Improvement of forage quality by means of molecular breeding in tropical grasses

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SUMMARY

Many important warm-season perennial grasses multiply either by vegetative propagation, or form their seeds by an asexual mode of reproduction called apomixes. Possibility of improving of these plants by conventional breeding method depends on availability of natural genetic variation and its manipulation through breeding and selection. However, there are naturally not many genetic variations in apomictic grasses to generate new genetic variation. Plant tissue culture techniques have complemented conventional plant breeding programs. The author established a system for plant regeneration from in vitro-cultured calluses, suspension cells and protoplasts in some tropical grass species including apomictic species. In addition, we focus on describing current and future applications and impact of genetic transformation in tropical grasses.

INTRODUCTION

Forage grasses can be grouped into two large categories; warm- and cool- season grasses. Warm-season grasses produce most of their growth during the warmer periods of the growing season, while the opposite is true for cool-season species. These grasses are utilized in many different agricultural production systems with greatest value as feeds for livestock. They are also useful for preventing soil erosion and maintaining soil fertility. A species may be grown alone, or in mixtures with other grasses or legumes at high or low levels of soil fertility. They may be grazed, or made into hay or silage for conservation.

Many important warm-season perennial grasses multiply either by vegetative propagation, or form their seeds by an asexual mode of reproduction called apomixes. Possibility of improving of these plants by conventional breeding method depends on availability of natural genetic variation and its manipulation through breeding and selection. However, there are naturally not many genetic variations in apomictic grasses to generate new genetic variation.

Recently, plant tissue culture techniques have complemented conventional plant breeding programs (Akashi 1991, Akashi and Adachi 1994, Akashi et al. 1994, Akashi and Kawamura 1998, Akashi et al. 2002). Major categories of these methods can be summarized as induction and screening of desirable mutants at cellular and tissue level, somatic hybridization between remotely related species, induction of haploid plants as breeding materials, and direct transformation in protoplasts, as well as micropropagation of unique genotypes.

Tissue culture methods ordinarily consist of two phases; firstly, initiation of callus and secondly, regeneration of plants from the callus. Many plant cells have been proved as totipotent, that is, for example, a non-embryogenic cell has the potential to differentiate into an embryogenic cell and then to develop into an entirely new plant. However, the requirements of each species for growth and regeneration are still unclear. Therefore, to make practical use of tissue culture, the ability to regenerate plants from callus and protoplast should be demonstrated in each appropriate species. The author established a system for plant regeneration from in vitro-cultured calluses, suspension cells and protoplasts in some tropical grass species including apomictic species. In addition, we focus on describing current and future applications and impact of genetic transformation in tropical grasses.

Plant Regeneration System from Embryogenic Callus and Cell Suspension in Some Tropical Grasses

Embryogenic callus was initiated from immature embryos on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 10 mg/L 2,4-D, 10% coconut water and Solidified

with 0.8% agar in guineagrass (*Panicum maximum* Jacq). Initially various types of callus were obtained and embryogenic responses were found to be correlated with the genotypes investigated (Fig.1a-1-a-5). For somatic embryos germination and plant formation MS medium supplemented with 1.0 mg/L GA_3 and 1.0 mg/L kinetin was used. The twelve genotypes analyzed can be classified into three groups by the frequency of somatic embryo formation and degree of apomixes. One of group consists of highly apomictic genotypes with a high embryogenic capacity (Akashi and Adachi 1991).

Plant regeneration from cultured immature inflorescences of dallisgrass (*Paspalum dilatatum* Poir.) was obtained by somatic embryogenesis. Embryogenic callus was initiated from immature inflorescences on MS medium supplemented with 10 mg/L 2,4-D and solidified with 0.2% Gellan Gum (Fig.1b-1). Somatic embryos developed and germinated precociously when embryogenic calli were transferred to a medium contained 1.0 mg/L each of kinetin and GA_3 (Fig.1b-2). All regenerants were successfully grown to maturity (Akashi and Adachi 1992a). The respective suspension cultures were initiated from immature inflorescence-derived embryogenic callus (Fig.1f-1). The suspension cells could be classified into small, starch-containing embryogenic cells with dense cytoplasm and large, vacuolated, non-embryogenic cells. Plant regenerated from cell suspensions were produced after 5 months of culture when 2-3 mL of pellets of centrifuged suspension cells were cultured on solid MS medium with 1.0 mg/L NAA and 0.2 mg/L BAP (Akashi and Adachi 1992b).

We have established a high-frequency plant regeneration system via somatic embryogenesis from seed-derived callus and cell suspension cultures in six genotypes of bahiagrass (*Paspalum notatum*). Embryogenic callus was initiated from mature seeds on MS medium supplemented with 2.0 mg/L 2,4-D, 3.0% sucrose and 0.3% Gellan Gum in the dark. Culture response was found to be correlated with genotype. “Pensacola” had the best response in embryogenic callus formation (Fig.1c-1-c-4), and 74% of the calli regenerated plants (Fig.1c-5, c-6). The suspension was composed of compact cell clustered. When smaller clusters were transferred to solid MS medium without hormones, plant regeneration was initiated at high frequency (28.6%). Morphological evidence is provided that regeneration of suspension cells occurred via embryogenesis (Akashi et al. 1993).

Protoplast Cultures in Some Tropical Grasses

Protoplasts were isolated from embryogenic suspension cells of apomictic dallisgrass. The respective suspension cultures were initiated from immature inflorescence-derived embryogenic callus (Akashi and Adachi 1992). Previous to protoplast isolation, suspension cells were treated with MS liquid medium without sucrose and hormones. Due to this pretreatment protoplast yield and viability were dramatically increased. A maximum protoplast yield of 5×10^6/g fresh weight was obtained (Fig.1f-2). Cell division and colony formation from pretreated protoplasts were found to be best in an agarose solidified KM8p medium (kao 1975). The plating efficiency, based on colony formation after 2 weeks of culture, was 0.5-0.8% (Fig.1f-3-f-6). Protoplast-derived colonies were transferred to a solidified MS medium containing 1.0 mg/L 2,4-D for callus proliferation. The calli formed embryonic structures which gave rise to green plants in 0.2% (w/v) Gellan Gum solidified MS medium with 1.0 mg/L NAA and 0.2 mg/L BAP (Fig.1f-7). The regenerated plants were transferred to 1/2 MS hormone-free medium for further growth and root formation. Rooted plants could be transferred to soil (Akashi and Adachi 1992a).

In apomictic guineagrass the suspension culture used as donor material was originally initiated from immature embryo-derived embryogenic callus. Prior to protoplast isolation, suspension cells were conditioned with MS liquid medium without sucrose and growth regulators. This pretreatment lead to a dramatic increase in protoplast yield and colony formation. Cell division and colony formation from such pretreated protoplasts were found to be best in agarose-solidified modified KM8p medium. Protoplast derived colonies developed into callus on solidified MS medium supplemented with 1.0 mg/l 2,4-D. After 2 months in culture, calli formed compact somatic embryos. Although some of the somatic embryos developed small leafy structures, whole plants could not be regenerated (Akashi et al. 1995).

Genetic Transformation in Tropical Grasses

A simple and inexpensive, self-built particle acceleration apparatus is described, and the special features of the device are emphasized (Fig.1g-1). We have employed this easy-to-use gene gun for efficient direct delivery of DNA to cultured cells of an important monocotyledonous forage crop, dallisgrass. High levels of transient expression of the ß-glucuronidase gene were obtained following bombardment of suspension cells. Furthermore, stable transformed cells of this grass have been obtained after intrusion of the *bar* (bialaphos resistance) gene at optimized delivery conditions. Tentative transgenic calli were selected on solidified medium, and one of these calli, after transfer to liquid selection medium, gave a stable suspension culture tolerant at 1.0 mg/L bialaphos. Integration of the transgene in suspended cells was confirmed by PCR amplification analysis (Akashi et al. 2002).

We have established a reproducible particle bombardment transformation protocol for bahiagrass. The improved culture system, embryogenic callus formed frequently and produced highly regenerative tissues (Fig.1d-1-d-6). These tissues were formed from embryogenic callus on MS medium containing 2.0mg/L 2,4-D, 0.1mg/L BAP and 50 mM copper sulfate at 31±1 under dim light conditions and used as transformation targets. This modified culture minimized the problems with loss of regenability and increases in albinism that frequently occur for requiring transformed plants (Fig.1e-1-e-6). Many of green transformants were obtained under 3.0 mg/L bialaphos selection pressure, and this frequency was 2.2% (8 transgenic lines/320 pieces of target tissue) (Fig.1g-2-g-7). Integrated transgenes (GUS and *bar* gene) were confirmed by PCR amplification analysis. Transgenes were stable transmitted to T1 progenies (Fig.1g-8), and localized expression of GUS gene was analyzed by histochemical assay (Gondo et al. 2003).

On the other hand, luminescent proteins, such as the green fluorescent protein (GFP) from the jellyfish (*Aequorea victoria*), have proven to be powerful tools in plant genetic transformation and gene expression studies due to their excellent sensitivity and rapid response. GFP has also the significant advantage that it does not require a substrate, and its expression can be detected in real time in living cells by light excitation. Therefore, GFP has been used as a reliable reporter for plant transformation. In this study, the plasmid modified pBI221 (pBI0809) containing the gfp reporter gene under control of the cauliflower mosaic virus (CaMV) 35S promoter was introduced into mature seed-derived embryogenic calli by particle bombardment. Sectors containing embryogenic calli with strong GFP fluorescence were identified using a fluorescence viewing system. Cell- and tissue-specific expressions of the gfp gene were also investigated in transgenic plants, and stable GFP expression was observed in transgenic calli (Fig. 2a-d). As a result of GFP selection, the total time required to produce transgenic calli was reduced by approximately 14 days compared to the time needed when selective agents such as the bar gene are used (50–60 days). Three months after bombardment, regenerated transformants were potted in soil, acclimatized and transferred to the greenhouse (Fig. 2o). A 480-bp fragment was amplified and identified as the gfp gene by polymerase chain reaction (Fig. 2m). The presence of the gfp gene in genomic DNA of three out of nine transgenic bahiagrass lines was confirmed by DNA gel blot hybridization analysis (Fig. 2n). Transformants showed hybridization patterns suggesting that the gfp gene was present in three copies per regenerant. The GFP expression could be visually detected in all tissues such as leaves, leaf sheaths, anthers and pollen (Fig. 2e-l). Overall, the gfp gene can be utilized not only as a reporter gene, but also as a visual selectable marker for bahiagrass transformation.

Prospect

Brachiaria is tropical forage grasses, which has been cultivated in tropical and subtropical region as pasture mainly. At present, *B. brizantha* (A. Rich.) Stapf, *B. decumbens* Stapf and *B. ruziziensis* Germain & Evrard have been important commercially because these species have many positive attributes, e.g., tolerance to acid soil, high productivity and high quality forage. Thus, these cultivars are also important as breeding material for development of *Brachiaria* breeding. In *Brachiaria* breeding, besides productivity and digestibility, persistence, adaptation to infertile acid soil and antibiotic resistance to spittlebugs are also important breeding objectives. However, it is very difficult to develop a *Brachiaria* breeding program through a traditional hybridization approach because

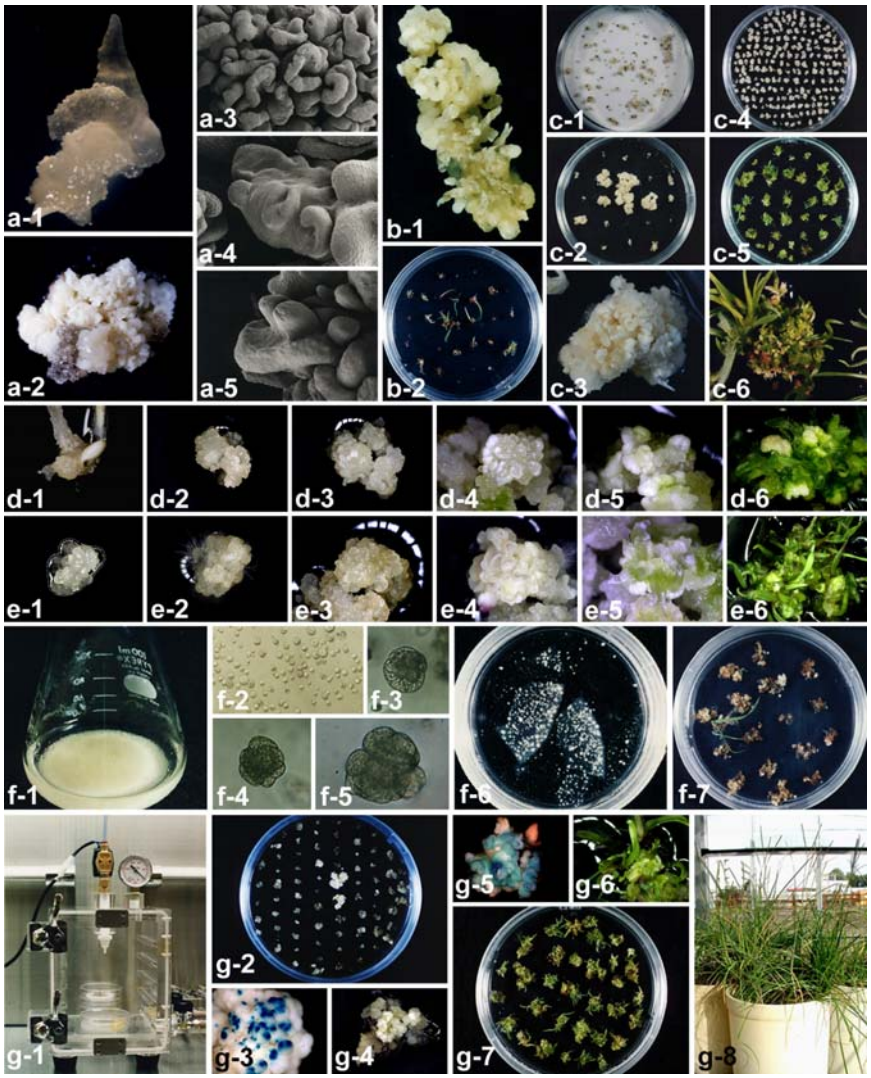

Figure 1. Tissue culture and genetic transformation in some warm season grasses. a) Embryogenic callus formation from immature embryos in guineagrass. 1) Immature embryo. 2) Embryogenic callus. 3-5) SEM of somatic embryos at different stages of development. b) Embryogenic callus formation from immature inflorescences and plant regeneration in dallisgrass. 1) Embryogenic callus. 2) Plant regeneration from somatic embryos. c) Embryogenic callus formation from mature seeds and plant regeneration in bahiagrass. 1) Primary callus after 14 days of culture. 2, 3) Embryogenic callus after 28 days of culture. 4) A subcultured micro-callus after 60 days culture. 5, 6) Plant regeneration from micro-callus. d) Developmental stages of seed-derived embryogenic callus and plant regeneration. 1-3) Embryogenic callus formation after 7 (1), 14 (2) and 21 (3) days of culture. 4) Somatic embryos formation. 5) Maturation of somatic embryos. 6) Germination of somatic embryos and plant regeneration. e) Developmental stages of highly regenerative embryogenic callus cultured on $CuSO_4$ additional medium and plant regeneration.1-3) Embryogenic callus cultured after 0 (1), 3 (2) and 14 (3) days on $CuSO_4$ additional medium. 4, 5) Shoot germination with scutellum formation. 6) Elongation of germinated shoot. f) Cell colony formation and plant regeneration from suspension protoplasts of dallisgrass. 1) Typical suspension cells. 2) Isolated protoplasts from suspension cells. 3-5) Cell division and cell colony formation from protoplasts after 5 (3), 7 (4) and 10 (5) days of culture. 6) Colonies formed from protoplasts after 20 days of culture. 7) Shoot formation from protoplast-derived callus. g) Stable transformation of bahiagrass mediated by particle inflow gun. 1) A simple self-built particle inflow gun. 2) Selection culture of bialaphos resistant callus. 3) Transient GUS expression 16 hours after bombardment. 4) Bialaphos resistant callus. 5) Stable GUS expression on bialaphos resistant callus. 6, 7) Plant regeneration from transformed callus. 8) Transgenic plants with setting seeds.

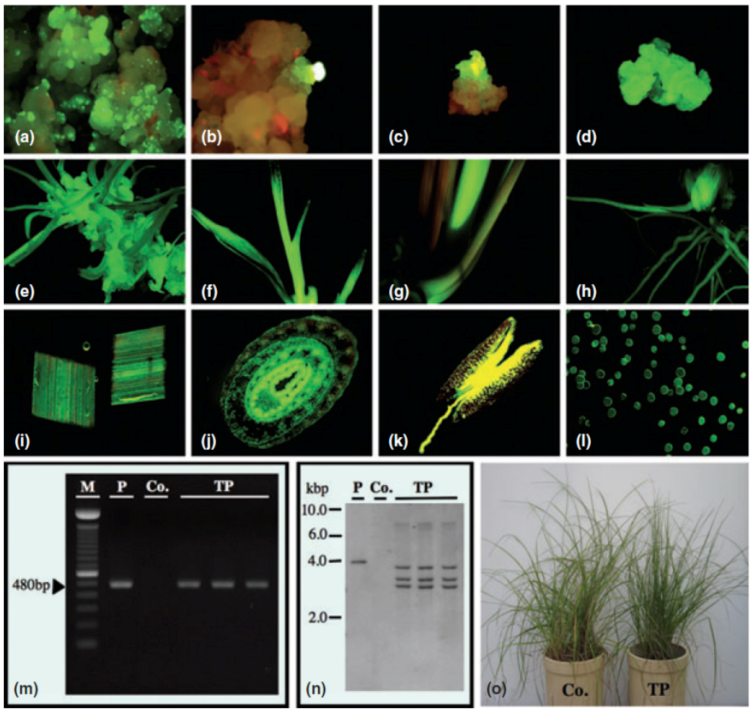

Figure 2. Expression of gfp gene in transgenic bahiagrass. (a) Transient green fluorescent protein (GFP) expression 16 h after bombardment. (b,c) GFP expressing callus 14 days after bombardment. (d,e) GFP expression from transformed callus to plant regeneration. (f–h) GFP expression of regenerated plant in vitro (f, leaf blade; g, tiller; h, underground part). (i–l) GFP expression in each tissue of a transgenic plant (i, leaf; j, leaf sheath; k, anther; l, pollen). (m) Detection of gfp gene in transgenic plants by polymerase chain reaction (M, marker; P, plasmid pBI221; Co., non-transformed plant; TP, transformed plant). (n) The number of transgenes in transgenic plants by DNA gel blot analysis (P, 5 pg Hind III-digested plasmid pBI221; Co., nontransformed plant; TP, transgenic plant). (o) Potted transgenic plants (Co., non-transformed plant; TP, transgenic plant).

almost all species are predominantly facultative apomictic tetraploids. Therefore, it is proposed that *B. ruziziensis* (ruzigrass) should be useful for *Brachiaria* breeding programs based on tissue culture system because this species has sexuality. We have established an effective method for plant regeneration through multiple-shoot formation or somatic embryogenesis from seed-derived shoot apical meristems of diploid ruzigrass firstly (Ishigaki *et al.* 2009a). Subsequently, tetraploid ruzigrass were produced by *in vitro*-colchicine treatment with multiple-shoot clumps or seedlings. The availability of these tetraploid ruzigrass individuals will likely also expand the breeding potential of the *Brachiaria* genus (Ishigaki *et al*. 2009b). Additionally, the author attempted to establish a particle inflow gun-mediated transformation protocol for ruzigrass using multiple-shoot clumps and embryogenic calli. This transformation system should be a valuable tool for use in *Brachiaria* breeding programs. These results can be applied to develop *Brachiaria* breeding programs in Japan.

Pennisetum purpureum is popular grass in tropical or subtropical area. This grass contains several positive characters, which are beneficial for animal feeding. They can be grown for years, having high yield, highly nutritious, selectively chosen as feed by animals, and can growth on a wide range of soil condition, drained soils, to some extent, drought tolerant of the deep root system. Napier grass does not tolerant flooding, but grows best in areas with high rainfall in excess of 1,500 mm per year. However, there are negative properties, which prevent this grass to develop as planed. First of all, their flowers are very small while the pollens are short-lived which result in low level of seed formation. Moreover, the flowering period of each type of napier grass is different making it more difficult and consuming to have them cross-pollinated. Because of a synchrony of male and female flower parts, the plant relies on cross-pollination by wind (Pongtongkam et al. 2006). This grass is an inconsistent seed producer and rarely develops seeds in some habitats, when seeds are produced, they are often of low viability. Dwarf napier grass, more leafier and higher nutrient contain than normal napier grass. These grass have high potential as forage in grazing area.

Recently, plant biotechnology and molecular biology have created unprecedented opportunities and promises in the field of agriculture. Methods have been developed for the propagation of genotype, more and efficient regeneration through micro-propagation (Ali et al. 2008). In our present research work was under taken by keeping in view the importance of tissue culture technology in dwarf napier grass improvement and establishment of efficient protocols for mass scale propagation of healthy, disease free and premium quality planting material through micro-propagation.

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