Biotechnology in Forage grasses: Tissue culture, genetic transformation, genome editing and risk assessment

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1. 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.

Plant tissue culture techniques have complemented conventional plant breeding programs in important Poaceae species (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. We have 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.

1.1. 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 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. 1.a1–a5). For somatic embryos germination and plant formation, MS medium supplemented with gibberellic acid and kinetin was used. The twelve genotypes analyzed can be classified into three groups by the frequency of somatic embryo formation and degree of apomixis. One of the 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 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 kinetin and gibberellic acid (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 centrifuged suspension cells were cultured on solid MS medium with NAA and 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 with supplements in the dark. Culture response was found to be correlated with genotype. ‘Pensacola’ had the best response in embryogenic callus formation (Fig. 1.c-1–c-4), and 74% of the calli regenerated plants (Fig. 1c-5, 1c-6). The suspension

was composed of clustered compact cells. Smaller clusters transferred to solid MS medium without hormones initiated plant regeneration at high frequency (28.6%). Morphological evidence indicated that regeneration of suspension cells occurred via embryogenesis (Akashi et al., 1993).

1.2. 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). In previous reports of 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.1d-2). Cell division and colony formation from pretreated protoplasts were found to be best in an agarose solidified KM8p medium (Kao and Michayluk, 1975). The plating efficiency, based on colony formation after 2 weeks of culture, was 0.5–0.8% (Fig. 1d-3–d-6). Protoplast-derived colonies were transferred to a solidified MS medium containing 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 NAA and BAP (Fig. 1d-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 led 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 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).

1.3. 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. 2a). 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 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. 2b–2). These tissues were formed from embryogenic callus on MS medium containing 2,4-D, BAP and 50 M copper sulfate at 31±1 °C under dim light conditions and used as transformation targets. This modified culture minimized the problems of loss of regenerability and increase in albinism that frequently occur in transformed plants (Fig. 2g). Many green transformants were obtained under bialaphos selection pressure, and this frequency was 2.2% (8 transgenic lines/320 pieces of target tissue) (Fig. 2h). Integrated transgenes (GUS and *bar* gene) were confirmed by PCR amplification analysis (Fig. 2j). Transgenes were stably transmitted to T_1 progenies (Fig. 2k, 2l), and localized expression of GUS gene on the leaf, pollen and seeds (Fig. 2m–2o) were analyzed by histochemical assay (Gondo et al. 2003).

Another important warm-season forage grass is Rhodes grass (*Chloris gayana*). With the establishment of transformation in bahiagrass, we used the same transformation protocol in rhodesgrass with modifications such as using multiple shoot clumps instead of embryogenic calli, osmotic treatment, and time before bialaphos selection culture (Fig. 3a–3c). A vector containing the herbicide-resistance (*bar*) with *β-glucorinidase* (GUS) was used in transformation for rhodesgrass (Fig. 3d, 3e). Following the selection of bialaphos-resistant multiple shoots, efficient recovery of bialaphos-resistant tissues was obtained when bombarded multiple shoot clumps were cultured in bialaphos-free medium for 2 weeks (Fig. 3f – 3h). Resistant tissues regenerated transgenic rhodesgrass expressing *GUS* gene in their genomic DNA (Fig. 3i). Transgenic multiple shoots were transferred in rooting medium and moved to pots for acclimatization (Fig. 3h). Further evaluation revealed GUS expression in the leaves and stem of transgenic rhodesgrass (Fig. 3k, 3l).

The use of biotechnological tools can enable us to overcome the breeding barriers in *Brachiaria*. First, we have established an efficient plant tissue culture technique for plant regeneration through multiple shoot formation and somatic embryogenesis from seed-derived shoot apical meristems in ruzigrass (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 also expand the breeding potential of the *Brachiaria* genus (Ishigaki et al., 2009b). We have established a particle inflow gun-mediated transformation protocol for ruzigrass using multiple-shoot clumps and embryogenic calli. Multiple-shoot clumps and embryogenic calli were initially

produced from diploid as well as tetraploid shoot apices (Ishigaki et al., 2009a, b). A vector carrying the GUS reporter gene and the bialaphos resistance gene (bar) both under control of maize ubiquitin promoter was used. Transient GUS gene expression 16 h after bombardment was assessed as the number of blue spots (Fig. 4a) wherein many of the spots formed in embryogenic calli were observed at crowded clusters of proembryos while no such specific pattern was found in shoot cultures. Compared to multiple-shoot clumps, bialaphos-resitant embryogenic calli showed significantly higher numbers of GUS expression spots per plate (Fig. 4b, 4c), The results indicate that embryogenic callus is the preferable culture type and a very suitable target tissue for the transformation of ruzigrass by particle bombardment (Ishigaki et al., 2012). Following bombardment and selection with bialaphos, herbicide-resistant embryogenic calli eventually regenerated shoots and roots in vitro, and mature transgenic plants have been raised in the greenhouse (Fig. 4e). Further, histochemical analysis revealed stable expression in roots, shoots and inflorescences (Fig. 4f, 4g).

Previously, tissue culture system in napiergrass was insufficient for the production of transgenic plants, one of which was due to the low quality of embryogenic callus and the low efficiency of plant regeneration. Following our established genetic transformation system for bahiagrass (Gondo et al., 2005) and ruzigrass (Ishigaki et al., 2012), we adapted similar transformation system in napiergrass. We have produced transgenic napier grass plants for the first time through the selection and adaptation of a genotype that leads to efficient embryogenic callus formation (Fig. 5a–5c). 'DL' is the best accession for callus induction, and high quality callus lines were screened on MS medium with 2,4-D, BAP and CuSO4. Subsequently, a highly regenerative callus which formed dense polyembryogenic clusters was obtained. A plasmid vector containing an herbicide-resistance gene (*bar*) and the β-glucuronidase (GUS) reporter gene was used in particle bombardment and bialaphos-resistant calli stably expressed the *GUS* gene. These herbicide-resistant napiergrass calli were regenerated, rooted, and grown into soil (Fig. 5 d–5h). Subsequent gel blot analysis analysis indicated the presence of the *GUS* gene in the genomic DNA and histochemical analysis confirm its expression in leaves of transgenic napier grass line.

Lignification of cell walls during plant development has been identified as the major factor limiting forage digestibility and consequently affecting ruminant productivity. In monotoctyledons, down-regulation of this enzyme has increased dry matter digestibility as observed in tall fescue (Chen et al., 2002; Chen et al., 2003) and sugarcane (Jung et al., 2013). Cinnamyl alcohol dehydrogenase (CAD) is a key enzyme involved in the last step of lignin biosynthesis and has been employed as target enzymes for manipulation of lignin content and composition by genetic engineering (Spangenberg et al., 2000). In order to understand the regulation of lignin biosynthesis and the potential to manipulate CAD expression,

we isolated and characterized cDNA of CAD in sorghum (Fig. 6). Our study found the coding region consisting of four exons and three introns, while putative some binding sites for transcription factors were identified (Fig. 6a). Cross-species comparison of CAD DNA sequences revealed evolutionary and structural variation among plants (Fig. 6b). Furthermore, we analyzed the expression profile of CAD in association with lignification in sorghum. CAD expression level was lower in *brown-midrib-6* mutant (*bmr*) than in normal isoline in root, stem, leaf, and midrib, thereby suggesting that the depression of CAD enzymatic activity in *bmr*-6 could affect enzymatic activity and regulation of lignin biosynthesis (Fig. 6c, 6d).

Many breeding efforts for improving forage quality have focused on identifying forage crops with altered lignification. Molecular breeding to reduce lignin content and increase digestibility is a strategic way to improve forage quality in warm-season grasses, such as bahiagrass. The genes in the lignin biosynthetic pathway have been elucidated, and using molecular breeding, the digestibility in economically important crops have been improved. CAD gene is in the last stage of the linin biosynthesis pathway and was preferred as the target gene for our research. Our study focused on the reduction of lignin content in bahiagrass by suppression of CAD gene expression (Fig. 7). An antisense vector and 4 RNAi vectors were constructed using either full length SbCAD 1098 or SbCAD 687 bp fragment of sorghum CAS cDNA that were assembled in opposite direction and inserted in a plant expression vector driven by the maize ubiquitin-1 promoter (Fig. 7a). Likewise, a CAD antisense vector was constructed by insertion of SbCAD (Fig. 7a). The transformation system used include tissue culture of seeds, the selection of embryogenic callus, introduction of vectors using particle bombardment, selection and regeneration of bialaphos-resistant transgenic plants, acclimatization in soil and growth and maturity in the greenhouse.

Calli resitant to bialaphos were obtained in 1.7% of the bombarded calli. PCR analysis of transgenic plants indicated the co-transformation with CAD construct confirmed half of the recovered calli. However, CAD down-regulation has hindered regeneration of callus into new plants, and only a total of 9 transformed lines were obtained. These lines were confirmed as seen on this blot analysis. The control plant showed only hybridized bands, which was derived from the endogenous CAD gene found in bahiagrass while the sorghum CAD gene used for silencing CAD was integrated at different sites and various copy numbers (Fig. 7b). Acid detergent lignin analysis was used to quantify the amount of reduction in lignin content and IVDMD or in vitro dry matter digestibility was used to quantify the digestibility of transgenic and control plants (Fig. 7c). Lignin content all the transgenic plants have reduced significantly differed relative to the control plant. This indicate that the suppression of CAD gene

allowed the reduction of the lignin content in bahiagrass. Moreover, we confirmed the digestibility levels of the experimental plants, and based on IVDMD analysis, almost all transgenic plants showed a significant increase in digestibility relative to the control plant (Fig. 7c). This is consistent with other reports involving transgenic plants belonging to the Gramineae where CAD suppression had increased digestibility. Our results succeeded to increase digestibility in bahiagrass through the downregulation of CAD.

As lignin is a structural polymer in plants, it was necessary to characterize the agronomic traits of the transgenic plants. Transgenic plants showed varied agronomic characteristics such as tendency of high leaf blade length, low number of tillers and percentage of tiller, and increase in DM yield. On the other hand, some lines exhibited a dwarf phenotype, low tiller number and percentage heading tiller (Fig. 7d). The genetic manipulation of the CAD gene in bahiagrass had led to the reduction of the lignin content and significantly increased the digestibility of the transgenic plants.

1.4. Environmental risk assessment in transgenic plants

Biotechnology is defined as the technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use. Recombinant-DNA derived plants or genetically modified organisms (GMOs), living modified organisms (LMOs, under the Cartagena Protocol of CBD), genetically engineered crops and transgenic crops, are often the cause of various debates. Although we recognize the possible advantages modern biotechnology offers to food, feed, and fuel, it is still important to ensure that safety comes first. As such, environmental risk assessment (ERA) needs to be implemented. In the world, many kind of GM crops were being commercialized such as transgenic cotton with herbicide-tolerant and insect-resistant traits. In 2014, transgenic varieties represented over 99% and 96% of all the cotton acres in Australia and the USA, respectively (James, 2014) while the majority of cottonseeds imported into Japan were derived from transgenic varieties. In 2015, Japan imported 103,247 tons of cottonseed mainly from Australia (42%) and the USA (30%) for food and feed use and (MAFF, 2016).

Transgenic crops are required to undergo safety assessments prior to importation and distribution under the laws concerning food safety (the Food Sanitary Law), feed safety (the Feed Safety Law) and environmental safety (the Law Concerning the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms, or the Cartagena Protocol Domestic Law) in Japan. As such, we conducted a field trial for insect-resistant and herbicide-tolerant transgenic cotton lines used for ERAs in Japan (Fig. 8). Confined field trials were conducted for the ERA of Lepidoptera-resistant and glufosinate-tolerant transgenic cotton (*Gossypium hirsutum* L.) lines GHB119 and T304-40

in Japan (Fig. 7b, 7c). GM lines were compared with conventional varieties for growth habit, morphological characteristics, seed dormancy, and allelopathic activity associated with competitiveness and production of harmful substances (Fig. 7d–7g). Slight statistical differences were observed between the GM line and its conventional comparator for some morphological characteristics; however, transgenes or transformation were not considered to be responsible for these differences. Based on our field trial for ERA, we demonstrated that these GM cotton lines were equivalent to conventional cotton varieties that have a long history of safe use in terms of competitiveness and production of harmful substances, and that GM cotton were observed to have no potential adverse effects to biosafety in Japan.

1.5. Genome editing in forage and turf grass

The genome editing is a breakthrough technology that can cut a targeted DNA at a pinpoint with artificial nucleases (ZFN, TALEN etc.) and RNA-inducible nucleases (CRISPR / Cas9), and can perform gene knockout and knocking of target genes. The technology is being used in various research fields such as medicine, science, and agronomics. In the field of plants, attempts are being made to plant breeding. It is focused as a new breeding technology different from genetic transformation technology. On the other hand, the way of regulation of genome editing is discussed in the world. In recent years, the US department of agriculture (USDA) has approved genome-edited mushrooms without under the GM regulation, and has shown its stance to accept the technology (Waltz, 2016) genome editing technology of plants is applied not only to model plants but also to crops such as corn, wheat and sorghum (Bortesi and Fisher, 2015). Although the research has been advanced for practical use, genome editing of forage and turf grass has not yet been successful.

In common, plant genome editing technology was performed to insert the CRISPR / Cas9 vector into the genome. The genome-edited mutant has the inserted vector in its genome, so the vector must be removed at the next generation by self-pollination. Therefore, this genome editing system is difficult to apply to forage grass or turfgrass, etc. which show vegetative propagation, apomictic and cross fertilization. In recent years, new genome editing systems have been developed which introduce editing vectors into plant cells and make genome editing with its transient expression. We apply this technique to forage and turfgrass, and have developed a system to create genome editing plants in T_0 stage (Fig. 9a). First target gene is CAD gene, we have isolated CAD gene from bahiagrass and constructed genome editing vector (Fig. 9b). Target tissues for particle bombardment is somatic embryo which is high potential plant regeneration and indicate high transient GUS expression (Fig. 9c). Heteroduplex PCR band patterns of CAD targeting site were recognized by transient expression of the editing vector after particle bombardment with somatic embryos, we confirmed that genome editing was induced at the cellular level

(Fig. 9d). Now, the somatic embryos have been regenerated plants and we will confirm genome editing in the plant. This is first research in warm-season grass, and this new technology is believed to accelerate the breeding of forage and turf grass.

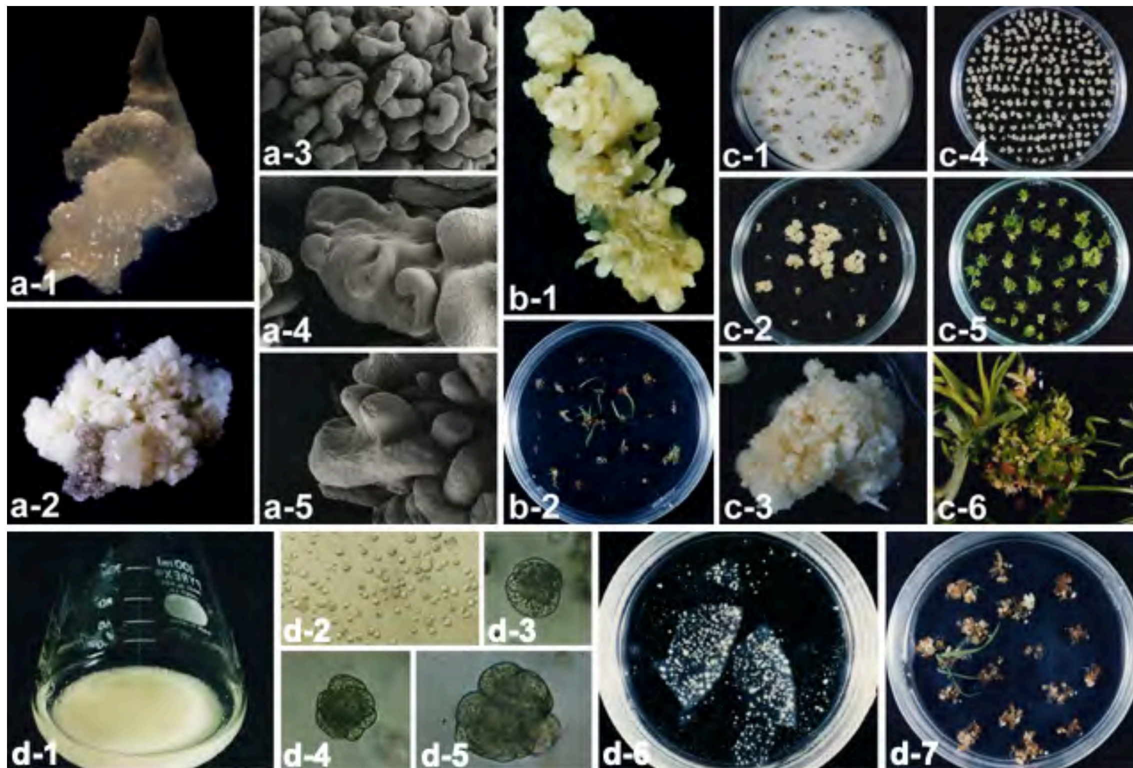

Figure 1. Tissue culture in some warm season grasses. (Grassland Science 2003, 49: 79–87)

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) 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.

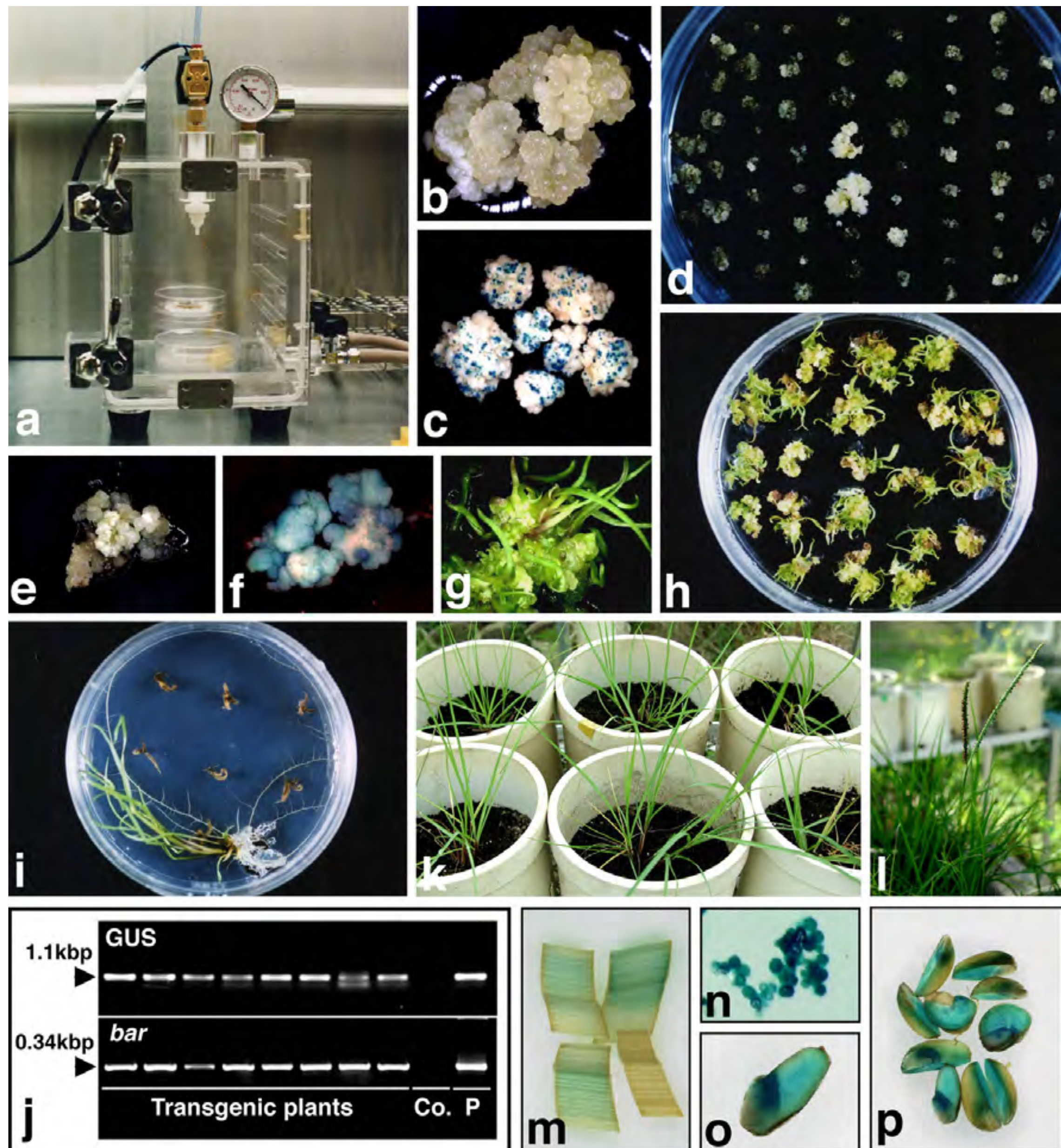

Figure 2. Transformation of diploid bahiagrass mediated by particle inflow gun. (Journal of Plant Physiology 2005, 162:1367–1375)

a) A simple self-built particle inflow gun. b) Highly regenerative embryogenic callus (target tissue). c) Transient GUS expression 16 h after bombardment. d, e) Bialaphos-resistant callus on MS-DBCH medium containing 3 mg/L. f) Stable GUS expression on bialaphos-resistant callus. g, h) Plant regeneration from bialaphos-resistant callus. i) Transgenic plants on rooting medium with 10 mg/L bialaphos. j) Detection of GUS and *bar* genes in transgenic plants by PCR (Co: non-transformed plant; P: plasmid pDBI). k) Potted transgenic plants. l) Flowering transgenic plants. m-p) GUS expression in leaves (m), pollen (n) and seeds (o, p).

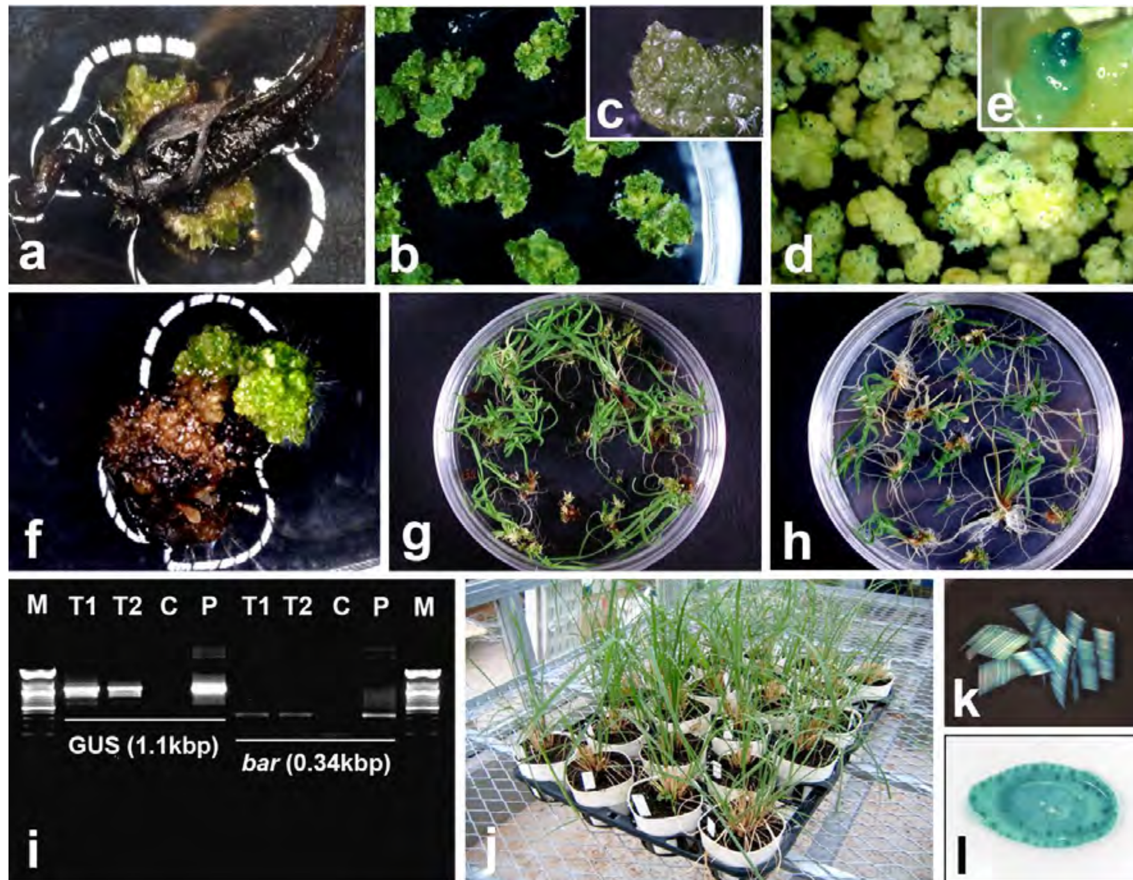

Figure 3. Multiple-shoot culture and genetic transformation of rhodesgrass mediated by a particle inflow gun. (Journal of Plant Physiology 2007, 166:435–441)

a) Primary multiple-shoot clumps after 30 d of culture on MS-D0.1B2 medium in the light. b, c) Compact and proliferating multiple-shoot clumps (target tissue). d, e) Transient GUS expression 16 h after bombardment. f) Bialaphos-resistant multiple-shoot clumps on MS-D0.1B2 medium containing 3mg/L bialaphos. g) Plant regeneration from bialaphos-resistant multiple-shoot clumps on hormone free MS medium containing 5mg/L bialaphos. h) Transgenic plants on rooting medium with 10mg/L bialaphos. i) Detection of GUS and *bar* genes in transgenic plants by PCR (M:DNAmarker; T1and T2:transformed plant; C: non-transformedplant; P: plasmid pAHC25). j) Potted transgenic plants. k, l) GUS expression in leaves and a stem of transgenic plants.

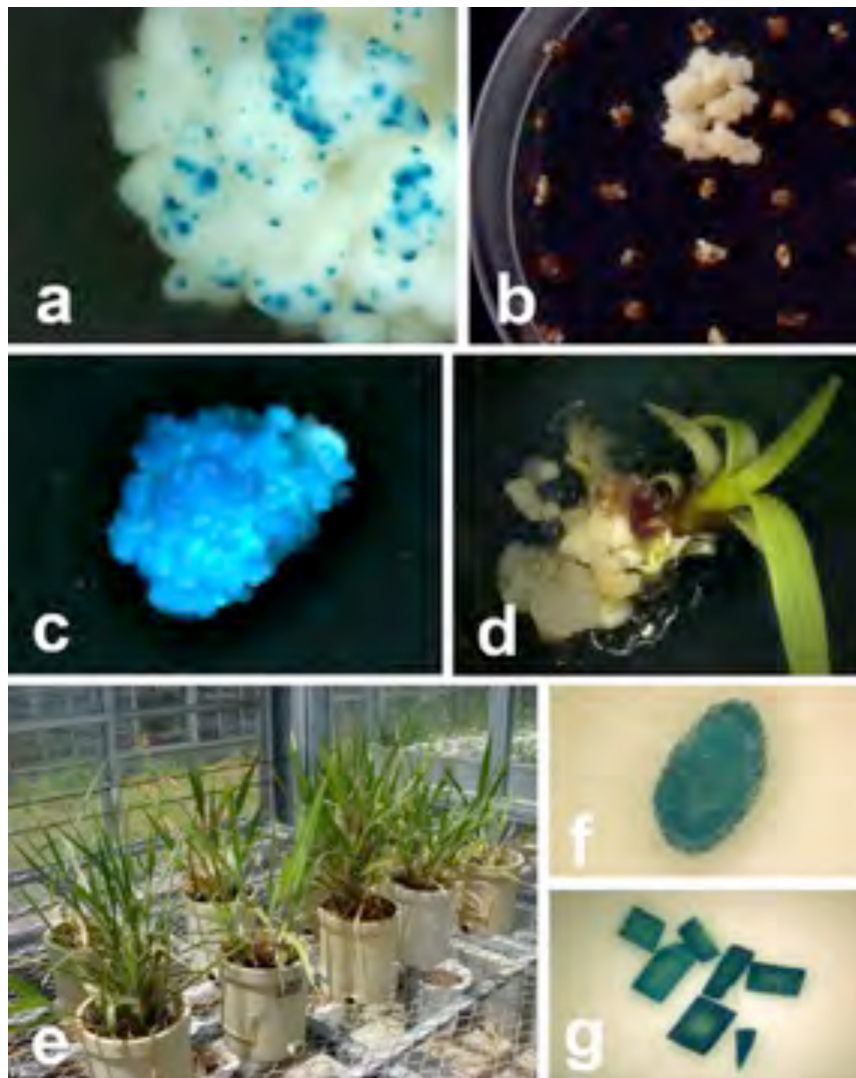

Figure 4. Transformation of diploid ruzigrass through particle bombardment. (Journal of Plant Physiology 2007, 166:435–441)

a) Transient GUS expression 16 h after bombardment. b) Bialaphos-resistant callus on MS medium containing 10 mg/L bialaphos. c) Stable GUS expression in bialaphos-resistant callus. d) Regenerated plant from bialaphos-resistant callus on medium with 10 mg/L bialaphos. e) Acclimated transgenic plants in greenhouse. f, g) GUS expression in stem (f) and leaves (g).

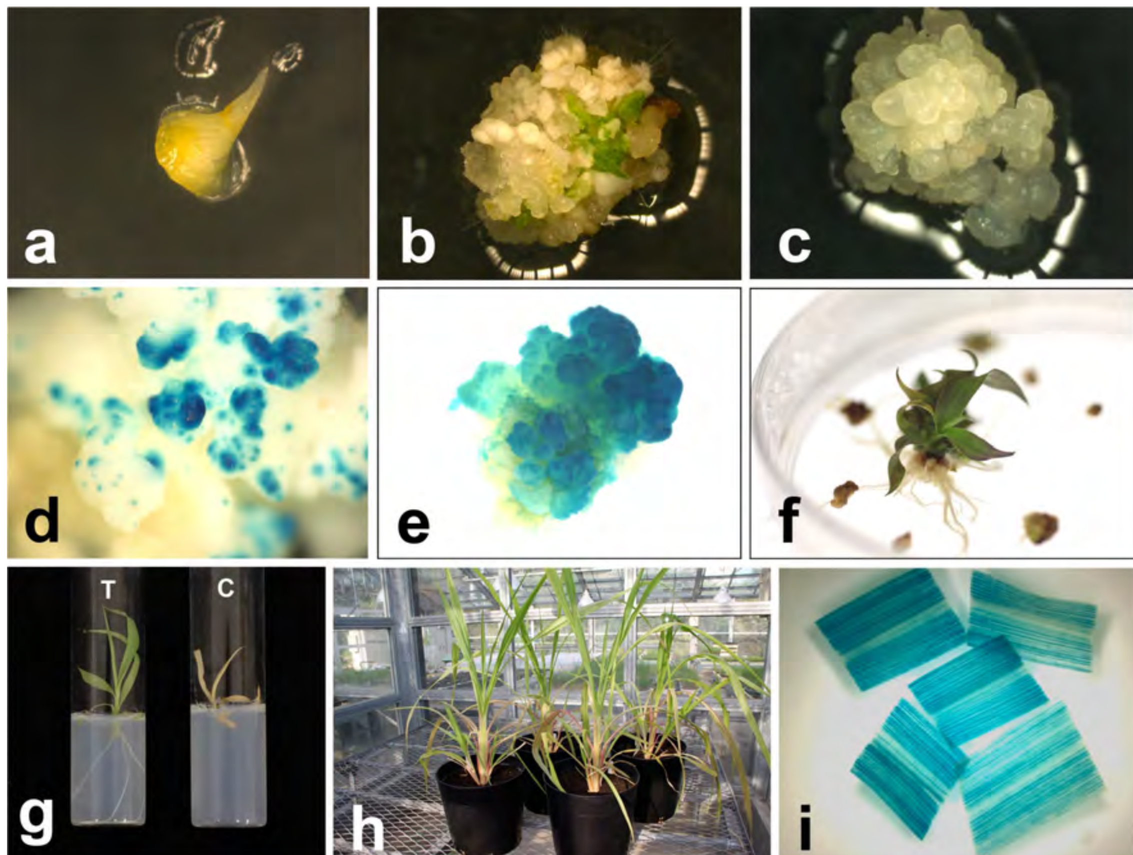

Figure 5. Formation of embryogenic callus and genetic transformation in dwarf napier grass line (DL) mediated by particle inflow gun transformation. (Plant Biotechnology 2017, 34: 143–150)

a) An excised shoot apex from a shoot tiller served as the initial explant. b) Primary embryogenic callus after 45 day of culture on MS medium containing 2.0 mg /L 2,4-D and 0.5 mg /L BAP. c) Compact and proliferating uniform embryogenic callus (target tissue). d) Transient GUS expression 16 h after bombardment. e) Stable GUS expression on bialaphos-resistant callus. f) Plant regeneration from transformed callus in MS medium containing 2.0 mg /L BAP, 0.1 mg /L NAA and 5.0 mg /L bialaphos. g) Rooting of transgenic plants on half-strength MS medium with 10.0 mg /L bialaphos (T, transgenic plant; C, non-transgenic plant). h) Potted and growing transgenic plants. i) GUS expression in leaves of transgenic plants.

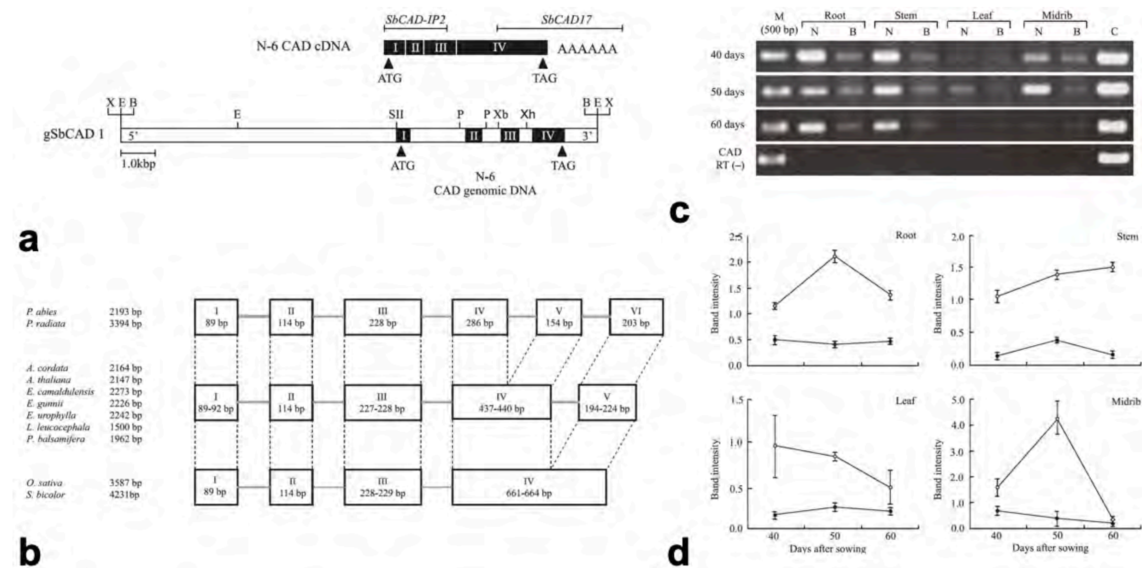

Figure 6. Structure of the sorghum N-6 CAD gene and Semi-quantitative RT-PCR expression analysis of CAD genes from sorghum N-6 and bmr-6. (Breeding Science 2010, 60: 314–323)

a) The upper part shows the structure of the *SbCAD* cDNA sequence. b) Comparison of the sorghum *CAD* gene with those of other species. Lengths run from the beginning of each start codon to the end of each stop codon. Exons are indicated by boxes and are numbered from the 5′ end in Roman numerals. The size of each exon is given in bp. Introns are indicated by solid lines. The *CAD* genes (GenBank accession number) are from *Picea abies* (AJ001926), *Pinus radiate* (AF060491), *Aralia cordata* (E07792), *Arabidopsis thaliana* (Z31715), *Eucalyptus camaldulensis* (GU109374), *Eucalyptus gunnii* (X75480), *Eucalyptus urophylla* (GQ387647), *Leucaena leucocephala* (AM263500), *Populus balsamifera* (AJ295837), *Oryza sativa* (AP004046), and *Sorghum bicolor* (AB565488; AB565489). c) A single representative experiment showing expression of the *CAD* gene in four different organs at three developmental stages. Total RNA isolated from root, stem, leaf, and midrib was subjected to RT-PCR semi-quantitative analysis using the sorghum actin gene as an internal control. RNA samples without reverse transcriptase (CAD RT [－]) were amplified as controls for DNA contamination. N, N-6; B, bmr-6; C, *SbCAD* cDNA; M, 500-bp ladder marker. d) Change of mRNA accumulation in four different organs at three developmental stages. Amplification products were quantified by densitometry using QuantiScan software. The sorghum actin gene (X79378) was used as a control for RNA input, and expression levels of each gene were adjusted by the corresponding amount of actin gene mRNA. The analysis was repeated at least three times. The values are the average of three replications; error bars indicate standard deviation. ○N-6; , ●bmr-6.

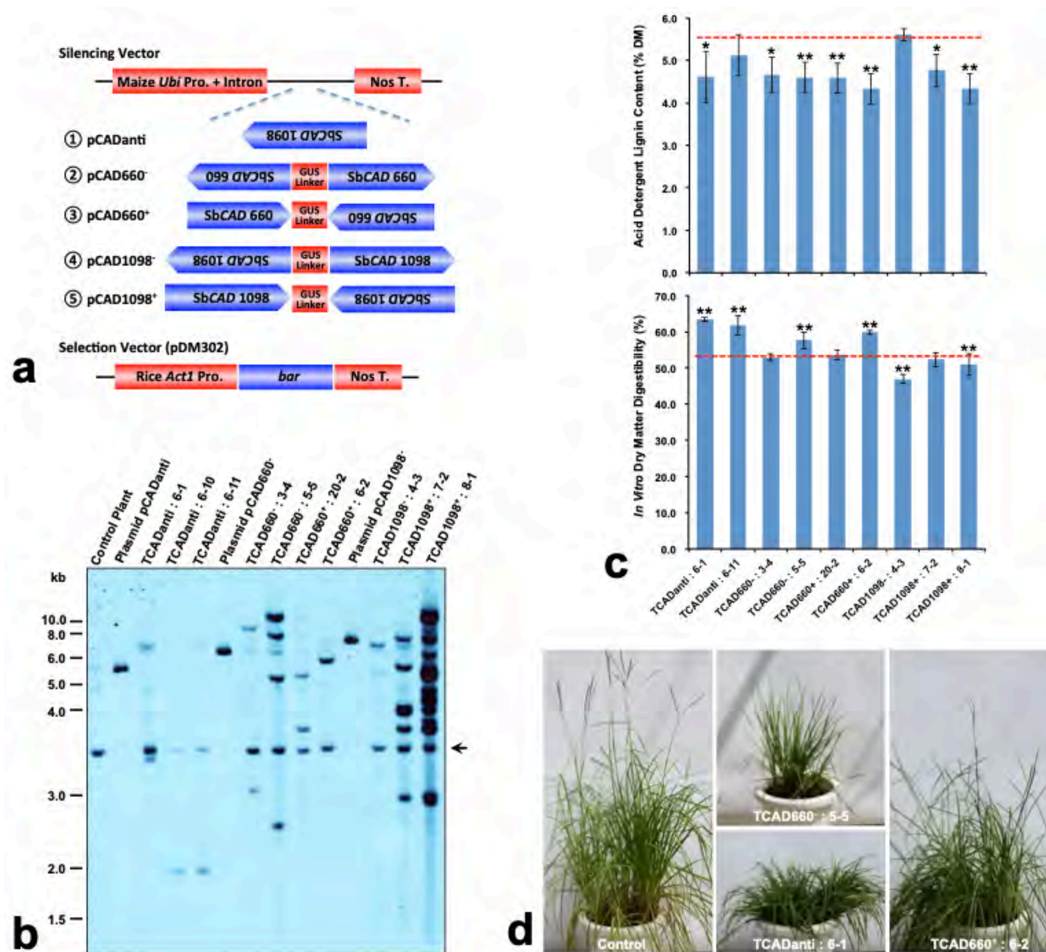

Figure 7. Production of high digestibility transgenic bahiagrass by genetic manipulation of cinnamyl alcohol dehydrogenase (CAD gene). (Asian Journal of Plant Sciences 2014, 13: 8–17)

a) Schematic diagram of sorghum *CAD* (*SbCAD*) antisense vector, RNAi vector and transgenic bahiagrass lines, each with their respective vectors. *SbCAD*1098: Full-length sorghum, *CAD*: cDNA, *SbCAD*687: 3'-fragment of sorghum *CAD* cDNA, GUS linker: Internal 370 bp sequence of *uid*A gene, Maize *Ubi* pro+intron: Maize ubiquitin promoter and first intron, Nos T: Nopaline synthase terminator. b) DNA gel blot analysis of transgenic bahiagrass plants. Two microgram genomic DNA, isolated from leaf tissue of non-transformed and 9 independent transformed lines, were digested with *Sac*I. Hybridization was carried out with a DIG-dUTP-labeled *SbCAD* gene probe. P1, P2 and P3 are positive control of 5 pg *Sac*I-digested plasmid, respectively. The arrow indicates the endogenous band derived from bahiagrass *CAD* gene. c) Acid detergent lignin content and in vitro dry matter digestibility in transgenic bahiagrass. The horizontal line indicates the relative level of the control plants. *,**Statistically significant difference between control and transgenic plants at $p<0.05$, 0.01 level, respectively by T-test d) Phenotypes of transgenic bahiagrass plants. Pot size was 25 cm in diameter.

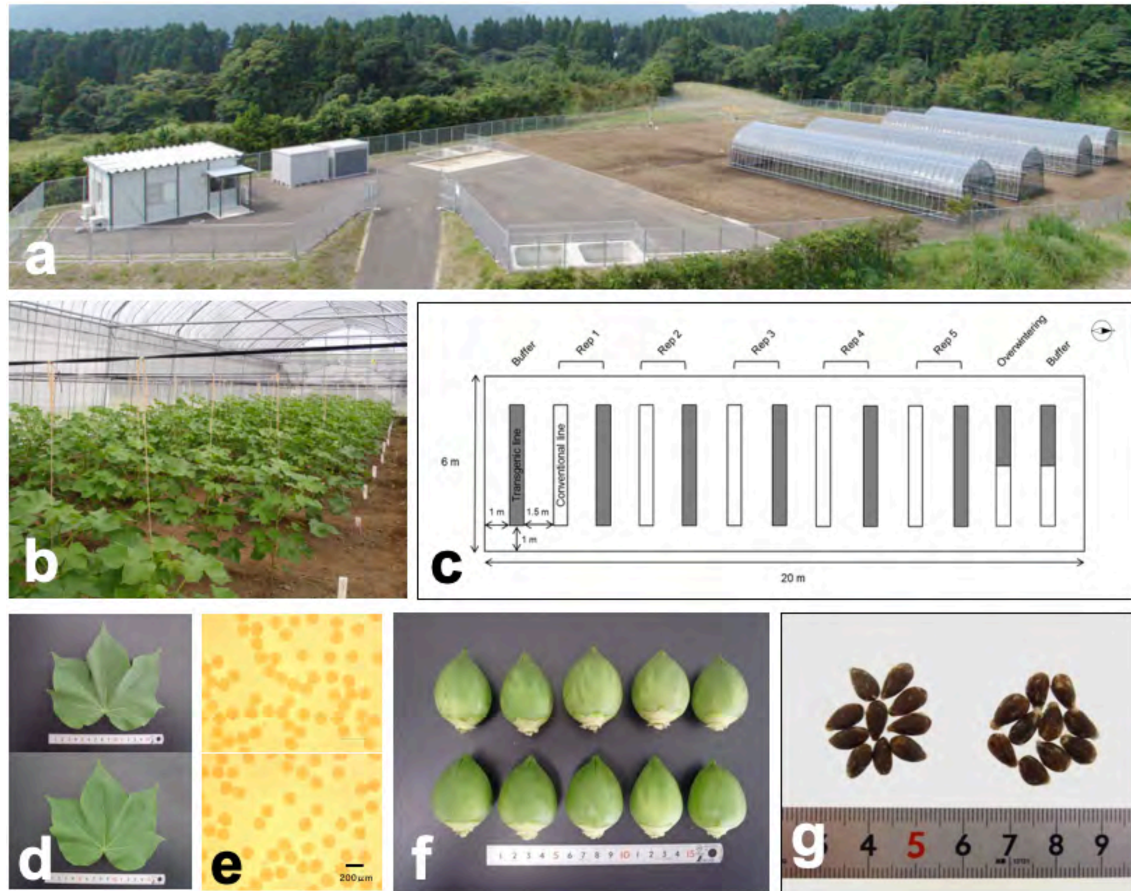

Figure 8. Field trial test of GM cotton (GHB119) in University of Miyazaki. (GM Crop & Food 2017, 8: 106–116) a) Overview of the confined field site at the University of Miyazaki. b) Schematic overview of the plot design. Each block is composed of eight plants. c) The test plot of GHB119 at 56 d after planting. d–g) Morphological characteristics of leaf (d), pollen (e), boll (f), seeds (g) (upper and left, GM line GHB119; lower and right, Non-GM line Coker 312).

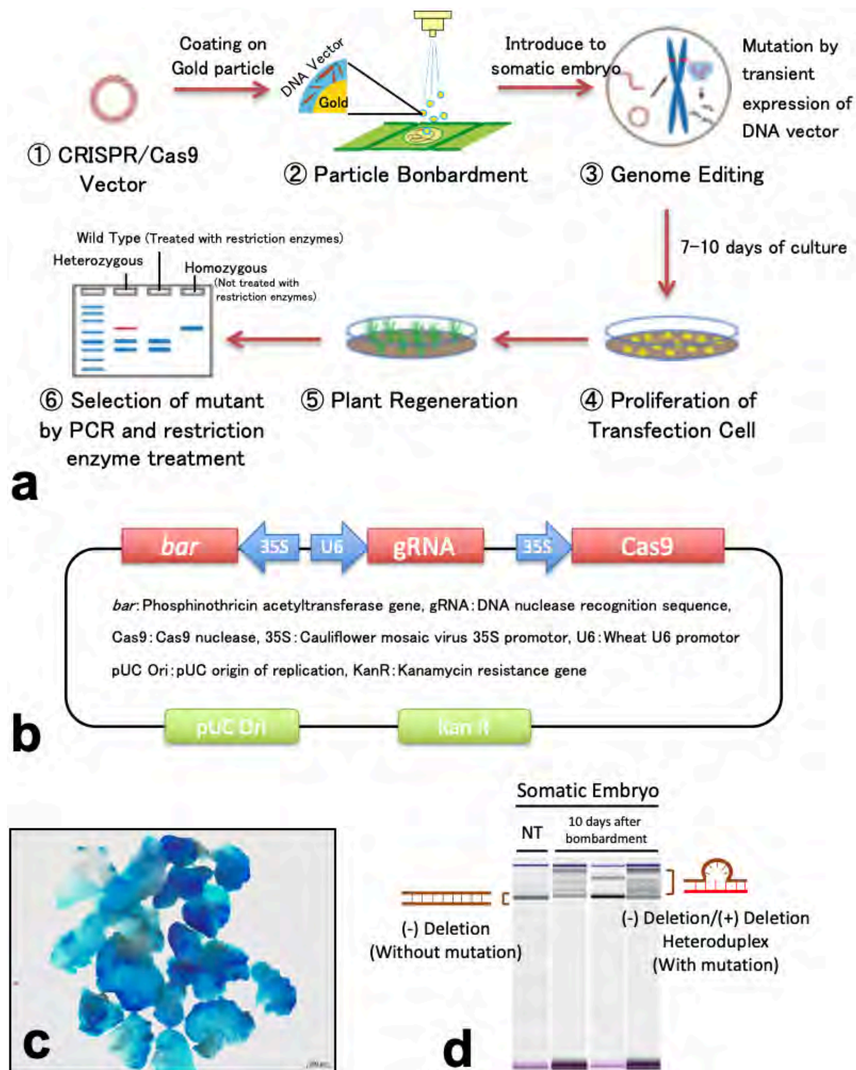

Figure 9. Genome editing of bahiagrass through transient expression of CRISPR/Cas9 vector on somatic embryos. (In progress.)

a) Scheme of genome editing through transient expression of CRISPR/Cas9 vector system. b) CRISPR/Cas9 vector used in this experiment. c) Transient GUS expression in somatic embryo after particle bombardment. d) Heterodupulex mobility assay (HMA) analysis of somatic embryo after 10 days of bombardment. Bombarded somatic embryos were extracted genome DNA after 10 days of culture. CAD targeting site were amplified by PCR and confirmed induction of deletion. Non-treatemnt (NT) indicate single clear band, but somatic embryo after 10 days of bombardment is shown heteroduplex band by deletion of targeting sequence.

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