Agrobacterium–mediated genetic engineering system for the C4 apomictic grass model species Cenchrus ciliaris L.

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Abstract

Cenchrus ciliaris L. (buffel grass) is a popular tropical range grass known for its perenniality and high palatability. Owing to its apomictic mode of reproduction, breeding this polymorphic grass is restricted to *in vitro* approaches of genetic manipulation. Apomixis facilitates genetic improvement by fixing heterosis as well as the changes made to the genome through transgenesis. Towards its genetic improvement, a robust protocol of plant transformation is desirable. Hence, first attempt to develop a rapid and efficient *Agrobacterium*– mediated transformation system for *Cenchrus ciliaris* genotype IG-3108 has been made. In this study, direct multiple shoot induction protocol using shoot apex explants was found to be suitable for transformation. Several factors such as inoculum of bacteria, co–culture time, co–cultivation duration, concentration of acetosyringone and effect of vacuum infiltration were optimized for achieving high transformation frequency using shoot apex explants. The shoot apex explants were co-cultured with EHA 105 harbouring the binary vectors pCAMBIA 1301 and 1305.1 containing the *hptII* gene as a selectable marker and *GUSA* as a reporter gene. The highest transformation frequency with pCAMBIA 1301 vector was 1.42% while with pCAMBIA 1305.1 vector, it was 1.37% when the explants were co-cultured for 30 min with cells of *Agrobacterium* at OD_{600} = 1.0 under vacuum (0.5 X 10^5 Pa) followed by co-cultivation for 3 days on MS with 3 mg/L TDZ and 400 µM acetosyringone. The status of transgene and its integration in the genome of regenerated putative transformed plants of *Cenchrus* were confirmed by PCR and Southern blot analysis.

Key message

A novel, efficient and reproducible transformation protocol using *Agrobacterium* has been developed for *Cenchrus* using shoot apices as explant.

Introduction

In the next 30 years, the world's population is expected to increase by 2 billion and the current breeding practices will be insufficient to meet the global food demands. Some of the approaches to accomplish this is through the use of novel biotechnological tools to accelerate breeding practices (Hojsgaard 2020) or exploring flex crops with multiple uses as food, feed, fuel, fibre and industrial materials (Kline et al. 2017). To accelerate crop breeding, researchers around the world are obsessed with the mechanism of apomixis, as it helps in fixing heterosis or hybrid vigor. To understand the genetic and molecular mechanisms controlling apomixis, *Cenchrus* has been used as a model plant (Goel et al. 2003; 2006; Conner et al. 2008) and is also a potential flex crop. *Cenchrus ciliaris* L. is an apomictic perennial semi–arid tropical and subtropical forage crop (Sanderson et al. 1999). It is tolerant to high temperature and drought conditions (Ayerza 1981) but also a highly nutritious and palatable fodder. Due to its resilience to harsh environmental conditions, it is cultivated as one of the major forage crops in India (Bhat et al. 2001; Griffa et al. 2006). It is predominantly cross-pollinated; the seed formation requires pollination known as pseudogamy. Since sexual plants are very rare in nature, the genetic improvement of this forage grass is mostly limited to selection methodologies hence, introgression of desirable genes from distant cultivars/ landraces has not been possible. Although *Cenchrus* is mostly resistant to biotic and abiotic stresses, improved cultivars often show vulnerability to salinity, diseases (such as smut) and pests (such as mites). Furthermore, forage quality in terms of its digestibility requires improvement which has not been possible till date.

Recent techniques of genetic manipulation has opened up new vistas for transferring desirable genes from alien lines and to overcome the limitations linked with conventional plant propagation. Genetic transformation of crop plants using *Agrobacterium* helps to make specific changes in the genome by the addition of one or some genes. A robust and efficient transformation protocol is also a pre-requisite for targeted genome editing to produce DNA-free lines using TALENs (transcription activator-like effector nuclease) and CRISPR-Cas (clustered regularly interspaced short palindromic repeats –CRISPR associated proteins) (Singh and Prasad 2016; Scheben and Hojsgaard 2020). Development of an efficient transformation system with *Agrobacterium* has been a main limitation in many grasses including buffel grass. Genetic crop improvement through conventional breeding methods is tricky, time consuming and limited to selection of genetic variants found in nature. The clones approach in apomictic species imposes a restriction on the genetic variability within a variety. A diminished hereditary base of any extensively used crop epitomizes a threat, providing pests and pathogens a chance to infect with single mutation. Once the pathogen becomes virulent, all other crops succumb to the infection, as they all are equally susceptible. This is evident from the epidemic outbreak of buffel grass blight caused by *Magnaporthe grisea* in 1996 (Rodriguez et al. 1999). Contrastingly, in case of apomixis additional breeding steps to fix the transferred character is not required, thus facilitating varietal change through DNA delivery system (Vielle Calzada et al. 1996). A proficient genetic transformation protocol of this grass, for instance, genetic manipulation of lignification to increase forage digestibility, would be exceptionally valuable (Cherney et al. 1991). Thus, a reliable transformation would be a potent tool to accelerate forward and reverse genetic studies for improvement of this meadow grass.

In buffel grass, few preliminary studies using biolistic particle delivery bombardment showing transient expression of a reporter gene have been published (Ross et al. 1995; Bhat et al. 2001; Laishram et al. 2020) Although, in cereals and grasses, transformation with microprojectile bombardment has been revolutionary, substantial differences have been observed in the stability, pattern of integration and expression of the introduced transgene (Kohli et al. 1999). Thus, there is a need for an effective and alternate method such as *Agrobacterium tumefaciens*-mediated DNA–delivery for regenerating transgenic plants with less copy number of transgenes. In addition, transgenic plants raised from *Agrobacterium* method are more stable over generations and express reduced levels of gene silencing (Barkat et al. 1997; Shou et al. 2004). The main drawback of *Agrobacterium*–mediated transformation in grasses is monocots are not their natural hosts, hence use of binary vectors with few varied host range virulent strains (AGL0 or 1, EHA101 or 105, LBA4404) is the only method for achieving high transformation efficiency in Graminaceous plants. Another important determining factor for successful transformation is the source of target plant tissue, growing medium and cellular and harvesting phase. The actively dividing cells of a tissue or organ are usually used as explant for regeneration of transgenic plant. Transformation efficiency with the type of explant used, differs from species to species or even within same species with diverse genotypes.

In major cereal crops, genetic engineering–based transgenic technology was adapted for the improvement of various traits in forage, turf and bioenergy grasses, including biomass yield and quality, ethanol production and abiotic and biotic stresses such as heavy metal tolerance and pathogen resistance. Successful *Agrobacterium tumefaciens*–mediated transformation have been reported in forage grasses, such as *Agrostis palustris* (Yu et al. 2000), *Panicum virgatum* (Somleva et al. 2002), *Zoysia japonica* (Toyama et al. 2003), *Festuca arundinacea*, *Lolium multiforum* (Bettany et al. 2003), *Agrostis tenuis* (Chai et

al. 2004), *Arostis stolonifera* (Luo et al. 2004), *Lolium perenne* (Altpeter et al. 2004), *Cynodon dactylon* (Hu et al. 2005), *Festuca arundinacea* (Dong and Qu 2005), *Poa pratensis* (She et al. 2005) and *Eragrostis curvula* (Ncanana et al. 2005). Initial attempts to transform *Cenchrus sp.* using *Agrobacterium tumefaciens* (Batra and Kumar 2002) were ineffective in terms of transformation efficiency due to the resistance to *Agrobacterium*. Till now, there have been no reports of stable transgenic plant in *Cenchrus*. Thus, there is a dire need to develop an efficient method to transfer desirable genes into *Cenchrus* for its genetic improvement.

The two most vital steps required for delivery of foreign DNA are entry into the plant cells and subsequent regeneration of plants from putative transformed cells (Yookongkaew et al. 2007). Somaclonal variations and genetic mutations are low in plantlets regenerated from direct shoot apices or an apical meristematic cell as compared to in vitro plantlet proliferated from induced calli (Bregitzer et al. 2002). The reason behind reduced somaclonal variation is lack of tissue or cell de-differentiation phases, which are common within the induced–calli and initiation of somatic embryos (Hirochika 1993). Hence, the direct shoot induction would be the popular method for *Agrobacterium*–mediated DNA–delivery protocol in order to reduce somaclonal variation and genotype dependency otherwise found in callus–mediated plant regeneration methods. Genetic transformation using shoot apex was first reported by McCabe and his group in 1988. Thereafter, various protocols have been optimised in other crop plants using shoot apex in combination with either *Agrobacterium*–mediated transformation or biolistic particle delivery system (Gould and Magallanes-Cedeno 1998; Zapata et al. 1999; Cho et al. 2003; Goldman et al. 2003; Yookongkaew et al. 2007; Zhang et al. 2010; Jha et al. 2011). Moreover, these transgenic plants showed identical genotypic and phenotypic characteristics compared to their wild type. The present study is the first report on *Agrobacterium*–mediated transformation in *Cenchrus ciliaris*. Multiple shoots were developed from shoot apices explant as previously described by Shashi and Bhat (2023) and various factors were optimised and evaluated to achieve a successful and efficient transformation system.

Materials and methods

Plant material

Mature seeds of open pollinated inflorescence of *Cenchrus ciliaris* genotype IG-3108 was used. The seeds were harvested from the plants grown in botanical garden, University campus area. Seeds were dried and stored for six months in room temperature. After seed dormancy period, seeds were used as experimental material.

Pre–treatment of seeds and isolation of shoot apices

The seeds were manually dehisced and surface disinfected with for 1 min with 70% (v/v) ethanol, followed by 5 min in 0.1% (w/v) $HgCl_2$. To eliminate the traces of $HgCl_2$ upon the surface of seeds were washed 4–5 times with sterile distilled water and then dipped overnight in 1 mg/L GA_3 to break dormancy and synchronize germination. The treated seeds were germinated on MS with 1 mg/L TDZ (Sigma, USA) media containing petri dish under aseptic conditions for 3 days at 25 ºC in light. Emerging 0.5 mm long shoots, comprising of shoot apical meristem with attached mesocotyl, were cut out from three-days old seedling using a sterile scalpel blade and cultured horizontally on semi–solid MS with 3 mg/L TDZ for 1 day at 25 ºC under 16 h photoperiod (40 $\mu mol^{-2}s^{-1}$). Pre-cultured shoot apices (0.7 mm- 1 cm) were used as explants for transformation.

Agrobacterium strain and binary vector used for foreign DNA–delivery

A supervirulent *Agrobacterium tumefaciens* strain EHA 105, containing binary vectors pCAMBIA 1301 and pCAMBIA 1305.1 were used in this study (Fig. 1). The T–DNA of pCAMBIA 1301 and 1305.1 contains *GUS* gene with a modified castor bean catalase intron as a reporter and *hptII* gene conferring resistance to Hygromycin as a selection marker driven by CaMV 35S promoter (Fig. 1). The *Agrobacterium tumefactions* strain was maintained on semi–solid LB agar media with 10 mg/L of Rifampicin and 50 mg/L of Kanamycin (Duchefa Biochemie).

Minimum inhibitory concentration of Hygromycin B for selection

Hygromycin is the most accepted selectable marker gene used in plant transformation vectors. For successful selection, the non–transformed plant cells must be susceptible to antibiotics. In this experiment Hygromycin sensitivity was tested to determine the threshold concentration of Hygromycin B required to be used for the selection of the transformants. Sixty number of shoot apices were placed on MS with 3 mg/L TDZ containing Hygromycin B (0, 5, 10, 15, 20, 25, 30, 50 mg/L) and lethal dose was determined for 2 week of I selection followed by transfer to fresh medium as II selection for another 2 week. Then subcultured into regeneration medium without Hygromycin B for shoot growth recovery for 1 week. Remaining shoots were counted to estimate survival rate. The concentration of Hygromycin B lethal for the explants was used in ensuant transformation experiments.

Preparation of Agrobacterium suspension culture

A single colony of *Agrobacterium* cultures from freshly streaked plate carrying the plasmids were grown overnight (14– 16 h) in small culture 10 mL of liquid YENB medium (7.5 mg/L yeast extract, 8 mg/L nutrient broth; pH 7.5) with 25 mg/L Rifampicin and 50 mg/L Kanamycin at 28°C on a thermo-rotatory incubator shaker at 180 rpm. From overnight small culture, 1 mL was re–suspended in 50 mL medium containing 400 µM acetosyringone, specified OD_{600} of 0.1 and then bulk grown for 2– 3 hours under above conditions till an OD_{600} of 0.8– 1.0 was reached. The *Agrobacterium* cells were centrifuged at 6000 rpm for 10 min at 4°C. The pellet was later re–suspended in liquid MS–infection medium to be used for co–culturing. Media composition used for *Agrobacterium tumefaciens*–mediated transformation and shoot regeneration given in Table 1.

Table 1
Tissue culture media composition used in the *Agrobacterium*-mediated transformation experiments

Medium	Purpose	Chemical composition
Seed sterilization	**Contamination free for in vitro culturing**	**70% ethanol, 0.1% mercuric chloride, distilled water**
Seed pre-germination treatment	Break dormancy and synchronize germination	1 mg/L Gibberellic acid solution
Seed germination	Obtained shoot apex explants	MS salts and vitamins + 1 mg/L TDZ + 30 g/L sucrose + 7 g/L agar (pH 5.8)
Shoot apices pre-cultured medium	Shoot meristem growth	MS salts and vitamins + 3 mg/L TDZ + 30 g/L sucrose + 8 g/L agar (pH 5.8)
MS-infection medium	*Agrobacterium* suspension and infection	MS + 68.5 g/L sucrose + 36.04 g/L glucose + 1 g/L casamino acid + 0.1 g/L glycine, 1.5 g/L arginine, 3.2 g/L aspartic acid (pH 5.2) and 400 µM acetosyringone (modified from Ishida et al. 1996)
Co-cultivation medium	*Agrobacterium* co-cultivation	MS + 3 mg/L TDZ + 30 g/L sucrose + 0.5 g/L MES + 400 µM acetosyringone + 0.5 g/L + 8 g/L agar (pH 5.8) (modified from Ishida et al. 1996)
Recovery medium	Shoot apices growth	MS + 3 mg/L TDZ + 30 g/L sucrose + 8 g/L agar + 500 mg/L cefotaxime (pH 5.8)
Selection medium	Hygromycin B-resistant shoot apices	MS + 3 mg/L TDZ + 30 g/L sucrose + 8 g/L agar + 250 mg/L cefotaxime + 30 mg/L hygromycin B (pH 5.8)
Pre-regeneration medium	Transformed shoot apices growth	MS + 3 mg/L TDZ + 30 g/L sucrose + 8 g/L agar + 250 mg/L cefotaxime (pH 5.8)
Regeneration medium	Transformed shoot apices regeneration	MS + 3 mg/L TDZ + 30 g/L sucrose + 8 g/L agar + 30 mg/L hygromycin B (pH 5.8)
Rooting medium	Transformed roots development	MS + 2 mg/L IBA + 15 g/L sucrose + 8 g/L agar + 30 mg/L hygromycin B (pH 5.8)
Hardening	Grown transgenics plant in fields	Sterile vermiculite or soilrite with coconut peat in pots and nutrient provide Hoagland's solution (Hoagland and Arnon 1950)

Infection, co–cultivation and selection of shoot apices with Agrobacterium tumefaciens

After one day incubation, shoot meristem explants were immersed in MS–infection medium for 30 min with gentle shaking (80 rpm at 25 ºC) on incubator shaker. Before agro–infection, the OD_{600} of *Agrobacterium* strain was adjusted 1.0 in liquid MS–infection medium. For Agro–infection, petri dish with agro–bacteria and shoot apices were placed in a vacuum chamber under negative pressure, (0.5 x 10^5 pa) by vacuum pump. The explants were taken out of bacterial suspension and were blot dried using sterile blotting paper to get rid of excess *Agrobacterium* and then placed on co–cultivation medium with 400µM acetosyringone for 3 days. Co–cultivation was carried out at 25°C in 16 h light on semi–solid medium. After co–cultivation, shoot apices were washed with sterile water thrice and then distilled water containing 250 mg/L Cefotaxime for 5 min, blot dried with sterile blotting paper and sub–cultured to recovery medium for 1 week to reduce the growth of *Agrobacterium*. Subsequently, they were cultured onto the I selection medium for 30 days with sub–culturing every 15 days for the proliferation of transgenic multiple shoots. After Hygromycin selection, surviving shootlets were cultured onto pre–regeneration medium for 2 weeks. Finally, these multiple shootlets were regenerated on regeneration medium in 2 weeks and plantlet were rooted on rooting medium. After 20 days, rooted plants were hardened and transferred to small cups with sterile vermiculite.

Optimization of parameters

The potential factors including Bacterial cell density (0.4–2.0 OD), under negative pressure (0.5 x 10^5 pa) using vacuum pump, duration of infection or co–culturing (10–60 min), co–cultivation period (1–6 days), acetosyringone concentration (100–500 µM) affecting the transformation efficiency. These factor were assessed by transient expression of reporter gene after the shoot apices were transformed as described above under heading. Shoot apices were transferred to GUS staining solution after 3 days of co–cultivation.

Histochemical GUS assay

After transformation with *Agrobacterium tumefaciens*, GUS assay was accomplished according to the protocol developed by Jefferson (1987), in order to observe transient GUS expression. For GUS assay, explants were immersed in 1 mM chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) in falcon tubes and they were vacuum infiltrated at 600 mm Hg for 1–2 min for better absorption of substrate solution by the plant tissue. Then the transformed plant parts (shoot meristem after co–cultivation and leaf fragment after selection) were incubated overnight at 37°C in dark. Subsequently, the shoot apices or leaves were decoloured in 70% ethanol overnight at room temperature. At the end of the incubation period, the explants were examined under Stereomicroscope and scored for the number of explants showing blue stain per treatment.

Statistical analysis

Each experiment was replicated 3 times and each set within a replicate consisted of at least 60 explants. The percentage of transformation efficiency was calculated as the total number of shoot apices expressing GUS gene per total number of explants infected by *Agrobacterium* x 100. For all the experiment,

statistical analyses were performed by SPSS 16.0 and using one way ANOVA and Duncan's multiple range test were used to determine the statistical significance differences among means at level $P < 0.05$.

Molecular analysis of putative transgenic

PCR-based confirmed transgenic plants

DNA was extracted from 1g young leaves of putative transformants and control (non–transformed) using method of Qiagen DNeasy Plant Maxi Kit. DNA was checked by running on 0.8% agarose gel for determine the concentration and pureness of DNA. To confirm the presence of transgene, the control and the putatively transformed plants were tested for the presence of *hptII* and *GUS* gene by PCR using specific primers and Southern blotting. The PCR reactions were carried out in a 25 µl reaction mixture having DNA (500 ng), 1X Taq Buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 1U of Taq DNA Polymerase 1µM of each forward and reverse primers specific to the *nptII* and *hptII* genes of both binary vector 1301 [(nptII; forward primer GCCATTTGAAGCCGATGTCACGCC; reverse primer GTATCGGTGTGAGCGTCGCAGAAC), (hptII; forward primer 5'CTATCGGCGAGTACTTCTACAC; reverse primer GTGTCACGTTGCAAGACCTA)] and 1305.1, [(nptII; forward primer TCATCAACAACAAACCGTTCTAC; reverse primer TCTTCCTCAGTCGCCGCCTCG), (hptII; forward primer GGCGTCGGTTTCCACTATCGG; reverse primer ATTGACTGGAGCGAGGCGATGTTC)]. These primers specifically amplified the 1029 bp, 406 bp *nptII* and 694 bp, 355 bp *hptII* genes, respectively. The PCR cycling conditions was programmed with initial denaturation of DNA at 94°C for 5 min, 30 cycles at 94°C for 30 sec, Annealing temperature (T_a) for 1 min, 72°C for 1 min/ Kb of fragment, a final extension at 72°C for 10 min. Plasmid of pCAMBIA 1301, pCAMBIA 1305.1 and control plant DNA was used as negative control for confirmation of transgene.

Southern blot analysis

Southern Blot was performed to verify the stable integration and copy number of *nptII* and *hptII* gene in the *Cenchrus* genome. Thirty micrograms of DNA from PCR–positive and control as negative plants were digested with restriction enzyme *EcoR1* overnight at 37 ˚C and then purified using Qiagen PCR purification kit. The digested DNA was electrophoresed on a 1% (w/v) agarose gel and the DNA from the gel was transferred onto a nylon membrane using the capillary transfer method as described in Sambrook et al. (1989). Briefly, the gel was treated with Depurination solution (0.2 N HCl) for 15 min, Denaturation solution (1.5 M NaCl, 0.5 N NaOH) for 30 min and Neutralizing solution (1M Tris-HCl, pH 7.4, 1.5 M NaCl) for 30 min. The DNA was transferred to nylon membrane (Amersham Pharamacia Biotech, UK) by capillary transfer in 20X SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) overnight and cross–linked by UV irradiation at 254 nm for 2 min. The membrane was pre–hybridised for 4 h at 45°C and DIG labeled full-length PCR-amplified *GUS* probe (1029 bp) and *hptII* probe (355 bp) were prepared by random priming method using the Roche DIG High Prime DNA Labeling and Detection Starter kit II. The DIG labeled probes were denatured and added to the fresh pre-hybridization solution for overnight incubation at 50–53°C. The membrane was washed sequentially in 2X SSC + 0.1% SDS; 0.5X SSC + 0.1% SDS at 68°C for 15 min each 3 times, then washed with blocking solution and with Anti–Digoxigenin for 30 min each. Finally for detection, detection buffer was used for 5 min and applied CSPD as substrate. The membrane was then wrapped in saran wrap and incubated for 2 h at 37°C. Detection of the signals and images was recorded using Night OWL instrument.

Results and discussion

Various explants have been used for the genetic transformation of grass species, including *Cenchrus*. Shoot apical meristems have, by far, been the most sustainable explants, due to ease of *in vitro* culturing and obtaining transgenics which are genetically identical to their parent (Sticklen and Oraby 2005). The cells in shoot apical meristem undergo constant cell divisions, therefore, regeneration of plants after transformation is usually rapid. Two strategies are typically used to develop transformants by transfer of foreign T–DNA into the shoot meristems. One is that transformed newly shoots developed directly from the meristematic cells (i.e., shoot and floral meristem), wherein, putative transformants produced are usually chimeric. The other method primarily involves multiplication of transgenic apical meristem cells using plant growth regulators (TDZ or BA or Kinetin), which re–programme the dividing cells under in vitro environment (Zhong et al. 1996). Therefore, genetic manipulation supplemented with plant growth regulators to induce multiple shoot formation from shoot meristem regenerate potentially more positive transformants (Yookongkaew et al. 2007). Therefore, in our study the instant pathway of shoot organogenesis from shoot meristems was adopted for transformation (Shashi and Bhat 2023).

The delivery process of T–DNA into plant cells is influenced by numerous factors (Jones et al. 2005; Matthysse 2006). Standardization of these factors is essential for the establishment of a successful *Agrobacterium* transformation system in monocot plants. In *Cenchrus ciliaris*, there has been no report of *Agrobacterium*–mediated transformation, hence it was crucial to study the effects of numerous factors on T–DNA delivery. Several factors affecting gene transfer were determined by assessing the expression of reporter GUS gene in the leaves of putatively transformed plants as reported in *Sorghum bicolor*, *Oryza sativa*, *Poa pratensis*, *Pennisetum glaucum* (Sairam et al. 2003; Yookongkaew et al. 2007; Zhang et al. 2010; Jha et al. 2011).

Cenchrus seed was germinated on 1 mg/L TDZ rather than MS medium alone because it breaks dormancy and induces synchronous germination (Fig. 2a; Chengalrayan and Gallo-Megher 2001; Sumlu et al. 2010). Pre-culturing shoot apices for 12–14 hours on MS media with 3 mg/L TDZ promotes cell division (Fig. 2b). These induced cell division are in turn responsible for subsequent events of transformation such as T–DNA delivery and its integration (Sangwan et al. 1992), inhibition of adverse effects of Agrobacteria and selection medium on cell differentiation (McHughen et al.1989). Similar reports in cereal crops such as *Hordeum vulgare*, *Triticum aesivum*, *Pennisetum glaucum*, pre-culturing of explants has proven to be beneficial for increasing the transformation efficiency (Trifonova et al. 2001; Wu et al. 2003; Jha et al. 2011).

Hygromycin B sensitivity in selection for transformants

During all transformation events, only a few cells are transformed and regenerating putative transgenic lines without selection marker is a monotonous process. Hence, the selection marker has been essential for the regeneration of transformants, as this permits only the cells or tissues expressing the

transgene to regenerate. Moreover, the selection marker also helps in subsequent screening and selection of the transgenic lines. A selectable marker such as hygromycin phosphotransferase (*hptII*) gene is usually used, which provides resistance against antibiotic Hygromycin B (Song et al. 2012; Hwang et al. 2014) and therefore allows screening of transgenic from non-transgenic tissues. A plasmid construct containing Hygromycin resistance gene in the T–DNA region was used for transformation protocol. The present investigation used different levels (0, 5, 10, 15, 20, 25, 30, 50 mg/L) of Hygromycin B and 30 mg/L was found to completely inhibit the growth of shoots whereas only 3% shoots survived at a concentration of 25 mg/L (Fig. 3a). Therefore, 30 mg/L Hygromycin B was used as optimum concentration for selection of putative transformants. Our results are in accordance with study by Jha et al. (2011) in *Pennisetum glaucum* and Padmanabhan and Sahi (2009) in *Sesbania drummondii* however, Li et al. (2011) found concentration of 20 mg/L was optimal for *Saussurea involucrata*.

Optimizing factors influenced in T-DNA delivery system

Agrobacterium tumefaciens DNA-delivery has turn into the most frequently used technique for introducing foreign gene into plant cells and subsequent regeneration of plantlets. Towards this, optimum conditions are required for conducting successful transformation experiments. Diverse factors such as bacterial cell density, duration of co–culture and co–cultivation period, use of Acetosyringone and effect of vacuum infiltration were optimised for *Agrobacterium*–mediated transformation in *Cenchrus*, based on transient GUS assay expression (Fig. 3). Standardization was performed using super–virulent *Agrobacterium* strain EHA 105 containing construct pCAMBIA 1301 and 1305.1 in shoot apices of genotype IG-3108. Subsequently, transgenic shoots were multiplied through direct shoot organogenesis.

Bacterial cell density

The first and most important factor for optimization of transformation efficiency is density of *Agrobacterium* cell suspension culture. To study the effect of most suitable OD_{600} for *Agrobacterium* culture, six different levels were tested; 0.6, 0.8, 1.0, 1.2, 1.5, 2.0. When using *Agrobacterium* strain EHA 105 with pCAMBIA 1301 and 1305.1, the highest number of GUS expressing shoot apices were observed with OD_{600} = 1.0 (Fig. 3b) similar to the findings in *Oryza sativa*, *Hordeum vulgare* and *Brachypodium distachyon* (Kumar et al. 2005; Shrawat et al. 2007; Behpouri et al. 2018). The number of shoot apices expressing GUS at OD_{600} = 1.0 was observed to be 15 ± 2.9 and 18 ± 3.7 for pCAMBIA 1301 and pCAMBIA 1305.1, respectively. The lowest number of GUS positive shoot apices was recorded at OD_{600} = 2.0 (0.66 ± 1.0) indicating cell density had significant influence on transformation efficiency. Higher concentration of bacterial cell density (OD_{600} > 1.0) is harmful to infected tissues leading to cell death, thereby decreasing the percentage of transformation. However, some workers found high transformation efficiency even at OD_{600} > 1.0 such as *Triticum aestivum* and *Pennisetum glaucum* (Amoah et al. 2001; Jha et al. 2011), however, in *Paspalum vaginatum*, *Panicum virgatum* and *Setaria italica* (Lin et al. 2017; Wu et al. 2018; Sood et al. 2020), OD_{600} < 1.0 was more effective. Optimization of the *Agrobacterium* cell density is essential for the reason that at high OD levels, the plant tissues are almost entirely dense with bacterial colony and hence its elimination becomes very challenging throughout the transformation process.

Duration of infection and co–culture

GUS expression frequency is influenced by the period of exposure of plant tissue with *Agrobacterium* cells hence the effect of changing the length of co–culturing period (10, 20, 30, 40, 50, 60 min) was considered. The shoot apices co–cultured with *Agrobacterium* cells (OD_{600} = 1.0) for 30 minutes showed highest efficiency of GUS expression compared to the one exposed to less than 20 min or over 40–60 minutes (Fig. 3c). The results obtained were similar to the reports in *Dactylis glomerata*, *Pennisetum glaucum*, and *Oryza sativa* (Lee et al. 2006; Jha et al. 2011; Zhao et al. 2011). Prolonged co–culture period negatively affected the plant tissues due to overgrowing cell density. However, few studies have indicated longer duration, i.e., 50 min in *Oryza sativa* (Sarker and Biswas 2002) and some shorter duration, i.e., only 5 min in *Zea mays* and *Sorghum bicolor* (Howe et al. 2006; Ishida et al. 2007), yielded better results.

Co–cultivation period

The time of T– DNA transfer and integration into the genome through *Agrobacterium tumefaciens* is a prolonged time-consuming process and differs widely from limited hours to few days depending upon the genotype, explants and culturing environment (Danilova and Dolgikh 2005). We conducted a transient expression experiment after co–culturing for 30 min and assessed the duration of co–cultivation period. Co–cultivation was carried out in 16/8 h light/ dark photoperiod at 25 ˚C from 0, 1, 2, 3, 4, 5 days. Initial co-cultivation period of 30 minutes did not yield any GUS blue spots, nonetheless, GUS expression was prominent after 3 days of co-cultivation. Prolonged incubation up to 5 days, did not increase the GUS expression, instead caused necrosis and damage to the tissues (similar to Li et al. 2015 report). Our results indicated that percentage of transient expression increased from 1 to 3 days (Fig. 3d). Co–cultivation period for 3 days is mostly appropriate for DNA–delivery in *Dichanthium annulatum*, *Zea mays*, *Paspalum vaginatum* (Kumar et al. 2005; Chen et al. 2014; Wu et al. 2018) whereas in *Dactylis glomerata*, highest efficiency was achieved after 2 days of co–cultivation (Ma et al. 2010).

Acetosyringone dosage on T–DNA insertion

Acetosyringone, a phenolic compound is well–known to enhance the virulence of *Agrobacterium cells*, to facilitate the T–DNA integration and also increase the percentage of transformation (Stachel et al. 1985; Dalton 2020). In monocotyledonous plants, these compounds are not naturally synthesized, hence addition of acetosyringone in the media during plant–bacterial interface supports the delivery of T–DNA (Cheng et al. 1997; Koichi et al. 2002). Using the optimal conditions (i.e., OD_{600} = 1.0, incubation period of 30 min), for transient expression of GUS, varying concentration of acetosyringone (0, 100, 200, 300, 400, 500 µM) in co–cultivation medium was assessed, after co–culturing with bacterial cells. It was observed that GUS expression efficiency was higher with increasing concentrations of acetosyringone (100 to 400 µM) and the maximum frequency of 23 ± 1.1 and 32.3 ± 1.4 for pCAMBIA 1301 and pCAMBIA 1305.1 respectively was obtained on co–cultivation medium with 400 µM acetosyringone (Fig. 3e). Addition of acetosyringone in co–cultivation medium significantly influenced the expression of GUS and was vital for successful *C. ciliaris* transformation but, did not improve the transformation efficiency with or without 200 µM in MS–infection medium (data not shown; also reported by Wu et al. 2018). This phenolic compound has been described to be a crucial constituent of transformation in *Sorghum bicolor*, *Brachypodium distachyon*, *Setaria italica* and *Setaria viridis* (Jeoung et al. 2002; Chen et al. 2019; Nguyen et al. 2020; Sood

et al. 2020). The variation in the effect of acetosyringone on plant transformation of graminaceous plants may be due to the differences in the cell density inoculum, co–cultivation period and the proficiency of target cells.

Infection under negative pressure using vacuum pump

Vacuum infiltration is one of the additional steps in *Agrobacterium*-mediated transformation to enhance transformation efficiency. In this experiment, shoot apices were immersed in MS-infection liquid medium with *Agrobacterium* cells and placed in vacuum desiccator, a negative pressure of 0.5 x 10^5 Pa was applied with vacuum pump for 30 minutes and incubated at 25 ºC and 80 rpm. This resulted in an increased GUS expression efficiency for *Cenchrus* transformation. Similar studies using vacuum infiltration for transformation have been reported in other graminaceous species such as *Festuca arundinacea* and *Panicum virgatum* (Wang and Ge 2005; Xi et al. 2009; Li and Qu 2011; Lin et al. 2017). Applying negative pressure to shoot apices immersed in *Agrobacterium* cells for more than 30 min, results in explants entirely inhabited by *Agrobacterium*, making it harder to remove in the pre-regeneration medium, which subsequently impairs shoots growth. In *Cenchrus*, the highest transformation percentage (17.3 ± 1.4) was obtained with a negative pressure of 0.5 x 10^5 Pa compared to 13.6 ± 1.3 without the pressure (Fig. 3f). Vacuum infiltration at 0.5 x 10^5Pa have been widely used by many workers (Quan et al. 2003; Yang et al. 2005; Zhang et al. 2010; Jha et al. 2011) for maximising transformation efficiency. In Seashore paspalum, Wu et al. (2018) reported that vacuum infiltration along with sonication helped increase the transformation rates.

Regenerate stable putative transformant by using Agrobacterium T–DNA delivery system

By using above optimal conditions, approx. 90 shoot apices for each plasmid were immersed in *Agrobacterium* MS- infection medium with OD_{600} = 1.0 for 30 min under vacuum with constant stirring at 80 rpm at 25ºC (Fig. 2c). Excess agrobacterial suspension was removed from surface of shoot apices by absorbing on sterile Whatman™ filter paper and after that infected shoot apices were cultured for 3 days on co–cultivation medium with 400 µM acetosyringone for 16/8 h light/dark photoperiod at 25 ˚C (Fig. 2d). If the infected shoot apices were transferred into first selection medium directly after co–cultivation stage, these shoot apices suffered from the biotic stress caused by *Agrobacterium* cells on selection medium. To avoid this, shoot apices were sub–cultured onto recovery medium after co-cultivation. In this medium, shoots meristem multiplied without selection agent but contained Cefotaxime to remove excess *Agrobacterium* growth. Shoot apices were rinsed with 250 mg/L Cefotaxime for 10 min, washed thoroughly with sterile double distilled water for 2–3 times, subsequently dry-blotted on filter paper and sub–cultured onto recovery medium for 1 week (Fig. 2e). After recovery stage, infected shoot apices were sub–cultured to first selection medium with 30 mg/L Hygromycin B for 2 weeks (Fig. 2f) and after that again sub–cultured on II selection medium for 2 week to permit the better multiplication of transformed shoot meristem (Fig. 2g, h). Transformed shoot apices alive after II selection (Fig. 2i) were cultured on pre–regeneration medium for 2 weeks to enhance the proliferation of putative transgenic shoots (Fig. 2j). Hygromycin B–resistant shoots and leaves was tested with GUS stain and indicated strong GUS expression (indicated by blue colour in the tissues). Of the transformed shoots, 14 plants showed GUS expression with pCAMBIA 1301 and 17 with pCAMBIA 1305.1 (Fig. 2s). On the other hand, leaves from non–transformed plantlets, used as control, did not show any blue colour. GUS as a reporter gene has been extensively used by several worker for confirming regenerants using *Agrobacterium* transformation (Luo et al. 2004; Li et al. 2005; Wang et al. 2011; Song et al. 2012). These transformed shoots were then multiplied on regeneration medium for 2 weeks (Fig. 2k, l). Healthy grown elongated shoots with adventitious shoots were separated (Fig. 2m) and single shoots were transferred into rooting medium (Fig. 2n, o). Surviving plantlets were hardened in pots filled with sterilized vermiculite and soilrite for 3 weeks (Fig. 2p). These transformed plants were covered with autoclaved transparent polythene to retain humidity and nutrients were provided Hoagland's solution through capillary system (Hoagland and Arnon 1950; Fig. 2q, r).

Overall data from 10 independent experiments are summarised in Table 2. The data shows a total of 706 explants were co–cultivated with *Agrobacterium* and 47 transferred to II selection medium, 14 survived from selection and 6.34 ± 0.86% multiplied on regeneration medium (Table 2). Stable transformation efficiency in these experiments for pCAMBIA 1301 averaged to 1.42 ± 0.34 and ranged between 1.3 to 2.8%. Similarly 699 shoot apices were co–cultured with *Agrobacterium* containing pCAMBIA 1305.1 and 46 plants were transferred to II selection medium and 17 survived the selection and 6.97 ± 1.2% plants regenerated on regeneration medium (Table 2). We obtained a transformation efficiency of 1.37 ± 0.54% with pCAMBIA 1305.1 and percentage ranged between 1.2 to 4.3% (Table 2). This efficiency of *Agrobacterium* mediated transformation in comparable to the efficiencies attained in other lower grass species such as *Festuca arundinacea* (8%), *Festuca pratensis* (2%), *Poa pratensis* (1.42%), *Eleusine coracana* (3.8%), *Eremochloa ophiuroides* (8%) and *Paspalum vaginatum* (9%) (Dong and Qu 2005; Gao et al. 2009; Zhang et al 2010; Ceasar and Ignacimuthu 2011; Liu et al. 2012; Wu et al. 2018). Regenerated plantlets were confirmed positive from screening by the transient expression of GUS assay (Fig. 2t) which were further used for PCR analysis.

Table 2
Summary of *Agrobacterium*– mediated transformation of 3 days old shoot apex explants of *Cenchrus ciliaris* genotype IG-3108

Binary Vector	Number of Experiment	Number of shoot apex evaluated	After co-cultivation period	Recovery medium	2 week after I selection	2 week after II selection	Percentage of regeneration	PCR analysis		Southern positive plants	Transformat frequency (%
								GUS	Hygromycin		
pCAMBIA 1301	1	70	64	62	40	6	8.5	2	2	2	2.8
	2	65	61	59	45	5	7.6	2	2	1	1.5
	3	87	83	80	64	10	11.4	3	3	2	2.2
	4	56	52	50	35	3	5.3	0	0	0	0
	5	90	82	80	45	7	7.7	2	2	2	2.2
	6	60	54	53	30	2	3.3	1	1	0	0
	7	74	69	66	42	4	5.4	1	1	1	1.3
	8	72	68	67	38	5	6.9	2	2	2	2.7
	9	63	60	60	32	1	1.6	1	1	1	1.5
	10	69	65	63	29	4	5.7	0	0	0	0
	Total	706	685	640	400	47	6.34 ± 0.86	14	14	11	1.42 ± 0.34
pCAMBIA 1305.1	1	65	63	60	31	7	10.7	1	1	0	0
	2	55	50	49	35	9	16.3	1	1	0	0
	3	83	78	76	30	4	6.0	6	6	3	3.6
	4	72	67	65	25	5	6.9	1	1	1	1.3
	5	69	65	63	27	3	4.3	3	3	3	4.3
	6	90	81	79	15	4	4.4	0	0	0	0
	7	60	56	52	20	2	3.3	2	2	2	3.3
	8	85	80	76	17	4	4.7	2	2	1	1.2
	9	50	48	45	12	3	6.0	0	0	0	0
	10	70	66	63	22	5	7.1	1	1	0	0
	Total	699	654	628	234	46	6.97 ± 1.2	17	17	10	1.37 ± 0.54

Shoot apex were co–cultured with EHA 105 harbouring pCAMBIA 1301 and 1305.1 at OD_{600} = 1.0 and an infection time of 30 minutes with vaccum and co–cultivated for 3 days on co–cultivation medium supplemented with 400µM acetosyringone

Molecular analysis of putatively transformants

The putatively transformed plants obtained after selection and regeneration were analyzed for the *nptII* (GUS) and *hptII* (Hygromycin) gene integration using PCR (Fig. 4). Genomic DNA from leaves of putatively transformed and non–transformed (control) were extracted following the Qiagen Kit method. Expected *GUS* PCR product (1029 bp for pCAMBIA1301 and 406 bp for pCAMBIA 1305.1) were amplified using DNA from 14 and 17 transformant lines while no amplified PCR product was observed in non–transformed plant (Table 2), thus verifying successful transgenics. Out of 31 GUS positive transgenic plants, 14 (pCAMBIA 1301; Fig. 4a, only 11 lines shown) and 17 (pCAMBIA 1305.1; Fig. 4c shows only representative 10 lines) transgenic plants were again tested using *hptII* gene primers and showed a fragment of size 694 bp (Fig. 4b shows only 9 lines) and 355 bp (Fig. 4d, shows only 8 lines) respectively. Contrastingly, genomic DNA of non–transformed plant used as negative control indicated no amplification with *hpt II* gene-specific primers.

Stable T–DNA integration of the transgene in PCR–positive transgenic plants was also established by Southern blot analysis. Genomic DNA was digested with *EcoR1* restriction enzyme which knows a single site within the T–DNA region of the pCAMBIA 1301and hybridized to a 1029 bp probe specific to *GUS* gene. Out of 14 plants identified through PCR, 11 plants contained single copy of *GUS* transgene in Southern Blot, thus confirming their stable transgenic status (Table 2 Fig. 4e represents 6 out of the 11 transgenic lines). On the other hand, in case of pCAMBIA 1305.1, genomic DNA was digested with enzyme *EcoRI* and 355 bp probe for *hpt II* gene coding region was used. No signals found in non–transformed plants after hybridization. However, out of 17 plants identified through PCR, 10 plants of *hptII* transgenic lines (Table 2; Fig. 4e shows representative 6 out of 10 transgenic line), indicated integration of *hptII* gene into the plant genome (Fig. 4e). This acquisition of low copy number is similar to the results obtained from other grasses transformed (Han et al. 2005; Bajaj et al. 2006; Gao et al. 2008; Lee et al. 2010; Xu et al. 2011; Lin et al. 2017).

Abbreviations

hptII Hygromycin phosphotransferase

nptII	Neomycin phosphotransferase
MS	Murashige and Skoog
TDZ	Thidiazuron
GA_3	Gibberellic acid
LB	Luria Bertani
GUSA	β–glucuronidase A

Conclusion

For the first time, we report a simple, efficient *Agrobacterium*–mediated T–DNA delivery system for highly recalcitrant *Cenchrus ciliaris* using shoot apices as the main explants. Previous reports of transformants from mature embryos were obtained using biolistic method only in this grass species. The present study evidently shows that optimisation of various factors such as concentration of growth regulators, phenolic compounds and duration of incubation strongly influence the transformation efficiency in *Cenchrus*. This optimized protocol could be used for harnessing useful apomixis related genes in *Cenchrus* and inducing apomixis in sexual plants through newer genetic engineering approaches such as CRISPR-Cas9 editing and sense-antisense transgenesis. Such studies are currently being conducted in our lab to express apomixis responsive genes for the genetic improvement of cereals crops.

Declarations

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Author contributions S conceived the ideas, conduct all of tissue culture experiments, data collection, analysis and drafted the manuscript, figures and tables. S and AD conducted DNA isolation, PCR analysis and Southern Hybridization. MA provided input for data analysis, edited the manuscript. All authors read and approved the final version of the manuscript.

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Conflict of interest All the authors declare that they have no conflict of interest.

References

1. Altpeter F, Fang YD, Xu J, Ma X (2004) Comparison of transgene expression stability after *Agrobacterium*–mediated or biolistic gene transfer into perennial ryegrass (*Lolium perenne* L). In: Hopkins A, Wang ZY, Mian R, Sledge M, Barker RE (eds) Molecular breeding of forage and turf. Kluwer Academic Publishers, Dordrecht, pp 255–260.
2. Amoah BK, Wu H, Sparks C, Jones HD (2001) Factors influencing *Agrobacterium* mediated transient expression of *uidA* in wheat inflorescence tissue. J Exp Bot 52:1135–1142. https://doi.org/10.1093/jexbot/52.358.1135
3. Ayerza R (1981) 'El Buffel grass: utilidad y manejo de una promisoria gramínea.' (Hemisferio Sur: Buenos Aires).
4. Bajaj S, Ran Y, Phillips J, Kularajathevan G, Pal S, Cohen D, Elborough K, Puthigae S (2006) A high throughput *Agrobacterium tumefaciens*–mediated transformation method for functional genomics of perennial ryegrass (*Lolium perenne* L). Plant Cell Rep 25:651–659. https://doi.org/10.1007/s00299-005-0099-9
5. Barkat A, Carels N, Bernardi G (1997) The distribution of genes in the genome of gramineae. Proc Natl Acad Sci USA 94:6857–6861.
6. Batra S, Kumar S (2002) In vitro high frequency plant regeneration in buffel grass (*Cenchrus ciliaris* L). J Plant Biol 29:191–194.
7. Behpouri A, Perochon A, Doohan FM, Ng CK-Y (2018) Optimizing Callus Induction and Proliferation for *Agrobacterium*-mediated Transformation of *Brachypodium distachyon.* Cereal Res Commun 46(2):221–231. https://doi.org/10.1556/0806.46.2018.04
8. Bettany AJ, Dalton SJ, Timms E, Manderyck B, Dhanoa MS, Morris P (2003) *Agrobacterium tumefaciens*–mediated transformation of *Festuca arundinacea* (Schreb) and *Lolium multiflorum* (Lam). Plant Cell Rep 21:437–444. https://doi.org/10.1007/s00299-002-0531-3
9. Bhat V, Dalton SJ, Kumar S, Bhat BV, Gupta MG, Morris P (2001) Particle in flow-gun–mediated genetic transformation of buffel grass (*Cenchrus ciliaris* L.): optimizing biological and physical parameters. J Appl Genet 42:405–412.
10. Bregitzer P, Zhang S, Cho M–J, Lemaux PG (2002) Reduced somaclonal variation in barley is associated with culturing highly differentiated, meristematic tissues. Crop Sci 42:1303–1308. https://doi.org/10.2135/cropsci2002.1303

11. Ceasar SA, Ignacimuthu S (2011) *Agrobacterium*-mediated transformation of finger millet (*Eleusine coracana* (L.) Gaertn.) using shoot apex explants. Plant Cell Rep 30:1759–1770. https://doi.org/10.1007/s00299-011-1084-0
12. Chai ML, Senthil KK, Kim DH (2004) Transgenic plants of colonial bentgrass from embryogenic callus via *Agrobacterium*–mediated transformation. Plant Cell Tissue Organ Cult 77:165–171. https://doi.org/10.1023/B:TICU.0000016823.94547.c7
13. Chen F, Liu Q, Vogel JP, Wu J (2019) *Agrobacterium*-mediated transformation of *Brachypodium distachyon*. Curr Protoc Plant Biol e20088. https://doi.org/10.1002/cppb.20088
14. Chen L, Cong Y, He H, Yu Y (2014) Maize (*Zea mays* L.) transformation by *Agrobacterium tumefaciens* infection of pollinated ovules. J Biotechnol 171:8–16. https://doi.org/10.1016/j.jbiotec.2013.11.019
15. Cheng M, Fry JE, Pang S, Zhou H, Hironaka CM, Duncan DR, Conner TW, Wan Y (1997) Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. Plant Physiol 115:971–980. https://doi.org/10.1104/pp.115.3.971
16. Chengalrayan K, Gallo–Meagher M (2001) Effect of various growth regulators on shoot regeneration of sugarcane. In Vitro Cell Dev Biol Plant 37:434–439 https://doi.org/10.1007/s11627-001-0076-0 .
17. Cherney JH, Cherney DJR, Akin DE, Axtell JD (1991) Potential of brown-midrib, low-lignin mutants for improving forage quality. Advances Agron 46:157–198. https://doi.org/10.1016/S0065-2113(08)60580-5
18. Cho MJ, Choi HW, Okamoto D, Zhang S, Lemaux PG (2003) Expression of green fluorescent protein and its inheritance in transgenic oat plants generated from shoot meristematic cultures. Plant Cell Rep 21:467–474. https://doi.org/10.1007/s00299-002-0542-0
19. Conner JA, Goel S, Gunawan G, Cordonnier-Pratt MM, Johnson VE, Liang C, Wang H, Pratt LH, Mullet JE, Debarry J, Yang L, Bennetzen JL, Klein PE, Ozias-Akins P (2008) Sequence Analysis of Bacterial Artificial Chromosome Clones from the Apospory-Specific Genomic Region of *Pennisetum* and *Cenchrus*. Plant Physiol 147(3):1396–1411. https://doi.org/10.1104/pp.108.119081
20. Dalton SJ (2020) A reformulation of Murashige and Skoog medium (WPBS medium) improves embryogenesis, morphogenesis and transformation efficiency in temperate and tropical grasses and cereals. Plant Cell Tissue Organ Cult 141:257–273. https://doi.org/10.1007/s11240-020-01784-8
21. Danilova S, Dolgikh YI (2005) Optimization of agrobacterial (*Agrobacterium tumefaciens*) transformation of maize embryogenic callus. Russ J Plant Physiol 52(4):535–541. https://doi.org/10.1007/s11183-005-0079-5
22. Dong S, Qu R (2005) High efficiency transformation of tall fescue with *Agrobacterium tumefaciens*. Plant Sci 168:1453–1458. https://doi.org/10.1016/j.plantsci.2005.01.008
23. Gao C, Liu J, Nielsen KK (2009) *Agrobacterium*-mediated transformation of meadow fescue (*Festuca pratensis* Huds.). Plant Cell Rep 28(9):1431–1437. https://doi.org/10.1007/s00299-009-0743-x
24. Gao C, Long D, Lenk I, Nielsen KK (2008) Comparative analysis of transgenic tall fescue (*Festuca arundinacea* Schreb) plants obtained by *Agrobacterium*-mediated transformation and particle bombardment. Plant Cell Rep 27(10):1601–1609. https://doi.org/10.1007/s00299-008-0578-x
25. Goel S, Chen Z, Conner JA, Akiyama Y, Hanna WW, Ozias-Akins P (2003) Delineation by fluorescence in situ hybridization of a single hemizygous chromosomal region associated with aposporous embryo sac formation in *Pennisetum squamulatum* and *Cenchrus ciliaris*. Genetics 163(3):1069–1082. https://doi.org/10.1093/genetics/163.3.1069
26. Goel S, Chen Z, Akiyama Y, Conner JA, Basu M, Gualtieri G, Hanna WW, Ozias-Akins P (2006) Comparative physical mapping of the apospory-specific genomic region in two apomictic grasses: *Pennisetum squamulatum* and *Cenchrus ciliaris*. Genetics, 173(1), 389–400. https://doi.org/10.1534/genetics.105.054429
27. Goldman JJ, Hanna WW, Fleming G, Ozias–Akins P (2003) Fertile transgenic pearl millet [*Pennisetum glaucum* (L.) R. Br.] plants recovered through microprojectile bombardment and phosphinothricin selection of apical meristem–, inflorescence–, and immature embryo–derived embryogenic tissues. Plant Cell Rep 21:999–1009. https://doi.org/10.1007/s00299-003-0615-8
28. Gould H, Magallanes–Cedeno M (1998) Adaptation of cotton shoot apex culture to *Agrobacterium*–mediated transformation. Plant Mol Biol Rep 16:1–10.
29. Griffa SDD, Ribotta A, Castelli SL, Munoz N, Colomba EL, Luna C, Grunberg K, Biderbost E (2006) Molecular genetic discrimination of Buffel grass genotypes and F1 hybrids for breeding purposes using amplified fragment length polymorphism analyses. Grass Forage Sci 61:454–458. https://doi.org/10.1111/j.1365-2494.2006.00546.x
30. Han N, Chen D, Bian HW, Deng MJ, Zhu MY (2005) Production of transgenic creeping bentgrass *Agrostis stolonifera* var. palustris plants by *Agrobacterium* tumefaciens–mediated transformation using hygromycin selection. Plant Cell Tissue Organ Cult 81:131–138. https://doi.org/10.1007/s11240-004-4042-5
31. Hirochika H (1993) Activation of tobacco retrotransposons during tissue culture. EMBO J 12:2521–2528.
32. Hoagland M, Arnon DI (1950) The water culture method to grow plants without soil. Calif Agric Expt Station Cir 347. Berkeley California.
33. Hojsgaard D (2020) Apomixis technology: Separating the wheat from the chaff. Genes 11(4): 411. https://doi.org/10.3390/genes11040411
34. Howe A, Sato S, Dweikat I, Fromm M, Clemente T (2006) Rapid and reproducible *Agrobacterium*–mediated transformation of *Sorghum*. Plant Cell Rep 25:784–791. https://doi.org/10.1007/s00299-005-0081-6
35. Hu F, Zhang L, Wang X, Ding J, Wu D (2005) *Agrobacterium*–mediated transformed transgenic triploid bermudagrass (*Cynodon dactylon* x *C. transvaalensis*) plants are highly resistant to the glufosinate herbicide Liberty. Plant Cell Tissue Organ Cult 83:13–19. https://doi.org/10.1007/s11240-005-3705-1
36. Hwang O-J, Cho M-A, Han Y-J, Kim Y-M, Lim S-H, Kim D-S, Hwang I, Kim J-I (2014) *Agrobacterium*-mediated genetic transformation of *Miscanthus sinensis*. Plant Cell Tissue Organ Cult 117(1):51–63. https://doi.org/10.1007/s11240-013-0419-7

37. Ishida Y, Hiei Y, Komari T (2007) Agrobacterium–mediated transformation of maize. Nat Protoc 2 (7):1614–1621. https://doi.org/10.1038/nprot.2007.241
38. Jefferson RA (1987) Assaying chimeric plant genes: the GUS fusion system. Plant Mol Biol Rep 4:387–405.
39. Jeoung JM, Krishnaveni S, Muthukrishnan S, Trick HN, Liang GH (2002) Optimization of *Sorghum* transformation parameters using genes for green fluorescent protein and β-glucuronidase as visual markers. Hereditas 137:20–8. https://doi.org/10.1034/j.1601-5223.2002.1370104.x
40. Jha P, Shashi, Rustagi A, Agnihotri P, Kulkarni V, Bhat V (2011) Efficient *Agrobacterium*–mediated transformation of *Pennisetum glaucum* (L.) R. Br. using shoot apices as explant source. Plant Cell Tissue Organ Cult 107:501–512. https://doi.org/10.1007/s11240-011-0001-0
41. Jones HD, Doherty A, Wu H (2005) Review of methodologies and a protocol for the *Agrobacterium*–mediated transformation of wheat. Plant Methods 1:5. https://doi.org/10.1186/1746-4811-1-5
42. Kline KI , Msangi S, Dale VH, Woods J, Souza GM, Osseweijer P, Clancy JS, Hilbert JA, Johnson FX, Mcdonnell PC, Mugera H (2017) Reconciling food security and bioenergy: priorities for action. GCB Bioenerg 9:557–576.
43. Kohli A, Gahakwa D, Vain P, Laurie DA, Christou P (1999) Transgene expression in rice engineered through particle bombardment: molecular factors controlling stable expression and transgene silencing. Planta 208:88–97. https://doi.org/10.1007/s004250050538
44. Koichi T, Bae CH, Seo MS, Song IJ, Lim YP, Song PS, Lee HY (2002) Overcoming of barriers to transformation in monocot plants. J Plant Biotech 4:135–141.
45. Kumar J, Shukla SM, Bhat V, Gupta S, Gupta MG (2005) *In vitro* plant regeneration and genetic transformation of *Dichantium annulatum*. DNA Cell Biol 24:670–679. https://doi.org/10.1089/dna.2005.24.670
46. Laishram SD, Goyal S, Shashi, Kulkarni VM, Kumar S, Bhat V (2020) Assessment of biolistic and *Agrobacterium*-mediated genetic transformation methods in *Cenchrus ciliaris*. Nucleus 63:303–312. https://doi.org/10.1007/s13237-020-00332-1
47. Lee KW, Choi GJ, Kim KY, Yoon SH, Ji HC, Park HS, Lim YC, Lee SH (2010) Genotypic variation of *Agrobacterium*-mediated transformation of Italian ryegrass. Electron J Biotechn 13:3.
48. Lee SH, Lee DG, Woo HS, Lee KW, Kim DH, Kwak SS, Kim JS, Kim H, Ahsan N, Choi MS, Yang JK, Lee BH (2006) Production of transgenic orchardgrass via *Agrobacterium*–mediated transformation of seed–derived callus tissues. Plant Sci 171:408–414. https://doi.org/10.1016/j.plantsci.2006.05.006
49. Li F, Li M, Zhan C, Wang S (2015) A reliable and high-efficiency *Agrobacterium tumefaciens*-mediated transformation system of *Pogonatherum paniceum* embryogenic callus using GFP as a reporter gene. Plant Cell Tissue Organ Cult 120(1):155–165. https://doi.org/10.1007/s11240-014-0589-y
50. Li L, Li R, Fei S, Qu R (2005) *Agrobacterium*-mediated transformation of common bermudagrass (*Cynodon dactylon*). Plant Cell Tissue Organ Cult 83(2):223-229. https://doi.org/10.1007/s11240-005-6838-3
51. Li M, Li H, Hu X, Pan X, Wu G (2011) Genetic transformation and overexpression of a rice Hd3a induces early flowering in *Saussurea involucrata* Kar. et Kir. Ex Maxim. Plant Cell Tissue Organ Cult 106(3):363–371. https://doi.org/10.1007/s11240-011-9927-5
52. Li R, Qu R (2011) High throughput *Agrobacterium*-mediated switchgrass transformation. Biomass Bioenerg 35:1046–54. https://doi.org/10.1016/j.biombioe.2010.11.025
53. Lin CY, Donohoe BS, Ahuja N, Garrity DM, Qu R, Tucker MP, Himme ME, Wei H (2017) Evaluation of parameters affecting switchgrass tissue culture: toward a consolidated procedure for *Agrobacterium*-mediated transformation of switchgrass (*Panicum virgatum*). Plant Methods 13:113. https://doi.org/10.1186/s13007-017-0263-6
54. Liu M, Lu S, Liu L, Tan J, Guo Z (2012) *Agrobacterium*–mediated transformation of centipedegrass (*Eremochloa ophiuroides* [Munro] Hack.). Plant Cell Tissue Organ Cult 109(3):557–563. https://doi.org/10.1007/s11240-012-0122-0
55. Luo H, Hu Q, Nelson K, Longo C, Kausch AP, Chandlee JM, Wipff JK, Fricker CR (2004) *Agrobacterium tumefaciens*–mediated creeping bentgrass (*Agrostis stolonifera* L.) transformation using phosphinothricin selection results in a high frequency of single copy transgene integration. Plant Cell Rep 22:645–652. https://doi.org/10.1007/s00299-003-0734-2
56. Ma X, Yu C, Tang S, Guo S, Zhang R, Wang Y, Zhu A, Zhu S, Xiong H (2010) Genetic transformation of the bast fiber plant ramie (*Boehmeria nivea* Gaud.) via *Agrobacterium tumefaciens*. Plant Cell Tissue Organ Cult 100:165–174. https://doi.org/10.1007/s11240-009-9633-8
57. Matthysse AG (2006) The genus *Agrobacterium*. Prokaryotes 5:91–114.
58. McCabe D, Swain W, Martinell B, Christou P (1988) Stable transformation of soybean (*Glycine max*) by particle acceleration. Biotechn 6:923–925.
59. McHughen A, Jordan M, Feist G (1989) A pre–culture period prior to *Agrobacterium* inoculation increases production of transgenic plants. J Plant Physiol 135:245–248.
60. Ncanana S, Brandt W, Lindsey G, Farrant J (2005) Development of plant regeneration and transformation protocols for the desiccation–sensitive weeping lovegrass *Eragrostis curvula*. Plant Cell Rep 24:335–340. https://doi.org/10.1007/s00299-005-0940-1
61. Nguyen DQ, Eck JV, Eamens AL, Grof CPL (2020) Robust and reproducible *Agrobacterium*-mediated transformation System of the C_4 genetic model species *Setaria viridis*. Front Plant Sci 11:1–12. https://doi.org/10.3389/fpls.2020.00281
62. Padmanabhan P, Sahi SV (2009) Genetic transformation and regeneration of *Sesbania drummondii* using cotyledonary nodes. Plant Cell Rep 28 (1):31–40. https://doi.org/10.1007/s00299-008-0618-6
63. Quan RD, Shang M, Zhang JR (2003) Optimization of transformation conditions in maize inbred line calluses via *Agrobacterium tumefaciens*. J Plant Physiol Mol Biol 29(3):245–250.
64. Rodriguez O, Gonzalez–Dominguez J, Creusz JP, Odvody GN, Wilson JP, Hanna WW, Levy M (1999) First report and epidemics of buffel grass blight caused by *Pyricularia grisea* in South Texas. Plant Disease 83:398. https://doi.org/10.1094/PDIS.1999.83.4.398D

65. Ross AH, Manners JM, Birch RG (1995) Embryogenic callus production, plant regeneration and transient gene expression following particle bombardment in the pasture grass, *C. ciliaris* (Graminae). Aust J Bot 43:193–199. https://doi.org/10.1071/BT9950193
66. Sairam RV, Parani M, Franklin G, Lifeng Z, Smith B, MacDougall J, Wilber C, Sheikh H, Kashikar N, Meeker K, Al–Abed D, Berry K, Vierling R, Goldman SL (2003) Shoot meristem: an ideal explant for *Zea mays* L. transformation. Genome 46:323–329. https://doi.org/10.1139/g02-120
67. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold spring harbor laboratory press.
68. Sanderson MA, Voigt P, Jones RM (1999) Yield and quality of warm season grasses in central Texas. J Range Manag 52:45–50.
69. Sangwan RS, Bourgeois Y, Brown S, Vasseur G, Sangwan–Noreel BS (1992) Characterization of competent cells and early events of *Agrobacterium*–mediated genetic transformation in *Arabidopsis thaliana*. Planta 188:439–456. https://doi.org/10.1007/BF00192812
70. Sarker RH, Biswas A (2002) In vitro plantlet regeneration and *Agrobacterium*– mediated genetic transformation of wheat (*Triticum aestivum* L.). Plant Cell Tissue Organ Cult 12(2):155–165.
71. Scheben A, Hojsgaard D (2020) Can we use gene-editing to induce apomixis in sexual plants? Genes 11(7):781. https://doi.org/10.3390/genes11070781
72. Shashi, Bhat V (2023) Development of rapid and genotype-independent *in vitro* adventitious shoot differentiation in C_4 bioenergy crop *Cenchrus ciliaris* L. Vegetos, https://doi.org/10.1007/s42535-023-00770-7
73. She JM, Liang LF, Zhang BL, He XL, Chen ZY, Ni WC (2005) Acquirement of Bt transgenic plants by *Agrobacterium tumefaciens* in Kentucky bluegrass (*Poa pratensis* L.). Jiangsu J Agr Sci 21:102– 105.
74. Shou H, Frame BR, Whitham SA, Wang K (2004) Assessment of transgenic maize events produced by particle bombardment or *Agrobacterium*– mediated transformation. Mol Breed 13:201–208. https://doi.org/10.1023/B:MOLB.0000018767.64586.53
75. Shrawat AK Becker D, Lo¨rz H (2007) *Agrobacterium tumefaciens*–mediated genetic transformation of barley (*Hordeum vulgare* L.). Plant Sci 172:281–290. https://doi.org/10.1016/j.plantsci.2006.09.005
76. Singh RK, Prasad M (2016) Advances in *Agrobacterium tumefaciens*-mediated genetic transformation of graminaceous crops. Proroplasma 253:691–707. https://doi.org/10.1007/s00709-015-0905-3
77. Somleva MN, Tomaszewski Z, Conger BV (2002) *Agrobacterium*–mediated genetic transformation of switchgrass. Crop Sci 42:2080–2087. https://doi.org/10.2135/cropsci2002.2080
78. Song GQ, Aaron W, Hancock JF (2012) Factors influencing *Agrobacterium*-mediated transformation of switchgrass cultivars. Plant Cell Tissue Organ Cult 108:445–453. https://doi.org/10.1007/s11240-011-0056-y
79. Sood P, Singh RK, Prasad M (2020) An efficient *Agrobacterium*-mediated genetic transformation method for foxtail millet (*Setaria italica* L.). Plant Cell Rep 39:511–525. https://doi.org/10.1007/s00299-019-02507-w
80. Stachel SE, Messens E, van Montagu M, Zambryski P (1985) Identification of the signal molecules produced by wounded plant cells that activate T–DNA transfer in *Agrobacterium tumefaciens*. Nature 318:624–629. https://doi.org/10.1038/318624a0
81. Sticklen MB, Oraby HF (2005) Shoot apical meristem: a sustainable explant for genetic transformation of cereal crops. In Vitro Cell Dev Biol Plant 41:187–200. https://doi.org/10.1079/IVP2004616
82. Sumlu S, Atar HH, Khawar KM (2010) Breaking seed dormancy of water lily (*Nymphaea alba* L.) under *in vitro* conditions. Biotechn Biotechnol Equip 24(1):1582–1586. https://doi.org/10.2478/V10133-010-0009-3
83. Toyama K, Bae CH, Kang JG, Lim YP, Adachi T, Riu KZ, Song PS, Lee HY (2003) Production of herbicide–tolerant zoysiagrass by *Agrobacterium*–mediated transformation. Mol Cells 16:19–27. https://doi.org/10.1016/s1016-8478(23)13760-5
84. Trifonova A, Madsen S, Olesen A (2001) *Agrobacterium*–mediated transgene delivery and integration into barley under a range of *in vitro* culture conditions. Plant Sci 161:871–880. https://doi.org/10.1016/S0168-9452(01)00479-4
85. Vielle Calzada JP, Crane CF, Stelly DM (1996) Apomixis: the asexual revolution. Science 274:1322–1323. https://doi.org/10.1126/science.274.5291.1322
86. Wang M-Z, Pan Y-L, Li C, Liu C, Zhao Q, Ao G-M, Yu J-J (2011) Culturing of immature inflorescences and *Agrobacterium*-mediated transformation of foxtail millet (*Setaria italica*). Afr J Biotechnol 10(73):16466–16479. https://doi.org/10.5897/AJB10.2330
87. Wang ZY, Ge Y (2005) *Agrobacterium*–mediated high efficiency transformation of tall fescue (*Festuca arundinacea*). J Plant Physiol 162:103–113. https://doi.org/10.1016/j.jplph.2004.07.009
88. Wu H, Sparks C, Amoah B, Jones HD (2003) Factors influencing successful *Agrobacterium*–mediated genetic transformation of wheat. Plant Cell Rep 21:659–668. https://doi.org/10.1007/s00299-002-0564-7
89. Wu X, Shi H, Chen X, Liu Y, Guo Z (2018) Establishment of *Agrobacterium*-mediated transformation of seashore paspalum (*Paspalum vaginatum* O. Swartz). In Vitro Cell Dev Biol Plant 54:545–552. https://doi.org/10.1007/s11627-018-9930-1
90. Xi Y, Fu C, Ge Y, Nandakumar R, Hisano H, Bouton J, Wang Z-Y (2009) *Agrobacterium*- mediated transformation of switchgrass and inheritance of the transgenes. Bioenerg Res 2:275–83. https://doi.org/10.1007/s12155-009-9049-7
91. Xu B, Huang L, Shen Z, Welbaum G, Zhang X, Zhao, B (2011) Selection and characherization of a new switchgrass (*Panicum virgatum* L.) line with high somatic embryogenic capacity of genetic transformation. Sci Horticult 129:854–861. https://doi.org/10.1016/j.scienta.2011.05.016
92. Yang AF, Duan XG, Gu XF, Gao F, Zhang JR (2005) Efficient transformation of beet (*Beta vulgaris*) and production of plants with improved salt–tolerance. Plant Cell Tissue Organ Cult 83:259–270. https://doi.org/10.1007/s11240-005-6670-9
93. Yookongkaew N, Srivatanakul M, Narangajavana J (2007) Development of genotype–independent regeneration system for transformation of rice (*Oryza sativa* ssp. indica). J Plant Res 120:237–245. https://doi.org/10.1007/s10265-006-0046-z

94. Yu TT, Skinner DZ, Liang GH, Trick HN, Huang B, Muthukrishnan S (2000) *Agrobacterium*–mediated transformation of creeping bentgrass using GFP as a reporter gene. Hereditas 133:229–33. https://doi.org/10.1111/j.1601-5223.2000.00229.x
95. Zapata C, Srivatanakul M, Park S, Lee B, Maria S, Smith R (1999) Improvements in shoot apex regeneration of two fiber crops: cotton and kenaf. Plant Cell Tissue Organ Cult 56:185–191. https://doi.org/10.1023/A:1006238924439
96. Zhang K., Wang J, Hu X, Yang A, Zhang J (2010) *Agrobacterium*–mediated transformation of shoot apices of Kentucky bluegrass (*Poa pratensis* L.) and production of transgenic plants carrying a *betA* gene. Plant Cell Tissue Organ Cult 102(2):135–143. https://doi.org/10.1007/s11240-010-9713-9
97. Zhao W, Zheng S, Ling H–Q (2011) An efficient regeneration system and *Agrobacterium*–mediated transformation of Chinese upland rice cultivar Handao297. Plant Cell Tissue Organ Cult 106(3):475–483. https://doi.org/10.1007/s11240-011-9946-2
98. Zhong H, Sun B, Warkentin D, Zhang S, Wu R, Wu T, Sticklen MB (1996) The competence of maize shoot meristems for integrative transformation and inherited expression of transgenes. Plant Physiol 110:1097–1107. https://doi.org/10.1104/pp.110.4.1097

Figures

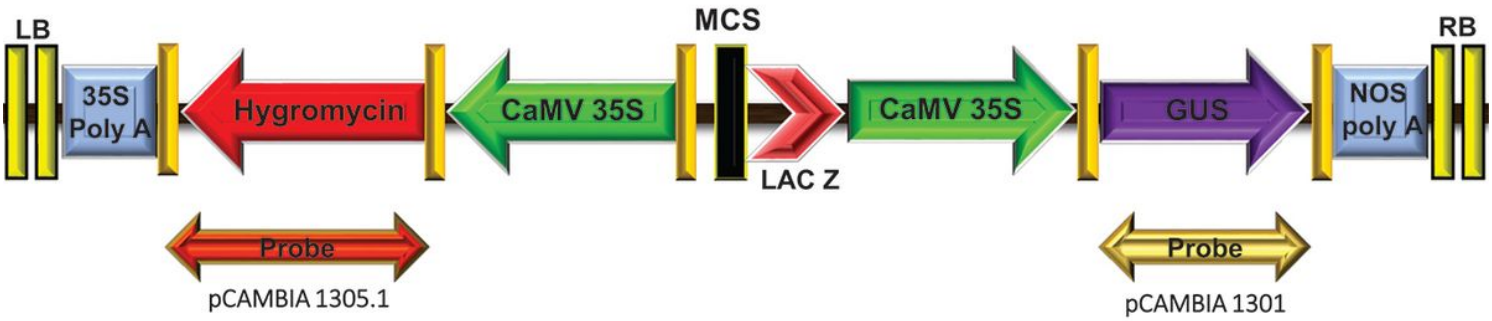

Figure 1

Schematic diagram of T–DNA region of pCAMBIA 1301 and 1305.1. The region of probe selected for Southern Hybridization for pCAMBIA 1301 (GUS) and pCAMBIA1305.1 (hygromycin) has also been indicated

Figure 2

Agrobacterium–mediated transformation and plant regeneration in *Cenchrus ciliaris.* **a** seeds grown on MS medium with 1 mg/L TDZ, **b** three days old shoot apices grown on pre-culture medium, **c** co-culturing *Agrobacterium* with shoot apices on MS-infection medium, **d** co-cultivation of shoot apices for 3 days; **e** seven days old plants on recovery medium, **f** shoots growing on hygromycin B supplemented I selection medium, **g, h** transformed plants after II selection medium, **i** regenerated transgenic plant; **j** transformed plants growing on regeneration medium, **k, l** shoot multiplication, **m** shoot elongation, **n, o** transformed plant on rooting medium, **p-r** hardening of transformed plants in green house, **s** No GUS expression of untransformed control shoot apices and transient GUS expression (visible as blue spots) in transformed shoots apices after co-cultivation, **t** GUS expression showed in transgenic leaves of Cenchrus

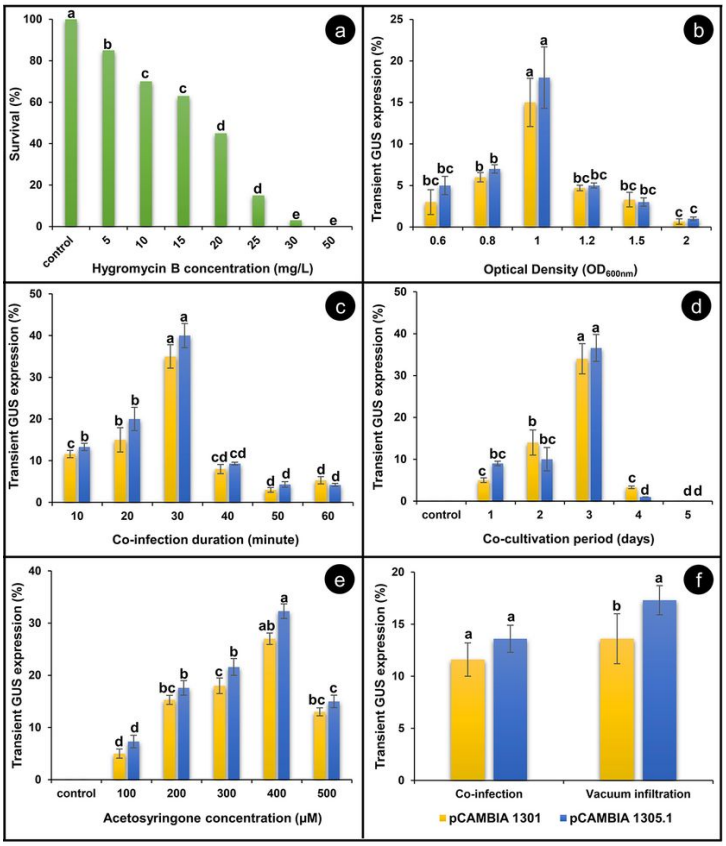

Fig. 3 Factors affecting on *Agrobacterium*-mediated transformation of *Cenchrus ciliaris*. **a** Influence of hygromycin B sensitivity test, **b** *Agrobacterium* cells density, **c** duration of co-culture, **d** co-cultivation period, **e** acetosyringone concentration, **f** vacuum infiltration on the transient GUS expression efficiency in shoot apices explant of *Cenchrus* genotype IG-3108 infected with *Agrobacterium tumefaciens* EHA-105 harboring pCAMBIA 1301 and pCAMBIA 1305.1 plasmid at OD_{600} 1.0 and an infection time of 30 min and co-cultivated for 3 days on co-cultivation medium with 400 μM acetosyringone

a-e Statistical analysis was carried out using one-way ANOVA. Mean within same columns followed by the same letter were not significantly different, as indicated by LSD ($P<0.05$) using Duncan's multiple range tests.

For each concentration studied, experiments were repeated three times with 60 explants.

Transient GUS expression frequency (%) = (Number of explants showing GUS-positive spots/ Total number of explants inoculated) X 100

Figure 3

See image above for figure legend

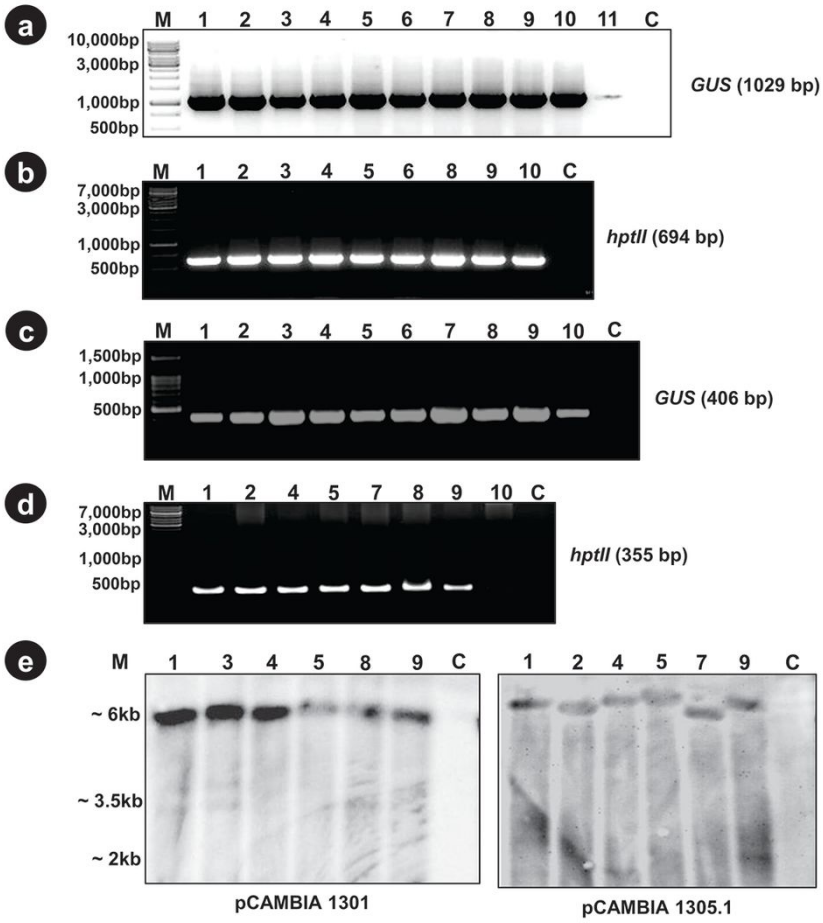

Figure 4

The prime screening for the presence of transgene in putative transgenic lines using genomic DNA PCR. **a**Amplification of detection of 1029 bp of the *GUS* gene in pCAMBIA 1301, **b** Amplification of 694 bp of the *hptII* gene in pCAMBIA 1301, **c**Amplification of 406 bp *GUS* gene in pCAMBIA 1305.1, **d** Amplification of 355 bp of *hptII* gene in pCAMBIA 1305.1 transformed transgenic Cenchrus plants. Confirmation to stable integration of transgene using southern blot analysis, **e** hybridized with GUS probe (1029 bp) in pCAMBIA 1301 and hptII probe (355 bp) in pCAMBIA 1305.1 transformed transgenic buffel grass. Genomic DNA was digested with *EcoR1*. c negative control