Volume 55(1):193-195, 2011
Acta Biologica Szegediensis
http://www.sci.u-szeged.hu/ABS

ARTICLE

Different approaches for *Agrobacterium*-mediated genetic transformation of *Brachypodium distachyon*, a new model plant for temperate grasses

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ABSTRACT Cereal crops belonging to temperate grasses are fundamental sources of human nutrition therefore their agronomical improvement is centrally important. For this purpose the establishment of an experimental model system for these monocot plants is essential in plant biology research. Considering its advantageous morphological and physiological virtues taken together with the results of the advanced genomics research the *Brachypodium distachyon* became the new model plant for the Poaceae. The aim of our work is to adapt and possibly improve the *Agrobacterium*-mediated transformation method using embryogenic callus from *in vitro* cultures and furthermore develop an *in planta* methodology via *Agrobacterium tumefaciens* for Brachypodium in order to simplify the transformation procedure.
Acta Biol Szeged 55(1):193-195 (2011)

KEY WORDS

Brachypodium distachyon
in vitro culture
in planta transformation
Agrobacterium-mediated gene transfer

One of the most important plant families for the human economy is the Poaceae, the true grasses, including the most of the forage and lawn species, and all cereal crops grown all around the world. Most of these species possess some limitations of being a good experimental organism in plant biology research. Arabidopsis was used for the model organism in plant biology for decades (Bevan et al. 2005), but due to the fundamental differences in growth and development between monocots and dicots, a grass model species that facilitates investigations comparable to those ones possible in Arabidopsis would find wide applicability for future grass crop improvement. The first grass species with available whole sequenced genome was the rice (*Oryza sativa*) (Yu et al. 2002), therefore it has been used as an experimental plant, whilst few phenotypic traits like plant height, generation time and the demanding growth conditions make this grass species uneasy model organism. The *Brachypodium* genus is located at the base of the four grass tribes that includes the majority of domesticated cereal and forage crops (Kellogg 2001), and considering that the grass genomes` gene organization shows significant colinearity, taken together with the other advantageous properties of the only annual species of the genus the *Brachypodium distachyon* – such as the compact and totally sequenced genome (International Brachypodium Initiative 2010), simple growth conditions, self-fertility, small plant size, and diploidity – could determine this grass as the potential experimental model plant of the Poaceae.

Accepted July 11, 2011
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Developing efficient transformation methods has key importance in various research fields so it is an absolute requirement for a modern model system. Both of the two methods – biolistic and *Agrobacterium*-mediated transformation – were proved to be effective in the case of Brachypodium, however the *Agrobacterium*-mediated method appeared to be more efficient as it had been observed on other plant species in general (Vogel et al. 2006). Although this technique is very feasible, the tissue culturing and the regeneration of the transformed plants can be time consuming. Development of an efficient *in planta* transformation procedures – as established for dicot Arabidopsis and tomato and for the monocot rice (Supertana et al. 2006) – would reduce the elapsed time to reach the T_1 generation by the avoidance of the callus induction, selection and plant regeneration steps of the transformation methodology, and sparing notable amount of demanding sterile labour. Furthermore this method also protects from the frequently occurring somatic mutation or somaclonal variation during *in vitro* culture in plant cells, and the casual failure of regeneration of the *in vitro* plants.

Materials and Methods

Plant Material

Brachypodium distachyon Bd21 and Bd21-3 inbred lines were used throughout the experiments.

Agrobacterium

The *Agrobacterium tumefaciens* LBA 4404 strain was chosen for the plant transformations, harbouring the pEGAD plant

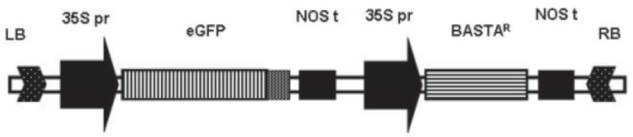

Figure 1. Schematic diagram of T-DNA of the pEGAD plant expression vector. LB, left border; 35S pr, cauliflower mosaic virus 35S promoter; eGFP, enhanced green fluorescent protein gene; NOS t, nopaline synthase terminator; BASTA^R, gluphosinate resistance gene; RB, right border.

expression vector. This plasmid contains a constitutive 35S promoter-driven enhanced GFP gene and herbicide (glyphosinate) resistance cassette between its border sequences (Fig. 1.).

Callus Initiation

Seeds from the Bd21 inbred line were collected 15 days after visible anthesis, surface sterilized by soaking in a solution of 10% household bleach plus 0.1% triton X-100 for 5 min with occasional mixing. The seeds were then rinsed three times with sterile distilled water and the immature embryos were excised under microscope in a laminar flow box and put immediately onto LS/R medium (basal LS salts plus 40 g/l sucrose and 7 g/l plant agar, pH 5.8) containing 2.5 mg/l 2,4-D with scutellar side down. Then the embryos were incubated in the dark at 25°C. After 3 weeks only the yellowish, solid embryonic calli were sub-cultured onto the same medium, and repeated 2 weeks later, respectively.

Transformation

Tissue culture

2 weeks after subculturing, the calli were harvested from the plates, chopped into pieces (cca. 1 mm in diameter), and put into R2/MED liquid medium (basal R2 salts plus 10 g/l glucose, pH 5.2) containing 2.5 mg/l 2,4-D, 100 µM acetosyringone and 500 µl *Agrobacterium* inoculum (OD_{600}= 0.8) into a Büchner flask. After 10 min of vacuum treatment, the flask was placed onto a shaker for 1 h shaking at 100 rpm at room temperature. The calli were taken out, dried using sterile filter paper disks, and transferred into Petri dishes containing R2/MED medium (as above plus 7 g/l plant agar, pH 5.2) with 2.5 mg/l 2,4-D, 100 µM acetosyringone for co-cultivation. The plates were kept 3 days in dark at 25°C, then the calli were harvested, and washed by shaking at 100 rpm for 30 min in the liquid R2/MED medium containing 10 mg/l Augmentin to eliminate the *Agrobacterium*. The calli were dried on sterile filter paper and put into Petri dishes containing R2/S medium (basal R2 salts plus 30 g/l sucrose, pH 6.0) with 2.5 mg/l 2,4-D, 1 mg/l Augmentin, 5 mg/l bialaphos, and kept in dark at room temperature. In order to prove that viable plants can be regenerated via the transformation procedure, control calli were prepared using the same method except the *Agrobacterium* infection and the herbicide selection.

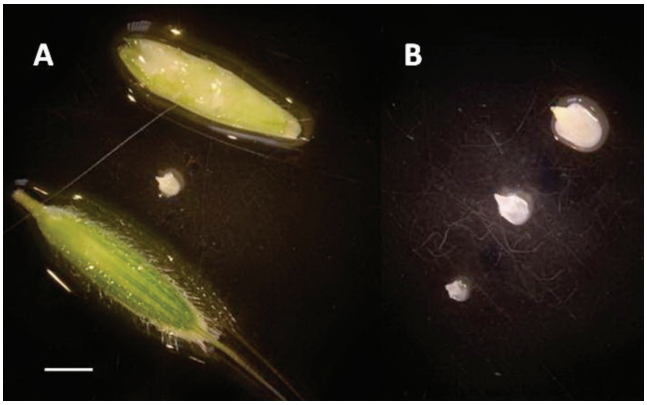

Figure 2. Isolation of the unmatured embryos of *Brachypodium distachyon*. Seeds with palea and after the embryo excision, Bar = 2 mm (A); isolated embryos with different size, harvested from the same plant (B).

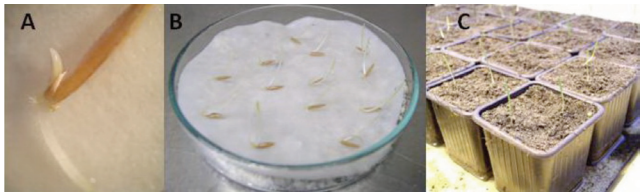

Figure 3. Steps of the *in planta* transformation. Developing root and shoot 2 days after the infection (A); 4 days after the transformation (B); 1 week old plantlets in the greenhouse (C).

In planta transformation

Seeds from Bd21-3 inbred line were husked and surface sterilized as previously described above. The sterilized seeds were put onto filter paper disks placed in Petri dishes and incubated at 4°C for 1 day. The embryo region of the seeds turns white and swollen. 2 ml of overnight-grown *Agrobacterium* culture was centrifuged and resuspend in 100 µl sterile water. The embryos were pierced with a needle (0.80 mm in diameter) dipped into the *Agrobacterium* suspension to the depth of 1 mm. For control plants water was used for inoculation. Then the seeds were put onto wet filter paper disks lying in Petri dishes filled up with wet perlite, and placed in dark, at 23°C for 3 days. The germinated plantlets were washed in 1000 ppm cefatoxamine to kill the *Agrobacterium*, rinsed with distilled water three times, and planted in the greenhouse.

Plant regeneration

The calli were put in Petri dishes containing LS/R medium supplied with 0.5 mg/l IAA and 0.3 mg/l BAP, and placed in

light conditions at 24°C. The calli turn into greenish colour and start to develop shoots and roots. The regenerating plantlets with 2-3 cm long roots were transferred into flasks containing 0.5xMS medium (half of the basal MS salts plus 10 g/l sucrose and 2.5 g/l Phytagel, pH 5.8) supplied with 100mg/l NAA. The plants grown well-developed root system were planted in the greenhouse.

Results and Discussion

During *in vitro* culture the Bd21 inbred line was used. The 90% of the isolated immature embryos produced calli successfully. The distribution of the embryo size was high, even though the spikes were harvested all at once (Fig. 2.). The size of the excised embryo is an important feature, it can determine the rate of the developed embryogenic calli, which is the source of the transformation material; the smaller embryos (0.3 mm in diameter) produce better quality. After the two sub-culturing, which multiplies the obtained calli from one embryo, we chopped the calli to gain more transformable material. This treatment does not harm the development of the *in vitro* plants as it was proved by the regeneration of the control calli, thus all the employed medium and the diverse plant hormones seems to be appropriate. The calli were able to grow on the selective medium supplied with 5 mg/l bialaphos are ready for the regeneration.

The Bd21-3 inbred line was selected for the *in planta* transformation experiment. 100% of the 40 pierced seeds were successfully germinated (Fig. 3.), and planted in the greenhouse. Thirty-six plantlets (90%) managed to survive the first week and produced spikes. Only 2 lines were unable to yield seeds, so overall 34 out of the 40 (85%) possible T_0 lines managed to crop. We would like to test the success of the gene transfer in the T_1 generation by application of genomic PCR technique, investigation of the expression pattern of the GFP gene by real-time qPCR and fluorescence assay, and herbicide (bialaphos) tests.

Acknowledgements

This work has been supported by the Hungarian Scientific Research Fund (OTKA 76273). The authors thank Ferhan Ayaydin (BRC of the HAS) for kindly providing us the pE-GAD vector. A part of this study was presented on the 10th Congress of the Hungarian Society for Plant Biology, August 31 - September 2, 2011, Szeged, Hungary.

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