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High throughput *Agrobacterium*-mediated switchgrass transformation

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ARTICLE INFO

Article history:
Received 20 July 2010
Received in revised form
1 November 2010
Accepted 8 November 2010
Available online 3 December 2010

Keywords:
Alamo
Colony
Genetic transformation
GFP
Panicum vertigam L
Performer

ABSTRACT

Switchgrass is one of the most important biomass/bioenergy crops. For its improvement as a feedstock through biotechnological approach, we have developed a high throughput *Agrobacterium*-mediated transformation system for cv. Alamo and two new elite cultivars, Performer and Colony. Highly regenerable and transformation-competent embryogenic calli were identified and used for genetic transformation. *GFP* reporter gene was employed to identify transformation events at early stages and to guide modifications at various stages for improvement of transformation efficiency. The modifications included infection under vacuum, co-cultivation at desiccation conditions, resting between co-cultivation and selection, and supplement of L-proline in the callus culture and selection media. Transformation efficiency over 90% was routinely achieved for Performer, and around 50% for Alamo and Colony. The new system substantially improved switchgrass transformation efficiency and will significantly contribute to the genetic improvement of this important biofuel feedstock via biotechnological approach.

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1. Introduction

Switchgrass (*Panicum virgatum* L.) is a C4 perennial grass native to North America [1]. It is recognized as a major biofuel crop because of its high yield of lignocellulosic biomass, which was estimated to yield 5.6 m^3 ha^{-1} of ethanol [2,3]. In the past, switchgrass breeding was targeted at its improvement as a forage grass [4,5]. Along with recognition of switchgrass as a major biomass/bioenergy crop, recent switchgrass breeding has been focused on its improvement for bioethanol production using conventional [6–8] and molecular approaches [9,10]. So far, a few reports regarding genetic transformation of switchgrass using particle bombardment or *Agrobacterium*-mediated approach have been published [11–14]. *Agrobacterium*-mediated transformation has been considered as a preferred technique for plant genetic engineering mainly due to its low transgene copy number and thus less chance to induce transgene silencing [15,16]. Great progress has been made for *Agrobacterium*-mediated transformation of grass species, once considered recalcitrant, such as rice [17], maize [18,19], wheat [20], sorghum [21], and creeping bentgrass [22]. Relatively high transformation frequencies were reported in tall fescue [23,24], *Brachypodium distachyon* [25], and maize [19]. However, most transformation efforts of monocots still suffer from inefficiency, which is also a major obstacle for switchgrass transformation. *Agrobacterium tumefaciens* strain AGL1 carrying binary vector pDM805 was utilized to transform somatic embryo or embryogenic callus of various selected genotypes of cv. Alamo using the *GUS* gene as a reporter gene and the *bar* gene for selection [12]. The transformation efficiency varied a great deal depending on the explants and genotypes used in the experiments. Certain genotypes from Alamo were reportedly more competent for *Agrobacterium*-mediated transformation. However, these materials seem to

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doi:10.1016/j.biombioe.2010.11.025

go to the private sector [13] and are no longer available to the public (J. Zale, personal communication). Antibiotic hygromycin B (hyg B) selection in *Agrobacterium*-mediated transformation of switchgrass was reported but it is not clear what the transformation efficiency was [14].

In this correspondence, we report a high-throughput transformation system of Alamo, together with two newly-released, elite cultivars, Performer [26] and Colony [8]. Performer has less lignin content and Colony has more cellulose content than Alamo. They could serve as excellent starting materials for further improvement by genetic engineering for their use as biofuel feedstock. The system we developed uses random seeds of a cultivar and does not require specific genotypes of the plants. It includes identification of embryogenic callus highly competent for transformation and regeneration, improvement of callus growth and selection by supplement of L-proline in the media, vacuum treatment at infection stage, desiccation treatment during co-cultivation stage, resting between co-cultivation and selection stages, and selection of transformants with hyg B. In addition, utilization of *GFP* as a reporter gene greatly facilitates early identification of transformation events and guides the development of the system. As the result, transformation efficiency reached 50% for Alamo and Colony, and was as high as 90% for Performer. Using this system, the time period from *Agrobacterium* infection to transplantation of transgenic plants to the soil could be as short as 3 months. The new system substantially improved switchgrass transformation efficiency and will significantly contribute to the genetic improvement of this important biofuel feedstock via biotechnological approach.

2. Materials and methods

2.1. Callus induction and culture

Seeds of cultivars Alamo, Performer, and Colony were kindly provided by Dr. J. Burns of NCSU and USDA/ARS Forage Program, surface-sterilized with full strength Clorox® (6% sodium hypochlorite, Clorox, Oakland, CA, USA) for two and an half hours with gentle stirring. After rinsing with distilled water three times, seeds were kept overnight in the dark at 26 °C and then sterilized again for another 80 min. With another round of rinsing with distilled water three times, seeds were placed on callus induction medium (MB) (MB and other media used in the experiments are listed in Table 1). Six to eight weeks later, the embryogenic calli were picked and subcultured on MP medium.

2.2. Regeneration test of subcultured callus

Embryogenic calli, which were subcultured once every 3 or 4 weeks, were placed in REG medium for regeneration. The cultures were kept under cool white fluorescent light (140 μmol m^{-2} s^{-1}) with a photoperiod of 16/8 h (light/dark) at 25 °C in a growth chamber. A month later, number of calli having green or albino shoots were recorded. In each experiment, 20 to 30 pieces of calli (0.5–1 cm in diameter) were used per replicate and the experiment was replicated three times.

Table 1 – Media composition used in the experiments.

Medium	Composition
MB	MS basic medium with 30 g l^{-1} maltose, 5 mg l^{-1} 2, 4-D, 1 mg l^{-1} BAP, 3 g l^{-1} phytagel, pH 5.8
MP	MB with 2 g l^{-1} L-proline
CTMP	MP with 200 mg l^{-1} carbencillin and 150 mg l^{-1} timentin
REG	MS with 0.2 mg l^{-1} NAA, 1 mg l^{-1} BAP, 0.5 mg l^{-1} GA, 30 g l^{-1} maltose, pH 5.8
½ TM	½ MS with 30 g l^{-1} maltose, 150 mg l^{-1} timentin

Sources of the chemicals: MS basal medium (Caisson Laboratories, North Logan, UT, USA), timentin (GlaxoSmithKline, Research Triangle Park, NC, USA), carbenicillin (Apollo Scientific, Stockport, UK), maltose (Caisson Laboratories North Logan, UT, USA), L-proline (Fisher, Fair Lawn, New Jersey, USA). All other chemicals used in the experiments were purchased from Sigma (St. Louis, MO, USA).

2.3. L-proline effects on callus growth and transformation selection

The effects of L-proline supplement with concentrations from 10 mg l^{-1} up to 32 g l^{-1} were tested on Alamo callus growth in MB medium in a pilot experiment. Based on the results, supplement of 2 g l^{-1} L-proline was chosen and formally evaluated on callus growth of both Alamo and Performer. Ten pieces of callus per plate, five plates per treatment, were weighed before the treatment and cultured in the dark at 25 °C. The calli were weighed again 4 weeks later. The growth index was calculated as the final fresh weight minus the initial fresh weight, and divided by the initial fresh weight. The experiment was replicated three times. The effect of proline supplement in callus selection medium during transformation was also evaluated.

2.4. Agrobacterium strain and vectors

Binary vector pJLU13 [27] was used in the transformation experiments. It is a derivative of pCAMBIA1300 containing the hyg B selectable marker gene, *hpt*, and a green fluorescent protein reporter gene *sGFP* (S65T) [28], driven by the rice *rubi3* promoter [29]. The freeze-thaw method [30] was used to mobilize pJLU13 and another plasmid, pTOK47, into *Agrobacterium* strain EHA105 [31]. Plasmid pTOK47 carries a 20 kb *Kpn*I fragment of Ti plasmid from pTiBo542, which contains *virB*, *virC*, and *virG* virulence genes [24]. The resulted *Agrobacterium* strain, EHA105 (pTOK47, pJLU13), was grown in YEP medium in the presence of 20 mg l^{-1} rifampicin, 5 mg l^{-1} tetracycline, and 50 mg l^{-1} kanamycin. The culture was grown at 28 °C with shaking (250 rpm) until the OD_{595} reading reached 0.8–1.0, and then centrifuged at 4000 g for 10 min. The pelleted cells were resuspended in liquid MP medium (Table 1) to adjust OD_{595} to 0.5–0.6. Acetosyringone (3′, 5′–dimethoxy-4′-hydroxyactophenone, Aldrich, Milwaukee, WI, USA) was then added to the suspension to 100 μM.

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