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Establishment of Sorghum BTx623 Immature Embryos Genetic Transformation and Regeneration System

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Abstract Sorghum is one of the world's important crops after wheat, rice, maize, and barley. Although the sorghum genome had been well-sequenced, genetic breeding and functional genome research in sorghum cultivar BTx623 is still limited due to the lack of efficient and stable genetic transformation and regeneration system in sequencing. In this study, the immature embryos of sorghum genome-sequencing cultivar BTx623 was used as the explants material, and the bar gene resistant to phosphoglyphosate was used as the screening marker for *Agrobacterium*-mediated sorghum genetic transformation. By screening the adaptability of callus to different concentrations of phosphoglyphosate, the appropriate concentration of phosphoglyphosate in the genetic transformation of sorghum cultivar BTx623 was determined to be 2.5 mg/L, and BTx623 immature embryo was used as explants to obtain resistant callus. After screening, regenerated plants were obtained by treating resistant callus with 0.0067 mg/L ZNC in regeneration medium. Therefore, this study successfully obtained resistant callus and regenerating plants, and established a genetic transformation and regeneration system in sorghum cultivar BTx623, which may have great significance for functional genome research and genetic breeding in sorghum.
Keywords *Sorghum bicolor*; BTx623; Immature embryo; *Agrobacterium*-transformation; Regeneration

Sorghum (*Sorghum bicolor*) is the fifth largest cereal crop in the world after wheat (*Triticum aestivum*), rice (*Oryza sativa*), corn (*Zea mays*), and barley (*Hordeum vulgare*), and it has a strong photosynthetic efficiency as a C4 plant. It's widely grown on six continents (Liu and Godwin, 2012). Sorghum is not only a high-quality forage crop, but also an important energy plant; it also has extensive acreage and wide range of uses. Therefore, it has great significance of genetic improvement in sorghum (Duan et al., 2018).

Recently, the genetic transformation methods of sorghum are also improved and optimized with the developments of genetic engineering technology, which associated with the improved transformation efficiency. The genetic transformation in sorghum mainly includes four methods: electroporation, pollen pipeline transformation, gene-gun transformation and *Agrobacterium*-mediated transformation (Shao et al., 2015). The *Agrobacterium*- mediated transformation uses the natural of *Agrobacterium* for introducing foreign nucleotides into plant genome, and it has many advantages such as simple operation and low cost, which makes it the most extensive transformation method in plants (Peng, 2018). In 1994, people successfully used *Agrobacterium*-mediated method to obtain genetically transformed plants in sorghum (Godwin and Chikwamba, 1994). Furthermore, young embryos were used as recipient tissue using the Agrobacterium-mediated method and transformed sorghum plants were obtained with an average 2.1% transformation efficiency (Zhao et al., 2000). Subsequently, many studies used this method to obtain genetically transformed sorghum plants but the transformation efficiency and regeneration rate were still very low (Zhu et al., 2011). Although the first research on genetically modified sorghum has been carried out for more than two decades, the transformation efficiency of sorghum is still far below 50%~90% of rice (Hiei and Komari, 2008), 50% of corn (Ishida et al., 2007) and 14.8% of barley (Ibrahim et al., 2010). In terms of tissue

culture and genetic transformation, sorghum has been widely regarded as a difficult crop for genetic transformation and regeneration (Grootboom et al., 2010).

There are many factors that affect the transformation efficiency of sorghum. Genotype, *Agrobacterium* strains, culture media, explant types and bacterial concentration are considered as main factors in sorghum transformation (Zhang, 2002). Sorghum cultivars that can be transformed by *Agrobacterium*-mediated methods include: P898012, Pioneer 8505, PHI391, Sensako 85/1191, Tx430, etc., and now is mainly concentrated in P898012 and Tx430 (Hiei et al., 2014; Shao et al., 2015). Meanwhile, it is difficult for sorghum to regenerate complete plants by callus, which has become a technical bottleneck for genetic transformation in sorghum (Liu et al., 2015; Peng, 2018). The study found that the success rate of callus induction and plant regeneration of immature embryos of sorghum varies greatly, especially depending on the plant genotype (Do and Zhang, 2015). Therefore, the transformation efficiency and regeneration efficiency of different genotypes are different, even use the same cultivation and transformation conditions (Do and Zhang, 2015). Although the sorghum cultivar BTx623 had been well-sequenced, which provided assistance for the study of sorghum gene function (Paterson et al., 2009), there are still few studies on the genetic transformation an d regeneration system of BTx623. In 2005, gene-gun transformation was performed to obtain genetically transformed plants by bombarding the apex of BTx623. Transferring Baby Boom and Wuschel2 genes, which is related to embryonic development, can promote the regeneration in Btx623. However, the complex operation and the low rate of genetic transformation limit the large-scale application of this technology (GirijaShankar et al., 2005; Nelson-Vasilchik et al., 2018). The current sorghum genome sequence is mainly derived from BTx623, so the lack of an effective BTx623 genetic transformation and regeneration system will hinder the development of sorghum genetic engineering and genome function research.

The establishment of a plant regeneration system is necessary for using plant callus to obtain genetically transformed plants. In sorghum tissue culture plant hormones, medium composition, and accumulation of phenols during the cultivation process have a great influence on the induction rate and survival rate of callus (Zhang, 2002; Polumahanthi et al., 2015). Zhineng Cong (ZNC) is an extract of *Paecilomyces variotii* and a highly effective plant immune inducer. It can enhance disease resistance and stress resistance by activating the molecular immune system and regulating metabolism in plants (Qiu et al., 2014). Previous studies have shown that low concentrations of ZNC can promote the elongation of roots and biomass accumulation in rice and mazie. ZNC has similar functions as auxin or its analogs in promoting plant growth (Lu et al., 2019). But at present, the application research of ZNC in sorghum has not been reported.

In this study, the immature embryos of sorghum cultivar BTx623 were used as explant materials. The bar gene of phosphoglyphosate-resistant was introduced into the genome of sorghum cultivar BTx623 by *Agrobacterium*-mediated, and the genetic transformation resistance callus of BTx623 was obtained. Sorghum callus regenerated plant are obtained by adding ZNC in the regeneration process to promote the regeneration. The genetic transformation and regeneration system of sorghum cultivar BTx623 was initially established, which laid the foundation for the development gene function group and genetic breeding work in sorghum.

1 Results and Analysis

1.1 Adaptability of callus to different concentrations of phosphoglyphosate

The *bar* gene has good resistance to phosphoglyphosate. At present, the concentration of BTx623 callus to phosphoglyphosate is not clear. In order to determine the concentration required for the screening of phosphoglyphosate after transformation, the sensitivity test of phosphoglyphosate on callus was conducted. Experimental results showed (Table 1): After 14 days of cultivation, the callus can survive when the phosphoglyphosate concentration is 0; the survival rates of phosphoglyphosate at 0.5 mg/L and 1.5 mg/L were (40.00±5.44)% and (13.99±5.03)% respectively. When the concentration of phosphoglyphosate was greater than 2.5 mg/L, the callus could not grow normally. The growth of callus in different phosphoglyphosate concentrations was significantly inhibited. As the

phosphoglyphosate concentration increased, the callus browned and died, and the survival rate gradually decreased. When the phosphoglyphosate concentration was 2.5 mg/L and 5 mg/L, the callus did not grow or stopped growing and died of browning in 5 days. Although a small amount of callus survived when the concentration was 0.5 mg/L or 1.5 mg/L, calli were mostly water-stained and poor quality. Therefore, 1.5 mg/L phosphoglyphosate was selected as the initial screening concentration in the transformation, and 2.5 mg/L was selected as the final concentration.

Table 1 Lethal test of phosphoglyphosate

Concentration of glyphosate (mg/L)	Number of Inoculation	Number of survivors	Survival rate (%)
0	46	46	100.00±0.00
0.5	45	18	40.00±5.44
1.5	42	6	13.99±5.03
2.5	36	0	0.00±0.00
5.0	41	0	0.00±0.00

1.2 *Agrobacterium*-mediated transformation and resistance callus obtained

After clarifying the screening phosphoglyphosate concentration of sorghum callus. In order to establish the BTx623 genetic transformation system, sorghum BTx623 immature embryos were inoculated with *Agrobacterium* AGL1-pEGAD with *bar* gene (Figure 1). To improve the coversion rate, the surfactants Silwet L-77 and L-Glutamine (L-Gln) were added to the inoculum and silver nitrate was added during co-cultivation.

A total of 1 827 calli were obtained after co-cultivation for 3 days (Figure 1de) and resting culture for 10 days (Figure 1fg), the average induction rate of immature embryos reached (96.04±0.5)3%. Transferred callus to the selection medium with phosphoglyphosate concentration of 1.5 mg/ L for 2 weeks as initial screening. Then it was transferred to a screening medium with phosphoglyphosate concentration of 2.5 mg/L and cultured for 2 weeks as rescreening. A total of 36 resistant calli were finally obtained through the three tests with an average conversion rate of (1.94±0.33%) (Figure 1i; Table 2). The DNA of resistant callus was extracted and PCR positive detection (Figure 2) showed and that the PCR amplified band of resistant callus were consistent with the positive control, and the negative control did not amplify the band, which indicated that the transformation was successful. It proved that the obtained resistant callus was a positive transformed callus.

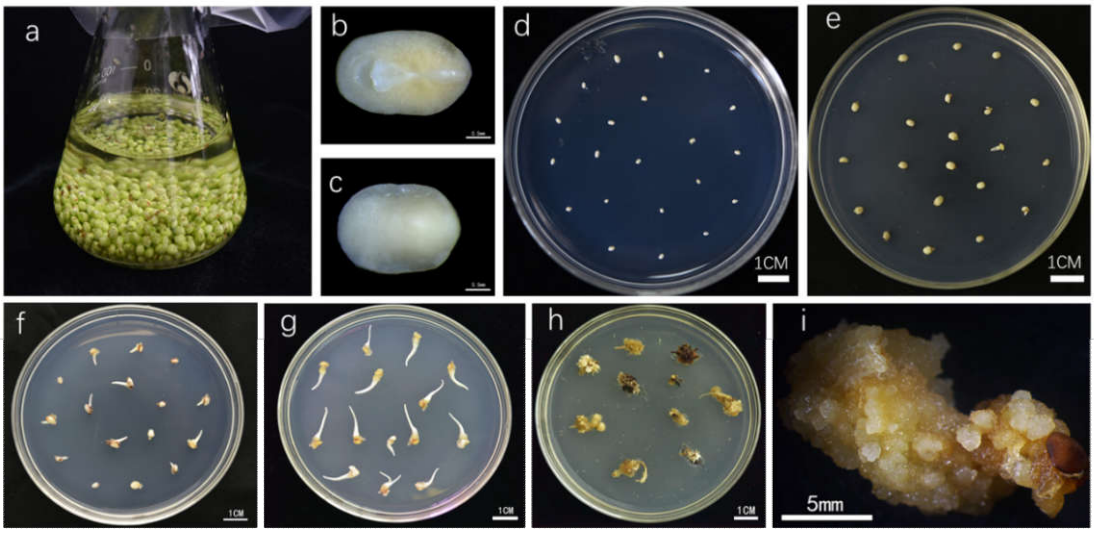

Figure 1 Infection transformation and screening of immature embryos

Note: a: Immature seeds; b, c: Immature embryos; d: 0 days of impregnation; e: Co-cultivate for 3 days; f: Resting for 5 days G: Resting for 10 days; H: Screening for 4 weeks; I: Resistant callus

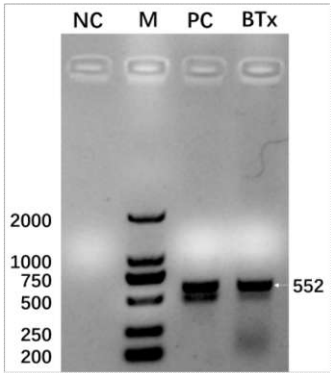

Figure 2 Positive amplification of BTx623 resistant callus

Note: M: DL2 000 marker; BTx: BTx623 resistant callus DNA; PC: as a positive control; NC: as a negative control

Table 2 Induction rate and transgenic rate of BTx623

Batch	Number of Inoculation	Number of callus	Number of resistant callus	Inductionrate	Transformation rate
1	704	671	15	95.31%	2.24%
2	557	536	8	96.23%	1.49%
3	642	620	13	96.57%	2.10%

1.3 Resistant callus differentiation and BTx623 callus regeneration

The regeneration of resistant callus is a key step of genetic transformation. In order to explore the sorghum cultivar BTx623 callus regeneration system, we inoculated resistant callus on the differentiation medium. It was found that during the differentiation process, phenols were secreted around the callus with the extension of the cultivation time, the medium became brown after 2 weeks of cultivation, and many plexiform roots grew on the surface of the callus. Eventually failed to obtain regenerated plants. The plant immune inducer ZNC has the effects of promoting growth, biomass accumulation and nutrient absorption in rice and mazie, so it is speculated that ZNC may promote the growth and regeneration of sorghum resistant callus. Therefore, we added different concentrations of ZNC to the differentiation medium to observe the effect of ZNC on the growth and regeneration of BTx623 callus, and the results showed that BTx623 callus produced more plexiform roots without ZNC addition (Figure 3A); with the increase of ZNC concentration, the production of plexiform roots decreased significantly, at ZNC concentration of 0.006 7 mg/L, 0.01 mg/L and 0.02 mg/L, there were obvious green shoots (Figure 3A). But at ZNC concentration of 0.01 mg/L and 0.02 mg/L, the green shoots of callus will gradually brown and die. Therefore, this study chosed to continue regeneration culture in regeneration medium with ZNC concentration of 0.006 7 mg/L. The callus was placed in a regeneration medium with a ZNC concentration of 0.006 7 mg/L, and the regenerated plants were finally obtained after 21 days of light cultivation (Figure 3B).

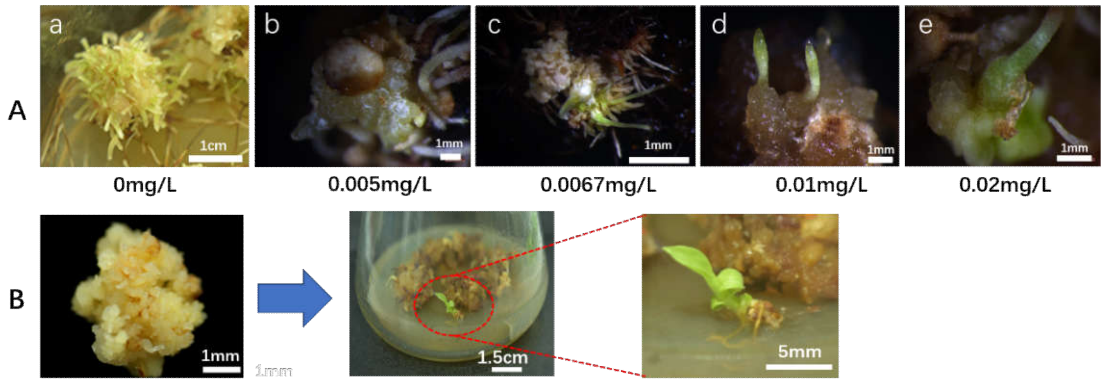

Figure 3 Establishment of BTx623 regeneration system

Note: A: Effects of different concentrations of ZNC on sorghum callus; B: Obtain of regenerated sorghum BTx623 plant

2 Discussion

In this study, the sorghum cultivar BTx623 resistant callus was tested for adaptability to different concentrations of phosphoglyphosate, and obtained that the concentration of phosphoglyphosate was 2.5 mg/L as the optimal screening concentration. Then BTx623-resistant callus was successfully obtained through *Agrobacterium*-mediated transformation, and ZNC was successfully added to the regeneration medium to obtain callus regeneration transformed plants. In this study, the genetic transformation and regeneration system of sorghum cultivar BTx623 was basically established, which provided technical support for genetic breeding and functional genomics research in sorghum.

Because sorghum has a strong genotype dependence, different genotypes have different genetic transformation systems. At present, Tx430 and P898012 have good regeneration ability, so they are the main genetic transformation materials in sorghum, but the genetic transformation and regeneration of other cultivars are more difficult (Do and Zhang, 2015). The genotype is different from other genotypes such as Tx430, which makes traditional transformation and regeneration are not fully applicable to BTx623. Plant necrosis caused by *Agrobacterium* infection in sorghum transformation is a common phenomenon, and adjusting the infection time, *Agrobacterium* concentration are the common control methods. This study foundy that it can improve the antioxidant capacity of explants, promote *Agrobacterium* transformation and reduce the plant tissue necrosis caused by adding L-cysteine (L-Cys), L-glutamine (L-Gln) and silver nitrate. In the callus culture and regeneration stage, using polyvinylpyrrolidone (PVP) instead of cross-linked polyvinylpyrrolidone (PVPP) also achieved the effect of preventing browning. PVP is more soluble in water than PVPP, and the amount of PVP is less (Do et al., 2016). In co-cultivation, the addition of vitamin C can not only reduce oxidation and reduce plant loss, but also help reduce browning. In previous studies, the sorghum cultivar BTx623 did not have a mature genetic transformation system. In this study, the sorghum cultivar BTx623 obtained a conversion rate of (1.94±0.33)%, and it provides new ideas for the establishment of sorghum genetic transformation systems.

In this study, sorghum cultivar BTx623 callus could not obtain regenerated plants and many plexiform roots grew when ZNC was not added. With the extension of culture time, no embryonic buds were produced, which indicated that the callus may lack differentiation and regeneration ability. When ZNC was added, the plexiform roots were significantly reduced and green shoots were produced, which indicated that ZNC may promote the embryogenic differentiation of sorghum cultivar BTx623 resistant callus, and it provided the possibility for its regeneration. The plant immune inducer *Paecilomyces variotii* extract ZNC has an auxin-like effect. The effective concentration of ZNC in this study is 0.0 067 mg/L, which is much lower than the effective concentration of auxin or its analogs (1~2 mg/L). ZNC is a mixture, so it is speculated that the differentiation and regeneration of sorghum cultivar BTx623 is likely to be the result of a combination of substances in ZNC, which promotes the growth and differentiation of sorghum cultivar BTx623 (Lu et al., 2019). Therefore, by adding metabolites, extracts of bacteria or fungi and breaking the restrictions of conventional hormones, it may be a new idea for future plant genetic transformation and tissue culture.

3 Materials and Methods

3.1 Experiment materials

The sorghum cultivar BTx623 seeds used in this experiment came from the Biotechnology Research Center of China Three Gorges University and were planted in the experimental field. They were bagged before flowering, and immature seeds 14 days after flowering were selected as test materials. The *Agrobacterium* strain was AGL1 and the plasmid was pEGAD (the reporter gene was GFP, the screening gene was bar), they are all provided by the Biotechnology Research Center of China Three Gorges University.

3.2 Treatment of immature seeds in sorghum

Immerse the sorghum immature seeds in tap water for 20 min (add a few drops of detergent), and rinse with running water for 30 min, then transfer to a sterile clean bench, sterilize with 75% alcohol for 5 min, wash twice

with sterile water; 0.1% mercuric dichloride (containing 0.1% Tween-20) was sterilized for 30 minutes, and wash three times with sterile water (about 5 minutes each time) (Figure 1a).

3.3 Adaptability test of phosphoglyphosate in sorghum callus

Put the immature seeds on the sterile filter paper in the clean bench, and use sterilized forceps, dissecting needles to take out the sorghum immature embryos (Figure 1b; Figure 1c), then put the scutellum upward into the induction medium to induced 14d (Figure 1c). Inoculated the induced callus on the medium containing different concentrations of phosphoglyphosate and cultured in the dark at 25℃ for 14 days to observed the growth of callus.

3.4 Preparation of *Agrobacterium*

Take the constructed *Agrobacterium* AGL1-pEGAD (already stored at -80℃) on a solid LB medium plate containing 50 mg/L of rifampicin and kanamycin, and cultivate at 28℃ for two days, then pick single colony on LB liquid medium (containing 50 mg/L each of rifampicin and kanamycin), perform shaker culture (100 r/min, 28℃), and culture for about 8h until OD_{600} reached 0.4~0.7, then Centrifuge for 5 minutes at 4℃, 5 000 r/min, discard the supernatant to collect the cells and resuspend the cells with 5 mL of inoculation medium, then transfer to 50 mL of inoculation medium and cultivate to OD_{600}=0.5 for dipping.

3.5 *Agrobacterium* infection, co-culture and resting sterilization

Put the treated immature seeds on sterile filter paper in the clean bench, and use sterilized forceps and dissecting needles to remove the sorghum immature embryos (Figure 1b; Figure 1c). Place the immature embryos in 50 mL EP tube with liquid medium in a 43℃ water bath for 3 minutes and palce for 2 minutes at 25℃, then add bacterial solution to infuse for 10 minutes. Remove the immature embryos, blot the surface liquid with sterilized filter paper, and place the scutellum upwards in the co-cultivation medium (Figure 1c; Figure 1d). Incubate at 25℃ in the dark for 3 days (Figure 1; Figure 1e). After co-cultivation, remove young embryos from the co-culture medium, then wash them with sterile cephalosporin-added water for 3 to 5 times, then absorb the water with sterile filter paper and place them in the resting medium incubate at 25℃ for 10 days in the dark (Figure 1; Figure 1f; Figure 1g).

3.6 Screening and differentiation regeneration

Take out immature embryos after co-cultivation, placed them on the selection medium and cultured in the dark at 25℃ for 14 days. Then they were cultured on high-concentration phosphoglyphosate-screening medium for 14 days (Figure 1h). After that, the obtained BTx623 resistant callus was inoculated on the subculture medium. After culturing for 14 days, part of the resistant callus was inoculated on the germination medium for differentiation culture and cultured for another 14 days at 25℃, light: dark=16:8 h. When the bud germinated and growed to three or four leaves, it was transferred to the rooting medium for rooting culture under the same growth conditions as the bud.

3.7 Medium formula and calculation formula

The medium formula is slightly changed on the basis of previous studies (Do et al., 2016), and ZNC is added to the germination medium at the callus regeneration stage to promote callus regeneration (Table 3).

1 B5 organic (1000X): inositol: 100 mg/mL; niacin (b3): 1.0 mg/mL; pyridoxine hydrochloride (b6): 1.0 mg/mL; thiamine hydrochloride (b1): 10.0 mg/mL.

2 Cephalosporin 200 mg/L should be added to the resting medium, and phosphoglyphosate (2.5mg/L) should be added to the screening medium and regeneration medium for screening.

The calculation formula is:
Induction rate = (number of callus induced/number of inoculated explants) ×100%
Resistance rate = (resistance callus number/induced callus number) ×100%

Table 3 Medium formula

Medium composition	Unit	Infection	Cocultivation	Resting	Selection	Budding	Rooting	Induction
MS powder	g/L	4.43	4.43	4.43	4.43	4.43	4.43	4.43
B5 organics (1000X)	mL/L	1	1	1	1	1	1	1
Sugar	g/L	68.5	20	30	30	30	30	30
Glucose	g/L	36	10	0	0	0	0	0
Vc	mg/L	0	10	0	0	0	0	0
AS	μmol/L	100	100	0	0	0	0	0
MES	g/L	0.5	0.5	0	0	0	0	0
Silwet L-77	mL/L	0.05	0	0	0	0	0	0
L-Gln	μmol/L	100	100	0	0	0	0	0
L-Cys	mg/L	0	200	0	0	0	0	0
$AgNO_3$	mg/L	0	0.85	0	0	0	0	0
2,4-D	mg/L	1.5	2	2	1	0	0	2
IAA	mg/L	0	0	0	0	1	0	0
IBA	mg/L	0	0	0	0	0	1	0
6-BA	mg/L	0	0	0.5	0.5	1	0	0
KH_2PO_4	g/L	0	0	1	1	0	0	1
L-Proline	mg/L	0	0.7	1	1	0	0	1
L-Asparagine	g/L	0	0	1	1	0	0	1
$CuSO_4·5H_2O$	mg/L	0	0	2.5	2.5	0.25	0.25	2.5
PVP	g/L	0	0	1	1	1	1	1
Agar	g/L	0	8	8	8	8	8	8
pH		5.2	5.8	5.8	5.8	5.8	5.8	5.8

Authors' contributions

Cheng Yunwei is the experimental designer and executive of this research; Lu Yelei, Han Shaopeng and Lu Yang completed the data analysis and the writing of the first draft of the paper; Deng Wei, Zhou Chao and Zhang Dechun participated in the experimental design and analysis of the test results; Shen Xiangling is the architect and person in charge of the project, directing experimental design, data analysis, and writing and revising papers. All authors read and approved the final manuscript.

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