Journal of Plant Physiology **166** (2009) 435—441

Available online at www.sciencedirect.com

ScienceDirect

JOURNAL OF PLANT PHYSIOLOGY

www.elsevier.de/jplph

Particle inflow gun-mediated transformation of multiple-shoot clumps in rhodes grass (*Chloris gayana*)

Takahiro Gondo[a], Jun Matsumoto[b], Shin-ichi Tsuruta[b], Midori Yoshida[c], Akira Kawakami[d], Fumihiro Terami[c], Masumi Ebina[e], Toshihiko Yamada[f], Ryo Akashi[a,*]

[a]*Frontier Science Research Center, University of Miyazaki, Miyazaki 889-2192, Japan*
[b]*Faculty of Agriculture, University of Miyazaki, Miyazaki 889-2192, Japan*
[c]*National Agricultural Research Center for Hokkaido Region, Sapporo, Hokkaido 062-8555, Japan*
[d]*National Agricultural Research Center, Tsukuba, Ibaraki 305-8666, Japan*
[e]*National Institute of Livestock and Grassland Science, Nasushiobara, Tochigi 329-2793, Japan*
[f]*Field Science Center for Northern Biosphere, Hokkaido University, Sapporo, Hokkaido 060-0811, Japan*

Received 10 March 2008; received in revised form 24 June 2008; accepted 24 June 2008

KEYWORDS
Bialaphos resistance;
Genetic transformation;
Multiple-shoot clump;
Particle inflow gun;
Rhodes grass

Summary

Rhodes grass (*Chloris gayana*) is one of the most important warm-season forage grasses. It is cultivated in tropical and subtropical parts of the world and is mostly used for grazing and hay production. We have established a particle-bombardment transformation protocol for rhodes grass using multiple-shoot clumps (MSCs) as the target tissue. A vector pAHC25 containing a herbicide-resistance gene (*bar*) together with the β-glucuronidase (GUS) gene was used in transformation experiments. The most efficient recovery of bialaphos-resistant tissue was achieved when the bombarded MSCs were first cultured for 15 d on bialaphos-free medium before being subjected to selection pressure. The resistant tissues regenerated transgenic plants that displayed GUS gene expression. Under optimized conditions, 251 target pieces yielded 46 transgenic plants from 4 independent transgenic lines.

Introduction

Perennial rhodes grass (*Chloris gayana*), one of the most important warm-season forage grasses, is cultivated in tropical and subtropical areas of the

Abbreviations: BAP, 6-benzylaminopurine; *Bar*, bialaphos resistance gene; GUS, β-glucuronidase; MS, Murashige and Skoog; MSC, multiple-shoot clump; PCR, polymerase chain reaction; X-Gluc, 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid.

*Corresponding author. Tel.:/fax: +81 985 58 7257.
E-mail address: rakashi@cc.miyazaki-u.ac.jp (R. Akashi).

0176-1617/$ - see front matter © 2008 Elsevier GmbH. All rights reserved.
doi:10.1016/j.jplph.2008.06.014

world (Bogdan, 1969; Loch et al., 2004), primarily for grazing and hay production. However, one of the major limitations for cattle production on rhodes grass and some other warm-season forage grasses is the low nutritional value and poor digestibility of these species in comparison to most temperate grasses or non-grass forage (Akin and Burdick, 1975; Reid et al., 1990).

The low digestibility of many warm-season grasses is due to their high lignin content (Akin et al., 1983; Jung and Vogel, 1986). Recent advances in genetic technology suggest possible strategies for the modification of the lignin content of plants (Akashi et al., 2003; Chen et al., 2003). There are, however, species-related technical difficulties to be overcome in warm-season forage grasses. Only few reports on the establishment of efficient transformation systems exist for this category of crops (Gondo et al., 2005).

The availability of an efficient and reproducible *in vitro* plant regeneration system is essential for the successful genetic engineering of plants (Vasil, 1990). Multiple-shoot clumps (MSCs) derived from shoot apical meristems are a promising explant source from which to develop regenerable tissue for particle gun-mediated transformation (Zhong et al., 1996; Zhang et al., 1999). Plant regeneration in rhodes grass has been reported from callus derived from mature seeds (Doi et al., 1985) and mesocotyl explants (Nishihira et al., 1989). Recently, we have established an *in vitro* regeneration system via MSC formation from seed-derived shoot apical meristems of rhodes grass (Gondo et al., 2007). This culture system carries a high potential for plant regeneration and can provide a continuous supply of high-quality target tissues for genetic transformation.

In this report, we describe a transformation method for rhodes grass using an MSC culture system. Transgenic plants were obtained using the *bar* gene as the selective marker. Under optimized conditions, 251 target pieces yielded 46 transgenic plants from 4 independent transgenic lines.

Materials and methods

Formation of multiple-shoot clumps

MSCs were induced as previously described (Gondo et al., 2007). Briefly, seeds of diploid rhodes grass (*Chloris gayana* Kunth) cv. Katambora, with lemmas and paleas removed, were surface-sterilized in 70% (v/v) ethanol for 1 min and in 2% (v/v) sodium hypochlorite for 15 min, followed by three washings with sterile water. The surface-sterilized seeds were germinated by placing 80–90 seeds on filter paper (Advantec, Japan) in a 90 mm Petri dish with 5 mL sterile water. The leaf base section (3–5 mm in length) of a 4 d old seedling containing an apical meristem and 2–3 leaf primordials was excised and cultured on MS medium (Murashige and Skoog, 1962) containing 3% sucrose and 0.3% gellan gum (Wako, Japan) supplemented with 0.1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 2 mg/L 6-benzylaminopurine (BAP) (MS-D0.1B2 medium). After 30 d in culture, primary MSCs were transferred to fresh MS-D0.1B2 medium. Single seed-derived, compact and dividing clumps were subcultured every 30 d onto the same medium. All media were adjusted to pH 5.6–5.8 prior to autoclaving at 121 °C for 15 min, and all cultures were incubated at 27 °C in light.

Bombardment with a particle inflow gun

The self-built particle bombardment apparatus (spray gun) was constructed as described previously (Akashi et al., 2002; Gondo et al., 2005). Three to 6-month-old MSCs (2–3 mm in diameter), approximately 50 clumps per bombardment, were used as target tissue. Embryogenic callus (EC), cultured on MS medium (MS-D2) containing 2 mg/L 2,4-D (Gondo et al., 2007), was used for comparison. Four hours prior to bombardment, the MSCs were transferred to MS-D0.1B2 medium supplemented with equimolar amounts of mannitol and sorbitol to yield 0–1.5 M and were left on this medium for 16 h after bombardment for osmotic (plasmolysing) post-treatment.

The plasmid pAHC25 (Christensen and Quail, 1996), containing the β-glucuronidase (GUS) gene and the bialaphos resistance gene (*bar*) under control of separate maize *ubi1* promoters and its first intron, was used for transformation experiments. Plasmid DNA was precipitated onto gold particles (1.5–3.0 μm diameter; Aldrich, USA) as described by Gondo et al. (2005). Bombardment was carried out at a reduced air pressure of −0.1 MPa, a target distance of 9.6 cm, helium pressure of 5 kg/cm^2 and single shots per plate.

The histochemical GUS assay was carried out according to a modified Jefferson (1987) method. To assay transient GUS activity, the bombarded tissue was incubated after 16 h for 18–24 h at 37 °C in 1.9 mM X-gluc, 50 mM phosphate-buffered saline (PBS) of pH 7.0, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.3% Triton X-100 and 20% methanol. The Fisher multiple comparison test at a $p < 0.05$ level of significance was used to analyze the data of this study.

Selection and regeneration of transgenic plants

Following bombardment and osmotic post-treatment, MSCs were placed on MS-D0.1B2 medium for 0–30 d and subsequently subcultured several times for 30 d periods on the same medium containing 3 mg/L bialaphos. After 60–70 d of subculture under selective conditions, bialaphos-resistant clumps were transferred to hormone-free MS medium containing 5 mg/L bialaphos for plant