pAHC20-transformed plants; NC, non-transgenic plant; PC, 100 pg pAHC20 plasmid digested with *Sph* I.

complex integration patterns (Dai et al., 2001; Shou et al., 2004; Travella et al., 2005). The different hybridization patterns obtained showed that the tested events were derived from independent transgenic events.

We examined phenotypic segregation to confirm expression of *bar* in the T_1 generation of 27 Basta-resistant T_0 events. Of these T_0 events, 51.9% (14/27) exhibited a 3:1 segregation ratio for expression of *bar* (Table S2), indicating that *bar* segregated as a single functional locus, though there could be more than one integrated copies. Approximately 14.8% (4/27) of the tested events gave a 15:1 segregation ratio, suggesting that segregation of *bar* occurred at two loci. In addition, 33.3% (9/27) of the T_0 events produced T_1 progeny with a non-Mendelian segregation ratio of 1:1, presumable due to aberrant gamete or seed formation (He et al., 2010). We obtained a higher frequency of 3:1 segregation ratios in the present data than did the previous analyses of transgene loci produced by wheat transformation *via* projectile bombardment (Jordan, 2000). Our results demonstrate that the herbicide trait in transgenic wheat plants is faithfully transmitted through successive generations following Mendelian rules.

In summary, the wheat transformation protocol presented here is reliable, reproducible and efficient, and the system has been used for producing transgenic wheat with improved quality traits, drought and cold tolerance and resistance to fungal diseases. During the past 5 years, we have produced nearly 20,000 independent transgenic wheat plants involving more than 200 genes of interest.

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SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jgg.2014.11.005.

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