



Novel protocol for isolation and transient transformation of Rhodes grass (*Chloris gayana*) protoplasts

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Abstract

Rhodes grass (*Chloris gayana*) is a warm-season C4 grass currently grown as a forage in some tropical regions, with anticipated future application in areas affected by climate change. However, there are few resources available for this grass, with few genomic resources and only one established transformation system, thus limiting the application of biotechnology methods for its improvement. Protoplast transformation can be used as a time- and resource-efficient way to examine gene pathways and functions of transcriptional elements *via* proteomics and transcriptomics, and to validate gene constructs. The aim of this work was therefore to establish the first Rhodes grass leaf mesophyll protoplast isolation and transient transformation protocol. A range of protoplast isolation factors were examined, including enzyme quantity and vacuum infiltration time. Up to 4.13×10^6 protoplasts were isolated per gram of fresh weight with an average 94.8% viability from an overnight digestion protocol with 4% (w/v) cellulase R10, 0.8% (w/v) Macerozyme R10, and no vacuum infiltration. Transformation conditions were optimized *via* Taguchi's orthogonal array, which compared combinations of three levels each of green fluorescent protein (GFP) plasmid DNA quantity, polyethylene glycol (PEG) concentration, and transformation time. A transient transformation efficiency of up to 27.88% was observed by GFP expression. DNA quantity was identified to be the only factor from those tested effecting transformation efficiency ($p < 0.001$) in a linear way. Work here represents the first report for Rhodes grass protoplast isolation and transformation, which could facilitate genome editing and transcriptome and proteome studies.

Keywords Linear mixed model · Mesophyll protoplasts · Orphan crop · Poaceae · Transfection

Introduction

Rhodes grass (*Chloris gayana*) is a warm-season, C4 grass currently grown as pasture in some tropical regions including northern Australia, Argentina, and parts of Africa (Loch *et al.* 2004). It is capable of growing in high salinity and drought-prone areas, with a deep-rooted nature which also makes it valuable for soil restoration purposes (Daba *et al.* 2019). Rhodes grass dry matter digestibility (DMD) typically ranges from 54.1 to 55.5% (Milford and Minson 1968; Minson 1972), which is substantially lower than that of the widely grazed temperate species perennial ryegrass, which range from 74.7 to 86.2% (Frandsen 1986). This low

digestibility has been a barrier to wider adoption; however, environmental shifts due to climate change are predicted to substantially increase the future relevance of warm-season forages like Rhodes grass in grazing industries (Cheng *et al.* 2022).

Regeneration and stable transformation systems have been developed for Rhodes grass (Gondo *et al.* 2007, 2009), which can be used to generate improved plants through genome editing. Biotechnology-facilitated plant improvement strategies, such as editing or molecular-based precision breeding, rely on gene function information, which for Rhodes grass is in part provided by the presence of a whole genome sequence and homology-based gene function information (Maybery-Reupert *et al.* 2023). Homology-based gene prediction models are capable of estimating a large proportion of genes present; however, protein functions and regulatory elements may not be adequately captured by this method (Punta and Ofran 2008). Gene regulatory networks have important roles; however, there is a gap in characterization tools. Computational approaches that

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rely on homology-based identification approaches require the existence of known sequences for regulatory elements; however, this data is often unavailable (Schlitt and Brazma 2007). While gene discovery pipelines are extensively developed and implemented (Solovjev *et al.* 2006; Humann *et al.* 2019), plant promoter computational tools are continuing to be improved (Chow *et al.* 2024). Development of protoplast isolation and transient transformation protocols for Rhodes grass could enable these characterization approaches.

Protoplasts are plant cells of which the cell walls have been removed, which simplifies the introduction of plasmid DNA constructs and proteins (Yang *et al.* 2022). Isolation and transient transformation of protoplasts has been achieved for many species (Ren *et al.* 2021; Akter *et al.* 2023), with these systems having potential use in elucidating gene functions, signal transduction pathways, and transcript regulation (Xu *et al.* 2022). Due to the low cost and rapid generation of results, protoplast systems have been used in gene functional and expression analyses (Jung *et al.* 2015), to characterize protein interactions (Yu *et al.* 2017), in metabolomics (Tohge *et al.* 2011) and for non-coding sequence characterization (Bai *et al.* 2014). Additionally, protoplast systems are a platform for validating genome editing constructs (Nadakuduti *et al.* 2019).

Due to the number of potentially influential factors, optimizing protoplast transformation can be challenging. While there are no existing protoplast protocols for Rhodes grass, isolation and transient transformation systems have been developed for other C4 grasses, including switchgrass (*Panicum virgatum*, Burris *et al.* 2016) and bermudagrass (*Cynodon dactylon*, Chen *et al.* 2023). Variation exists in optimal isolation and transformation factors between protoplast isolation methods. Differences in digestion time, tissue type, and cell wall digestion enzyme type and concentration are major factors for protoplast isolation. Major differences in published grass protoplast transformation protocols are polyethylene glycol (PEG) concentration, total DNA template quantity used, and transformation time. For validation of genome editing experiments, 1 million cells per reaction is preferred for reliable molecular analysis post-transformation. However, most of the C4 grass protoplast isolation protocols only yield 2–5 million protoplasts per gram of leaf tissue (Burris *et al.* 2016; Kim *et al.* 2022; Chen *et al.* 2023), thus limiting the number of transformation conditions that can be evaluated within the same experiment. The use of Taguchi's orthogonal array can condense the total number of reactions needed to compare multiple levels of multiple factors (Kacker *et al.* 1991; Taguchi 1995). Experiments designed with orthogonal arrays are not truly replicated; thus, results are typically analyzed by calculation of the signal to noise ratio (S/N; where signal is the controllable factor and noise is the uncontrollable or variable factor) to predict the ideal experimental conditions (Karna and Sahai 2012; Fraley *et*

Table 1. Rhodes grass (*Chloris gayana*) protoplast isolation conditions and resulting cell yield and viability.

Treatment	Enzyme digest ^a	Vacuum infiltration time (min)	Total yield (× 10 ⁶ , per 0.5 g FW)	Viability (%)
1	E1	0	1.32	82
2	E1	30	2.22	84
3	E2	0	1.94	95
4	E2	30	1.78	96

^aEnzyme digest 1 (E1): 1.5% (w/v) cellulase R10, 0.3% (w/v) macerozyme R10; Enzyme digest 2 (E2): 4% (w/v) cellulase R10, 0.8% (w/v) macerozyme R10

al. 2024). Linear mixed models (LMMs) could also be used to analyze the data as it incorporates both fixed and random effects to represent non-independent data groupings (Bolker 2015). This study reports the first protoplast isolation and transformation system for Rhodes grass. Same-day and overnight isolation protocols were evaluated, along with a variety of enzyme concentrations. Taguchi's orthogonal array was used to determine optimal transformation conditions (Taguchi 1995) for the following factors: PEG concentration, transformation time, and DNA quantity. The system developed here has future applications in functional gene characterization, transcriptomics, and gene construct validation.

Materials and Methods

Plant material Tissue culture leaf material was derived from the Rhodes grass cv. Tolgar clonal line Tolgar 108 (T108), which was established in a previous work (Maybery-Reupert *et al.* 2024). Shoots were maintained and multiplied in 947-mL tubs containing approx. 75 mL Murashige-Skoog (MS) medium in light growth conditions (16-h light photoperiod, photon flux density of 80 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ at 24°C), with 1–2-wk-old leaves from these plants used as tissue sources for protoplast isolation.

Protoplast isolation and quantification All protoplast isolation and transformation steps were conducted under aseptic conditions. Six combinations of conditions were initially assessed for overnight digestion (Table 1). For each set of conditions, 0.5 g fresh weight leaf tissue from 1–2-wk-old tissue culture plants was collected and cut into 0.5–1-mm strips in a 90-mm-diameter Petri dish.

The digestion mixes consisted of 50 mL of a base solution (0.6 M mannitol, 10 mM mannitol, adjusted to pH 5.7 and 0.22- μm filter sterilized) with Cellulase R10 and Macerozyme R10 (both enzymes manufactured by Yakult Pharmaceutical Industry Co., Ltd, Tokyo, Japan) in varying quantities. Enzyme digestion mix 1 (E1) contained

Table II. Orthogonal (L9) Rhodes grass (*Chloris gayana*) protoplast transformation factor optimization and transformation efficiency determined by replicate and overall mean values.

Treatment	Factors	Transformation efficiency (%) ^a					
		Transformation time	PEG	DNA	R1 ^b	R2	R3
1	1 (10 min)	1 (30%)	1 (10 µg)	1.61	12.34	0.56	4.84 (± 5.32)
2	1 (10 min)	2 (40%)	2 (20 µg)	24.80	21.09	1.48	15.79 (± 10.23)
3	1 (10 min)	3 (50%)	3 (30 µg)	15.23	22.34	2.90	13.49 (± 8.03)
4	2 (15 min)	1 (30%)	2 (20 µg)	7.88	17.11	3.13	9.37 (± 5.8)
5	2 (15 min)	2 (40%)	3 (30 µg)	18.28	25.27	4.32	15.96 (± 8.71)
6	2 (15 min)	3 (50%)	1 (10 µg)	2.11	7.69	0.85	3.55 (± 2.97)
7	3 (20 min)	1 (30%)	3 (30 µg)	18.34	27.88	2.04	16.09 (± 10.67)
8	3 (20 min)	2 (40%)	1 (10 µg)	1.76	7.83	0.64	3.41 (± 3.16)
9	3 (20 min)	3 (50%)	2 (20 µg)	10.23	15.88	0.46	8.86 (± 6.37)

^aTransformation efficiency calculated as proportion of cells expressing GFP out of the total visible cells, as counted with a hemocytometer

^bReplicate (R)

^cStandard deviation (SD)

1.5% (w/v) Cellulase R10 and 0.3% (w/v) Macerozyme, and enzyme digest mix 2 (E2) contained 4% (w/v) Cellulase R10 and 0.8% (w/v) Macerozyme. E2 was based on enzyme quantities from the established bermudagrass protoplast isolation protocol (Chen *et al.* 2023). To dissolve the enzymes, the digestion mix was heated at 50°C for 10 min in a water bath, then cooled to room temperature. The following components were added: 1 mM calcium chloride dihydrate (CaCl₂·2H₂O), 1% (w/v) bovine serum albumin, 0.025% (v/v) pluronic acid (10%), and 5 mM β-mercaptoethanol; with pH adjusted to 5.7 and sterilized with a 0.22-µm filter. Petri dishes were placed in a vacuum chamber (250–450 psi) for 0 or 30 min (Table I), then sealed and placed in an incubator overnight (approx. 16 h, 24°C, no agitation).

Protoplasts were collected by gentle swirling of the Petri dish, with the digestion mix collected in a sterile 50-mL tube after passing through 100-µm and 40-µm sieves (pluriS-trainer, pluriSelect Life Science, Leipzig, Germany). Leaf wash buffer (WB: 0.6 M mannitol, 20 mM KCl, 4 mM MES hydrate; Sigma-Aldrich (St. Louis, Missouri), pH 5.6 and filter sterilized) was added, and the digested leaf material was washed by gentle swirling and agitation of the tissue by gentle mixing through aspiration into a 25-mL pipette (Greiner Bio-One, Kremsmünster, Austria). Protoplasts suspended in wash buffer were then passed through 100-µm and 40-µm sieves and collected in a 50-mL tube. The suspended protoplasts were then transferred to round-bottom 12-mL tubes and centrifuged (70 g for 10 min at 15°C), with subsequent removal of supernatant. Protoplasts were resuspended in 7 mL WB, then gently agitated and centrifuged (70 g for 10 min at 15°C). The supernatant was removed, and the wash was repeated. Following removal of the supernatant, protoplasts were resuspended in 1 mL Qiao's medium (liquid MS, 3 mM MES hydrate, 0.6 M glucose, 4.5 µM 2,4-D; pH 5.7).

Cells were counted using a Countess II FL automated Cell Counter (Thermo Fisher, Waltham, MA) as per the manufacturer's instructions. To accurately set the Countess II cell counting protocol for the Rhodes grass cell size, protoplasts were initially, manually counted with a Reichert Bright-Line hemacytometer (0.1-mm depth, Hausser Scientific, Horsham, PA) with an Olympus BX41 microscope (Olympus Life Science, Tokyo, Japan) after staining with Evans blue (Huang *et al.* 1986). The same sample was then used to set parameters for total cell count and viability assessment in the Countess II, with viability measured as a proportion of fluorescent cells post staining of a 10 µL protoplast sample, with 2.5 µM Sytox Red Dead Cell stain (Thermo Fisher). For cell counting, 10 µL protoplast samples were similarly stained, with two samples measured per isolation. Total cells and dead cells were measured as the average of the two measurements, with cell viability calculated as the total number of cells minus dead cells.

PEG-mediated transformation The green fluorescent protein (GFP) reporter vector (pDPI-94) which contains the turbo GFP expression cassette (*ZmUbi-p:turboGFP:nos-t*) was used for transformation (Supplementary Figure 1).

For transformation, leaf tissue was digested with 10–15 mL of E2 for 16 h in dark conditions, with isolation and cell counting completed as earlier described. The transformation factors PEG concentration, DNA quantity, and transformation time were set at three levels each, with combinations of the factors determined according to Taguchi's orthogonal array (Table II). According to cell counting results, 1×10⁶ cells were aliquoted into a 12-mL round-bottom tube, along with 10, 20, or 30 µg of plasmid DNA (Table II) and made up to 230 µL with WB. Subsequently, 1 mL of PEG (0.6 M mannitol, 135 mM CaCl₂, 30, 40, or 50% (w/v) polyethylene glycol, Table II) was added dropwise with gentle

agitation of the tube until components were homogenous. The transformation mix was left at room temperature for 10, 15, or 20 min (Table II), and then 1 mL wash buffer was added with simultaneous gentle agitation to stop the reaction. Following this, 2 mL WB was added and gently mixed as before, and then a further 6 mL WB was similarly added. The nine distinct tubes containing the orthogonal array treatment combinations were centrifuged (70 g for 10 min at 15°C), the supernatant was removed, and the protoplasts were resuspended in 10 mL of WB then centrifuged and washed as described previously. The supernatant was removed, and the cells were resuspended in 1 mL of Qiao's medium and incubated for 48 h (dark conditions, 24°C). The same nine transformation condition combinations were repeated on three separate days, with each repetition termed a biological replicate.

Following incubation, total and dead cell counts were determined with the Countess II as previously described. To determine transformation efficiency, the protoplasts were centrifuged (70 g for 10 min at 15°C) and 10 mL concentrated protoplasts were microscopically counted with a hemacytometer as previously described. The number of transformed cells was determined by counting the total number of GFP-expressing cells, and the transformation efficiency was determined as the proportion of GFP-expressing cells of the total viewable in the hemocytometer. Lysed and egg-shaped or otherwise misshapen cells were excluded from the cell count.

Statistical analysis The data used for statistical analyses were the cell viability percentages (calculated as the total number of cells minus the number of dead cells) and the transformed cell percentages (calculated as the proportion of GFP-expressing cells). Cell viability values were analyzed via a single-factor ANOVA in Excel (Microsoft, Redmond, WA) of both raw and z-transformed data.

Initial analysis of the transformation data calculated the S/N ratio for each treatment. Relevant terms for this analysis are as follows:

Treatment: combination of the three factors evaluated here (e.g., treatment 1 is 10-min transformation time, 30% PEG and 10 µg DNA)

Factor: one experimental variable in a given treatment (e.g., PEG concentration)

Level: amount of a given factor (e.g., transformation time has levels of 10, 15, and 20 min)

The S/N formula used was for experiments where larger values are preferred (here, a higher/larger value for transformation efficiency is preferred), with S/N ratio for each treatment (T1-9) calculated as per (Rashid 2023):

$$\frac{S}{N} = -10 \log_{10} \left[\frac{1}{n} \times \sum_{i=1}^n \frac{1}{x_i^2} \right]$$

where n is the number of biological replicants (here, $n = 3$), i is the experiment number, and x is the response variable (here, transformation efficiency).

Following this, the average S/N for each level of each factor was determined as the sum of response variables for a level of a factor (e.g., the S/N for 30% PEG is the average S/N for treatments 1, 2, and 3). Subsequently, the S/N values for each level of each factor were tabulated, with the range (R) determined as the maximum S/N minus the minimum S/N for a factor (Supplementary Table I).

Transformation efficiency data were also analyzed using an LMM, which was fitted using restricted maximum likelihood (REML), with the individual test being unit of analysis. The LMM used to analyze transformation efficiency data can be written in the following form:

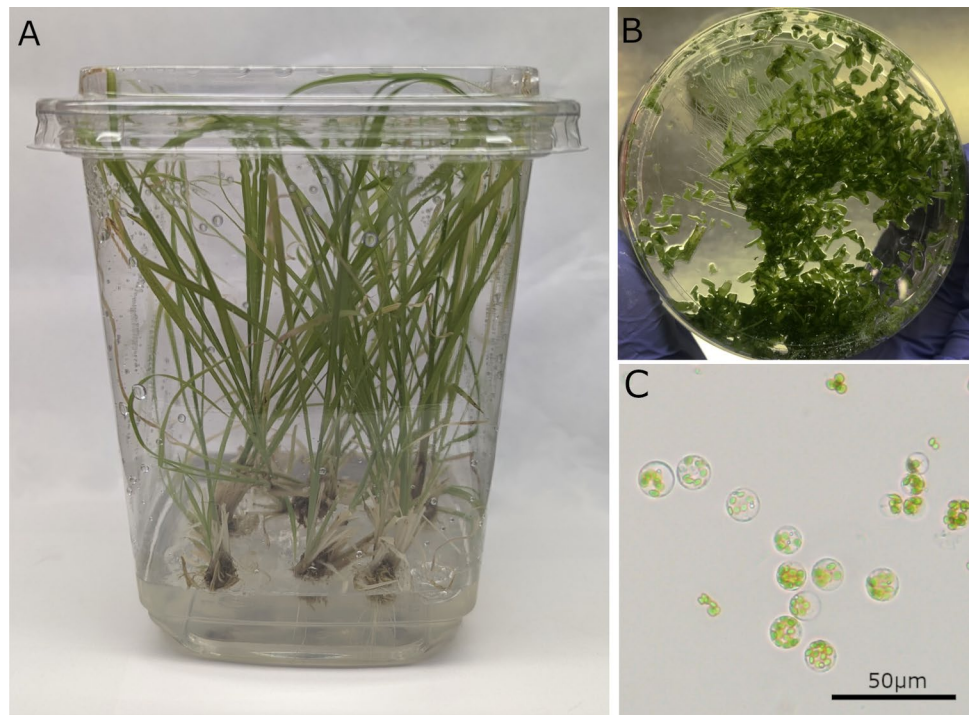
$$y = \mu + DNA + PEG + TIME + rep + \epsilon$$

where y is the response variate (transformation efficiency), μ is the overall mean, and DNA, PEG, and Time are the fixed main effects. The rep and ϵ were random effects, with rep being the effect of biological replicate and ϵ the random error term. The random effects were assumed to follow normal distribution with zero mean and constant variance. Only the main effects were fitted and assessed based on Wald's F -tests, because the Taguchi's orthogonal array design does not allow for testing of the interactions due to interactions being confounded. The P -values less than 0.05 were considered statistically significant. Histograms of residuals and plots of residuals *versus* fitted values were examined for normality of distribution with constant variance. All LMM statistical analyses were undertaken using Genstat (Genstat release 23, VSN International Ltd., Hemel Hempstead, UK).

Results and Discussion

Isolation Protoplast isolation systems offer a rapid, *in vitro* platform to assess construct viability and to pursue various -omics studies. In order to develop a transformation system, a reliable and robust isolation protocol that yields large quantities of viable cells must first be established. Prior to this work, no protoplast protocols had been developed for Rhodes grass. Therefore, multiple common experimental conditions from established grass protoplast isolation protocols were evaluated to determine optimal conditions. Previously established leaf mesophyll protoplast isolation methods for grasses including *Brachypodium distachyon*

Figure 1. Rhodes grass (*Chloris gayana*) leaf mesophyll protoplast isolation. (A) 1–2-wk-old Rhodes grass cv. Tolgar plants grown in tissue culture. (B) Rhodes grass leaf tissue cut to 2–3 mm slices, digested in enzyme mix. (C) Isolated Rhodes grass protoplasts.



(Hong *et al.* 2012), *Panicum virgatum* (Burris *et al.* 2016), *Lolium perenne* (Yu *et al.* 2017), *Zoysia japonica* (Kim *et al.* 2022), and *Cynodon dactylon* (Chen *et al.* 2023) used isolation times between 3 and 16 h, vacuum times between 0 and 60 min, and various types and quantities of cell wall digestion enzymes to isolate large amounts of viable cells. Here, a preliminary test of a 4-h isolation protocol yielded no protoplasts from either glasshouse or tissue culture–derived leaf material (data not shown). An overnight (approx. 16 h) digestion protocol was therefore assessed, along with different vacuum times (0 or 30 min) and enzyme digestion mixes containing different quantities of cell wall digestion enzymes (Table I).

All treatments yielded over a million protoplasts per gram of leaf tissue (Table I). Samples digested with E1 had lower viability (between 82 and 84%) than those digested with E2 (95–96%). Vacuum infiltration time had no obvious impact on isolation rates. Additional vacuum times (60 min) were evaluated, but the isolation rates were unreliable (data not shown). The highest quantity of protoplasts isolated from 0.5 g leaf tissue was obtained with use of digest mix 1 and a 30-min vacuum time (Table I, treatment 2: 2.22×10^6 from 0.5 g fresh leaf weight (FW)); however, the viability for these isolation conditions was lower (84%) than for other conditions with comparable cell counts (Table I, treatment 3, cell count 1.94×10^6 , viability 96%). The conditions from treatment 3 were used for subsequent isolations, yielding an average of $2.8 \pm 0.95 \times 10^6$ protoplasts per g FW, with an average $94.8 \pm 4.3\%$ viability (Figure 1).

As these sets of isolation conditions were evaluated only once, statistical analyses cannot be applied; however, the range of protoplast yields and viability between treatments was similar. Additionally, the total viable cell count was comparable to other established grass protoplast isolation systems, with isolation protocols yielding 2.42×10^6 protoplasts per g FW for switchgrass (Burris *et al.* 2016) and 4.72×10^6 protoplasts per g FW for bermudagrass (Chen *et al.* 2023), respectively. While the isolation system was dependable and efficient, there was a large quantity of debris observed in the isolated cell samples. This could potentially be mitigated in the future by use of sucrose floatation, which is commonly used to purify protoplast samples (Saka *et al.* 1987), but however can also result in lower yields as cells can be lost in this process. The exemplification of the purification process has, however, not been validated in this study. Overall, this novel protoplast isolation system was robust and yielded large enough cell quantities for use in transformation.

Transformation Protoplast transformation systems have several conditions that can affect transformation efficiency. The quantity of PEG and DNA used and transformation times are variables that can have large impacts on transformation efficiency and often require optimization (Shen *et al.* 2014). However, protoplast transformation protocols can be challenging to optimize due to the large quantity (generally 1×10^6) of cells required per set of transformation conditions. Orthogonal arrays can be used to compare multiple levels of multiple factors in as few combinations as possible and

have previously been used to optimize protoplast isolation and transformation protocols (Ma *et al.* 2020; Matchett-Oates *et al.* 2021). An orthogonal array was therefore used to optimize three levels for each of the three variables: PEG quantity (30, 40, or 50%), DNA quantity (10, 20, or 30 µg), and transformation time (10, 15, or 20 min) in nine total combinations (Table II). If this diversity of experimental conditions were optimized conventionally, with every possible combination of each component level evaluated, a total of 27 different combinations would be required, and 27 million protoplasts would be needed to examine each possible reaction. Compared to the orthogonal array approach, this would be a threefold increase in both leaf tissue biomass and experimental time required to isolate and transform these protoplasts. Use of the orthogonal array therefore greatly reduces experimental load.

By statistical analysis of Taguchi's orthogonal array results, the importance of individual factors can be elucidated. Comparing the transformation efficiencies of individual tubes and treatments gives only a comparison of a set of conditions, whereas the use of the REML model indicates which factors and level are important to transformation efficiency. While the orthogonal array approach is useful to examine the "main effects" influencing a system (here, DNA quantity was a main influence on transformation efficiency), this approach cannot be used to determine interactions between factors. In order to determine any potential interactions, additional experimentation with all factor level combinations would be required.

The standard approach for an orthogonal array result analysis from existing studies is to examine the signal to noise (S/N) ratio, where the signal is a controllable factor (here, transformation time and PEG and DNA concentrations), and noise is an uncontrollable factor (examples here would be operator effects, tissue health, cell health) (Karna and Sahai 2012; Fraley *et al.* 2024). This method was used for a preliminary examination of factors influencing transformation efficiency and was used to calculate S/N ratios for each level of each factor, as well as the ranges (*R*) of S/N values for each factor (Table III). Larger ranges and larger

Table III. The signal to noise (S/N) ratios and ranges (*R*) for Rhodes grass (*Chloris gayana*) protoplast transformation factors.

Level	Factor 1 (transformation time)	Factor 2 (PEG concentration)	Factor 3 (DNA quantity)
1	7.06	8.01	0.74
2	11.24	8.53	6.69
3	3.08	4.82	13.94
R	8.16	3.71	13.20

With levels 1, 2, and 3 for each factor as follows:

Time: 10, 20, and 30 min; PEG: 30, 40, and 50%; DNA: 10, 20, and 30 µg

Table IV. *P*-values for Rhodes grass (*Chloris gayana*) protoplast transformation efficiency factors derived from a linear mixed model.

Fixed term	N.d.f	D.d.f	<i>F</i> statistic	<i>P</i> -value
Transformation time	2	18	0.5	0.614
PEG	2	18	1.06	0.368
DNA	2	18	14.49	<0.001

N.d.f., numerator degrees of freedom; *D.d.f.*, denominator degrees of freedom

Where rep. level is used as a residual term

S/N values indicate a larger effect of a factor or a level of a factor on transformation efficiency, with DNA quantity having the largest overall impact ($R=13.20$) and PEG concentration having the smallest impact ($R=3.71$). The highest DNA amount used (30 µg) had the largest S/N value (13.94, Table III). One weakness in this analysis method is that it gives only relative results, without indicating the significance of each factor used (Fraley *et al.* 2024).

The data were analyzed using a LMM fitted using the REML method. The LMM manages unbalanced data as well as missing values and is best suited to account for various random sources of variability in the response variable. When used here, the REML analysis showed similar results to the S/N method, with DNA quantity found to have a significant effect ($p<0.001$) on transformation efficiency, with a higher quantity of DNA resulting in a higher transformation efficiency (Figure 2, Table IV). This effect was found to be linear, with a 0.56% increase in transformation efficiency for a 1 µg increase in DNA. While the use of excess DNA in protoplast transformation can be toxic and result in cell death, here, no difference in viability was observed between different DNA quantities ($p>0.05$, Supplementary file 1). The graph of the relationship between DNA quantity and transformation efficiency (Figure 2) shows the beginning of a plateau in efficiency between 20 and 30 µg DNA. Similarly, a previously established switchgrass protoplast system determined a plateau for 10–20 µg DNA and then a decrease in transformation efficiency when 40 µg DNA was used (Burris *et al.* 2016). Subsequent work could further optimize the system by testing more experimental variables, including plant tissue age and health, as well as the use of higher quantities of DNA; however, improvements to efficiency should consider the increased effort required to produce high-quality plasmid DNA preparations and the anticipated reduction in efficiency.

Use of REML analysis also allows for inclusion of biological replicate as a factor influencing transformation efficiency. The transient transformation system for Rhodes grass protoplasts, as determined by GFP expression (Figure 3), obtained a maximum 27.88% transformation efficiency (Table II, treatment 7 in Rep 2). However, high variability was observed between experimental replicates

Figure 2. Effect of plasmid DNA quantity (μg) on Rhodes grass (*Chloris gayana*) protoplast transformation efficiency (percentage). Vertical bars on data points indicate the standard error.

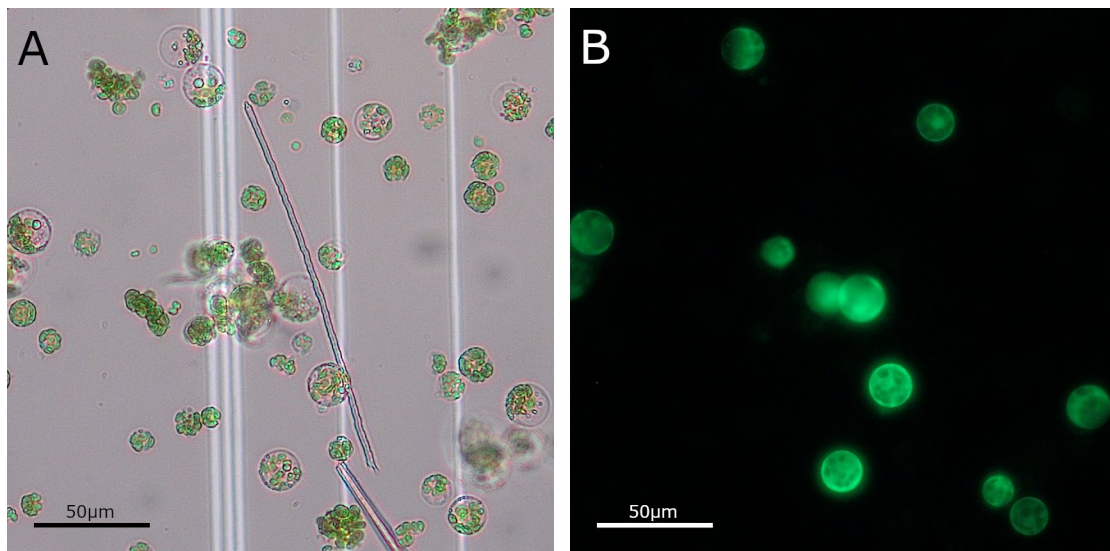
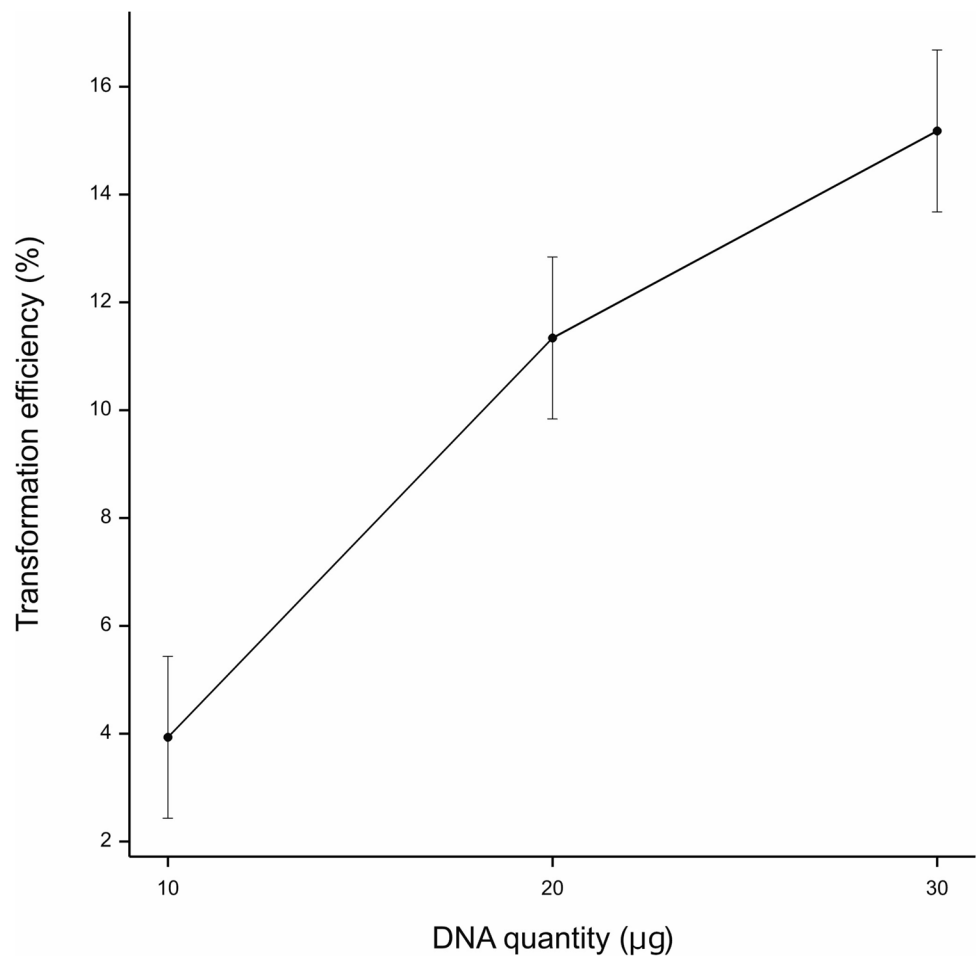


Figure 3. Isolated and transformed Rhodes grass (*Chloris gayana*) protoplasts. (A) Brightfield isolated protoplasts and debris. (B) Transformed protoplasts expressing GFP.

and treatment combinations (Table II), with the highest average TE occurring for treatment 7 ($16.06\% \pm 10.67\%$), and the lowest occurring for treatment 8 ($3.41\% \pm 3.16\%$). The REML model used here showed that the variability in transformation efficiency due to the biological replicate was three times higher than residual variability. Highly variable transformation efficiencies in protoplast systems are common, with previously established transformation efficiencies for bermudagrass (Chen *et al.* 2023) and *Populus* hybrid (Yang *et al.* 2024) varying by 10–20% for the same conditions. The variability between biological replicates could be due to factors outside the transformation treatments. Protoplast systems rely on good quality, viable cells, with leaf tissue quality and age having a large impact on transformation outcomes (Reyna-Llorens *et al.* 2023). Subsequent work could therefore ensure the tissue used is of consistently high quality across replicates.

The transformation efficiency was not sensitive to the narrow range of levels evaluated for PEG and transformation time. While some protoplast transformation protocols have shown that PEG concentrations of 50% lead to cell damage and negatively impact transformation efficiency (Han *et al.* 2023), other protocols have shown efficient transformation rates from a range of concentrations. Burris *et al.* (2016) established a switchgrass protoplast system for which transformation factors were flexible, with no significant difference in transformation rates for PEG concentrations between 20 and 50% or for transformation times between 10 and 40 min, suggesting that protoplasts from some species can be transformed in a robust range of conditions. The results obtained here indicate that the transformation system is robust enough to allow for variation in some transformation factors.

Conclusions

The methods reported here are the first established protoplast protocols for Rhodes grass. The optimal mesophyll protoplast isolation method was determined through comparison of multiple common isolation factors, resulting in high yields of viable cells. The transformation system variables were optimized with use of an orthogonal array, with up to 27.88% transformation efficiency obtained. As this system needs minimal resources and is a rapid approach to obtain transformed cells, it can be applied in construct validation and transcriptome studies.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11627-024-10494-1>.

Declarations

Conflict of interest The authors declare no competing interests.

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References

- Akter N, Shim J, Lee S (2023) Optimized protocol for isolation and transient expression of placenta originated protoplast in pepper (*Capsicum annuum* L.). *In Vitro Cell Dev Biol-Plant*. <https://doi.org/10.1007/s11627-023-10404-x>
- Bai Y, Han N, Wu J *et al* (2014) A transient gene expression system using barley protoplasts to evaluate microRNAs for post-transcriptional regulation of their target genes. *Plant Cell Tiss Organ Cult* 119:211–219. <https://doi.org/10.1007/s11240-014-0527-z>
- Bolker BM (2015) Linear and generalized linear mixed models. In: Fox GA, Negrete-Yankelevich S, Sosa VJ (eds) *Ecological statistics*, 1st edn. Oxford University Press, Oxford, pp 309–333
- Burris KP, Dlugosz EM, Collins AG *et al* (2016) Development of a rapid, low-cost protoplast transfection system for switchgrass (*Panicum virgatum* L.). *Plant Cell Rep* 35:693–704. <https://doi.org/10.1007/s00299-015-1913-7>
- Chen S, Liu J, Zhang B (2023) Efficient leaf mesophyll protoplast isolation and transient gene expression in bermudagrasses (*Cynodon* spp.). *Plant Cell Tiss Organ Cult* 152:229–235. <https://doi.org/10.1007/s11240-022-02394-2>
- Cheng M, McCarl B, Fei C (2022) Climate Change and Livestock Production: a Literature Review *Atmosphere* 13:140. <https://doi.org/10.3390/atmos13010140>
- Chow C-N, Yang C-W, Wu N-Y *et al* (2024) PlantPAN 4.0: updated database for identifying conserved non-coding sequences and exploring dynamic transcriptional regulation in plant promoters. *Nucleic Acids Res* 52:D1569–D1578. <https://doi.org/10.1093/nar/gkad945>
- Daba AW, Qureshi AS, Nisaren BN (2019) Evaluation of some Rhodes grass (*Chloris gayana*) genotypes for their salt tolerance, biomass yield and nutrient composition. *Appl Sci* 9:143. <https://doi.org/10.3390/app9010143>
- Fraley S, Zalewski J, Oom M, Terrien B (2024) Design of experiments via Taguchi methods - orthogonal arrays. In: Woolf P (ed) *Chemical process dynamics and controls*. LibreTexts, University of Michigan, Michigan, USA, section 14.1
- Frandsen KJ (1986) Variability and inheritance of digestibility in perennial ryegrass (*Lolium perenne*), meadow fescue (*Festuca pratensis*) and cocksfoot (*Dactylis glomerata*): II. F₁ and F₂ progeny. *Acta Agriculturae Scandinavica* 36:241–263. <https://doi.org/10.1080/00015128609436528>
- Gondo T, Matsumoto J, Tsuruta S *et al* (2009) Particle inflow gun-mediated transformation of multiple-shoot clumps in Rhodes grass (*Chloris gayana*). *J Plant Physiol* 166:435–441. <https://doi.org/10.1016/j.jplph.2008.06.014>

- Gondo T, Matsumoto J, Yamakawa K *et al* (2007) Somatic embryogenesis and multiple-shoot formation from seed-derived shoot apical meristems of Rhodes grass (*Chloris gayana* Kunth). *Grassland Sci* 53:138–142. <https://doi.org/10.1111/j.1744-697X.2007.00084.x>
- Han X, Rong H, Feng Y *et al* (2023) Protoplast isolation and transient transformation system for *Ginkgo biloba* L. *Front Plant Sci* 14:1145754. <https://doi.org/10.3389/fpls.2023.1145754>
- Hong S-Y, Seo PJ, Cho S-H, Park C-M (2012) Preparation of leaf mesophyll protoplasts for transient gene expression in *Brachypodium distachyon*. *J Plant Biol* 55:390–397. <https://doi.org/10.1007/s12374-012-0159-y>
- Huang C-N, Cornejo MJ, Bush DS, Jones RL (1986) Estimating viability of plant protoplasts using double and single staining. *Protoplasma* 135:80–87. <https://doi.org/10.1007/BF01277001>
- Humann JL, Lee T, Ficklin S, Main D (2019) Structural and functional annotation of eukaryotic genomes with GenSAS. In: Kollmar M (ed) *Gene prediction: methods and protocols*. Springer, New York, New York, NY, pp 29–51
- Jung H, Yan J, Zhai Z, Vatamaniuk OK (2015) Gene functional analysis using protoplast transient assays. In: Alonso JM, Stepanova AN (eds) *Plant functional genomics*. Springer, New York, New York, NY, pp 433–452
- Kacker RN, Lagergren ES, Filliben JJ (1991) Taguchi's orthogonal arrays are classical designs of experiments. *J Res Natl Inst Stan* 96:577. <https://doi.org/10.6028/jres.096.034>
- Karna SK, Sahai R (2012) An overview on Taguchi method. *Int J Eng Mathematical Sci* 1:11–18
- Kim JH, Doan PPT, Lee H-Y, Kim J (2022) Transient gene expression system in zoysiagrass leaf mesophyll protoplasts. *Plant Biotechnol Rep* 16:113–121. <https://doi.org/10.1007/s11816021-00726-w>
- Loch DS, Rethman NFG, Van Niekerk WA (2004) Rhodes grass. In: Moser LE, Burson BL, Sollenberger LE *et al* (eds) *Warm-season (C4) grasses*. American Society of Agronomy Inc, Crop Science Society of America Inc, Soil Science Society of America Inc, Wisconsin, USA, pp 833–872
- Ma W, Yi F, Xiao Y *et al* (2020) Isolation of leaf mesophyll protoplasts optimized by orthogonal design for transient gene expression in *Catalpa bungei*. *Sci Hortic* 274:109684. <https://doi.org/10.1016/j.scienta.2020.109684>
- Matchett-Oates L, Mohamaden E, Spangenberg GC, Cogan NOI (2021) Development of a robust transient expression screening system in protoplasts of Cannabis. *In Vitro Cell Dev Biol-Plant*. <https://doi.org/10.1007/s11627-021-10178-0>
- Maybery-Reupert K, Isenegger D, Hayden M, Cogan N (2023) Development of genomic resources for Rhodes grass (*Chloris gayana*), draft genome and annotated variant discovery. *Front Plant Sci* 14:10. <https://doi.org/10.3389/fpls.2023.1239290>
- Maybery-Reupert K, Wijesinghe N, Hayden M *et al* (2024) Regeneration and transformation of Rhodes grass (*Chloris gayana*). Agriculture Victoria and La Trobe University, Victoria, Australia, unpublished manuscript
- Milford R, Minson D (1968) The digestibility and intake of six varieties of Rhodes grass (*Chloris gayana*). *Aust J Exp Agric* 8:413. <https://doi.org/10.1071/EA9680413>
- Minson DJ (1972) The digestibility and voluntary intake by sheep of six tropical grasses. *Aust J Exp Agric* 12:21. <https://doi.org/10.1071/EA9720021>
- Nadakuduti SS, Starker CG, Ko DK *et al* (2019) Evaluation of methods to assess *in vivo* activity of engineered genome-editing nucleases in protoplasts. *Front Plant Sci* 10:110. <https://doi.org/10.3389/fpls.2019.00110>
- Punta M, Ofra Y (2008) The rough guide to *in silico* function prediction, or how to use sequence and structure information to predict protein function. *PLoS Comput Biol* 4:e1000160. <https://doi.org/10.1371/journal.pcbi.1000160>
- Rashid KMJ (2023) Optimize the Taguchi method, the signal-to-noise ratio, and the sensitivity. *Int J Stat Appl Math* 8:64–70. <https://doi.org/10.22271/math.2023.v8.i6a.1406>
- Ren R, Gao J, Yin D *et al* (2021) Highly efficient leaf base protoplast isolation and transient expression systems for orchids and other important monocot crops. *Front Plant Sci* 12:626015. <https://doi.org/10.3389/fpls.2021.626015>
- Reyna-Llorens I, Ferro-Costa M, Burgess SJ (2023) Plant protoplasts in the age of synthetic biology. *J Exp Bot* 74:3821–3832. <https://doi.org/10.1093/jxb/erad172>
- Saka K, Katterman FR, Thomas JC (1987) Cell regeneration and sustained division of protoplasts from cotton (*Gossypium hirsutum* L.). *Plant Cell Rep* 6:470–472. <https://doi.org/10.1007/BF00272785>
- Schlitt T, Brazma A (2007) Current approaches to gene regulatory network modelling. *BMC Bioinformatics* 8:S9. <https://doi.org/10.1186/1471-2105-8-S6-S9>
- Shen J, Fu J, Ma J, *et al* (2014) Isolation, culture, and transient transformation of plant protoplasts. *CP Cell Biology* 63:2.8.1–2.8.17. <https://doi.org/10.1002/0471143030.cb0208s63>
- Solovyev V, Kosarev P, Seledsov I, Vorobyev D (2006) Automatic annotation of eukaryotic genes, pseudogenes, and promoters. *Genome Biology* 7:10.1–10.12
- Taguchi G (1995) Quality engineering (Taguchi methods) for the development of electronic circuit technology. *IEEE Trans Rel* 44:225–229. <https://doi.org/10.1109/24.387375>
- Tohge T, Ramos MS, Nunes-Nesi A *et al* (2011) Toward the storage metabolome: profiling the barley vacuole. *Plant Physiol* 157:1469–1482. <https://doi.org/10.1104/pp.111.185710>
- Xu Y, Li R, Luo H *et al* (2022) Protoplasts: small cells with big roles in plant biology. *Trends Plant Sci* 27:828–829. <https://doi.org/10.1016/j.tplants.2022.03.010>
- Yang D, Zhao Y, Liu Y *et al* (2022) A high-efficiency PEG-Ca²⁺-mediated transient transformation system for broccoli protoplasts. *Front Plant Sci* 13:1081321. <https://doi.org/10.3389/fpls.2022.1081321>
- Yang P, Sun Y, Sun X, *et al* (2024) Optimization of preparation and transformation of protoplasts from *Populus simonii* × *P. nigra* leaves and subcellular localization of the major latex protein 328 (MLP328). *Plant Methods* 20:3. <https://doi.org/10.1186/s13007-023-01128-5>
- Yu G, Cheng Q, Xie Z *et al* (2017) An efficient protocol for perennial ryegrass mesophyll protoplast isolation and transformation, and its application on interaction study between LpNOL and LpNYC1. *Plant Methods* 13:46. <https://doi.org/10.1186/s13007-017-0196-0>