



Research article

5-Azacytidine promotes shoot regeneration during *Agrobacterium*-mediated soybean transformation

Qiang Zhao^a, Yanli Du^a, Hetong Wang^b, Hilary J. Rogers^c, Cuimei Yu^a, Wan Liu^d, Mingzhe Zhao^{a,*}, Futi Xie^{a,**}

^a Agricultural College, Shenyang Agricultural University, Shenyang, 10866, PR China

^b College of Life Science and Bioengineering, Shenyang University, Shenyang, 110044, PR China

^c Cardiff University, School of Biosciences, Cardiff, CF10 3TL, UK

^d Key Laboratory of Pollution Ecology and Environmental Engineering, Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang, 110016, PR China

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ABSTRACT

Agrobacterium-mediated soybean transformation has been greatly improved in recent years, however the transformation efficiency is still low and highly genotype-dependent when compared to other species. Here, we characterized seventeen soybean genotypes based on their genetic transformation efficiencies, i.e., high and low, during *Agrobacterium*-mediated transformation. To reveal the molecular basis of this transformation difference, we constructed a highly efficient transient transgene expression system using soybean cotyledon protoplasts and then assess the methylation levels of promoter and coding regions of an *EYFP* (enhanced yellow fluorescent protein) gene introduced into the protoplast cultures of various soybean genotypes using BSP (bisulfite sequencing PCR). Increased methylation was found to be associated with the considerably decreased transfection efficiency (as percentage of *EYFP* fluorescent protoplasts) in low-efficacy genotypes as compared with those in high-efficacy on three DAT (day after transfection). 5-Azacytidine (5-Azac), a demethylating reagent commonly applied in epigenetic researches, significantly improved the transient transfection efficiency and transgene expression level in low-efficacy genotypes. Furthermore, the shoot regeneration efficiency in low-efficacy genotypes was substantially increased by 5-Azac treatment in an *Agrobacterium*-mediated soybean transformation system. Taken together, we concluded that lower methylation level in transgene contributed to enhanced shoot regeneration in *Agrobacterium*-mediated soybean transformation.

1. Introduction

Transgenic technology has the potential to promote revolutionary changes in agricultural production, since it provides an effective approach to breaking the species barrier and of being applied for facilitating genetic improvement of crops, leading to accelerating the selection process of high-quality/high-yield crop varieties (Miklos et al., 2007). GM (genetically modified) crops provide enhanced characters, such as multi-resistance to herbicides and pesticides and increased nutritional value, by recombination and direct transfer of plant genes (Halpin, 2005). Since the first transgenic tobacco plants were obtained in 1983 (Fraley et al., 1983), great success has been achieved in improving crops using transgenic technologies (Wang et al., 2016).

Soybean (*Glycine max* (L.) Merr.) is the main edible oil, edible

protein and feedstock crop, leading to a key need for enhancing its yield and quality. Since GM soybean was first commercially cultivated in 1996, it has been the major commercialized biotech crop with 90.7 million hectares planted globally till now (Aldemita et al., 2015). Currently, herbicide-resistance is one of the most commonly used traits in commercialized GM soybeans. With the completed soybean genome sequence (Williams 82) published in 2010, gene structural and functional identification, molecular marker exploitation and genetic variation research have been successively carried out, for which genetic transformation is a frequently used research tool (Chen et al., 2006). Increasing evidence suggests that most agronomic traits are quantitative traits (QT) and regulated by multi-genes, whose co-transformation is usually required to obtain the phenotypic trait, but accompanied by lower transformation/expression efficiency and poorer stability

* Corresponding author.

** Corresponding author.

E-mail addresses: zqiang0416@hotmail.com (Q. Zhao), dyl0305@sina.cn (Y. Du), tony.w.china@hotmail.com (H. Wang), RogersHJ@cardiff.ac.uk (H.J. Rogers), yucimei@163.com (C. Yu), liuwan63@hotmail.com (W. Liu), mzhao718@hotmail.com (M. Zhao), snsoybean@sohu.com (F. Xie).

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Abbreviations

5-Azac	5-Azacytidine
CONS	Constitutive expression vector 35S::EYFP
DAT	Day after transformation
HIGH	Soybean genotypes with high transformation efficiency
INDU	Inducible expression vector XVE-35S::GFP
LOW	Soybean genotypes with low transformation efficiency
MIDDLE	Soybean genotypes with middle transformation efficiency
UNKNOWN	Soybean genotypes with unknown transformation efficiency

(Halpin, 2005). In fact, the bottleneck of low transformation efficiency is a serious problem in soybean gene functional research when compared with other species, restricting its commercial development (Cao et al., 2009). Thus, establishing an efficient genetic transformation system will promote functional genomics research and the development of transgenic technology in soybean.

Agrobacterium-mediated genetic transformation has been proven to be the most efficient and widely used method for soybean transformation compared to other transformation systems (Travella et al., 2005; Tzfira and Citovsky, 2006). However, previous studies have shown that *Agrobacterium*-mediated soybean transformation efficiency is strongly dependent on soybean genotypes (Bailey et al., 1993; van de Lagemaat et al., 2007) because of the variation in susceptibility to *Agrobacterium* (Song et al., 2013), which significantly limits biotechnological application in certain elite and commercial genotypes. Till now, most soybean genetic engineering efforts have focused on deployment of soybean genotypes more efficient for transformation. And not much is known about the association between the transgene expression status and *in vitro* soybean plant regeneration after *Agrobacterium*-mediated genetic transformation.

Integrated transgenes in plants are not always expressed as expected. Moreover, there exists variation in expression of transgene between transformed individuals, which is independent of copy number but dependent on both strength of the promoter and epigenetic effects (Jaenisch and Bird, 2003). Transgene silencing has been reported frequently in transgenic plants and their progeny, and the methylation level of promoter and coding region of transgene in transgenic plants was reported to be associated with transgene silencing (Nan et al., 1998; Bird, 2002; Wu and Ho, 2004). It is well known that 5-mC methylation of CpG, CpNpG and CpHpH sites is observed in higher plants and normally contributes to 20–30% of all cytosines in the plant genome, which was confirmed as a mechanism for inactivation of chimeric transgenes (Hobbs et al., 1990; Gelvin, 2017; Testroet et al., 2017). Moreover, an inverse correlation has been noted between transgene activity and its methylation level (Martienssen and Richards, 1995). However, little information is available about impact of transgene methylation on *in vitro* plant regeneration ability among different soybean genotypes after *Agrobacterium*-mediated transformation.

5-Azacytidine (5-Azac) is a nucleotide analogue which can incorporate into a newly synthesized DNA strand by replacing cytosines. Since the incorporation of 5-Azac in DNA can result in the decrease of genomic cytosine methylation at random sequences, it is commonly applied in epigenetic researches as a global demethylating reagent (Issa and Kantarjian, 2009). DNA methylation is an important regulatory mechanism for various plant developmental processes. Demethylation through 5-Azac application could cause complex expression changes (including both the stimulation and repression) of plant endogenous genes. For instance, embryogenic tissue callus from *Arabidopsis* and *Medicago* were incapable of being induced into somatic embryogenesis process once treated with 5-Azac (Santos and Fevereiro, 2002; Grzybkowska et al., 2018). Similarly, the formation of carrot

embryogenic cell clumps from epidermal cells was also suppressed by 5-Azac treatment (Yamamoto et al., 2005). On the other hand, a more recent study has suggested that an increase in DNA methylation decreased the shoot-forming ability and that 5-Azac can partially recover this ability (Tokuji et al., 2011). In addition, 5-Azac demethylation has been reported to change transgene expressions distinctly in transformed plants. For example, when transformed tobacco cells/tissues were treated with 5-Azac, notable increased transgene activities were observed (Zhu et al., 1991; Palmgren et al., 1993; Shao and Hughes, 1995). Furthermore, 5-Azac treatment had been shown to result in the demethylation of the Ubi1 promoter and reactivation of *BAR* transgene in rice (Kumpatla et al., 1997). Baldes et al. (1987) found that the expression of the T-DNA nopaline-synthase gene was repressed in fast growing soybean protoplast cells which could be strongly increased by 5-Azac. However, since then, there has been no published study to assess the effects of 5-Azac demethylation on soybean regeneration and transformation. Here, we describe the promoting role of 5-Azac in shoot regeneration of soybean during soybean transformation.

In the current *Agrobacterium*-mediated soybean transformation system, the selective reagents are usually added into the shoot-regeneration medium after co-cultivation with *Agrobacterium* to screen the resistant shoots. It is very difficult to trace transformed cells and separate them from un-transformed cells from the same explants during this progress. Therefore, it is almost impossible to collect adequate number of *Agrobacterium*-transformed cells for performing studies on molecular mechanisms underlying the soybean genotypic differential transformation efficiency during the shoot-regeneration under real *Agrobacterium*-mediated transformation experimental conditions. This technical barrier becomes even more significant especially with soybean genotypes with low transformation efficiencies. Additionally, there are always false-positive transformation events which would complicate interpretation of gene expression data. To avoid above described technical difficulties, we developed a strategy for identification of transformation-affecting molecular factors by taking advantages of a soybean protoplast transient expression system. Transient assays using protoplasts have provided an ideally convenient system for high-throughput genomics and proteomics analyses in recent years (Dekeyser et al., 1990; Sheen, 2001). In most cases, the results from protoplast transient expression were consistent with transgenic plant/mutant research (Sheen, 2001). In addition, the transient assay system is rapid, simple, safe and efficient compared with the relatively expensive and time-consuming processes of obtaining transgenic lines (Chen et al., 2006). The establishment of highly-efficient PEG-mediated transformation method and sensitive and economical reporter assays, combined with abundant genetic resources of model plants (Yoo et al., 2007; Zhang et al., 2011), has made protoplast transient expression systems important and versatile tools in genomic studies.

The principal objectives of the current study were to (1) establish and optimize a PEG-mediated soybean protoplast transient system, which is suitable for both constitutive (35S-) and inducible (XVE-35S-) gene expression; (2) examine the variation in transfection efficiency using the transient protoplast system; (3) analyze transfection efficiency variation at transcriptional and post-transcriptional levels under applications of the DNA methylation inhibitor (5-Azac) and protease inhibitors (MG-115, MG-132 and AEBSF), using the transient protoplast system, and (4) validate whether 5-Azac can improve the shoot regeneration and the transformation of low-efficiency soybean genotypes using the *Agrobacterium*-mediated genetic transformation system.

2. Materials and methods

2.1. Materials and growth conditions

Soybean (*Glycine max* L.) Merr.) used in this study were obtained from The Soybean Institute, College of Agriculture, Shenyang Agricultural University (Table S1). For the *Agrobacterium*-mediated

soybean genetic transformation, the soybean seeds were sterilized using chlorine gas (made by mixing 4 ml 12 M HCl and 100 mL 5.25% hypochlorite) in a glass desiccator for 8–10 h. Then sterilized seeds were sown in the B5 solid medium (Caisson, USA) with 2% (w/v) sucrose (pH 5.8) and 0.7% (w/v) agar, and cultivated at $28 \pm 1^\circ\text{C}$ in darkness for 3 days. Homogeneously germinated seeds were selected for the subsequent experiments.

To isolate protoplasts from etiolated soybean seedlings, soybean seeds were sown in the soil mixture of vermiculite and turfy soil (3:1) with about 50–60% water. The seeds were cultivated for 3–4 days at $28 \pm 1^\circ\text{C}$ in darkness, until the hypocotyls of soybean seedlings were elongated to 5 cm. Then germinated soybean seedlings were transferred into 150 ml flask with water changed at every the other day and allowed to grow in the dark for 7–12 days until true leaves completely expanded.

2.2. Plasmid construction

Plant binary expression vector pCAMBIA3301-1 (35S::GUS-BAR) was kindly donated by Jilin Academy of Agricultural Sciences, China (Jia et al., 2015). The constitutive expression vector pEarleyGate-104 (35S::EYFP) and the inducible vector pER8 were kindly donated by Dr. Mingzhe Zhao and Dr. Xiaoxue Wang from the College of Agriculture, Shenyang Agricultural University (Zuo et al., 2000; Earley et al., 2006). In our study, a GFP fragment was inserted into the pER8 plasmid between XhoI and SpeI restriction sites to construct the pER8-GFP (XVE-35S::GFP) vector (as shown in Fig. S1). Plasmids for protoplast transient expression were isolated by E.Z.N.A Endo-Free Plasmid Kit (Omega Bio-Tek, Norcross, USA).

2.3. Agrobacterium-mediated soybean genetic transformation

The *Agrobacterium* strain EHA105 transformed with pCAMBIA3301-1 which carried a GUS and a BAR gene, was used for *Agrobacterium*-mediated soybean cotyledons transformation as described in our previous study (Jia et al., 2015). The formulas for all medium were listed in Table S2.

2.4. Isolation of soybean protoplasts

The method described by Sheen (2001) was adopted with minor modifications. Tissues from etiolated soybean seedlings were cut into 0.5 mm strips, transferred into CPW-10M solution immediately, and incubated on a rotator at 90 rpm in darkness at 28°C for 30 min. Then the CPW-10M solution was filtered out with a cell strainer (150 mesh) and replaced by a digestion solution (0.8–1 g/20 ml) to continue the above rotation incubation for another 10 h. After digestion, the mixture was filtered with a cell strainer (300 mesh) again and the flow-through was centrifuged at 100 g at room temperature for 10 min. The precipitate (protoplast cells) was re-suspended in the W5 solution and washed twice. The yield of protoplasts was calculated by counting on a hemocytometer under the microscope (Zeiss fluorescence microscope Axio Lab A1). Protoplast viability was measured by ratio of fluorescein diacetate (FDA) (0.01% v/v) stained cells among all visible protoplasts (Larkin, 1976). For each protoplast cell-suspension, three slides (40 μL of suspension on each slide) were prepared and examined for FDA staining. For each slide, at least five pictures were taken at random positions under both bright light and the excitation of 488 nm. Data was collected from five biological replicates. Formulas for above solutions were listed in Table S3.

2.5. Transformation of soybean protoplasts

The transient transformation protocol for Arabidopsis protoplasts (Yoo et al., 2007) was modified for the transformation of soybean protoplasts. Protoplasts were collected from the W5 solution by

centrifugation and re-suspended in an equal volume of MMG solution, then placed on ice for 30 min. Plasmid DNA of 20 μg was mixed gently with 200 μL (2×10^5) protoplasts first and then with 220 μL of a freshly prepared 40% PEG4000 solution. The mixture was incubated for 20 min and inverted gently to mix at each 5 min. After incubation, 800 μL of the WI solution was added to stop the transformation. The mixture was centrifuged at 100 g for 5 min to remove the supernatant. The transformed protoplasts were gently re-suspended in 1 ml WI solution (with 0.1% BSA) and incubated at 28°C in darkness on tissue culture plate which was prewashed with WI solution.

2.6. Transfection efficiency measurement

The transfection efficiency was calculated by the equation: Transfection efficiency (%) = (num. of fluorescent protoplasts/num. of total protoplasts) \times 100%. The measurement was performed at 1, 2, 3, 4 day after the protoplast transformation (DAT) by fluorescence observation under a Zeiss fluorescence microscope (Axio Lab A1). For each transformation (1 ml of mixture), three slides (50 μL of transformation mixture on each slide) were prepared and examined for fluorescent protoplasts. For each slide, at least five pictures were taken at random positions under both bright light and the excitation of 488 nm. Transformation experiments were repeated five times for each soybean cultivar to calculate its transfection efficiency statistically.

2.7. Transgene expression

The EYFP and BAR genes were used to detect the transgene expression and gene activity after transformation by qRT-PCR. Soybean *Tubulin A* (NM_001250372) or *Actin* (NM_001289231) gene were used for signals normalization, and the information of the primers for amplifying specific genes can be found in Table S5.

For different soybean genotypes, approximately 10^7 protoplasts were collected as described above, and flash frozen in liquid nitrogen prior to storage at -80°C . Total RNA was isolated and purified using Plant Total RNA Isolation Kit (Qiagen, Hilden, Germany). First-strand cDNA was synthesized from 1 μg of total RNA using a TransScript® All-in-One First-Strand cDNA Synthesis SuperMix for qPCR Kit (TransScript, Beijing, China) according to the manufacturer's instructions. Then, 1 μL of the reaction mixture was subsequently used for qRT-PCR in a 20 μL reaction volume using TransScript® Top Green qPCR SuperMix (TransScript, Beijing, China). The following thermal cycle conditions were used: 95°C for 30 s, followed by 35 cycles of 95°C for 5 s, 58°C for 20 s, and 72°C for 20 s. The qRT-PCR experiments were performed with three biological replications, and each biological replication was measured in three technical replications. Following the PCR, a melting curve analysis was performed. Threshold cycle was used for relative quantification of the input target number. The operational formula $2^{-\Delta\Delta\text{Ct}}$ was used to calculate the genes relative expression between different treatments (Livak and Schmittgen, 2001).

2.8. Protease inhibitors influence assay

Protease inhibitors MG115, MG-132 and AEBSF (Sigma, USA) were resolved in appropriate Dimethyl sulfoxide (DMSO) to make concentrated solutions, which were never kept for > 1 month at -20°C . They were added into the WI solution (with 1% BSA) for soybean protoplasts culture on 2 DAT to the final concentration of MG132 (20 μM) (Huang et al., 2013), MG115 (200 μM) (Nakagami et al., 2006) and AEBSF (50 μM) (Mazel and Levine, 2001), respectively. An equal dosage of DMSO was used as blank test.

2.9. Protoplast DNA preparation and bisulfite sequencing PCR (BSP) analysis

Genomic bisulfite sequencing was performed to analyze the

methylation level in promoter and coding regions of transgene *EYFP*. First, genomic DNA was extracted from soybean protoplast cells using a Universal Genomic DNA Extraction Kit (TaKaRa, Japan) according to the manufacturer's instructions. Amount of 500 ng genomic DNA was treated with sodium bisulfite using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA, USA). The plasmid pEearlygate 104 was treated with bisulfite in parallel with soybean protoplast genomic DNA to estimate the efficiency of converting C to T with the sodium bisulfite treatment. The primers for the BSP were designed using the MethPrimer program (<http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi>) (Li and Dahiya, 2002), and listed in Table S5. The PCR program was 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 40 s. The BSP products were cloned into a pMD19-T-simple vector which was subsequently transferred into *E. coli* strain *JM109* (TaKaRa, Japan). For each line and each gene, ten positive clones were randomly selected and purified for sequencing. In the end, the sequences of amplicons were aligned to the pEearlygate 104 reference sequence, and the methylation level was calculated by dividing all CpGs analyzed by the total number of methylated CpGs detected.

2.10. Treatment of 5-Azacytidine

The 5-Azacytidine (5-Azac) was formulated into different concentrations of 0, 10, 30 and 50 μM with distilled water. For the protoplast transformation system, soybean protoplasts were treated with different concentrations of 5-Azac for one day before measurement of activities of transgenes. For the *Agrobacterium*-mediated transformation system, soybean explants were treated with 5-Azac for seven days on the shoot regeneration medium.

2.11. Dosage-effect analysis of 5-Azacytidine

Agrobacterium-mediated soybean cotyledons transformation (with pCAMBIA3301-1, 35S::GUS-BAR) was carried out with Kottman (Kt) genotype and different concentrations of 5-Azac administrated by 0–1W (10 μM 5-Azac was added into the shoot regeneration medium only during the 2nd week of cultivation). The number of regenerated shoots (under 5 mg L^{-1} PPT selection) was calculated at the end of the 3rd week of cultivation. Then shoots were moved to shoot elongation medium. At the end of shoot elongation period (4th - 6th week), Percentage of shoot elongation was calculated by: No. of shoots elongation/No. of shoots \times 100%. Then, rooting was carried out with elongated shoots from 7th - 10th week on root induction medium for calculation of percentage of rooting by: No. of rooting/No. of elongated shoots \times 100%. Experiments were repeated for five times, and more than 50 explants were used in each replication.

Table 1
Agrobacterium-mediated transformation efficiencies of different soybean genotypes.

Genotype	Total explants	Total regenerated shoots		Transplants	shoots/explant		Transplants/100 explants
		No selection	Selection		No selection	Selection	
Dongnong 50	325	1464	1214	24	4.50 \pm 0.28 a	3.74 \pm 0.66 a	7.38 \pm 0.68 a
Williams 82	320	1188	943	20	3.71 \pm 0.75 ab	2.95 \pm 0.49 b	6.25 \pm 0.85 b
Bert	312	997	806	20	3.20 \pm 0.32 c	2.58 \pm 0.51 b	6.41 \pm 0.78 ab
Shennong 9	307	1122	852	18	3.65 \pm 0.66 ab	2.78 \pm 0.48 b	5.86 \pm 0.99 b
Kottman	310	962	149	3	3.10 \pm 0.36 c	0.48 \pm 0.04 c	0.97 \pm 0.35 c
General	301	1142	109	2	3.79 \pm 0.55 ab	0.36 \pm 0.07 c	0.66 \pm 0.23 c
Zhonghuang 35	311	997	57	2	3.21 \pm 0.32 c	0.18 \pm 0.03 c	0.64 \pm 0.33 c
Liaodou 16	317	978	33	0	3.09 \pm 0.56 c	0.10 \pm 0.02 c	0.00 \pm 0.00 d

Explants; soybean cotyledons as explants used for transformation. Shoots; the shoots which were induced from soybean cotyledons explants. Selection; 5 mg L^{-1} PPT was added into the shoots induction medium for selection. Transplants; regenerated plants survived after soil potting and tested positive by GUS staining. Data was collected from three repeated transformations and summed as total numbers. Standard deviations were calculated with three repeated transformations. Letter indicated significant differences ($P < 0.05$) according to the LSD test.

2.12. β -Glucuronidase (GUS) staining

During the *Agrobacterium*-mediated transformation, the regenerated shoots were separated from explants and washed in sterile water. Then, the shoots were transferred into the GUS histochemical staining solution (Jia et al., 2015), vacuumed for 30 min and incubated at 37 °C for 16 h. After staining, the shoots were washed in 70% and 100% ethanol respectively. The number of shoots that stained into blue color was counted to calculate the rate of transformation.

2.13. Statistical analysis

SPSS (version 23.0) was used for statistical analysis of the experimental data. Results are recorded in the format of means \pm standard deviation (SD). Statistical differences between the control and treatments were calculated using one-way analysis of variance (ANOVA), taking $P < 0.05$ as significantly according to the least significant differences (LSDs) tests.

3. Results

3.1. Shoot regeneration efficiencies of various soybean genotypes under selective conditions

To screen suitable soybean genotypes for downstream molecular studies, a total of seventeen American and Chinese genotypes were assessed for transformation efficiency by the *Agrobacterium*-mediated transformation. *Agrobacterium* EHA105 harboring binary construct pCAMBIA3301 that contains *GUS* and *BAR* gene expression cassettes (Fig. S1) was used for soybean transformation, and phosphinothricin (PPT) was used for selecting regenerated shoots in seventeen soybean genotypes (Table S1). There existed an obvious variation in the shoot regeneration efficiency among the seventeen soybean genotypes after 5 mg L^{-1} PPT selection. Dongnong 50 (DN50), Williams 82 (Wm82), Bert (Br) and Shennong 9 (SN9) had higher shoot regeneration efficiencies (82.9%, 79.4%, 80.8% and 75.9%, respectively, calculated from three repeated experiments with total of more than 300 explants) than Kottman (Kt), General (Gr), Zhonghuang 35 (ZH35) and Liaodou 16 (LD16) that showed lower than 20% shoot regeneration rates. Of these latter genotypes, ZH35, a currently widely planted cultivar in China, displayed only 5.72% shoot regeneration rate. However, there was no obvious difference in the efficiency of shoot regeneration without PPT selection among the above genotypes, suggesting that the difference in shoot regeneration efficiencies was caused by transgene expression levels (Table S1). Similarly, the transformation efficiencies (defined as the number of GUS-positive and herbicide resistance events divided by the number of explants to start with) of DN50, Wm82, Br and SN9 were 7.4%, 6.3%, 6.4% and 5.9% for T0 plants, respectively,

about 7–10 times higher than those in Kt, Gr, ZH35 and LD16. Based on the above data, we selected four highly-efficient (HIGH) genotypes including DN50, Wm82, Br and SN9, and four low-efficient (LOW) genotypes including Kt, Gr, ZH35 and LD16 for further study (Table 1).

3.2. Isolation of protoplasts and establishment of transient expression system

Since there were few studies about soybean protoplast gene expression system available for molecular studies, we followed the current Arabidopsis protoplast isolation and transient expression protocols (Lin et al., 1987; Cheng et al., 2015; Kidokoro et al., 2015) and optimized them by using unifoliolate leaves, stems and cotyledons of soybean etiolated seedlings (Fig. 1A) as described in the Methods section. Results indicated that the protoplasts isolated had a high viability (Fig. 1B), yet with different yields from the different genotypes and tissues used. Compared with other tissues, the protoplasts from cotyledons showed the highest yield of about 20×10^6 g-FW⁻¹ protoplast cells, with > 94% viability by fluorescein diacetate (FDA) staining (Fig. 1D). The viability of the protoplast isolated from each different tissue of soybean genotypes was shown in Fig. S4.

Protoplasts from Wm82 cotyledons were isolated and used for transient expression with the CONS plant expression plasmid (pEarleyGate104) carrying the EYFP gene under the control of CaMV35S promoter (Fig. S1). EYFP fluorescence was clearly detected in transfected protoplasts on 1 DAT (day after transfection) (Fig. 1C, Fig. S3) and the highest fluorescence percentage appeared on 2 DAT with $75.7 \pm 0.82\%$ (Fig. 1E). After this peak, the percentage declined gradually. Furthermore, similar EYFP fluorescence of protoplasts occurred in the unifoliolate leaves, stem and cotyledon from different soybean genotypes on 2 DAT, with the percentage of expressing protoplasts routinely greater than 70% (Fig. S4). Overall, protoplasts isolated from

different tissues showed similar viability and transfection efficiency, whereas the cotyledon protoplasts showed the highest yield. Thus, protoplasts from soybean cotyledons were used for subsequent experiments.

3.3. Differential expression of transgene in the cotyledon protoplasts of various soybean genotypes

To determine the correlation between expression levels of transgene in cotyledon protoplasts of various soybean genotypes with different genetic transformation efficiency, EYFP and GFP fluorescence were assayed using the CONS or INDU (pER8-GFP) plant expression vector on 1–5 DAT. The highest percentage of EYFP/GFP fluorescence was detected on 2 DAT with the scored range between 74–79% and 65–69%, respectively, and there was no significant difference between HIGH and LOW genotypes (Table S4). However, the transformation efficiency decreased substantially (1.57–2.01 fold) in the four LOW genotypes than that in the four HIGH genotypes between 2 DAT and 3 DAT (Fig. 2A and C). In addition, qRT-PCR analysis indicated that expression levels of the transgene were significantly down-regulated in protoplasts from the LOW soybean genotypes from 2 DAT to 3 DAT, but not in protoplasts of HIGH soybean genotypes (Fig. 2B and D). These results indicate that in protoplasts from LOW genotypes the expression of transgene genes fell off more rapidly than in HIGH genotypes.

3.4. Transgene methylation correlated to low transgene expression in the protoplasts of low efficient genotypes

Gene expression can be regulated on transcriptional and post-transcriptional levels. Since the application of protease inhibitors MG115 (Z-Leu-Leu-Norvalinal), MG-132 (Z-Leu-Leu-Leu-al) and AEBSF (serine protease inhibitors) exerted no significant influence on protoplast

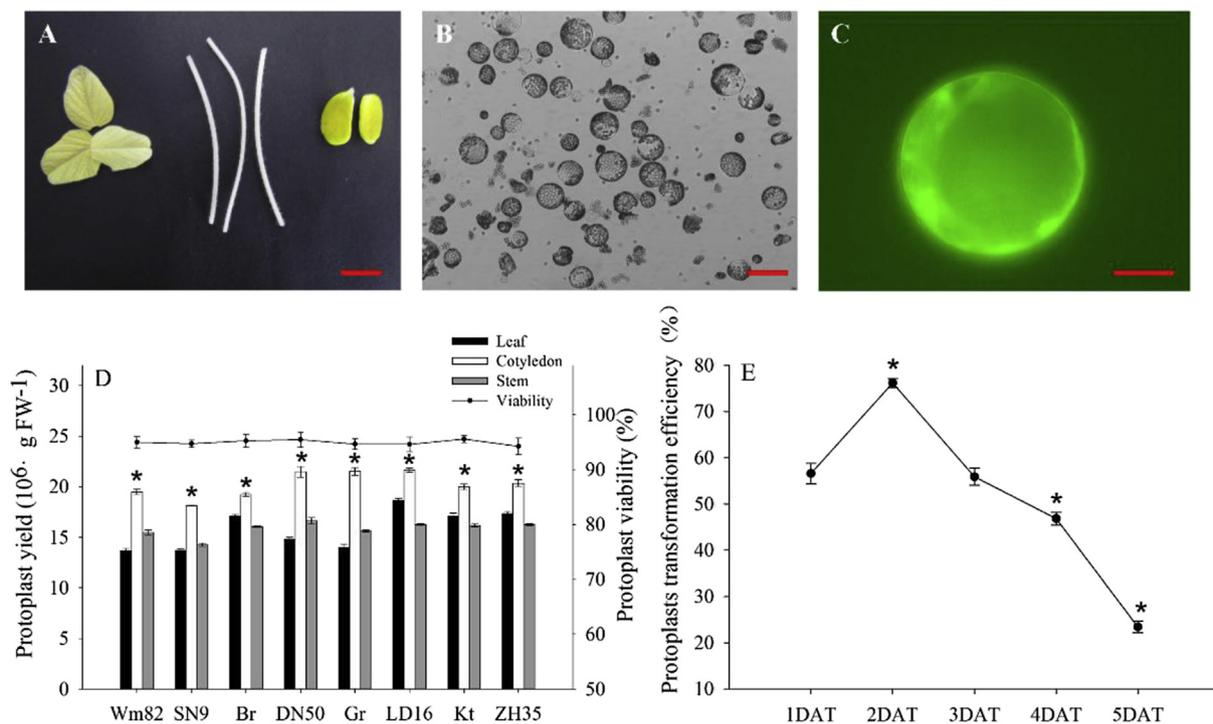


Fig. 1. Transient expression of EYFP in protoplasts isolated from soybean cotyledons. (A) Leaves, stems and cotyledons from etiolated soybean seedlings. (B) Bright-field microscopic image of the suspension of soybean cotyledon protoplasts. (C) Fluorescence-field merged images of soybean protoplasts over-expressing EYFP. (D) The yield and viability of soybean protoplasts. Vertical bars: protoplast yields from different soybean tissues and genotypes; the line above histogram: percentages of freshly isolated living cotyledon protoplasts from different soybean genotypes. Protoplast viability = (FDA-stained fluorescent protoplasts/total protoplasts) × 100%. (E) Time-course of transfection efficiency (trans. effic.) of Williams 82 (Wm82) cotyledon protoplasts (the percentage of EYFP fluorescent protoplasts). DAT: day after transfection. Standard deviations were calculated with five biological replicates. * Statistical significant difference at $P < 0.05$. Bar: A-1cm, B-50 μ m, C-10 μ m.

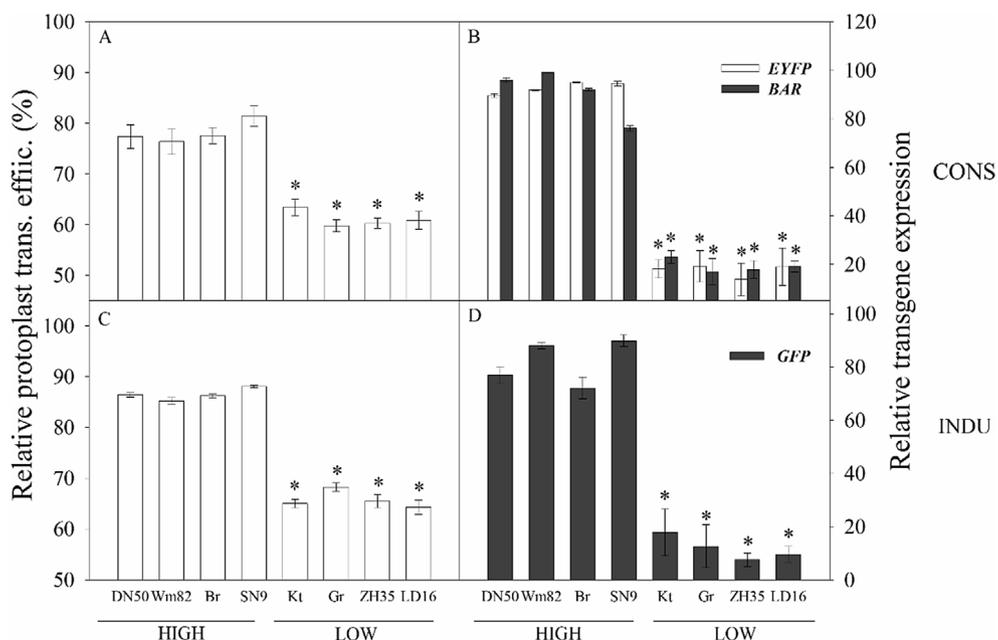


Fig. 2. Relative transfection efficiency and transgene expression in different soybean genotypic protoplasts. (A, C) Protoplast transfection efficiency was measured as the percentage of EYFP or GFP fluorescent protoplasts. (B, D) Transgene expression levels were measured by qRT-PCR normalized to Tubulin A as the internal control gene. Relative protoplast transfection efficiency and gene expression were calculated using the equation: $(3 \text{ DAT}/2 \text{ DAT}) \times 100\%$. CONS: constitutive expression of 35S::EYFP (with PearleyGate-104 vector); INDU: inducible expression of XVE-35S::GFP (with PER-8 vector), as described in the methods. The induction was performed by adding 17- β -estradiol to the protoplast suspension (final concentration of 10 μM) at indicated DATs. HIGH: high-efficiency soybean genotypes; LOW: low-efficiency soybean genotypes. Standard deviations were calculated with five biological replicates. * Statistical significant difference to the HIGH soybean genotypes in A-D ($P < 0.05$).

transfection efficiency of HIGH genotype Wm82 and LOW genotype Kt, it could be concluded that an endogenous protease digestion pathway was not the main reason for low transfection efficiency in the low-efficient genotype (Fig. S5).

To investigate the association between DNA methylation level of the transgene and its reduced transcription from 2 DAT to 3 DAT in the protoplasts, the promoter and coding region of EYFP gene were subjected to BSP (bisulfite sequencing PCR) using the CONS expression vector (Fig. 3A). The BSP sequencing result indicated that the methylation levels of the transgene EYFP promoter and coding region in the LOW genotype were much higher than that in the HIGH genotype on 3 DAT (Fig. 3B and C). In addition, qRT-PCR analysis indicated that the

relative abundance ($3 \text{ DAT}/2 \text{ DAT} \times 100\%$) of the soybean MET1 (XM_014776536) transcripts (ortholog of *A. thaliana* MET1: cytosine-DNA-methyl-transferase) increased significantly in LOW genotypes than that in HIGH genotypes (Fig. 3D). In contrast, the relative abundance of methyltransferases genes CMT and DRM transcripts were similar in all genotypes (Fig. 3E). Same findings on the relative expression of MET1, CMT (XM_003542738) and DRM (XM_006603704) in protoplasts on 2 DAT and 3 DAT were also observed when the INDU expression vector was used (Fig. S7), which indicated that the increased methylation did not require the expression but rather likely was caused by the insertion of transgene into the soybean genome. Taken together, these results suggest that the transferred transgene leads to the up-

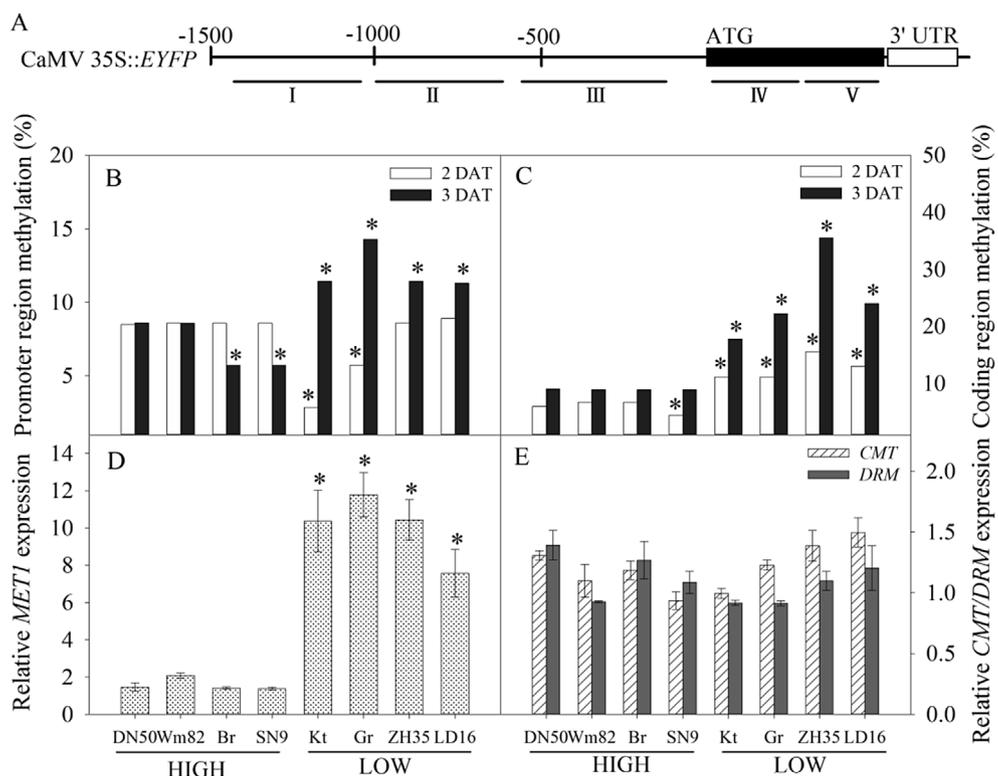


Fig. 3. Transgene methylation in soybean cotyledon protoplasts. (A) The structure of 35S::EYFP gene. Black and white boxes represented the coding region and 3'UTR, respectively. I–V indicated the regions subjected to BSP sequencing. (B and C) DNA methylation levels of promoter (region I–III) and EYFP coding region (region IV–V) on 2 DAT and 3 DAT. (D and E) qRT-PCR analysis of the relative abundance of MET1 CMT and DRM transcripts ($3 \text{ DAT}/2 \text{ DAT} \times 100\%$). Standard deviations were calculated with three biological replicates. * Statistical significant difference to the HIGH soybean genotypes ($P < 0.05$). No significant difference was identified between HIGH and LOW genotypes in E.

regulation of *MET1* gene, and increased methylation levels of both promoter and coding regions in transgene *EYFP*, which decreased expression of *EYFP* gene in LOW genotypes.

3.5. 5-Azac improves the transgene expression and transfection efficiency in soybean cotyledon protoplasts

To further determine whether DNA demethylation affected the expression of the transgene, the CONS plasmid was co-transformed into soybean protoplasts with 10 μM 5-Azac. Methylation levels of transgene in HIGH genotype Wm 82 and LOW genotypes were tested by BSP sequencing after 5-Azac treatment. Results showed that the 5-Azac treatment significantly ($P < 0.05$) reduced the methylation levels of the 35S promoter and the coding region of transgene *EYFP* in LOW genotypes (Fig. 4A and B). The transfection efficiency and expression levels of the *EYFP* and *BAR* genes were measured by fluorescence microscopy and qRT-PCR, respectively. 5-Azac treatment led to increased protoplast transfection efficiency both in LOW and HIGH genotypes compared to the untreated protoplasts (Fig. 4C). However, 5-Azac treatment resulted in enhanced transcription levels of *EYFP* and *BAR* genes in the LOW but not in the HIGH efficiency genotypes (Fig. 4D and E). Transcript levels of *MET1* were not affected by the 5-Azac treatment in any of the genotypes (Fig. 4F). Therefore, the significantly lower transfection efficiency in LOW genotypes should be attributed to higher levels of transgene DNA methylation.

3.6. 5-Azac promoted the shoot regeneration in Agrobacterium-mediated soybean transformation system

To elucidate whether transgene methylation could be correlated to the low shoot regeneration efficiency in LOW genotypes, soybean cotyledon explants were treated with 10 μM 5-Azac during the shoot

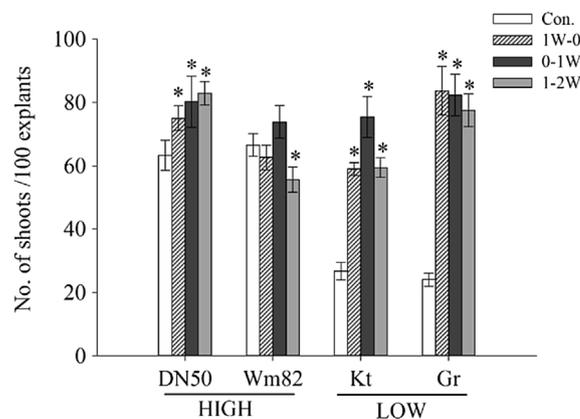


Fig. 5. Improvement of shoot regeneration by 5-Azac treatment during soybean transformation. Explants were cultivated on the shoot regeneration medium with 5 mg L⁻¹ PPT as selective reagent for a total three weeks. The medium was changed for every week. 1W-0: 5-Azac (10 μM) was added into the SIM medium only during the 1st week of cultivation; 0-1W: 5-Azac (10 μM) was added into the SIM medium only during the 2nd week of cultivation; 1-2W: 5-Azac (10 μM) was added into the SIM medium during the 1st and 2nd weeks of cultivation; The number of regenerated shoots was calculated at the end of the 3rd week of cultivation. Standard deviations were calculated with five experimental replicates. * Statistical significant difference to the control at $P < 0.05$.

regeneration stage using three different treatment regimes (Fig. 5). All three treatment regimes with 5-Azac significantly enhanced the shoot regeneration efficiency in LOW (Kt and Gr) and HIGH (DN50) explants, but not in soybean genotype Wm82 explants. The shoot regeneration efficiency of Kt and Gr showed the greatest enhancement with 0-1W 5-Azac treatment. Moreover, we observed that the shoot

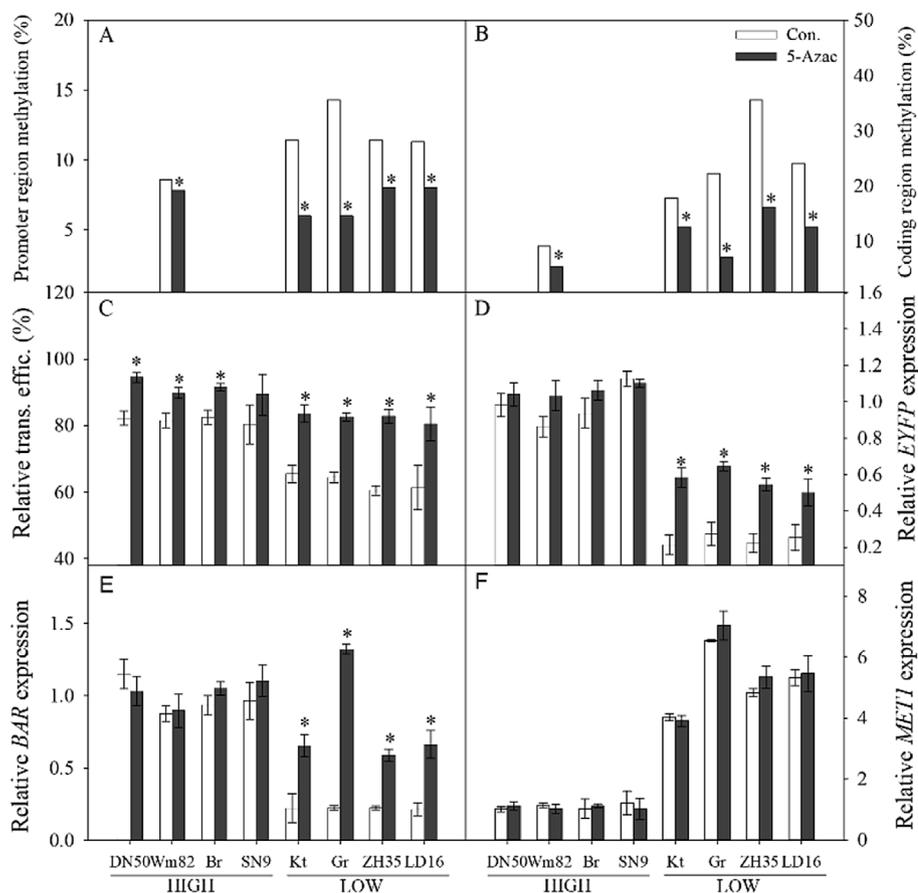


Fig. 4. Increase of transgene expression in LOW soybean protoplasts by 5-Azac treatment. (A and B) DNA methylation levels of promoter (region I-III) and *EYFP* coding region (region IV-V) on 3 DAT. (C) Relative transfection efficiency of soybean cotyledon protoplasts (3 DAT/2 DAT × 100%) with or without 5-Azac treatment. (D, E, F) qRT-PCR analysis of the relative abundance of *EYFP*, *BAR* and *MET1* transcripts in soybean cotyledon protoplasts (3 DAT/2 DAT × 100%) with or without 5-Azac treatment. Standard deviations were calculated with three biological replicates. * Statistical significant difference to the control (Con.) ($P < 0.05$). No significant difference was identified between 2 DAT and 3 DAT in F.

regeneration efficiency in Wm82 was significantly reduced with the 1–2W 5-Azac treatment regime. GUS staining indicated that almost all the regenerated shoots were indeed transgenic (Table S6).

To determine whether there was a dose-dependent relationship between 5-Azac treatment and the shoot regeneration efficiency, the LOW soybean genotype Kottman (Kt) was transformed with *Agrobacterium* carrying pCAMBIA3301-1 35S::GUS-BAR and treated with 5-Azac at different concentrations (0, 10, 30 and 50 μ M) using the 0–1W regime during the shoot regeneration stage. Similar shoot regeneration efficiency in Kt occurred at 5-Azac levels of 10–50 μ M (Table 2). In addition, there was no significant effect of the above 5-Azac levels on shoot elongation and subsequent rooting.

The dosage-effect was analyzed in LOW soybean genotype Kottman. Experiments were repeated for five times. Data collection and analysis were described in the “Dosage-effect analysis of 5-Azac” methods section. a and b indicated significant differences ($P < 0.05$) according to the LSD test.

To define the effects of 5-Azac treatment on shoot regeneration efficiency in other soybean genotypes, twenty-two soybean genotypes were selected and treated with 5-Azac using the 0–1W regime (Table S1). The results showed that the shoot regeneration efficiency was significantly enhanced in most MIDDLE and LOW genotypes by 10 μ M 5-Azac treatment, but did not change in HIGH genotypes (Fig. 6).

4. Discussion

4.1. Different soybean genotypes respond differently to transgene methylation, affecting shoot regeneration and *Agrobacterium*-mediated transformation efficiencies

Previous experiments had shown that *Agrobacterium*-mediated genetic transformation efficiency was strongly dependent on soybean genotype (Paz et al., 2004; Song et al., 2013). The results from the present study are, in agreement with these previous studies, showing a significant difference in induction rate of shoot regeneration (under 5 mg L⁻¹ PPT selection) amongst seventeen soybean genotypes during *Agrobacterium*-mediated transformation (Table 1 and Table S1). It is well-known that a single stranded T-DNA molecule is transferred from the *Agrobacterium* to the host cell and further integrated into the host genome with the help of Vir proteins and host-specific proteins (Tzfira and Citovsky, 2006). After entering the host cell, transgene expression can be regulated at DNA, transcriptional, post-transcriptional, translational and post-translational levels (Kavitha et al., 2010; Ziemienowicz, 2014; Shilo et al., 2017). Transgene transcription could be regulated by DNA methylation, histone modification, non-coding RNA (ncRNA), activation of transcription factors (TFs), etc. Our present study suggested that soybean genotypes could be grouped into different groups based on their regeneration efficiency, e.g., high and low-efficiency, which likely were influenced by differential methylation status in transgenes. This finding would allow effective selection of those soybean genotypes which have more efficient regeneration due to the low level of transgene methylation in a transformation effort.

4.2. Protoplast transient expression system is effective for evaluating transgene expression and regeneration and transformation potential of various soybean genotypes

Obtaining transgenic soybean lines is a time-consuming and laborious work. Furthermore, it is very difficult to trace and collect transformed cells for performing studies on molecular mechanisms of transgene expression. In contrast, protoplast transient assays provide a quick method for studying gene functional expression, and have been widely used for measuring promoter activity and for analyzing plant gene activity (Kapila et al., 1997). Additionally, the transient protoplast system has shown similar results to methods of transformation using intact tissues and plants (Sheen, 2001). Here, we have established an

efficient protoplast isolation and PEG-mediated transient system for transgene expression and activity analysis, ultimately used for evaluation of regeneration and transformation efficiency in soybean. Based on the assays on percentages of fluorescence protoplasts, the transfection efficiency of all selected cotyledon protoplasts reached the peak on 2 DAT, and then decreased with increasing culture time (Table S2). On 3 DAT, significant differences in transfection efficiency were detected between HIGH and LOW genotypes using transgene expression and EYFP fluorescence percentage (Fig. 2A and B), which is considered as a key criterion for evaluation of genotype-dependent transfection efficiency in the soybean transient expression system. On the basis of the above variation, an equation, 3 DAT/2 DAT \times 100%, is proposed for transient evaluation of soybean transfection efficiency, which can be used to assess both EYFP fluorescence and transgene expression in the protoplasts. The more rapid decreasing of transgene expression and transfection efficiency in protoplasts also indicated that transgenes might gradually lose their activities during the *Agrobacterium*-mediated soybean transformation process.

4.3. Transgene methylation affects soybean regeneration and transformation

Various factors have been proposed to explain variation in transgene activity among transformants, which is independent of copy number (Stam et al., 1997). Expression and translation of transgene in host cells could be considered as similar to an “antigen” that activates various endogenous defense mechanisms, leading to product degradation. Evidence has shown that protective proteins, including inhibitors of serine, cysteine, and aspartyl proteinases, can play active roles in plant defense reactions (Ryan et al., 1998). In this study, however, treating with proteasome inhibitors MG132, MG115 and AEBSF did not enhance the fluorescence percentage in Wm 82 and Kt on 3 DAT (Fig. S5), which indicates that proteolysis is not involved in the defense mechanism against foreign genes in host cells as might have been expected. Therefore, another mechanism was investigated involving DNA methylation of foreign genes.

In eukaryotes, DNA methylation levels are often found to be associated with transgene inactivation (Vilperte et al., 2016). In plant genomic DNA, 5-mC can occur in CpG, CHG and CHH sites. Previous studies have shown that DNA methylation in gene promoters and coding sequences is associated with transcriptional gene silencing and post-transcriptional gene silencing, accounting for transgene silencing (Liu et al., 2017; Zha et al., 2017). In this study, our transient expression system uncovered higher DNA methylation levels in promoter and coding regions responsible for the EYFP gene expression in LOW compared to HIGH genotypes on 3 DAT by BSP sequencing (Fig. 3B and C). Furthermore, significantly increased methylation only occurred on 3 DAT in LOW genotypes, which is in agreement with the variation in *Agrobacterium*-mediated transformation efficiency. Although the specific regulation mechanism is not clear, it strongly suggests that the differences in transformation efficiency between LOW and HIGH genotypes may derive from DNA methylation of transgene in soybean. As is well known, hypermethylation in the promoter region can result in transcriptional repression because of inhibiting transcription factor binding and initiation of transcription. On the other hand, hypermethylation in the coding sequence region may affect the transcriptional process,

Table 2
Dosage-effect of 5-Azac treatment on soybean tissue culture.

5-Azac (μ M)	No. of shoots/100 explants	Percentage of shoot elongation (%)	Percentage of rooting (%)
0	31.86 \pm 1.93 a	84.90 \pm 1.52 a	83.94 \pm 4.93 a
10	72.93 \pm 1.17 b	84.27 \pm 3.41 a	85.71 \pm 3.19 a
30	74.77 \pm 1.87 b	83.27 \pm 2.98 a	83.72 \pm 2.89 a
50	73.71 \pm 1.88 b	83.36 \pm 2.99 a	86.79 \pm 1.05 a

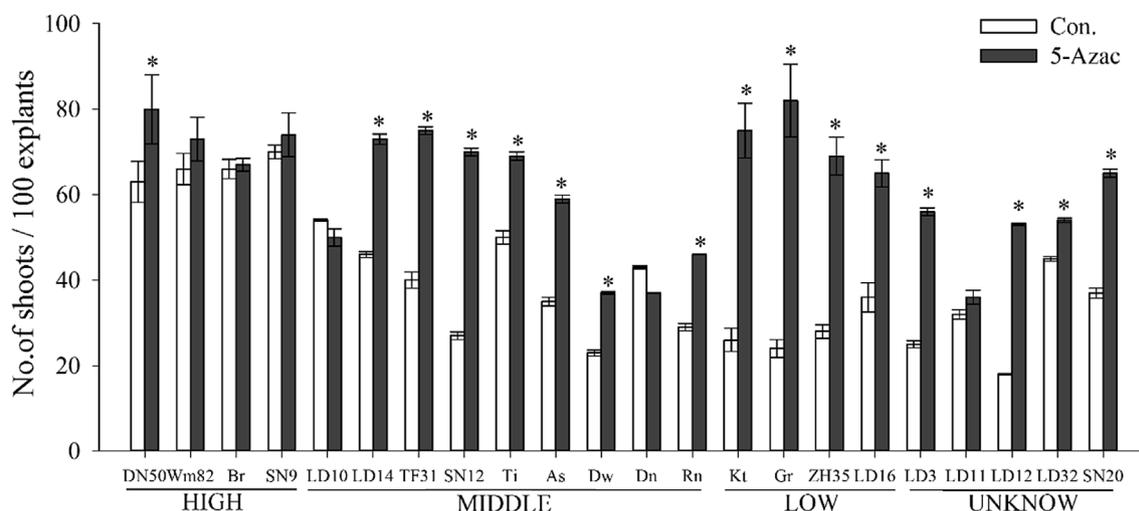


Fig. 6. Improvement of shoot regeneration by 5-Azac treatment during soybean transformation in twenty-two different soybean genotypes. Explants for each soybean genotype were transformed with pCAMBIA3301-1(35S::GUS-BAR) and cultivated for shoot regeneration as described in Fig. 6 with 0–1W 5-Azac treatment. MIDDLE: soybean genotypes of “middle” transformation-efficiency ($2 >$ transgenic plants/100 explants $>$ 0); UNKNOWN: soybean genotypes of “unknown” transformation-efficiency. Data collection was performed as described in Fig. 6 from three experimental replicates. More than 50 explants were used in each replicate for each genotype. Standard deviations were calculated. * Statistical significant difference to the control treatment ($P <$ 0.05).

leading to premature termination of transcription and having an important influence on exon recognition in alternative splicing (Maunakea et al., 2013; Maor et al., 2015). Further, homologous ncRNAs will then be produced to regulate target transgene. Additionally, the relative transcript level of the *MET1* gene was analyzed because of its methyltransferase function in higher plants (Gianoglio et al., 2017). Significant up-regulation of *MET1* was found in LOW genotypes (Fig. 3D) in agreement with data on transgene expression (Fig. 2A and B), DNA methylation (Fig. 3B and C) and effects of treatment with a DNA methylation inhibitor (Fig. 4), supporting this proposed mechanism.

4.4. 5-Azac application promotes soybean shoot regeneration

5-Azac is a well-known DNA methylation inhibitor, which has been commonly applied to induce DNA demethylation in plant studies and in cancer researches (Unnikrishnan et al., 2018). Previous studies have shown that 5-Azac treatment could enhance the transgene expression in transformed plant cells/tissue (Zhu et al., 1991; Palmgren et al., 1993; Song et al., 2017). In an early study with soybean protoplasts, the activity of transgene nopaline-synthase was strongly increased by 5-Azac to a variable extent (Balades et al., 1987), which implicated that DNA methylation might be the repressive mechanism on transgene expression in soybean. Firstly, in this experiment 5-Azac has been demonstrated to be capable of dramatically promoting the expression of transgenes and transfection efficiency in LOW genotype protoplasts (Fig. 5). Therefore, 5-Azac is a transfection-promoting agent in the PEG-mediated transient evaluation system (Fig. 4). To validate that 5-Azac would also promote soybean transformation, analysis of soybean shoot regeneration with 5-Azac was performed in the *Agrobacterium*-mediated soybean transformation system. The number of regenerated shoots was significantly increased by 5-Azac treatment in LOW genotypes to comparable levels as in HIGH genotypes (Fig. 5). Since the low shoot regeneration was shown to be the first bottleneck for transforming LOW soybean genotypes (Table 1), our findings have suggested that 5-Azac may be an applicable transformation-promoting agent especially for those transformation-recalcitrant soybean genotypes. In addition, the optimization of 5-Azac application concentrations was performed (Table 2) because it was reported that excessive 5-Azac treatment could cause negative effects on plant growth (Hohn et al., 1996; Zhao et al., 2006). We found that various concentrations of 5-Azac between 10 and 50 μ M displayed similar shoot regeneration-promoting effects on a

representative LOW genotype Kottman and caused no obvious negative defects in the following shoot elongation and rooting (Table 2).

Genotype-dependent transformation efficiency imposes severe restrictions on the transformation of soybean genotypes with excellent traits, such as ZH35, LD16 and TF31. However, when treated with 5-Azac, shoot regeneration efficiency of these genotypes can reach that of common varieties used in genetic transformation, such as Wm82, DN50 and Br, which would potentially cause transformation-efficiency increase in the T0 generation. This could be of significant benefit for the commercial development of transgenic soybean lines. Therefore, we analyzed twenty-two commercial Chinese and American soybean production genotypes with treatment of 5-Azac. Obvious promotion of shoot regeneration efficiency occurred in most LOW, MIDDLE and UNKNOWN genotypes, except LD10, Dn and LD11 (Fig. 6). We speculate that the reasons for the lack of increased shoot regeneration efficiency in HIGH genotypes may be that (1) little room for improvement is left in HIGH genotypes; or (2) genotypic differences lead to a different mechanism of transgenic silencing in these genotypes. Therefore, 5-Azac is available for soybean genotypes tested above to improve the shoot regeneration and eventually facilitate conquering the transformation-recalcitrant issue. For demethylation-insensitive commercial soybean genotypes, further study should be conducted on the mechanisms of transgene silencing and then screening for new transformation-promoting agents.

5. Conclusions

The present report reveals that variations in transformation efficiency correlate with the expression and methylation levels of the transgene in different soybean genotypes. Our protoplast transient assay revealed that the methylation level of promoter and coding regions in the transgene *EYFP* increased significantly, while corresponded substantially decreased transfection efficiency in LOW genotypes compared with HIGH genotypes on 3 DAT. Transfection efficiency was significantly improved by a 10 μ M 5-Azac treatment. In addition, the shoot regeneration efficiency (under 5 mg L⁻¹ PPT selection) in LOW genotypes was greatly enhanced with 5-Azac treatment of 0–1W, while it was not enhanced in HIGH soybean genotypes using an *Agrobacterium*-mediated soybean genetic transformation system. Moreover, there was no dose-dependent relationship between 5-Azac treatment of 10–50 μ M and the shoot regeneration efficiency, and 5-

Azac concentrations above 50 μM had no significant influence on shoot elongation and rooting. Since shoot regeneration is the key for high efficiency of *Agrobacterium*-mediated soybean transformation, these results provide new venue for improving the transformation efficiency of LOW soybean genotypes in *Agrobacterium*-mediated genetic transformation systems.

Contributions

Futi Xie and Mingzhe Zhao conceived the study and designed the experiments; Qiang Zhao and Yanli Du performed the experiment and analyzed the data; Qiang Zhao wrote the manuscript; Hetong Wang, Hilary J. Rogers, Wan Liu and Cuimei Yu revised the manuscript.

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Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plaphy.2019.05.014>.

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