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Establishment of a Bivector Genetic Transformation System in Recalcitrant Maize Inbred Lines

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Abstract: Maize is an important grain crop with high nutritional value. An effective transformation system is crucial for the genetic improvement of maize traits, but many important maize inbred lines remained recalcitrant to transformation. In this study, we developed a bivector transformation system that worked well in two recalcitrant maize inbred lines. This system included an induction vector (ZmBBM-ZmWUS) and an indicator vector (GFP), using microprojectile bombardment technology combined with *Agrobacterium*-mediated transformation. We found that the Zheng58 and Mo17 recalcitrant inbred lines could be transformed with this system. The whole transformation cycle lasted only 52 days, 38 days less than the traditional transformation cycle. Additionally, it was possible to eliminate inference of the induction vector and obtained progenies with only the target gene. Our results suggested that the bivector system was an optimization of the current maize transformation methods and could potentially be used in genetic improvement of maize inbred lines.

Keywords: maize; bivector system; genetic transformation; Zheng58; transformation cycle

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1. Introduction

Maize is the most heavily produced cereal crop in the world [1]. It has high nutritional value [2]. The exploration of genetic transformation has contributed to the genetic improvement of maize inbred lines. Thus, establishing an efficient genetic transformation system is important for the improvement of maize.

Successful genetic transformation depends on the traits and genotypes of the maize inbred lines [3]. It was found that the Hi-II line produced calli and regenerated somatic embryos from immature embryos [4,5]. The Hi-II hybrid line is then widely utilized in commercial maize genetic transformation [3,4,6]. The regenerated maize A188, W117, W59E, A554, W153R'H99, HKI 163 and Mo17 were also developed from immature embryos through *Agrobacterium tumefaciens*-mediated mechanisms [7–9]. However, many maize inbred lines remained recalcitrant to transformation, such as Zheng58 and Chang 7–2.

In the past few years, many important maize inbred lines have been transformed effectively with morphogenetic genes. *BABY BOOM (BBM)* is a morphogenetic gene that promotes cell proliferation and morphogenesis during embryogenesis [10]. *BBM* is widely employed to obtain transgenic plants from tobacco (*Nicotiana tabacum* L.) [11], sweet pepper (*C. annuum*) varieties [12], and *Theobroma cacao* [13]. *WUSCHEL (WUS)* is the central meristem identity of shoot and floral meristems and plays an important role in maintaining structural and functional integrity [14]. *WUS* is widely utilized to obtain transgenic plants from tobacco [15], *Coffea canephora* [16], and cotton (*Gossypium hirsutum* L.) [17]. Co-expression of *BBM/WUS* can successfully induce transgenic plants from the maize B73 inbred line [18], sorghum (*Sorghum bicolor*) immature embryos [19], sugarcane

(*Saccharum officinarum*) calli, and indica rice (*Oryza sativa* ssp *indica*) calli [20]. However, the strong expression of many morphogenetic genes often leads to the production of pleiotropic and harmful phenotypes, resulting in low fertility in transgenic T0 plants and subsequent offspring [21]. At present, effectively mitigating this situation is challenging.

This study reported the establishment of a bivector genetic transformation system for recalcitrant maize inbred lines. By employing *Agrobacterium*-mediated transformation and microprojectile bombardment technology, the bivector was transferred into immature embryos of recalcitrant maize inbred lines to successfully obtain transgenic plants. Our results showed that this system could successfully produce transgenic plants from the Zheng58 and Mo17 recalcitrant maize inbred lines with a significantly shorter transformation cycle. In addition, transgenic Zheng58 produced separated progenies without the existence of *ZmBBM-ZmWUS* morphogenetic genes.

2. Materials and Methods

2.1. Plant Materials

Four common inbred lines, namely, Zheng58, Mo17, Chang7-2 and B73, were maintained in the laboratory. After 14–16 days of pollination, when the young embryo grew to 1.8 mm–2.2 mm, the immature embryos of these inbred lines were harvested as acceptor materials for transformation. All plants were grown in the experimental field at the campus of Shanghai University.

2.2. Bivector Construction

The *BABY BOOM (BBM)*, *WUSCHEL* (*WUS*) and *Green fluorescent protein (GFP)* genes were amplified using gene-specific primers. The maize *Chitinase A1* promoter ($CTA1_{pro}$), *Nopaline synthase* promoter (NOS_{pro}), Auxin-inducible promoter ($Axig1_{pro}$) and *Phospholipid transferase protein* promoter ($PLTP_{pro}$) were reduplicated from the maize genome. The pHB vector was modified by our laboratory and stored long-term in our laboratory. The pHB vector contained the restriction sites *Eco*RI, *Hin*dIII, *Bam*HI, and *Xba*I. The pCAMBIA3301 vector contained the *Pst*I site. The bivector system contained an induction vector of pHB-ZmBBM-ZmWUS co-expression and an indicator vector of pCAMBIA3301-GFP (Table S1). The *$ZmCTA1_{pro}$::BBM* cassette was ligated into the pMD18T vector to form the pMD18T-CB (pMD18T-ZmCTA1$_{pro}$::BBM). Via the *Eco*RI and *Hin*dIII sites, the pMD18T-ZmCTA1$_{pro}$::BBM vector was digested and ligated into the pHB vector to form the pHB-CB (pHB-ZmCTA1$_{pro}$::BBM). The *NOS_{pro}::WUS* cassette was ligated into the pMD18T vector to form the pMD18T-NW (pMD18T-NOS$_{pro}$::WUS). Via the *Hin*dIII and *Bam*HI sites, the pMD18T-NW (pMD18T-NOS$_{pro}$::WUS) was digested and ligated into the pHB-CB vector to form the pHB-CB-NW (pHB-ZmCTA1$_{pro}$::BBM-NOS$_{pro}$::WUS). The *$Axig1_{pro}$::WUS* cassette was ligated into the pMD18T vector to form the pMD18T-AW (pMD18T-Axig1$_{pro}$::WUS). Via the *Hin*dIII and *Bam*HI sites, the pMD18T-AW (pMD18T-Axig1$_{pro}$::WUS) was digested and ligated into the pHB-CB vector to form the pHB-CB-AW (pHB-ZmCTA1$_{pro}$::BBM-Axig1$_{pro}$::WUS). The *$ZmPLTP_{pro}$::BBM* cassette was ligated into the pMD18T vector to form the pMD18T-PB (pMD18T-ZmPLTP$_{pro}$::BBM). Via the *Eco*RI and *Hin*dIII sites, the pMD18T-ZmPLTP$_{pro}$::BBM vector was digested and ligated into the pHB vector to form the pHB-PB (pHB-ZmPLTP$_{pro}$::BBM). Via the *Hin*dIII and *Xba*I sites, the pMD18T-AW (pMD18T-Axig1$_{pro}$::WUS) was digested and ligated into the pHB-PB vector to form the pHB-PB-AW (pHB-ZmPLTP$_{pro}$::BBM-Axig1$_{pro}$::WUS). The *GFP* gene was recombined between the 35S$_{pro}$ promoter and the RbsT terminator into the pCAMBIA3301 vector to form the pCA-GFP (pCA-35S$_{pro}$::GFP).

The linkage vectors contain *ZmBBM*, *ZmWUS* and *GFP* (Table S1). The pCA-GFP was recombined into the pHB-CB-NW (pHB-ZmCTA1$_{pro}$::BBM-NOS$_{pro}$::WUS) vector between the *ZmCTA1$_{pro}$::BBM* cassette and *NOS$_{pro}$::WUS* cassette to form the pHB-CB-NW-GFP vector (*ZmCTA1$_{pro}$::BBM-NOS$_{pro}$::WUS-35S$_{pro}$::GFP*). The pCA-GFP was recombined into the pHB-CB-AW (pHB-ZmCTA1$_{pro}$::BBM-Axig1$_{pro}$::WUS) vector between the *ZmCTA1$_{pro}$::BBM* cassette and *Axig1$_{pro}$::WUS* cassette to form the pHB-CB-AW-GFP vector (*ZmCTA1$_{pro}$::BBM-Axig1$_{pro}$::WUS-35S$_{pro}$::GFP*). The pCA-GFP was recombined into the pHB-PB-AW (pHB-ZmPLTP$_{pro}$::BBM-Axig1$_{pro}$::WUS) vector between the *ZmPLTP$_{pro}$::BBM* cassette and *Axig1$_{pro}$::WUS* cassette to form the pHB-PB-AW-GFP vector (*ZmPLTP$_{pro}$::BBM-Axig1$_{pro}$::WUS-35S$_{pro}$::GFP*). Information of the primers is provided in Table S2.

2.3. Microprojectile Bombardment Technology

Plasmid DNAs are coated on the surface of tungsten powder. Under high pressure, coated plasmids were accelerated at high speed into specific maize tissues.

The pHB-CB-NW, pHB-CB-AW and pHB-PB-AW vectors were transformed into immature embryos of four inbred lines by microprojectile bombardment technology, according to El-Itriby et al. [22].

2.4. Agrobacterium-Mediated Transformation

The pHB-CB-NW-GFP, pHB-CB-AW-GFP, pHB-PB-AW-GFP, and pCA-GFP vectors were transformed into immature embryos of four inbred lines by *Agrobacterium*-mediated transformation. *Agrobacterium*-mediated transformation of maize immature embryos was performed, as described by Frame et al. [23].

2.5. Culture Media and Conditions for the Plant Genetic Transformation System

Immature embryos of Zheng58, Mo17, Chang7-2, and B73 inbred lines were placed at 4 °C overnight before transformation. After dissecting immature embryos, the embryos were transferred to hypertonic medium and cultured at 25 °C for 3 h. After the immature embryos were bombarded with the microprojectile bombardment technology, the embryos were cultured in hypertonic cultivation medium at 25 °C for 3 h. The immature embryos were transferred to IM medium and washed twice with 1 mL fresh IM medium. One milliliter of *Agrobacterium tumefaciens* liquid-infected immature embryos was mixed well in the dark for 5 min. The young embryos were transferred to cultivation medium to remove the excess bacterial liquid, their scutellas were kept upright, they were blown dry for 38 min in the super clean platform, and they were sealed into plates and cultured in the dark at 19 °C for 3 days. The immature embryos on cultivation medium were transferred to resting cultivation medium in the dark at 28 °C for 7 days. When calli formation occurred, the calli were transferred to pre-regeneration cultivation medium and cultured in the dark at 25 °C for 14 days. The calli were transferred from pre-regeneration cultivation medium to dark regeneration cultivation medium and later sealed with breathable film at 25 °C. The somatic embryos were transferred to light regeneration medium, sealed with light regeneration, and placed in a light incubator (25 °C, light intensity 80–100 me/m^2/s, photoperiod 16:8). After somatic embryos grew into 3–5-cm seedlings, these embryos were placed in a light incubator with double distilled water.

After 2–3 days, the seedlings were transferred to small pots for culture (photoperiod 16:8, temperature 26 °C/22 °C (day/night), 350em-2s^{-1}); approximately 10–14 days later, the seedlings identified as positive were transferred to a large pot for culture and to await pollination. After pollination, the seeds of the T1 generation were harvested over 35–40 days and dried in an oven at 30 °C. Hypertonic medium formulation was derived from El-Itriby et al. [22]. All other medium formulations were as described by Frame et al. [24].

2.6. Genomic DNA Extraction and PCR Assay

The hexadecyltrimethylammonium bromide method was used for genomic DNA extraction in the maize plants [25]. Through PCR, we determined whether the transgenic plants were positive. The existence of pHB vectors was determined by amplifying *HPT*, and the pCA-GFP vector was directly and specifically amplified with specific primers targeting *GFP* (Table S2). In general, the gene was amplified with Taq mix in a PCR thermocycler. The PCR products were electrophoresed in a 1% agarose gel with ethidium bromide and imaged by the Gel Doc XRS system (Bio-Rad). The sizes and locations of specific gene bands were compared to determine the existence of the relevant genes.

2.7. Southern Blot Analyses

To determine if transgenes were stably integrated in transgenic plants, Southern blotting was used after preliminary PCR analysis of the plants. For Southern blot hybridization, genomic DNA was extracted, digested, gel-fractionated, and transferred to nylon membranes, as described previously [26]. In the preparation of the molecular probe, primers from the pCA-GFP vector were used to amplify a PCR product as the template to generate a digoxigenin-dUTP-labeled probe (Table S2). Hybridization and signal detection were subsequently performed, utilizing the PCR DIG Probe Synthesis Kit and the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Basel, Switzerland), according to the manufacturer's instructions.

2.8. RNA Extraction and RT-qPCR Analysis

Total RNA of the maize tissues was extracted with TRIzol reagent (Tiangen, Beijing, China), according to a previously described protocol [27], and DNA was removed through treatment with RNase-Free DNase I (Takara, Shiga, Japan). Two micrograms of total RNA was reverse-transcribed into complementary DNA using ReverTra Ace reverse transcriptase (Toyobo, Osaka City, Japan). RT-qPCR primers were designed using the NCBI program (Premier Biosoft, Palo Alto, CA, USA).

RT-qPCR experiments were performed on three independent RNA sample sets, with *Actin* serving as an internal control gene. RT-qPCR was performed with SYBR Green Real-Time PCR Master Mix (Toyobo) using a Mastercycler ep realplex 2 (Eppendorf, Framingham, MA, USA), according to the standard protocol [28]. Relative quantifiable differences in gene expression were analyzed, as described previously [29].

3. Results

3.1. Bivector Construction for Genetic Transformation of Recalcitrant Maize Inbred Lines

Due to the harmful pleiotropic effects of morphogenetic genes [21], we constructed a bivector system containing an induction vector (*ZmBBM-ZmWUS*) and an indicator vector (*GFP*) to eliminate the influence of morphogenetic *ZmBBM-ZmWUS* in progeny generations.

We employed the strategies of calli-specific expression of *ZmBBM* and general expression of *ZmWUS* to construct the induction vector (*ZmBBM-ZmWUS*). Two promoters were used to drive *ZmBBM*. We found that, compared with the maize calli-specific *Phospholipid transferase protein* gene (*ZmPLTP*) [30], the maize *Chitinase A1* gene (*ZmCTA1*) was also specifically expressed in calli but at a lower expression level (https://www.maizegdb.org/, accessed on 1 August 2019). Through reverse transcrition-quantitative PCR (RT-qPCR) analysis, we discovered that the *ZmCTA1* and *ZmPLTP* gene had the highest expression in the calli followed by the silk, anther, and other tissues (such as root, stem, leaf, ear, immature seed, and embryo tissues) (Figure 1a,b). Therefore, we used *ZmBBM* driven by the promoter of the *ZmCTA1* and *ZmPLTP* genes in the experimental group ($ZmCTA1_{pro}$*::BBM* and $ZmPLTP_{pro}$*::BBM*). Two promoters were used to drive *ZmWUS*. Compared with the maize auxin-inducible promoter ($Axig1_{pro}$) [31], the *Nopaline synthase* promoter (NOS_{pro}) [32] could produce a general expression without the induction of any hormone. Thus, we used *ZmWUS* driven by NOS_{pro} and $ZmAxig1_{pro}$ in the experimental group (NOS_{pro}*::WUS* and $ZmAxig1_{pro}$*::WUS*).

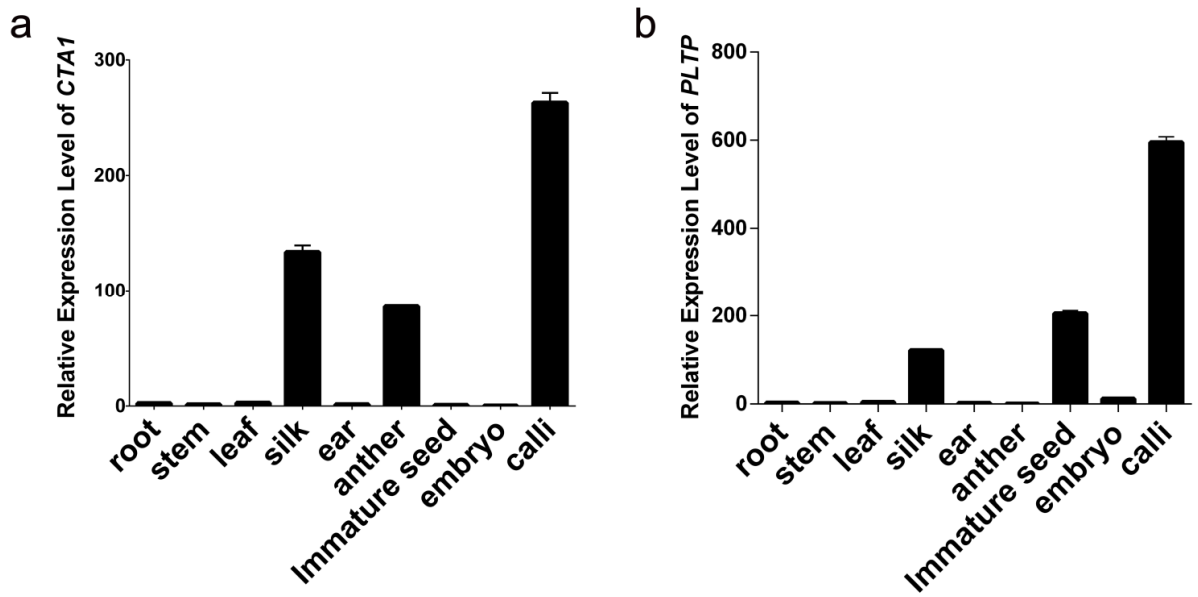

Figure 1. Gene expression level of *ZmCTA1* and *ZmPLTP* in various maize tissues. (**a**,**b**) RT-qPCR confirmed the expression level of *ZmCTA1* and *ZmPLTP* in the root, stem, leaf, silk, ear, anther, immature seed, embryo and calli of maize. *Actin* was used as an internal control. Date are the mean values with standard error of mean (SEM), $n = 3$ individuals.

The bivector system contained an induction vector of pHB-ZmBBM-ZmWUS co-expression and an indicator vector of pCAMBIA3301-GFP (Figure 2a–d). In the pHB-CB-NW vector, $ZmCTA1_{pro}$ drives *ZmBBM* and NOS_{pro} drives *ZmWUS* (*ZmCTA1*$_{pro}$*::BBM-NOS*$_{pro}$*::WUS*) (Figure 2a). In the pHB-CB-AW vector, $ZmCTA1_{pro}$ drives *ZmBBM*, and $ZmAxig1_{pro}$ drives *ZmWUS* (*ZmCTA1*$_{pro}$*::BBM-Axig1*$_{pro}$*::WUS*) (Figure 2b). In the pHB-PB-AW vector, $ZmPLTP_{pro}$ drives *ZmBBM* and $ZmAxig1_{pro}$ drives *ZmWUS* (*ZmPLTP*$_{pro}$*::BBM-Axig1*$_{pro}$*::WUS*) (Figure 2c). The reporting pCAMBIA3301 vector (pCA-GFP), including *Green fluorescent protein* (*GFP*) [33], is that the cauliflower mosaic virus 35S promoter ($35S_{pro}$) drives *GFP* (*35S*$_{pro}$*::GFP*) (Figure 2d).

We also constructed linkage pHB vectors expressing ZmBBM, ZmWUS, and GFP as the pHB-CB-NW-GFP vector (*ZmCTA1*$_{pro}$*::BBM-NOS*$_{pro}$*::WUS-35S*$_{pro}$*::GFP*) (Figure 2e), the pHB-CB-AW-GFP vector (*ZmCTA1*$_{pro}$*::BBM-Axig1*$_{pro}$*::WUS-35S*$_{pro}$*::GFP*) (Figure 2f), and the pHB-PB-AW-GFP vector (*ZmPLTP*$_{pro}$*::BBM-Axig1*$_{pro}$*::WUS-35S*$_{pro}$*::GFP*) (Figure 2g). These pHB-related vectors contain the *Hygromycin phosphotransferase gene* (*HPT*) [34] as the selectable marker gene of positive transgenic plants.

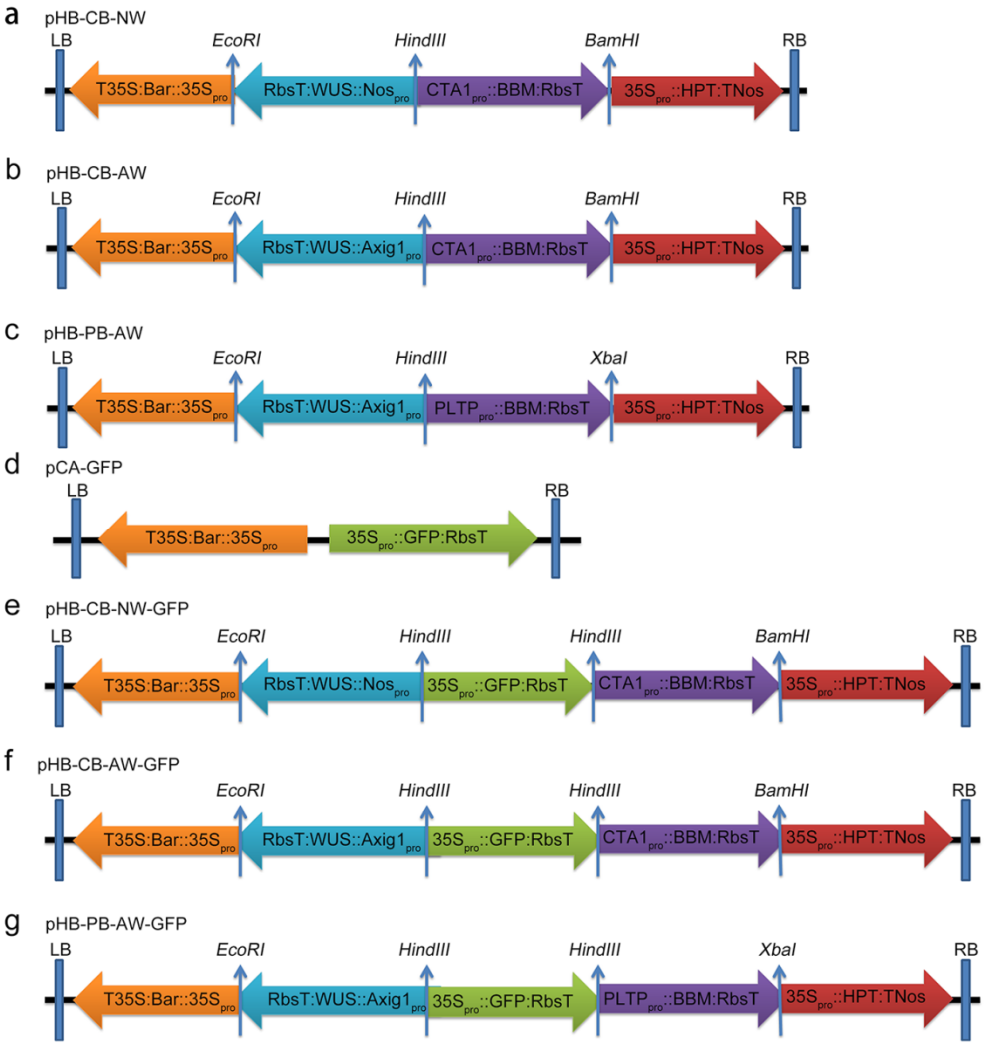

Figure 2. Vector maps for the genetic transformation system. *BBM*, *BABY BOOM*; *WUS*, *WUSCHEL*; *GFP*, *Green fluorescent protein*; NOS$_{pro}$, *Nopaline synthase* promoter; CTA1$_{pro}$, maize *Chitinase A1* promoter; Axig1$_{pro}$, Auxin-inducible promoter; PLTP$_{pro}$, *Phospholipid transferase protein* promoter; 35S$_{pro}$, the cauliflower mosaic virus 35S promoter; TNOS, *Nopaline synthase* terminator; TRbst, Rbst terminator; T35S, cauliflower mosaic virus 35S terminator; *BAR*, *Bialaphos resistance*; *HPT*, *Hygromycin phosphotransferase*; LB, left border; RB, right border. A summary of the primers is given in Table S2. (**a**) T-DNA composition for the pHB-CB-NW vector. This vector shows that ZmCTA1$_{pro}$ drives *ZmBBM* and that NOS$_{pro}$ drives *ZmWUS*. (**b**) T-DNA composition for the pHB-CB-AW vector. In this vector, ZmCTA1$_{pro}$ drives *ZmBBM* and ZmAxig1$_{pro}$ drives *ZmWUS*. (**c**) T-DNA composition for the pHB-PB-AW vector. This vector shows that ZmPLTP$_{pro}$ drives *ZmBBM* and that ZmAxig1$_{pro}$ drives *ZmWUS*. (**d**) T-DNA composition for the pCA-GFP vector. This vector is a directive reporting pCAMBIA3301 vector including *GFP* driven by the 35S promoter (*35S$_{pro}$::GFP*). (**e**) T-DNA composition for the pHB-CB-NW-GFP vector. In this vector, ZmCTA1$_{pro}$ drives *ZmBBM*, 35S$_{pro}$ drives *GFP*, and NOS$_{pro}$ drives *ZmWUS*. (**f**) T-DNA composition for the pHB-CB-NW-GFP vector. In the pHB-CGA vector, ZmCTA1$_{pro}$ drives *ZmBBM*, 35S$_{pro}$ drives GFP, and ZmAxig1$_{pro}$ drives *ZmWUS*. (**g**) T-DNA composition for the pHB-PB-AW-GFP vector. This vector shows that PLTP$_{pro}$ drives *ZmBBM*, 35S$_{pro}$ drives *GFP*, and ZmAxig1$_{pro}$ drives *ZmWUS*.

3.2. Culture Process of Genetic Transformation in Recalcitrant Maize Inbred Lines

For each of the four inbred lines (Zheng58, Mo17, Chang7-2 and B73), we designed three experimental groups for the bivector system test (pHB-NC+pCA-GFP or pHB-AC+pCA-GFP or pHB-AP+pCA-GFP), three experimental groups for the linkage vector test (pHB-NGC or pHB-AGC or pHB-AGP), and a control group (only pCA-GFP). So there were 24 experimental groups and 4 control groups (Table S3). Using microprojectile bombardment technology combined with *Agrobacterium*-mediated transformation, we successfully obtained transgenic Zheng58, Mo17 and Chang7-2 plants (Table 1). The target gene *GFP* was detected by amplifying a 410-bp band with specific primers (Table S2) in the transgenic T0 plants.

Table 1. Transformation ratio of the recalcitrant maize inbred lines.

Maize Inbred Lines	Vector	Number of Immature Embryos	Number of Positive T0 Plants	Transformation Ratio (%)
Zheng58	pHB-CB-NW pCA-GFP	238	7	2.94
Zheng58	pHB-CB-AW pCA-GFP	695	1	0.14
Mo17	pHB-CB-AW pCA-GFP	301	2	0.66
Mo17	pHB-PB-AW-GFP	509	8	1.57
Chang7-2	data pHB-CB-AW-GFP	692	4	0.58

The transformation ratio was calculated based on the number of transgenic positive T0 plants [(number of T0 plants ÷ number of embryos) × 100].

For the entire process of transformation culture, the transformation cycle for obtaining positive transgenic seedlings required approximately 52–66 days (Figure 3). In particular, we observed that the recalcitrant Zheng58 inbred line successfully produced transgenic plants after treatment with the bivector genetic transformation system, with the shortest period being 52 days, 38 days less than that required by traditional transformation [35]. Embryos were dissected from the seeds 14–16 days after self-pollination (DAP) of inbred lines. The length of immature embryos was 1.8–2.2 cm. After 3h in hypertonic cultivation medium, the pHB-CB-NW vector was transformed into immature embryos using microprojectile bombardment technology. After 3 h in hypertonic cultivation medium, the pCA-GFP vector was integrated into the maize genome via *Agrobacterium* infection. Transgenic embryo culture was undertaken in cocultivation medium for 3 days, resting cultivation medium for 7 days, no screening cultivation medium for 12 days (not a necessary step), pre-regeneration cultivation medium for 14 days (Figure 4a), dark regeneration cultivation medium for 14 days (Figure 4b), and light regeneration cultivation medium for 14–16 days (Figure 4c), and transgenic plants were grown in the experimental field (Figure 4d).

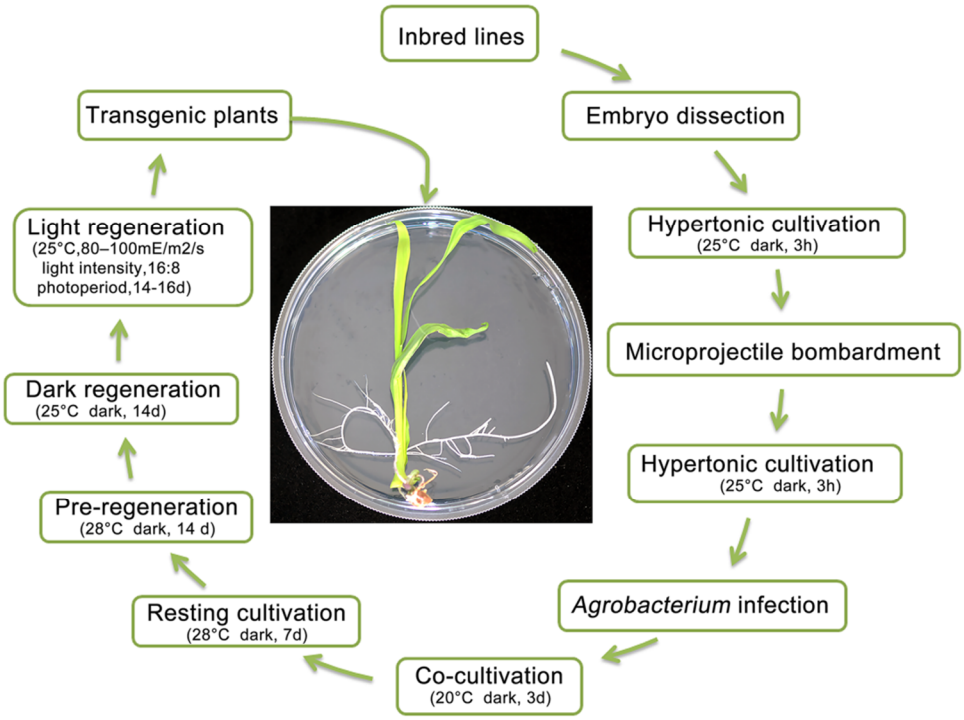

Figure 3. Culture process of the bivector maize genetic transformation system. Embryos were isolated from the seeds of the Zheng58 inbred line at 14–16 DAP. The length of immature embryos was 1.8–2.2 cm. The pHB-CB-NW vector was transformed into immature embryos by microprojectile bombardment technology. The pCA-GFP vector was integrated into the maize genome via *Agrobacterium* infection. There are a series of culture media and conditions to obtain transgenic plants.

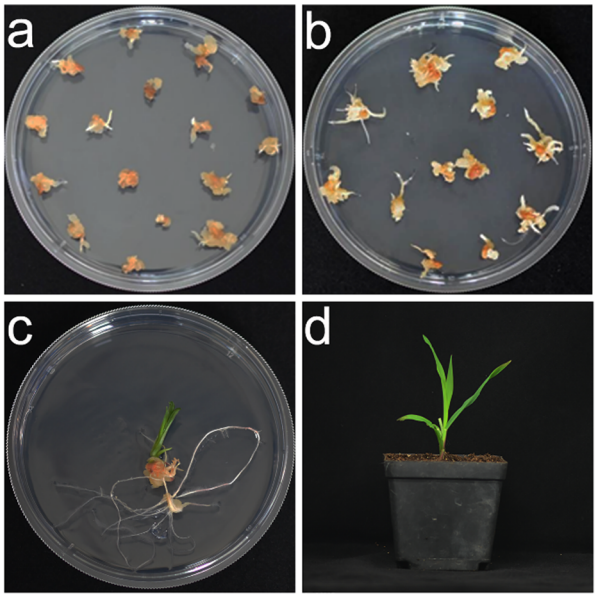

Figure 4. Development of transgenic plants. (**a**) The calli were sprouted on the pre-regeneration medium. (**b**) Most embryos initially induced roots on dark regeneration medium. (**c**) Embryos absorbed light to grow plants on light regeneration medium and were cultured in growth chambers. (**d**) Transgenic plants were successfully obtained from immature maize embryos of Zheng58 in a greenhouse.

3.3. Transformation Frequencies of Maize Recalcitrant Inbred Lines

Three out of four inbred lines were able to produce transgenic plants (Zheng58, Mo17 and Chang7-2) (Table 1). Using the pHB-CB-NW vector and pCA-GFP vector, the recalci-

trant inbred lines of Zheng58 successfully produced transgenic plants, with transformation ratios of 2.94%. With the pHB-CB-AW vector and pCA-GFP vector, transgenic plants were successfully obtained from the recalcitrant inbred lines Zheng58 and Mo17, with transformation ratios 0.14% and 0.66%, respectively. Via the linkage vector pHB-CB-AW-GFP, the recalcitrant inbred lines of Chang7-2 successfully produced transgenic plants, and the transformation ratios of Chang7-2 were 0.58%. Via the linkage vector pHB-PB-AW-GFP, the recalcitrant inbred line Mo17 successfully produced transgenic plants, and the transformation ratio of Mo17 was 1.57%.

Generally, we found that transgenic Zheng58, Mo17, and Chang7-2 all contained *ZmCTA1*$_{pro}$*::BBM*. Furthermore, Zheng58 transformed with the bivector system, including *ZmCTA1*$_{pro}$*::BBM-NOS*$_{pro}$*::WUS* and *35S*$_{pro}$*::GFP*, exhibited the highest transformation ratio of all experimental groups (Table S3). Transformation frequencies [(number of T0 plants ÷ number of immature embryos) × 100] ranged from 0.14 to 2.94% (Table 1).

3.4. Progeny Analysis of Transgenic Zheng58 Plants

The transgenic Zheng58 plants carried the bivector system, including the induction vector (*ZmCTA1*$_{pro}$*::BBM-NOS*$_{pro}$*::WUS*) and the indicator vector (*35S*$_{pro}$*::GFP*), exhibiting the highest transformation ratio. We identified the integration and expression of *GFP* in these positive transgenic lines of Zheng58. Via PCR, we detected the *GFP* gene by amplifying a 410-bp band with specific primers targeting *GFP*, and we detected *ZmCTA1*$_{pro}$*::BBM-NOS*$_{pro}$*::WUS* by amplifying a 546-bp band with specific primers targeting *HPT* (Table S2). By PCR identification, we detected both *GFP* and *ZmCTA1*$_{pro}$*::BBM-NOS*$_{pro}$*::WUS* in the 1#, 2#, 3#, and 4# transgenic lines (Figure 5a). Via Southern blot analysis, we detected the integrated copies of the *GFP* gene. The gel blot was probed with the DIG-labeled *GFP* cDNA fragment without the restriction enzyme sites of *HindIII* and *EcoRI*. By digesting the genome of the 1#, 2#, and 3# transgenic lines with *HindIII* (Figure 5b) and *EcoRI* (Figure 5c), the copy number of *GFP* was three, three, and one, respectively. RT-qPCR analysis showed that the *GFP* gene expressed in the 1#, 2#, 3#, and 4# transgenic lines (Figure 5d).

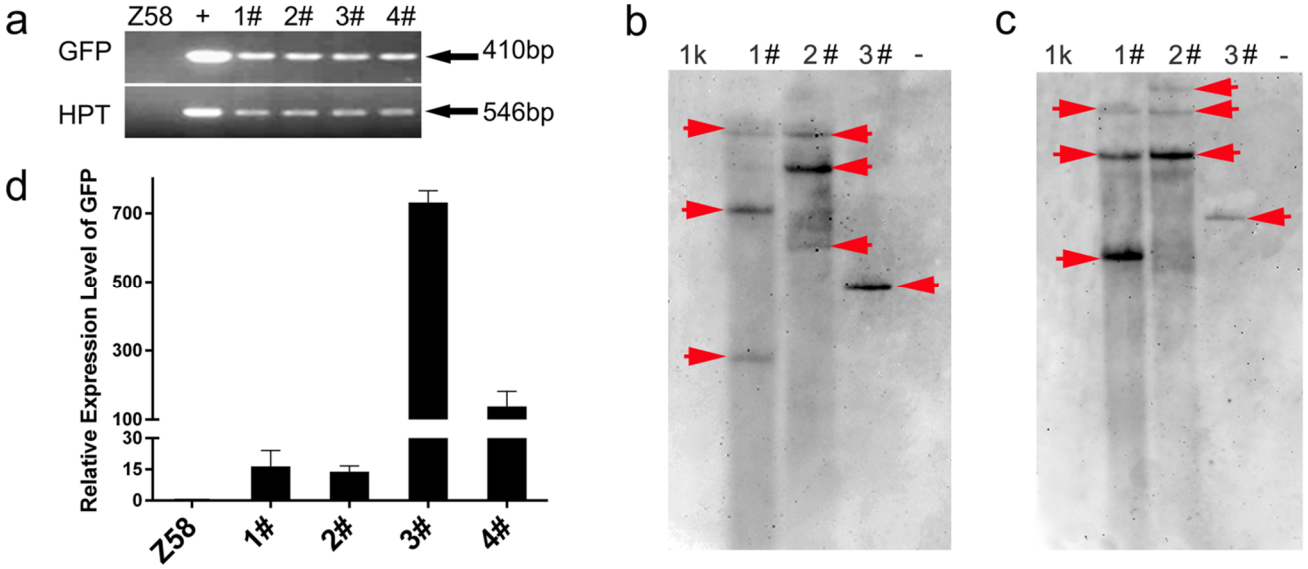

Figure 5. Progeny analysis of transgenic Zheng58 plants. Zheng58 (Z58) served as a negative control. (**a**) PCR identification concerning the 4 lines of transgenic Zheng58. The plasmid of the pCA-GFP vector was treated as a positive control. (**b**) Genomic DNA of transgenic Zheng58 plants digested with *Hin*dIII for Southern blot analysis. (**c**) Genomic DNA of transgenic Zheng58 plants digested with *EcoRI* for Southern blot analysis. Red arrows identify the gene copies. (**d**) Comparison of the *GFP* expression level in the leaf tissue of Z58 and transgenic Zheng58. The transcript levels were normalized according to *Actin*. Values are the means with SEM; n = 3 individuals. See Table S2 for primers.

3.5. Progeny Segregation Analysis of Transgenic Zheng58 Lines

For the progeny seeds of lines 1#, 2#, 3#, and 4# of transgenic Zheng58 (T2-1#, T2-2#, T2-3# and T2-4#), we detected the target gene *GFP* by amplifying a 410-bp band with primers specific for *GFP*, and we detected *ZmCTA1*$_{pro}$*::BBM-NOS*$_{pro}$*::WUS* by amplifying a 546-bp band with specific primers targeting *HPT* (Figure 6, Table S2). The results showed that we obtained separated progenies only with the presence of *GFP* but not with *ZmCTA1*$_{pro}$*::BBM-NOS*$_{pro}$*::WUS*. In T2-1#, T2-2#, T2-3#, T2-4#, the numbers of segregated seeds were four, three, three, and three, respectively. Further, statistical analysis showed that the segregation ratio [(number of separated seeds ÷ number of T2 seeds) × 100] was in the range of 2.03 to 6.78% (Table 2).

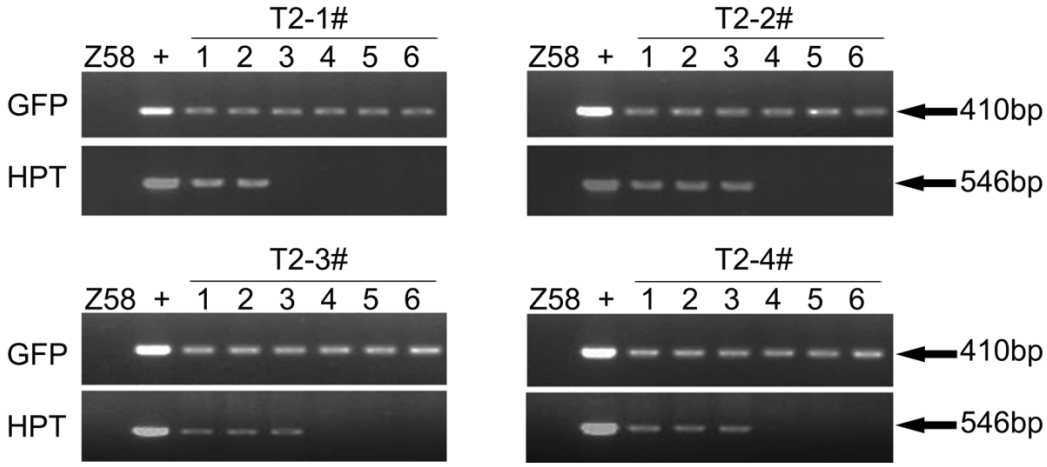

Figure 6. Progeny segregation of the transgenic Zheng58 lines. Progeny transgenic Zheng58 of lines 1#, 2#, 3#, and 4# contained *ZmCTA1*$_{pro}$*::BBM-NOS*$_{pro}$*::WUS* or *35S*$_{pro}$*::GFP*. Seeds were recognized by amplifying *GFP* and *HPT* in the descendants of each line. The pCA-GFP vector and pHB vector were treated as positive controls. Zheng58 (Z58) was served as a negative control. See Table S2 for primers.

Table 2. Segregation ratio of T2 transgenic Zheng58 seeds.

Maize Inbred Line	Event	Number of Detected T2 Seeds	Number of Coexistent Seeds	Number of Separated Seeds	Segregation Ratio (%)
Zheng58	1#	59	55	4	6.78%
	2#	107	78	4	2.80%
	3#	68	38	3	4.41%
	4#	148	118	4	2.03%

T2 seeds were progenies of transgenic Zheng58 of lines 1#, 2#, 3# and 4#. The number of coexisting seeds was the number of T2 transgenic Zheng58 seeds, including *ZmCTA1*$_{pro}$*::BBM-NOS*$_{pro}$*::WUS* and *35S*$_{pro}$*::GFP*. The number of separated seeds was the number of T2 transgenic Zheng58 seeds containing only *35S*$_{pro}$*::GFP*. The segregation ratio was calculated based on the number of separated seeds. [(Number of separated seeds ÷ number of T2 seeds) × 100].

4. Discussion

Maize plays a vital role as a food and feed crop worldwide [36]. Genetic transformation of many important maize inbred lines remains a challenge. Many recalcitrant maize inbred lines are still difficult to be transformed [21]. Enhanced maize genetic transformation procedures would help us to improve recalcitrant inbred lines. Therefore, we created a bivector genetic transformation system.

With the bivector system, we transferred *ZmBBM-ZmWUS* by microprojectile bombardment and transferred *GFP* via *Agrobacterium*-mediated transformation into maize immature embryos. Constitutive expression of *ZmBBM* and *ZmWUS* has severe pleiotropic effects, such as wrinkled leaves [37] and root thickening [10]. To reduce those effects on offsprings, we bombarded immature embryos with microprojectile bombardment technol-

ogy to make induction genes express instantaneously. We used *Agrobacterium*-mediated transformation to infect immature embryos to ensure stable expression of indicator gene. After cultivation, the Zheng58 and Mo17 maize inbred lines yielded transgenic plants. In all experimental groups, the highest transformation frequencies were 2.94% for transgenic Zheng58, which were induced by *ZmCTA1*$_{pro}$*::BBM-NOS*$_{pro}$*::WUS* of the bivector genetic transformation system to successfully obtain transgenic plants. Unfortunately, the induction genes were also stably transformed but not instantaneously expressed (Figure 5). The different genotypes affect somatic embryogenesis [38]. The transformation ability of maize is controlled by nuclear genes [39]. At least one gene or gene cluster affects the formation of somatic embryos in tissue culture [40]. The Zheng 58 inbred line is not only tolerant of close planting, fertilizer, drought, lodging, and bacterial resistance, but is also the most widely used inbred line and exhibits a high yield [41]. Recalcitrant Zheng58 inbred lines successfully obtained transgenic plants by the bivector genetic transformation system, which may establish a foundation for applications in agriculture and commerce.

Expression of morphogenetic genes provides a reliable method for producing fertile transgenic plants. The whole process involves a lot of induction genes, such as using the combination of embryonic regulatory factors and meristem regulatory factors to enhance embryonic response [21]. In our study, we used the embryonic regulatory factor of *ZmBBM* and the meristem regulatory factor of *ZmWUS* as the induction genes. Under the expression of *ZmBBM* and *ZmWUS*, embryo formation was accelerated and the transformation cycle was shortened. The whole transformation cycle was 52–66 days for the transformation of Zheng58 with the bivector system, which was 38 days less than the period required by traditional transformation. In the traditional transformation cycle, A188, A634, H99, and W117 inbred lines could effectively produce transgenic plants by *Agrobacterium tumefaciens*-mediated procedures, and the transformation cycle was approximately 90 days [35]. Recently, using *Agrobacterium*- and biolistic-mediated methods, immature embryos of maize inbred B104 were observed to produce transgenic plants within 112–147 days [42]. The establishment of a bivector regeneration system considerably reduces the time of the transformation cycle.

It has been reported that ectopic expression of morphogenetic genes presents a harmful pleiotropic phenotype [21]. To eliminate the pleiotropic effect caused by continuous expression of *BBM*, heat shock-induced FLP gene expression was used to remove the *BBM* gene sequence between two FRT sites, thereby resulting in normal transgenic plants of Chinese white poplar (*Populus tomentosa* Carr.) [43]. In our study, the expression of *BBM* under the control of ZmCTA1$_{\mathrm{pro}}$ was weaker than that of ZmPLTP$_{\mathrm{pro}}$, and the expression of Zm*WUS* was driven by Zm*Nos*$_{pro}$. The expression pattern of *ZmCTA1*$_{pro}$*::BBM-Nos*$_{pro}$*::WUS* was beneficial for eliminating the pleiotropic effect of morphogenetic genes. Utilizing the strengths of the bivector genetic transformation system, we could separate *GFP* and *ZmBBM-ZmWUS*. We detected only *GFP* but not *ZmBBM-ZmWUS* in T2 generation seeds, effectively mitigating the impact of *ZmBBM-ZmWUS*. High-copy-number transgenes are associated with functional and structural instability [44,45]. Based on the T2 generation seeds of transgenic Zheng58, the progeny segregation ratio was low. This low ratio might be related to the high-copy phenomenon.

Our research provided new ideas for the genetic transformation of maize and established a foundation for further research attempting to develop better maize lines and promote the development of maize breeding.

5. Conclusions

This study is the first attempt to establish a bivector genetic transformation system, which was successfully employed to obtain transgenic Zheng58 plants. This system not only provides a means of genetically transforming more recalcitrant maize inbred lines, but may also significantly reduce the time required by the transformation cycle. In addition, this system was able to eliminate the pleiotropic effects of *ZmBBM-ZmWUS*. This bivector

maize genetic transformation system would contribute to the improvement of maize genetic breeding.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agriculture11070663/s1, Table S1: The construction of the vectors. Table S2: Primers used in this study; Table S3: Summary data for Transformation Ratio of all trial groups.

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