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Full Length Article

Development of a Simple and Efficient Method for *Agrobacterium*-Mediated Transformation in Sorghum

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Abstract

Sorghum is considered a recalcitrant crop based on genetic transformation. The main technical problem is callus browning in culture tissue and genetic transformation for sorghum. In this study, we developed a simple and effective method for sorghum transformation excluding tissue culture process. Sorghum mature embryos were pierced by a needle and then soaked in an *Agrobacterium* inoculum with acetosyringone. The best-performing concentration of acetosyringone was 100 μM in inoculation with pierced seeds. When these inoculated seeds grew to three leaves, a 1‰ hygromycin solution was used to brush the second and third leaf of inoculated seedlings to identify transformants. The identification results showed that the highest efficiency of transformation was 4% in the study. Molecular analysis for T_0 and T_1 plants identified that target gene was integrated into sorghum genomic DNA. And target gene can normally express in transformants' leaves. Genetic analysis of T_1 transformants showed that most of T_1 transformants fitted to 3:1 Mendelian inheritance. All these results showed that the presented method is an easy and successful method for sorghum transformation. In the future, the method will be used for broad functional genomics studies and for biotechnological application of sorghum. © 2016 Friends Science Publishers

Keywords: Acetosyringone; *Agrobacterium tumefaciens*; Sorghum transformation; Green fluorescent protein; Transformants

Introduction

Sorghum (*Sorghum bicolor* (L.) Moench) is the world's fifth most important crop, after wheat, rice, maize and barley. This crop is grown throughout arid and semiarid regions because of its yield stability and well-adaptability under drought conditions (Grenier *et al*., 2004). Sorghum is widely used for food, especially in India and Africa, and for forage and as a biofuel energy crop in Americas and Australia (Carpita and McCann, 2008). The sorghum genome contains approximately 730 Mb of DNA, which is larger than that of the C3 crop rice but 3-folds smaller than that of the C4 crop maize (Paterson *et al*., 2009). The small genome of this species makes sorghum an attractive model for the functional genomics of C4 crop.

An efficient genetic transformation is foundation of functional genomics research. The first study on sorghum transformation was published thirty-two years ago (Gritz and Davies, 1983). In fact, sorghum transformation has lagged far behind rice, maize. Until now, most of researches for sorghum transformation are based on tissue culture. The main problem for sorghum transformation is callus browning in culture tissue and genetic transformation (Gurel *et al*., 2009). Many studies for sorghum transformation have been done, but the average transformation efficiency has remained below 20% (Zhao *et al*., 2000; Gao *et al*., 2005a, b; Gurel *et al*., 2009; Liu and Godwin, 2012). Additionally, these methods based on tissue culture will take six months to get transformed seedlings. So, it is important for the researches of sorghum functional genomics to develop a faster and simpler method.

The method of "in planta" transformation was first developed by Felmann and Marks (Feldmann and David, 1987). Today, the method is applied to many plants, including *Arabidposis*, rice, maize and wheat (Feldmann and David, 1987; Supartana *et al*., 2005, 2006). This method eliminates tissue culture process and only requires soaking mature seeds in *Agrobacterium tumefaciens*. Lin *et al*. (2012) and Fursova *et al*. (2012) respectively identified piercing seeds that were inoculated with *Agrobacterium tumefaciens*. However, to the best of our knowledge, this method for the transformation of sorghum has not yet been reported.

In this study, we developed and optimized a simple method for the transformation of sorghum that does not require the use of tissue culture and combines piercing embryo with *Agrobacterium*-mediated transformation. We also developed a simple method for screening transgenic plants. This method can make sorghum transformation more effective and much easier.

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Materials and Methods

Plant Materials and Vectors

Sorghum varieties U68 and Tx623B were used in this study. U68 was provided by J. F. Pedersen; Tx623B was provided by Qiuwen Zhan.Two binary vectors, pCUbi1390 and pCAMBIA1305.1-GFP, were used in the research. The sorghum *CAD2* gene Sb04g005950 was cloned into the KpnI and SpeI sites of the pCUbi1390 vector, which was thereafter called pCUbi1390-CAD2, containing the select marker hygromycin resistance (*hph*) gene (Fig. 1A). The other vector pCAMBIA1305.1-GFP contained hygromycin resistance (*hph*) gene and GFP (green fluorescent protein) gene (Fig. 1B). The constructs were introduced into *Agrobacterium tumefaciens* strain EHA105 by heat shock method.

Transformation Procedure

Agrobacterium cultures (100 mL) containing harboring pCAMBIA1305.1-GFP or pCUbi1390-CAD2 were grown in LB medium containing 50 mg L^{-1} kanamycin and 100 mg/L rifampicin at 28°C to OD600=0.6. The cultures were used as *Agrobacterium* inoculum.

The sorghum seeds were sterilized with 70% ethanol for 1 minute and then with 0.1% bleach solution for 30 minutes. Sterilized seeds were washed three to five times with sterile water for elimination bleach. Then, sterilized seeds were completely rinsed and soaked in sterile water at 25°C for 12 h. After soaking, embryos of these seeds turned white and were pierced once or twice by a needle (dimeter 1.0 mm). The piercing depth was approximately 1 mm. These pierced seeds were immersed in *Agrobacterium* inoculum, to which was added acetosyringone at four different concentrations. These seeds were transferred onto wet papers in petri and incubated in dark at 22°C for 4 days.

These seeds were soaked in a 250 mg/L carbenicillin solution for 1 h to kill *Agrobacterium* after 4 days of incubation. These seeds were washed 5 times with sterile water. Subsequently, seeds were planted in Potting Mix soil (PeiLei, China) and grown in a constant incubator for two weeks. The seedlings were normally grown to 3 leaves, and were then used for hygromycin resistance assays. Finally, positive seedlings were transplanted to Sanya, Hainan province in China. The control was untransformed plants.

Hygromycin Resistance Assays

To minimize use of hygromycin, we developed a new method for hygromycin resistance called brush-painting method. The second and third leaf were painted with a 1‰ hygromycin solution by a brush and marked with a marker pen to identify brushed leaves with hygromycin solution.

PCR and Sequencing Analysis

Sorghum genomic DNA was isolated from the flag leaves of transformants with hygromycin-resistant plants by an extraction method with sodium dodecyl sulfate (SDS). PCR was performed by KOD FX polymerase (TOYOBO, Japan). The fragment for vector pCUbi1390-CAD2 was amplified by the next primers: forward primer (F) 5'-CGGACTAGTATGGGGAGCCTGGCG-3' and reverse primer (R) 5'-CGCGGATCCGTTGCTCGGCGCATC-3'. The forward sequence was located in 35S promoter region, and the reverse sequence was located in Sb04g005950 gene region. A 759-bp fragment of GFP gene was amplified by the next primers: forward primer (F) 5'-GAATTCATGGTGAGCAAGGGC-3' and reverse primer (R) 5'- AAGCTTGGATCCGTGATGGTG-3'.

The amplified fragments were purified using DNA Gel Extraction Kit and sent to GenScript Company for sequencing.

Western Blot Analysis

The first and second leaves of T_1 seedlings were ground in liquid nitrogen. Samples was homogenized in protein extraction buffer [2 mM EDTA, 100 μM phenylmethylsulfonyl fluoride (PMSF),10 $\mu g \cdot mL^{-1}$ pepstatin A , 330 mM sorbitol, 10 μg/mL leupeptin and 50 mM HEPES/KOH pH 8.0] before centrifugation at 15,000 g at 4°C for 15 min to collect the soluble proteins. The proteins were transferred electrophoretically to a polyvinylidene difluoride membrane. The membrane was then incubated with antibodies. The first and second antibodies (GFP antibody) came from BGI Company (Beijing, china).

Inheritance Analysis

The seeds from hygromycin-resistant T_0 plants were immersed in water for 12 h at 25°C for germination. The germinated seeds were transferred to wet paper in a Petri plate to which 1% hygromycin solution was added. The solution was frequently added to the Petri so that the roots of seedlings were always soaked in hygromycin solution; 15 days later, the roots of transgenic plants were observed and analyzed. The non-transformed seeds were cultured in water as control.

Results

The Factors that Affected the Efficiency of Sorghum Transformation

Piercing embryos will hurt seed vitality and decrease the percentage of seed germination. To understand the effects of piercing embryos, the seedling emergence rate of pierced embryos and non-pierced embryos was measured using 300 seeds. The seedling emergence rate of the pierced embryos was 73.2%, while that of non-pierced embryos was 83.5%, suggesting that piercing embryos decreased the seedling emergency rate.

The ideal amount of acetosyringone will increase the

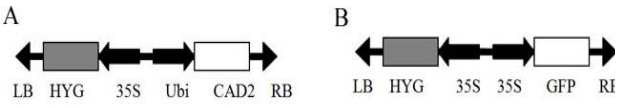

Fig. 1: Schematic diagram of the T-DNA of pCUbi1390-CAD2 (A) and pCAMBIA1305.1-GFP (B) expression vector. LB, left border; RB, right border; 35S, cauliflower mosaic virus 35S promoter; GFP, green fluorescent protein

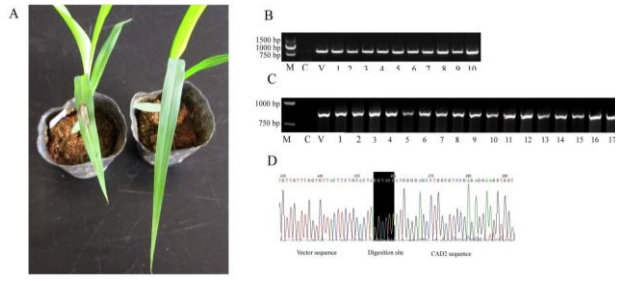

Fig. 2: Antibiotic resistance and PCR analysis of the T_0 transformants. A, Antibiotic resistant analysis of T_0 transformants; B, PCR analysis of 10 T_0 transformants that were inoculated with pCUbi1390-CAD2; C, PCR analysis of 17 transformants that were inoculated with pCAMBIA1305.1-GFP; and D, Sequencing analysis of T_0 transformants that were inoculated with pCUbi1390-CAD2. M, marker; V, vector

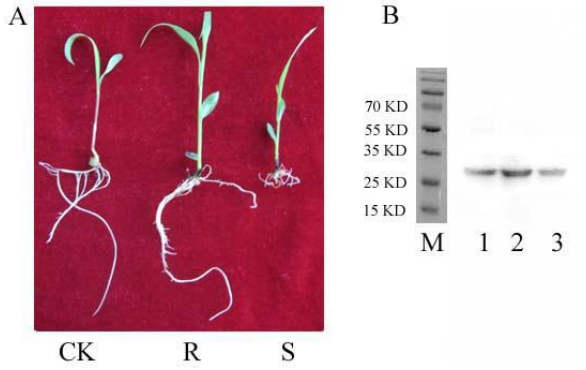

Fig. 3: Resistant and Western blot analysis of the T_1 transformants. A, Resistant analysis of the T_1 lines (R, resistant seedling; S, susceptible seedling) and B, Western analyses of the T_1 transformants (M, marker)

rate of transformation in crops (Sheikholeslam and Weeks 1987). To verify the ideal concentration of acetosyringone, four different acetosyringone concentrations were added during the transformation process. The results are shown in Table 1. The transformation efficiencies ranged between 1.00% and 3.00% that these seeds inoculated with pCUbi1390-CAD2. The total number of transformants was 15, and the average transformation efficiency was 1.875%. The transformation efficiencies ranged between 1.00% and 4.00% inoculated with pCAMBIA1305.1-GFP. The total number of transformants was 21, and the average transformation efficiency was 2.50%. These results indicate that adding acetosyringone could increase the transformation efficiency using the piercing embryo method. In addition, the best concentration of acetosyringone for transformation was 100 μM.

Hygromycin Resistance and Molecular Analysis of T_0 Transformants

Usually, leaves of transformants were cut and soaked in 1‰ hygromycin solution for resistance analysis. However, this method is costly and laborious when many plants are used in a resistance analysis. For ease, we developed a method called brush-paint method. The leaves of non-inoculated plants (control) showed necrosis 3 days after brushing 1‰ hygromycin solution. However, a leaf of transformants was healthy and green with hygromycin resistance (Fig. 2A). The plants with hygromycin resistance were considered as transgenic plants. These results also indicate that piercing mature embryos with *Agrobacterium* is an effective method for sorghum transformation.

To further confirm transformants at a molecular level, PCR analysis was conducted on the CAD2 and GFP genes that were integrated in the genomic DNA for these plants with hygromycin resistance. Genomic DNA was isolated from flag leaf of each transgenic plant with hygromycin resistance when these plants grew to maturation. 5 and 4 hygromycin-resistant plants that were respectively inoculated with pCUbi1390-CAD2 and pCAMBIA1305.1-GFP died in transport to Hainan Province for planting. A total of 10 hygromycin-resistant plants that were inoculated with pCUbi1390-CAD2 were amplified and produced the same size band with vector, while non-transformed plants did not amplify this band (Fig. 2B). Similarity, 17 hygromycin-resistant plants that were inoculated with pCAMBIA1305.1-GFP contained transgene with the expected band size (Fig. 2C). The amplified bands were purified and sequenced, demonstrating that the amplified band is the target gene (Fig. 2D). The results further confirm that T-DNA was transferred into sorghum and that target gene was integrated into the genomic DNA.

Genetic and Western Blot Analysis of T_1 Transformants

To verify the inherited model of transformants, a genetic analysis was conducted in the transgenic T_1 generation using hygromycin selection. The root length of the transgenic plants was normal, while the non-transgenic plants were short (Fig. 3A). Genetic analysis identified only 3 lines with hygromycin-resistant T_1 seedlings in 10 T_1 plants that were transformed with pCUbi1390-CAD2. The two lines C1 and C2 fitted 3:1 Mendelian inheritance, but line C3 did not. Similarly, 5 lines were had hygromycin-resistant T_1 seedlings in the 17 T_1 lines that were transformed with pCAMBIA1305.1-GFP. All of these lines fitted 3:1

Table 1: Effects of acetosyringone on transformation frequency of sorghum mature embryo

Vector	AS	Seeds Number	Average transformation efficiency
pCUbi1390-CAD2	0	200	1.00%
	100	200	3.00%
	200	200	1.50%
	300	200	2.00%
pCAMBIA1305.1-GFP	0	200	1.00%
	100	200	4.00%
	200	200	2.50%
	300	200	2.50%

Table 2: Segregation of transgenic (resistant) and non-transgenic (susceptible) seedlings in the T_1 plants by hygromycin selection

Line No.	Number of resistant seedling	Number of susceptible seedling	χ^2
C1	42	12	0.10
C2	33	19	3.1
C3	52	3	10.18**
G1	38	6	2.45
G2	62	24	0.55
G3	54	12	1.29
G4	15	10	2.25
G5	57	24	0.69

Note: **means significant at p 0.01

Mendelian inheritance, indicating that most of T_1 transformants underwent Mendelian inheritance for the two vectors (Table 2).

To further detect the expression of transgenes, we performed a Western blot analysis on three independent transgenic T_1 plants that were inoculated with pCAMBIA1305.1-GFP. An expected protein band was detected in the three independent transgenic plants (Fig. 3B). Our results showed that the transgenes had integrated into sorghum genomic DNA and also could be expressed normally in the leaves.

Discussion

The sorghum transformation method based on tissue culture has been widely considered challenging since the first research on sorghum transformation was published (Gritz and Davies, 1983; Liu and Godwin, 2012). Recently, the highest efficiency of sorghum transformation was reported at more than 20% using *Agrobacterium*-mediated and microprojectile transformation (Liu and Godwin, 2012; Wu *et al*., 2014). However, both of these studies used TX430 immature embryos as explants to induce callus. Although the transformation efficiency was higher than that in our research, this value is limited by the genotype and the time required generating immature embryos. In the study, we used two sorghum varieties for the transformation. The transformation efficiency of the two varieties was almost the same, suggesting that our method was less limited by genotype.

Normally, transformed method based on tissue culture will take six months get transformed seedlings (Liu and Godwin, 2012). Our method eliminates tissue culture process and only requires piercing mature embryos. So, we only need two months to get transformed seedlings. This method simplifies the entire transformation procedure and shortens the subsequent analytical process of transgenic plants. Therefore, our method shortens the time to get transformants. It provides faster solution to get transformed seedling for sorghum functional genomics research.

The method of piercing mature seeds for transformation has been applied to wheat and rice (Lin *et al*., 2009; Supartana *et al*., 2006). The usual method is laborious when identifying resistant plants. In the research, we developed a new resistant analysis method called the brush-painting method. Our studied also found that all of the resistant plants that were identified by the brush-painting method also amplified the same band with the vector. Therefore, this method is effective in resistant analysis. Compared to the ordinary method in resistant analysis, this method is simpler and cheaper for using hygromycin B.

Genetic transformation technology plays an important role in modern agriculture. A simpler and easier method will improve the application of this technology. Our research found that piercing sorghum mature seeds is an effective method for transformation. Although the transformation efficiency of our method is lower than that of the methods that are based on tissue culture, it is simpler and shortens the process of sorghum transformation. We believe that the method will facilitate monocot biology research, including for biotechnology applications of other cereal crops.

Conclusion

In the research, we developed a simple and effective transformation method for sorghum. The transformation method excludes culture process. In this method, the mature

embryos were pierced by a needle and then immersed in an *Agrobacterium* solution with 100 μM acetosyringone. When these inoculated seeds grew to three leaves, 1% hygromycin solution was used to brush the second and third leaf of inoculated seedlings to identify transformants. The highest efficiency of transformation was 4% in the study. Genetic and molecular analysis identified that transgene had been transferred into sorghum genome. In the future, the method will be used for broad functional genomics studies and for biotechnological application of sorghum.

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References

Carpita, N.C. and M.C. McCann, 2008. Maize and sorghum: genetic resources for bioenergy grasses. *Trends Plant Sci*., 13: 415–420

Feldmann, K. and M. David Marks, 1987. Agrobacterium-mediated transformation of germinating seeds of Arabidopsis thaliana: A non-tissue culture approach. *Mol. General Genetics,* 208: 1–9

Fursova, O., G. Pogorelko and O.A. Zabotina, 2012. An efficient method for transient gene expression in monocots applied to modify the Brachypodium distachyon cell wall. *Ann Bot*., 110: 47–56

Gao, Z., J. Jayaraj, S. Muthukrishnan, L. Claflin and G.H. Liang, 2005a. Efficient genetic transformation of Sorghum using a visual screening marker. *Genome*, 48: 321–333

Gao, Z., X. Xie, Y. Ling, S. Muthukrishnan and G.H. Liang, 2005b. Agrobacterium tumefaciens-mediated sorghum transformation using a mannose selection system. *Plant Biotechnol. J*., 3: 591–599

Grenier, C., P.J. Bramel, J.A. Dahlberg, A. El-Ahmadi, M. Mahmoud, G.C. Peterson, D.T. Rosenow and G. Ejeta, 2004. Sorghums of the Sudan: analysis of regional diversity and distribution. *Genet. Resour. Crop Evol*, 51: 489–500

Gritz, L. and J. Davies, 1983. Plasmid-encoded hygromycin B resistance: the sequence of hygromycin B phosphotransferase gene and its expression in Escherichia coli and Saccharomyces cerevisiae. *Gene*, 25: 179–188

Gurel, S., E. Gurel, R. Kaur, J. Wong, L. Meng, H.Q. Tan and P.G. Lemaux, 2009. Efficient, reproducible Agrobacterium-mediated transformation of sorghum using heat treatment of immature embryos. *Plant Cell Rep.*, 28: 429–444

Lin, J., B. Zhou, Y. Yang, J. Mei, X. Zhao, X. Guo, X. Huang, D. Tang and X. Liu, 2009. Piercing and vacuum infiltration of the mature embryo: a simplified method for Agrobacterium-mediated transformation of indica rice. *Plant Cell Rep*., 28: 1065–1074

Liu, G. and I.D. Godwin, 2012. Highly efficient sorghum transformation. *Plant Cell Rep*., 31: 999–1007

Paterson, A.H., J.E. Bowers, R. Bruggmann, I. Dubchak, J. Grimwood, H. Gundlach, G. Haberer, U. Hellsten, T. Mitros, A. Poliakov, J. Schmutz, M. Spannagl, H. Tang, X. Wang, T. Wicker, A.K. Bharti, J. Chapman, F.A. Feltus, U. Gowik, I.V. Grigoriev, E. Lyons, C.A. Maher, M. Martis, A. Narechania, R.P. Otillar, B.W. Penning, A.A. Salamov, Y. Wang, L. Zhang, N.C. Carpita, M. Freeling, A.R. Gingle, C.T. Hash, B. Keller, P. Klein, S. Kresovich, M.C. McCann, R. Ming, D.G. Peterson, Mehboob-ur-Rahman, D. Ware, P. Westhoff, K.F. Mayer, J. Messing and D.S. Rokhsar, 2009. The Sorghum bicolor genome and the diversification of grasses. *Nature*, 457: 551–556

Sheikholeslam, S.N. and D.P. Weeks, 1987. Acetosyringone promotes high efficiency transformation of Arabidopsis thaliana explants by Agrobacterium tumefaciens. *Plant Mol. Biol*., 8: 291–298

Supartana, P., T. Shimizu, M. Nogawa, H. Shioiri, T. Nakajima, N. Haramoto, M. Nozue and M. Kojima, 2006. Development of simple and efficient in Planta transformation method for wheat (*Triticum aestivum* L.) using Agrobacterium tumefaciens. *J. Biosci. Bioeng*., 102: 162–170

Supartana, P., T. Shimizu, H. Shioiri, M. Nogawa, M. Nozue and M. Kojima, 2005. Development of simple and efficient in planta transformation method for rice (*Oryza sativa* L.) using Agrobacterium tumefaciens. *J. Biosci. Bioeng*., 100: 391–397

Wu, E., B. Lenderts, K. Glassman, M. Berezowska-Kaniewska, H. Christensen, T. Asmus, S. Zhen, U. Chu, M. Cho and Z. Zhao, 2014. Optimized Agrobacterium-mediated sorghum transformation protocol and molecular data of transgenic sorghum plants. *In Vitro Cellular Develop. Biol. Plant,* 50: 9–18

Zhao, Z.Y., T. Cai, L. Tagliani, M. Miller, N. Wang, H. Pang, M. Rudert, S. Schroeder, D. Hondred, J. Seltzer and D. Pierce, 2000. Agrobacterium-mediated sorghum transformation. *Plant Mol. Biol*., 44: 789–798

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