



Embryogenic callus cultures and genetic transformation of Rhodes grass (*Chloris gayana*) cv. 'Tolgar'

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Abstract

There is emerging interest in tropical C4 forage grasses due to their potential value in changing pasture conditions caused by climate change. In this study, a robust regeneration and transformation system was developed for Rhodes grass (*Chloris gayana*). From elite commercial cultivar 'Tolgar' initial screening for embryogenic callus (EC) responses were generated from cut mesocotyl explants, with 20 µM of 2,4-D being most effective at generating EC from cut mesocotyl explants, with an EC induction rate of 3.89% for cv. 'Tolgar'. From these EC cultures, four isogenic lines were recovered via shoot regeneration and maintained by micropropagation. EC-derived lines were then validated for production of isogenic lines for use in plant transformation, using macerated basal meristem explants from tillers of micropropagated plantlets, with one line (T108) having reliable and efficient regeneration. The most efficient transformation efficiency (2.16%) was obtained using hygromycin selection with isogenic line T108. As determined by ddPCR among all recovered transgenic plants, 12% were single copy and 53% had low transgene copy number (2–4), with 47% of transformed plants expressing the reporter gene (DsRED2). The work here provides an updated transformation protocol for Rhodes grass which will enable future improvement of the species by molecular informed breeding or genome editing.

Key message

A tissue culture and transformation system for Rhodes grass was developed by evaluating factors for somatic embryogenesis induction and two selectable markers.

Keywords *Chloris gayana* · Orphan crop · Particle bombardment · Genetic transformation · Plant regeneration

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
ANOVA	Analysis of variance
BAP	6-benzylaminopurine
<i>bar</i>	Bialaphos resistance gene
CIM	Callus induction media
ddPCR	Digital droplet polymerase chain reaction
DMD	Dry matter digestibility

EC	Embryogenic callus
GUS	Beta-glucuronidase gene
<i>hyg</i>	Hygromycin resistance gene
MS	Murashige and Skoog
OSM	Osmotic shock media
PDS	Phytoene desaturase gene
PPT	Phosphinothricin
RM	Rooting media
SIM	Shoot induction media

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Introduction

Rhodes grass (*Chloris gayana*, NCBI: txid110876) is widely grown as a pasture grass in tropical regions including Kenya, Argentina and parts of Australia (Loch et al. 2004; Ponsens et al. 2010; Ruolo et al. 2022; Maweu et al. 2024). It is a tropical species originating from Africa, with the development

of commercial cultivars starting in the early 1900s (Loch et al. 2004; Loch 2007). Rhodes grass is an important species to consider as a potential future forage because of its beneficial characteristics, including tolerance to salinity and drought, as well as a deep-rooting habit which has enabled its use in soil restoration (Skerman and Riveros 1990; Daba et al. 2019; Singh et al. 2022). There are both diploid and tetraploid Rhodes grass cultivars, with diploids enabling a less complex approach for crop improvement via breeding and biotechnology such as genome editing (Anderson 1974; Loch et al. 2004). The introduction of desirable traits can be hampered in many C4 grasses due to apomixis, which can be a barrier to crop improvement due to the lack of sexual recombination (Bellido et al. 2021). However, unlike other C4 forage grass species, including *Paspalum notatum* and *Brachiaria brizantha*, Rhodes grass is solely outcrossing (Alves et al. 2001; Loch et al. 2004; Ortiz et al. 2013).

Of the 38% of global land surface currently used for agriculture, two thirds is used for grazing or growing feed for livestock industries (FAO 2020). In order to adapt and mitigate risk of productivity loss caused by climate change in temperate regions, a shift in grazing practices and use of C4 forage species may be required (Cheng et al. 2022). The impacts of climate change are expected to shorten and challenge the growth and productivity of temperate species as well as growing seasons in subtropical pastoral regions (Cullen et al. 2009). This anticipated change in pasture species distribution will impact forage quality which may also increase methane emissions from ruminant animals farmed on this land (Howden et al. 2008; Cullen et al. 2009). C4 plants have potential for a larger role in grazing systems as they are naturally adapted to warmer climates and are generally more tolerant to drought, conditions which both can be exacerbated by climate change (Ehleringer and Cerling 2002; Valeria and Santiago 2011). C4 plants have evolved to partition photosynthetic processes between two cell types; mesophyll and bundle sheath cells (Chen and Blankenship 2021). This partitioning generally results in higher cell wall content and lower dry matter digestibility (DMD) compared to temperate C3 forages, contributing to lower nutritive quality and animal productivity (Wilson and Hacker 1987; Wilson and Hattersley 1989).

In order to more effectively act as a substitute or supplement for cool-season forages, herbage quality traits must be improved in C4 grasses. Higher quality pasture grass leads to an increase in milk production by grazing dairy cows (Brink et al. 2008), and in beef cattle a 1% increase in in vitro DMD can lead to a 3.2% increase in live-weight daily gains (Casler and Vogel 1999). Additionally, the improvement and intensification of pasture can greatly improve land use efficiency (Cardoso et al. 2016). Forage digestibility also effects enteric methane emissions, whereby ruminant

animals fed C4 grasses have been shown to produce 10–17% more methane than those fed C3 grasses (Ulyatt et al. 2002; Boadi et al. 2004; Archimède et al. 2011). Indeed, the typical DMD of Rhodes grass (54.1–55.5%) is substantially lower in comparison to perennial ryegrass (74.7–86.2%) (Milford and Minson 1968; Minson 1972; Frandsen 1986).

The application of biotechnology and plant breeding can improve grass biomass yield, quality, digestibility, and disease resistance (Jung et al. 2012; Kingston-Smith et al. 2013; Jiang 2015; Kaur et al. 2017; Subedi et al. 2022). Biotechnology applications in C4 species have previously been demonstrated, with genome editing being applied in *Eragrostis tef* to reduce lodging (Beyene et al. 2022), to a chlorophyll biosynthesis gene in *Paspalum notatum* (May et al. 2023), and via a CRISPR-mediated knockout of a gene in *Setaria italica* to prevent seed shattering (Yu et al. 2023). However, genome editing to improve forage quality traits in C4 grasses is lacking. At the time of writing, two papers focussing on C4 forage grass herbage quality improvement via gene regulation or editing have been published (Gior-dano et al. 2014; Park et al. 2017).

For Rhodes grass, traits to improve include digestibility and increased tolerance to abiotic stressors such as extreme heat and cold (Sustek-Sánchez et al. 2023). Additionally, genome editing could focus on engineering pathways to produce condensed tannins in vegetative herbage, which could help with animal bloat and reduce ruminal methane production (Eugène et al. 2021; Katoch 2022). Although biotechnology resources are limited for Rhodes grass, the need to future proof temperate grazing regions has resulted in emerging interest in developing these resources, as supported by a draft genome and genetic markers to facilitate future breeding (Maybery-Reupert et al. 2023).

To facilitate forage improvement via biotechnology, a robust transformation system should be established with commercially available cultivars. The development of tissue culture systems with highly efficient regeneration is fundamental for all transformation systems that use in vitro regeneration (Smith et al. 2002; Schrauf et al. 2022). For Rhodes grass, there are only two established regeneration systems reported; wherein secondary somatic embryogenesis was induced via callus using shoot apical explants (Nishihira et al. 1989; Gondo et al. 2007), as well as one particle bombardment transformation system that used multi shoot clump explants derived from an adventitious shoot regeneration pathway (Gondo et al. 2009). The present study aimed to establish a robust regeneration and transformation system for commercial cultivars of Rhodes grass. Here, we evaluated the use of different explants to induce embryogenic callus (EC) and shoot regeneration, as well as different selectable markers for optimal transformation

efficiency. The study will provide invaluable resources for the genetic improvement of Rhodes grass for livestock industries worldwide.

Materials and methods

Screening and recovery of regenerable genotypes

Seeds of a diploid commercially sourced Rhodes grass cultivar (*Chloris gayana* Kunth, cv. ‘Tolgar’) were surface sterilised using a 1 min wash in 80% ethanol and rinsed with sterile distilled water. The seeds were then agitated at 300 RPM in 2% v/v sodium hypochlorite in commercial bleach solution (Domestos, Unilever) for 30 min and rinsed in sterile distilled water under aseptic conditions. After two days on germination/Murashige and Skoog basal media (hereon described as “MS media”, containing MS, 30 g/L sucrose, and 3 g/L phytagel; pH 5.8), seeds were visually screened and non-contaminated seeds were transferred to fresh MS media.

Cut mesocotyls explants from in vitro germinated seedlings were evaluated for EC induction, initiated on callus induction media (CIM; MS, 30 g/L sucrose, 3 g/L phytagel, 20 μM 2,4-D; Table 1). To generate seedlings for the cut mesocotyl explants, sterilised seeds were germinated on MS media in dark growth conditions at 24 °C for seven days, after which the etiolated seedlings were transferred to a growth chamber for 2–3 days under light conditions (16 h light photoperiod and photon flux density of 80 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at 24 °C) until the mesocotyl regions were visible. The mesocotyls of these seedlings were cut longitudinally and placed cut side down (Fig. 1c) on CIM. Following transfer for callus induction, explants were placed in dark growth conditions with a constant temperature of 24 °C.

After 5–8wks on CIM, EC were then transferred to shoot induction media (SIM; MS, 30 g/L maltose, 3 g/L phytagel, 3.6 μM BAP) and cultured under light conditions. EC was identified by compact, nodular structures as depicted in Fig. 1d. Following shoot regeneration, individual isogenic lines were micropropagated on MS media and multiplied in order to provide uniform clonal tillers as a source of basal meristems for induction of isogenic EC explants for transformation.

To validate regeneration and use of these lines for transformation, 12–18 day old in vitro grown tillers from lines of recovered regenerable genotypes from cv. ‘Tolgar’,

including line Tolgar 108 (T108), were used as an explant source to induce EC from macerated basal meristems. These basal meristems were excised from mature tissue culture grown plants (Fig. 1f), then finely macerated using a scalpel blade and placed on CIM (Fig. 1g) as outlined by Panter et al. (2017). EC derived from macerated basal meristems were isolated after 4–6 weeks and multiplied by dividing EC into smaller 2–3 mm pieces and grown on CIM for an additional 4–6 weeks.

Plant transformation

For transformation, two in-house plasmids, pDPI-77 and pDPI-95 were used. The plasmid pDPI-77 contains the bialaphos resistance gene (*bar*) (*OsAct1-p: bar: CaMV35S-t*) as a plant selectable marker, with reporter gene expression cassette comprised of the DsRED2 gene (*ZmUbi-p: DsRED2:AtNos-t*) (Supplementary Fig. 1). The second plasmid, pDPI-95, contained the same DsRED2 reporter expression cassette, with the plant selectable marker, hptII conferring hygromycin resistance (*hyg*) (*OsAct1-p: hptII: CaMV35S-t*) (Supp. Figure 2). The plant expression cassettes were in-house designed, codon optimised for monocots and synthesised by a commercial service (GeneArt, Thermo Fisher Scientific, Massachusetts, USA) and cloned into a pUC19-derived plasmid backbone using Gateway cloning (Thermo Fisher Scientific, Massachusetts, USA).

In preparation for particle bombardment, small 8–12 wk old EC explants (2–3 mm diameter) from line T108 were placed on osmotic shock media (OSM; MS, 30 g/L maltose, 64 g/L mannitol, 20 μM 2,4-D) and bombarded twice per plate, introducing an equivalent of 1 μg plasmid DNA per plate with transformation conditions as described by Spangenberg et al. (1995). Briefly, the DNA was prepared for bombardment by combining plasmid DNA, 0.6 μm gold particle solution, CaCl_2 and spermidine, and subsequently vortexed and allowed to precipitate, then spun down in a microcentrifuge. The particles were washed with 100% ethanol, which was removed via centrifugation, before the gold mix was resuspended in 100% ethanol and 10 μL was pipetted onto each macrocarrier.

Rupture disks rated at 900PSI were used for bombardment, subsequent to which calli were incubated on OSM overnight. During this incubation and the subsequent callus growth stage, two growth temperatures (24 °C and 28 °C) were evaluated to optimise callus growth.

Table 1 Summary of embryogenic callus lines induced from cut mesocotyl explants of Rhodes grass cultivar ‘Tolgar’

Cultivar	Explant source	2,4-D concentration (μM)	Number explants initiated	Number embryogenic calli	Number of EC regenerated
Tolgar	Cut mesocotyl	20	283	11 (3.89%)	4 (36%)

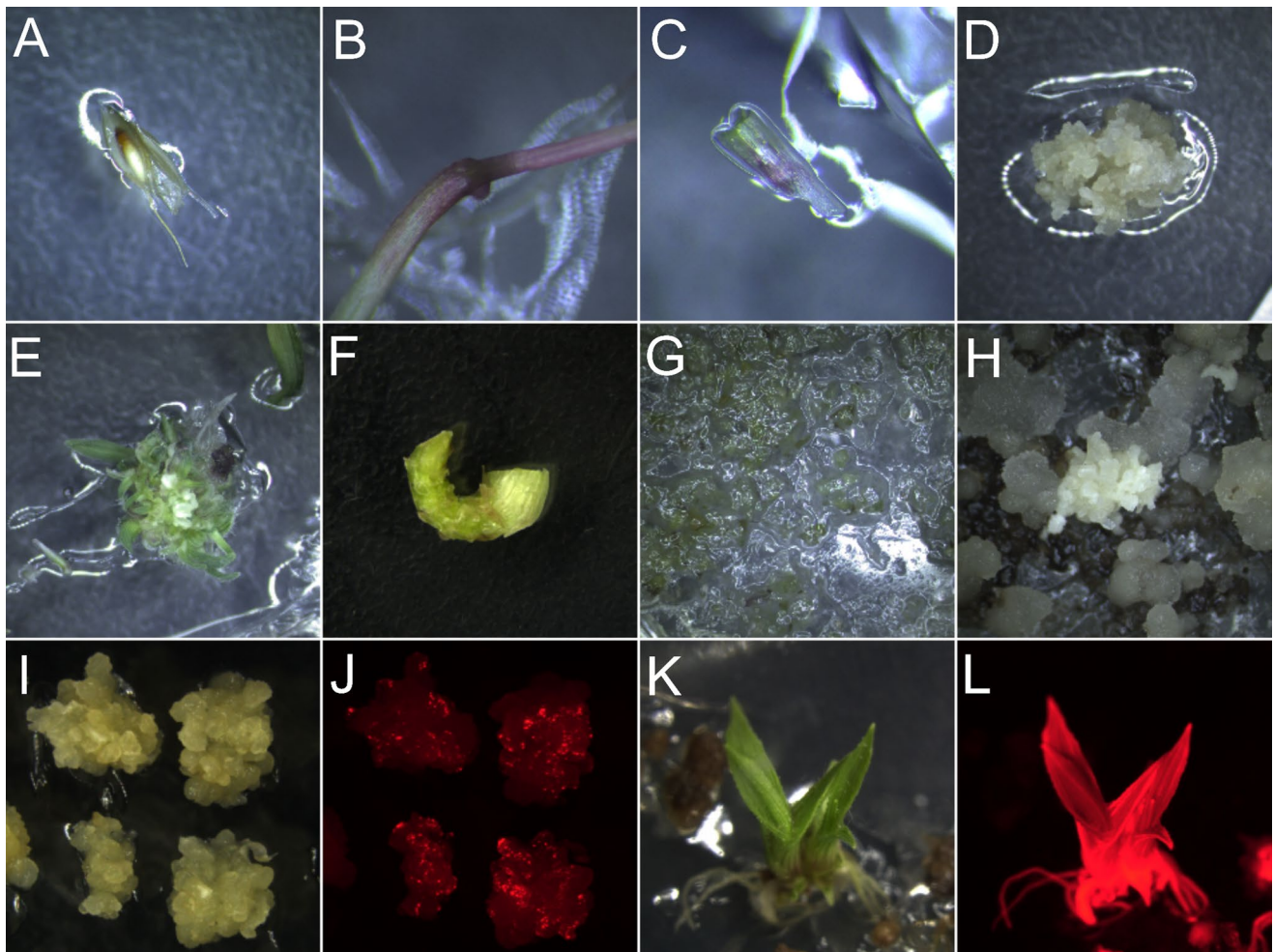


Fig. 1 Overview of Rhodes grass (*Chloris gayana*) regeneration and transformation system. **(a)** Sterilised Rhodes grass seed; **(b)** Rhodes grass seedling with visible mesocotyl region; **(c)** cut mesocotyl region on CIM; **(d)** embryogenic callus induction after 5–8 wks; **(e)** regenerating callus after 3–4 wks cultured on SIM; **(f)** excised basal meristem

of mature plantlet; **(g)** macerated basal meristem; **(h)** embryogenic callus from macerated meristems after 2–6 wks on CIM; **(i)** DsRED2 expression foci observed on bombarded callus at 7d and **(j)** ~5 wks post-bombardment; **(k)** regenerated transgenic shoot on brightfield and **(l)** fluorescence microscopy

Evaluation of selectable markers

Established selection conditions for *bar* were initially evaluated as outlined by Gondo et al. (2009). Callus was cultured on selection-free CIM for 15 days post bombardment, followed by 60–70 days on CIM supplemented with 3 mg/L phosphinothricin (PPT). The calli were then transferred to light growth conditions for regeneration on SIM supplemented with 5 mg/L PPT for 4 weeks, or until regenerating plantlets were approximately 1 cm long. Regenerating plantlets were then transferred to rooting media (RM: 0.5x (half concentration) MS media) with no PPT.

These PPT concentrations were potentially sub-optimal due to abnormal shoot growth observed upon regeneration of putative transgenic plants. Thus, an experiment to evaluate the dose response to PPT was conducted.

Non-transgenic EC explants from isogenic line T108 (five plates per PPT concentration with nine pieces of callus per plate) were subject to varying concentrations of PPT in both CIM (0, 1, 3, 5 mg/L) and SIM (0, 0.25, 0.5, 1, 3, and 5 mg/L) over a period of 5 weeks (Figs. 4 and 5; Tables 2 and 3), with efficacy of selection determined for callus media by measuring size changes over time and in SIM by number of shoots regenerated. The callus growth over time was measured using ImageJ software (Abramoff et al. 2004) from photographs taken with a Canon EOS 500D camera (Canon, Ota City, Tokyo, Japan) at two timepoints: initiation of callus on CIM and callus growth after four weeks on CIM. Scale was set as per a ruler photographed alongside the samples, and callus area was measured using the Wand tool with manual adjusting of selected areas. The number of shoots regenerated was determined by manual observation, with

Table 2 Response of Rhodes grass (*Chloris gayana*, Cv. ‘Tolgar’) line T108 embryogenic callus explants to phosphinothricin (PPT) in callus induction media (CIM*)

PPT concentration (mg/L)	Number of explants	Wk 0 Mean callus area ($\pm$ standard deviation (sd), mm ²)	Wk 4 Mean callus area ($\pm$ sd, mm ²)	Mean callus area change over time (Wk4 – Wk0; $\pm$ sd, mm ²)**
0	45	4.39 $\pm$ 1.47	72.06 $\pm$ 21.80	67.67 $\pm$ 22.71 a
1	45	4.21 $\pm$ 1.61	12.40 $\pm$ 4.18	8.19 $\pm$ 3.28 b
3	45	4.25 $\pm$ 1.62	9.30 $\pm$ 3.19	5.05 $\pm$ 1.88 b
5	45	4.04 $\pm$ 1.26	7.98 $\pm$ 2.27	3.94 $\pm$ 1.41 b

*CIM: MS, 30 g/L sucrose, 3 g/L phytagel, 20 μ M 2,4-D, supplemented with PPT as specified in the table**lowercase letters indicate significance by ANOVA and Tukey’s post-hoc analysis at $P < 0.05$ **Table 3** Response of Rhodes grass (*Chloris gayana* Cv. ‘Tolgar’) line T108 embryogenic explants to phosphinothricin (PPT) in shoot induction media (SIM*)

PPT concentration (mg/L)	Number of explants	Total number of explants with shoot regeneration after 5 wks	Average number of shoots per explant ($\pm$ sd)**
0	90	83 (92.2%)	8.43 $\pm$ 6.05 a
0.25	45	29 (64.4%)	4.07 $\pm$ 5.34 b
0.5	45	19 (42.2%)	1.9 $\pm$ 3.61 bc
1	90	2 (2.2%)	0.02 $\pm$ 0.15 c
3	45	0	0 c
5	45	0	0 c

*SIM: MS, 30 g/L maltose, 3 g/L phytagel, 3.6 μ M BAP, supplemented with PPT as specified in the table**lowercase letters indicate significance by ANOVA and Tukey’s post-hoc analysis at $P < 0.05$

counting performed under a dissecting microscope (Leica MZ6, Wetzlar, Germany) following five weeks of shoot regeneration.

The recovery time on nonselective media post bombardment with pDPI-77 was assessed as either zero or two weeks. Following this, the calli were transferred to CIM media supplemented with 1 mg/L PPT for approx. 8 wks. Calli were subsequently transferred to SIM supplemented with 1 mg/L PPT, and once plantlets were established (3–4 wks) they were transferred to RM supplemented with 1 mg/L PPT).

The use of hygromycin as a selectable marker was also preliminary evaluated based on Bajaj et al. (2006) and Panter et al. (2017) without further optimisation. Following callus bombardment with the *hyg* selectable marker vector (pDPI-95), calli were incubated on OSM at 28 °C overnight. The calli were then transferred to CIM supplemented with 50 mg/L hygromycin and incubated at 28 °C in dark conditions for two weeks. Calli were then transferred to CIM supplemented with 80 mg/L hygromycin and incubated at 28 °C in dark conditions for an additional four weeks, with subculturing at two-week intervals. The explants were then transferred to SIM supplemented with 25 mg/L hygromycin (16 h photoperiod and photon flux density of 80 μ mol. m⁻²s⁻¹ at 24 °C) for 6 wks. For rooting, regenerating shoots were transferred to RM supplemented with 20 mg/L hygromycin.

Statistical analysis for PPT selection optimisation

The CIM dataset consisted of the difference in callus size between Wk4 and Wk0 as calculated in ImageJ, while the SIM dataset consisted of the total number of shoots regenerated per calli explant after five weeks on SIM with PPT. These analyses were completed using R Statistical Software (v. 4.2.0) with packages ggplot and tidyr (Wickham 2016; R Core Team 2022; Wickham and Girlich 2022). A single factor analysis of variance (ANOVA) was performed to determine if there were statistically significant differences between any of the PPT concentrations. Additionally, Tukey’s multiple comparison of means was completed to determine which conditions/concentrations differed significantly.

Characterisation of transgenic plants

Putative transgenic shoots regenerating on selection media were screened for DsRED2 expression at approximately 4–6 wks, or when shoots were approx. 1 cm in length. This was done via visual observation under a fluorescence microscope (Leica M205 FA microscope with DsRED2 filter set, 510–560 nm excitation barrier; Wetzlar, Germany). For use as an internal reference for transgene copy number estimations using ddPCR, the *Chloris gayana* phytoene desaturase gene (CgPDS) was identified via a BLAST search (Zhang et al. 2000) of the *Zea mays* PDS gene (ZmPDS; GenBank NM_001352010.1) to the Rhodes whole genome sequence (NCBI BioProject: PRJNA974075). Primers and probes were designed with Primer3Plus (Untergasser et al. 2012) according to the assay design guidelines in Droplet Digital PCR Applications Guide (BioRad, Hercules, CA, USA) for the target sequences of the selectable marker genes (*hyg* and *bar*) and for the reference gene, CgPDS. The primers and probes were synthesised by Integrated DNA Technologies (IDT, Coralville, IA, USA), and their sequences are provided in Supplementary Table 1.

DNA was extracted from approx. 50 mg leaf tissue sampled from recovered plantlets using a DNeasy 96 Plant Kit according to the manufacturer’s instructions (Qiagen, Hilden, Germany). DNA template from non-transgenic

Tolgar-108 was used as a negative control for ddPCR and plasmid samples (pDPI-77 and -95) as positive controls. The ddPCR used (per sample) 12 µL ddPCR Supermix for Probes (BioRad, Hercules, CA, USA), 600 nM of each primer (forward and reverse primers for the reference and target), 200 nM each probe (reference and target) and nuclease free water for a total volume of 22 µL. Transgenic plant template DNA (10 ng) was added per well, and positive and negative controls were prepared (untransformed 10 ng T108 DNA and approx. 0.1×10^{-3} ng pDPI-95 or pDPI-77). Following addition of the DNA, droplets were generated in an Automated Droplet Generator, after which the plate was sealed with a pierceable foil seal in a PX1 PCR Plate Sealer (BioRad, Hercules, CA, USA). The PCR was completed on a T100 Thermal Cycler (BioRad, Hercules, CA, USA) with the following conditions: 95 °C for 10 min, followed by 39 cycles of 95 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min, then 72 °C for 5 min, 98 °C for 10 min. Results were read on a QX200 Droplet Reader using the software QuantaSoft v. 1.7.4 (BioRad, Hercules, CA, USA).

Results

Assessment of recovered regenerable genotypes

Cut mesocotyl explants were observed to be capable of forming EC, with 3.89% of cv. ‘Tolgar’ cut mesocotyl explants generating EC to potentially isolate totipotent isogenic lines and eliminate recalcitrant genotypes within the seed batch (Table 1). Induction of EC response was observed after 5–8 weeks on CIM, with eleven total ‘Tolgar’ lines generated from cut mesocotyl explants, of which four grew into plantlets. Each piece of EC obtained from a single explant was considered an individual genotype. The EC regenerated shoots after 2–4 wks on SIM. In order to maintain clonal lines and assess for repeatable embryogenic competency for regeneration and transformation, the shoots regenerated from the initial EC were maintained and multiplied by dividing tillers. These tillers were then used as the primary source of donor material for macerated basal meristems to induce EC explants suitable for transformation. The isogenic line T108 that was isolated had demonstrated reproducible embryogenesis and regeneration and was maintained for transformation studies.

Plant Transformation

Selectable marker evaluation and characterisation

The *bar* selection conditions that were initially assessed resulted in low transformation efficiency (0.34%, Table 4).

Table 4 Summary of Rhodes grass (*Chloris gayana*, Cv. ‘Tolgar’) transformation experiments

Plasmid	Total replicates of conditions	Selection conditions	Selectable marker (concentration)*	Total number of explants bombarded	Total number of independent regenerated plants	Average regeneration and range (%)	Independent PCR confirmed events**	Percentage independent PCR confirmed events (average and range)***	Number DsRed2 positive plants
pDPI-77	2	Direct selection	<i>bar</i> (CIM, SIM and RM: 1 mg/L)	545	1	0.18 (0-0.44)	1	0.18 (0-0.44)	1
pDPI-77	5	2wks recovery	<i>bar</i> (CIM, SIM and RM: 1 mg/L)	1505	5	0.33 (0.21–0.60)	2	0.13 (0-0.24)	1
pDPI-77	1	2wks recovery (Gondo)	<i>bar</i> (CIM: 1 mg/L, SIM: 3 mg/L, RM: 5 mg/L)	297	2	0.67	1	0.34	1
pDPI-95	3	Direct selection	<i>hyg</i> (CIM: 50 mg/L then 80 mg/L, SIM: 25 mg/L, RM: 20 mg/L)	881	53	6.02 (4.55–8.33)	12	1.36 (0.41–2.16)	6

*The selectable marker present in the plasmid and the concentration of selective agent used in the following media: CIM- callus induction media; SIM- shoot induction media; RM- rooting media

**PCR confirmation for the presence of *hyg*, *bar* or DsRED2 selectable marker genes

***The average value is calculated as the total percentage of independent events from all replicates of the transformation conditions, and the range is the maximum and minimum percentage of independent events obtained from individual transformation experiments

The single transgenic plant obtained under these conditions with clear DsRED2 expression showed distinct signs of abiotic stress and abnormal growth on SIM containing 5 mg/L PPT and only improved in condition after transfer to media containing no selective agent. This indicated that the concentration of PPT was too high and therefore, a dose response experiment was conducted to evaluate the concentration of PPT in both CIM and SIM in attempt to improve recovery of transgenic plants.

All PPT concentrations (0, 0.25, 0.5, 1, 3 and 5 mg/L) in CIM showed reduced callus growth compared to the control ($P < 0.05$, Table 2). Between the different PPT concentrations there was no major effect, however as the concentration increased callus growth was observed to be less prolific (Table 2). Additionally, for explants on SIM supplemented with PPT, 1 mg/L PPT did not inhibit shoot growth when compared to 0.5 mg/L or to the higher concentrations tested ($P < 0.05$, Table 3). Based on these results, 1 mg/L PPT was used for both CIM and SIM in subsequent transformations.

The use of 1 mg/L PPT in both CIM and SIM increased the average number of plantlets recovered from 0.18 to 0.33%, however the transformation efficiency did not change substantially (avg. 0.13%, maximum of 0.24%, Table 4). Subsequently, the recovery time post-bombardment was omitted to determine if more immediate selection would increase transformation efficiency, which was shown to be marginally effective (avg. 0.18%, maximum of 0.44%, Table 4).

This work indicated that the best conditions for transformation of line T108 with the *bar* selectable marker required direct selection post-bombardment, with incubation of callus on CIM and SIM supplemented with 1 mg/L PPT.

From the preliminary evaluation of transformation with the *hyg* selectable marker, 53 independent putative transgenic shoots were recovered. However, only twelve putative transgenic plants (23% of recovered plants, 1.36% of all bombarded explants) were confirmed to be transgenic by PCR analysis, with six expressing the DsRED2 reporter gene (Table 4).

From microscopic examination of the putative transformed plants, a total of ten independent events were identified with DsRED2 expression, four of which were transformed with *bar* and the remainder six with *hyg*. No albino plants were observed.

Of the recovered putative transgenic events, twelve of the *hyg*-resistant plants and five of the *bar*-resistant plants were confirmed to be transgenic via ddPCR. The ddPCR results were used to determine nature of transgenesis by quantifying the transgene integration by copy number. Copy number variation (CNV) of the events ranged from 1 to 38. With *hyg* selection, two of the events generated were single copy (17%), five were low copy (< 5 , 42%) and the remaining

Table 5 Copy number variation and expression of the DsRED2 reporter gene and selectable marker genes *hyg* and *bar* in transformed Rhodes grass (*Chloris gayana*) plants

Plasmid	Event number	Selection (<i>bar</i> or <i>hyg</i>) copy number	DsRED2 copy number	Reporter gene expression
pDPI-95	1–1*	4	3	+
pDPI-95	1–2*	3	4	+
pDPI-95	2–1**	2	3	+
pDPI-95	2–2**	2	4	+
pDPI-95	7	≥ 5	≥ 5	-
pDPI-95	10	1	0	-
pDPI-95	15	≥ 5	≥ 5	-
pDPI-95	28	≥ 5	≥ 5	-
pDPI-95	34	1	1	-
pDPI-95	39	≥ 5	≥ 5	+
pDPI-95	45	1	1	-
pDPI-95	48	≥ 5	≥ 5	+
pDPI-77	61	2	2	+
pDPI-77	62	3	1	+
pDPI-77	63	≥ 5	4	+
pDPI-77	64	2	1	+
pDPI-77	65	2	1	-

*Events 1–1 and 1–2 were recovered from the same explant; **Events 2–1 and 2–2 were recovered from the same explant

events (5) had 5 or greater (42%, Table 5). With *bar* selection, four low copy events (80%), and one high copy (≥ 5) event were identified (Table 5).

Discussion

For many crop species, an efficient and reliable regeneration system is an essential and interacting component for genetic engineering to deliver new and novel traits to industry using transgenesis and gene editing. Plant regeneration via adventitious shoots or organogenesis and somatic embryogenesis are typically used with explants that are also competent for gene delivery. In particular, meristematic organs have been shown to be competent explants for regeneration via the somatic embryogenesis pathway (Williams and Maheswaran 1986; Deo et al. 2010; Piqueras et al. 2010).

Auxins, such as 2,4-D, have been well documented to induce embryogenic callus from various explants (Bhaskaran and Smith 1990), including Rhodes grass (Gondo et al. 2007). The reported Rhodes grass embryogenesis system used 2 mg/L (approx. 10 μ M) 2,4-D to induce callus growth from cv. Katambora shoot apical meristem explants, with an EC induction rate of 29.5% (Gondo et al. 2007). Here, in a preliminary experiment, this concentration was not as effective in inducing EC from mesocotyl explants, however, was capable in inducing EC responses in mature embryo explants (data not shown). In this study, it was found that

2,4-D (20 µM) induced EC-like structures in eleven (3.89%) Tolgar explants, four of which were found competent for plant regeneration (Table 1). The contrast in 2,4-D concentrations required to induce an efficient EC response are probably due to the difference in explant competency and the cultivars used (Gondo et al. 2007). EC induction responses can be variable for different explants and genotypes, as demonstrated in other outcrossing species such as lucerne (*Medicago sativa*) (Brown and Atanassov 1985).

Preliminary work showed EC responses from another cultivar of Rhodes grass (cv. Finecut), however these lines did not respond as reliably when compared to Tolgar lines, such as T108 (data not shown). The variability in callus responses when using genetically distinct individuals has been reported, with different perennial ryegrass (*Lolium perenne*) cultivars showing EC induction rates varying from 2.3 to 21% (Bradley et al. 2001). The work here adapts the existing Rhodes grass tissue culture protocol for use with the elite, commercial Tolgar.

Rhodes grass is an outcrossing species, with high levels of genetic diversity expected between different individuals (Maybery-Reupert et al. 2023). Therefore, screening and isolating a regenerable isogenic line is advantageous for reliable and efficient transformation, as well as for evaluating traits introduced from genetic engineering because the wild type can be reliably maintained and used for direct comparison (Anami et al. 2013). Using this screening approach, an isogenic line, Tolgar-108, was found to be prolific in EC formation and was selected as a suitable donor line for transformation. Furthermore, transformation of a clonally maintained line can facilitate simpler approaches to genome editing, alleviating the need to target multiple unique sites across multiple genotypes with high within-cultivar allelic diversity, such as Rhodes grass. Using this screening approach, an isogenic line, Tolgar-108, was found to be prolific in EC formation and was selected as a suitable donor line for transformation.

A reported Rhodes grass transformation system (Gondo et al. 2009) obtained a transformation efficiency of 1.59% using *bar* selection. When implemented, the previously described conditions (3 mg/L PPT in CIM, 5 mg/L PPT in SIM) resulted in unhealthy DsRED2 expressing transformed plants. Therefore, a lower PPT concentration was thought to improve transformation efficiency, thus a dose response experiment was designed to determine optimal concentration. The concentration of selective agent in media should be high enough to suppress growth of non-transformed cells, but not so high that transformed callus and plant growth is affected (Low et al. 2018). The data generated (Tables 2 and 3) indicate 1 mg/L was the preferred concentration in both CIM and SIM, because it was the lowest concentration tested that impeded growth. Despite these adjustments

to selection conditions, transformation efficiency using the *bar* selectable marker was low (0–0.44%), with a total 2,347 explants transformed under a range of conditions resulting in a total of five independent transgenic events (Table 4). The difference in efficiencies reported was probably due to different protocols, genotypes and explants used (Kausch et al. 2019).

Different selectable markers can produce different transformation efficiency within the same genotypes, for example, an evaluation of *bar*, *aphA*, or *hyg* selectable markers in wheat (*Triticum aestivum* L.) ranged in efficiency from 0 to 1.2% (Witrzens et al. 1998). Similarly, Indica rice (*Oryza sativa* subsp. *Indica*) transformed with *bar* displayed poor growth and lower transformation efficiency compared to using *hyg* (Li et al. 1997). Due to the low transformation efficiency with the *bar* selectable marker, a preliminary evaluation using *hyg* was carried out using transformation conditions previously established for other forage grasses without any further optimisation. The *hyg* selectable marker showed potential utility for Rhodes grass. A higher transformation efficiency was obtained compared to *bar* (avg. 1.36%, range of 0.41–2.16, Table 4), however, a large proportion of non-transgenic shoots survived selection which indicated that selection pressure needs further optimisation.

It was observed that some of the bombarded callus regenerated a mix of transgenic and non-transgenic shoots, which may be due to transient expression of the selectable marker in early transformation or protection of non-transformed tissue by surrounding transformed tissue (Domínguez et al. 2004). EC is a multicellular mass wherein each cell is capable of regeneration into an independent event (Fehér 2019), and poor selection pressure may have contributed to survival of non-transgenic shoots or escapes mixed with transgenic events. On the other hand, it is also possible to generate multiple independent events from same piece of callus which can only be confirmed by molecular characterisation such as ddPCR CNV, sequencing or Southern blot analysis (e.g., Events 1–1 and 1–2; Events 2–1 and 2–2, Table 5). It can be observed in Fig. 1j that plasmids are delivered to many cells across an explant as shown by the transient dsRED2 foci expression, each of which has the potential to regenerate into an independent event.

Integration of fragmented plasmids as indicated by ddPCR CNV analysis of two elements of the construct was observed in multiple events (Table 5). This is not unusual, as direct DNA delivery via particle bombardment can lead to fragmented plasmid integrations and can potentially be mitigated through further optimisation of the transformation system or by generating more events and screening for clean, single, or low-copy integrations (Altpeter et al. 2005; Gao and Nielsen 2013).

From transformation with *hyg*, a total of two single copy and five low copy events were obtained. There were an additional four low copy events recovered from the transformation with *bar* (Table 5). The generation of single and low copy events with clean integrations is generally preferred for regulatory stewardship, as stable transmission simplifies future breeding and tracking while stably expressing transgenes are potentially less prone to gene silencing (Joung et al. 2015; Cosgrove 2019). For transgene-free gene editing, single-copy or single-locus integrations (which can contain multiple copies) are also favoured as removal of a single locus transgene integration via breeding is more straightforward than removing multiple transgene loci (Jiang 2015; Jaganathan et al. 2018). Another strategy to consider is *Agrobacterium tumefaciens* mediated transformation which has often shown to generate simpler transgene integrations (Matsumoto and Goncalves 2012). *Agrobacterium* transformation has been useful with similar explants in *Lolium perenne* (Bajaj et al. 2006).

This study reports a method for Rhodes grass transformation using particle bombardment. To enable this, a stable isogenic line T108 from elite germplasm was isolated and shown to be responsive to tissue culture and competent for repeatable regeneration from EC derived from macerated basal meristems explant sources. EC explants derived from T108 was found suitable for transformation with two different selectable markers, *hyg* and *bar*, and capable of generating low and single-copy events. Future work should include optimisation of the *hyg* system and potentially evaluating other selection methods and gene delivery conditions to improve efficiency and recovery of quality events. These resources will facilitate genetic improvement and functional genomics studies through conventional transgenesis and genome editing.

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Declarations

Competing interests The authors declare that they have no known competing interests.

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