

ORIGINAL RESEARCH



Electroporation-based delivery of proteins in *Penium margaritaceum* and other zygnematophycean algae

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Abstract

Zygnematophycean algae represent the streptophyte group identified as the closest sister clade to land plants. Their phylogenetic position and growing genomic resources make these freshwater algae attractive models for evolutionary studies in the context of plant terrestrialization. However, available genetic transformation protocols are limited and exclusively DNA-based. To expand the zygnematophycean toolkit, we developed a DNA-free method for protein delivery into intact cells using electroporation. We use confocal microscopy coupled with fluorescence lifetime imaging to assess the delivery of mNeonGreen into algal cells. We optimized the method to obtain high efficiency of delivery and cell recovery after electroporation in two strains of *Penium margaritaceum* and show that the experimental setup can also be used to deliver proteins in other zygnematophycean species such as *Closterium peracerosum-strigosum-littorale* complex and *Mesotaenium endlicherianum*. We discuss the possible applications of this proof-of-concept method.

1 | INTRODUCTION

From within the streptophytes, the clade containing the land plants and their algal relatives, an important step in planetary evolution occurred around half a billion years ago – plant terrestrialization (Morris et al., 2018). Yet it is still unresolved which molecular, anatomical, and physiological features present in the streptophyte last common ancestor allowed these photosynthetic organisms to adapt to a terrestrial lifestyle (One Thousand Plants Initiative, 2019; Rieseberg et al., 2023). In recent years, deep phylogenetic analyses have contributed to inferring the evolutionary relationships within Streptophyta, identifying Zygnematophyceae as the sister clade to land plants among streptophyte algae (Wickett et al., 2014).

In recent years, the growing availability of Zygnematophyceae genomes and transcriptomes is uncovering molecular traits that may have played a role during the terrestrialization process (Cheng et al., 2019; Feng et al., 2023; Jiao et al., 2020; Sekimoto et al., 2023). Some traits seem to be land plant-specific, such as F-box hormone-

mediated signalling pathways (Blázquez et al., 2020; Bowman et al., 2019). Other traits may have predated the emergence of land plants, such as stress responses to irradiance and temperature (Cheng et al., 2019; Dadras et al., 2023). Hypotheses regarding the contribution of genetic innovations to aspects of terrestrialization are being developed, but for rigorous testing of such hypotheses, genetic model systems are urgently needed (Zhou and von Schwartzberg, 2020).

There are only a few reports of successful and reproducible genetic transformation methods tested among the zygnematophycean algae [Reviewed in (Zhou and von Schwartzberg, 2020)]. Current protocols are based on recombinant DNA delivered to intact cells or protoplasts. The delivery methods include *Agrobacterium tumefaciens*-mediated transformation (Sørensen et al., 2019), particle bombardment (Abe et al., 2008; Kanda et al., 2017; Regensdorff et al., 2018; Vannerum et al., 2010), PEG-mediated transfection (Zhou and von Schwartzberg, 2020) or electroporation (Kawai et al., 2022), and have been tested in six species. Despite this diversity of approaches, there is a lack of reports using these for reverse genetics in multiple species, suggesting that other alternatives,

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such as DNA-free approaches, might be helpful to extend the toolkit and accomplish robust genetic model systems.

Delivery of recombinant proteins into cells is a DNA-free tool used in mammalian cells for functional and structural studies or genome editing (Baek et al., 2016; Bonucci et al., 2020; Naduthodi et al., 2019; Torricella et al., 2022). In recent years, similar approaches have been explored for other eukaryotes, such as land plants (Kim et al., 2017; Wang et al., 2023; Woo et al., 2015), chlorophyte/phytoflagellate microalgae (Nomura et al., 2020), brown algae (Badis et al., 2021) and fungi (Hao and Su, 2019). Inspired by these approaches, we developed a DNA-free alternative to deliver recombinant proteins inside intact cells of three zygnemathophyceae species with available genome assemblies. Our method is based on square-pulse electroporation using the recombinant green fluorescent protein mNeonGreen (mNG) (Shaner et al., 2013) as a delivery cargo. We assess the presence and localization of mNG fluorescence qualitatively and quantitatively using confocal microscopy coupled to fluorescence lifetime imaging (FLIM) and show efficiencies of delivery and cell viability after electroporation mainly focused on the desmid *Penium margaritaceum*. We discuss the considerations that allowed us to develop the protocol and its limitations.

Providing that the method can be used in different species, we propose this method as the first proof of principle step towards the development of CRISPR-Cas ribonucleoprotein (RNP) systems for genome editing in the Zygnemathophyceae.

2 | MATERIALS AND METHODS

2.1 | Algal material and culture conditions

Penium margaritaceum strains Skidmore isolate #8 (PmSkd) (Jiao et al., 2020; kindly provided by David Domozych, Skidmore College, NY, USA), *Penium margaritaceum* NIES-217 (PmNIES217), *Closterium peracerosum-strigosum-littorale* complex NIES-67, both obtained from the NIES Collection (<https://mcc.nies.go.jp/>), and *Mesotaenium endlicheriarum* SAG 12.97, obtained from the Göttingen Algal Culture Collection (SAG, <https://sagdb.uni-goettingen.de>). All the above-mentioned cultures were grown in 100 mL of liquid Woods Hole medium (WHM) 2014 at pH 7.2 with gentle agitation (60 rpm) and maintained in a walk-in temperature-controlled chamber at 20°C with a 16/8 h light/dark cycle, 30–50 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of light irradiance, in 1 L Erlenmeyer flasks. Cultures were inoculated in a 1:10 ratio with starting cultures obtained from a single colony and grown for two weeks to an absorbance at 750 nm of 0.2–0.3 (growth phase).

2.2 | Expression and purification of recombinant mNeonGreen

The coding sequence of mNeonGreen (mNG) was inserted in a pET expression vector (Novagen), which contains a C-terminal 6 $\times$ His tag and a linker. The coding sequence contained an SV40 nuclear localization signal (SV40^{NLS}) followed by a maltose binding protein (MBP) at

the N-terminal and a second SV40^{NLS} at the C-terminus. The expression vector was introduced in the *E. coli* strain Rosetta2 (DE3) (Novagen). Expression of the SV40^{NLS}-MBP-mNG-SV40^{NLS} fusion protein was induced with 300 μM IPTG in Terrific Broth (TB) medium supplemented with kanamycin and chloramphenicol at 20°C overnight. The protein was then purified by amylose affinity chromatography followed by size exclusion purification using an ÄKTA Pure 25 system. A 5 mL amylose column and 2 MBPTrapTM HP columns connected sequentially were used for affinity purification. The excess cell-free extract was washed from the columns using a 50 mM Tris-HCl pH 7.5 and 300 mM NaCl buffer. Next, the protein was eluted in the same buffer containing 20 mM maltose. The eluted mixture was concentrated and injected on a Superdex 200PG column for gel filtration using 50 mM Tris-HCl pH 7.5 and 100 mM NaCl buffer. The 71 kDa protein was concentrated to the desired concentration using Amicon spin filters and stored at -80°C until use.

2.3 | Fluorescent staining of nuclei

Fluorescent labelling of the nucleus of electroporated cells was done using 1 mg mL^{-1} of Syto 11 (Thermo Fischer) for 3 min. Cells were washed before imaging with WHM by centrifugation at 1620 g for 3 min.

2.4 | Electroporation

Cell cultures of all species were harvested (200 mL per experiment), collected by centrifugation in 50 mL tubes at 1620 g for 3 min, and washed five times with WHM to remove the extracellular polysaccharide (EPS) layer. Next, cells were washed three times with WHM supplemented with 0.3 M sucrose. The concentration of cells was adjusted with a hemocytometer (5 technical replicates, 3 independent experiments) to $2.6 \pm 1 \times 10^6$ cells mL^{-1} for PmSkd, $4.4 \pm 1.4 \times 10^6$ cells mL^{-1} for Pm NIES217, $3.5 \pm 1.4 \times 10^6$ cells/mL for *C. peracerosum-strigosum-littorale* complex, and $3.5 \pm 0.7 \times 10^7$ cells mL^{-1} for *M. endlicherianum*. The cells were maintained on ice until electroporation to avoid EPS accumulation. 70 μL of washed cells were transferred to electroporation cuvettes with a 2 mm gap (Nepagene, Japan), together with 20 μL of OPTI-MEM I Reduced Serum Medium (OPTI-MEM, Thermo Fischer) and 2 μL of 100 μM recombinant mNG. Control experiments without protein contained 2 μL of 50 mM Tris-HCl pH 7.5, 100 mM NaCl buffer. Cuvettes were placed into an ELEPO21 system (NEPA GENE) based on multiple electroporation pulses, poring, and transfer pulses. For exploratory experiments, we varied the poring pulse conditions previously used in *Closterium* for plasmid-based genetic transformation and maintained the transfer pulse conditions (Kawai et al., 2022). The poring pulse tests were performed at 2.5 ms pulse length and 300, 325, 350, 400, 500, 600 V; 3.5 ms at 300, 325, 350, 400, 500, 600 V and 5 ms at 325, 350, 400, 500, 600 V (Figure S1) and transfer pulses at 50 ms pulse duration, 15 V, 50 ms pulse interval, 10 pulses for both (+) and (–)

polarity. Final experiments were conducted at 3.5 ms pulse length and 400 V, maintaining the above-mentioned transfer pulses. Immediately after electroporation, 1 mL of WHM 0.25 M sucrose was added to the cuvettes, and the mixture was carefully transferred to 50 mL tubes to a final volume of 5 mL WHM 0.25 M sucrose and incubated at room temperature for 2 h in darkness. Then, 5 mL of WHM was added to the mixture, followed by incubation in darkness in a growth chamber (20°C) for two days. Cells were then washed three times with WHM for microscopy analyses.

For preliminary experiments, two days after electroporation, the cells were washed three times with WHM and transferred to 24-well plates to assess cell recovery. The number of living cells was counted by fluorescence ten days after electroporation.

2.5 | Confocal live cell imaging and FLIM analysis

To validate the delivery of mNG in intact cells of zygnematophycean species, we used a confocal laser scanning microscope (Leica TCS SP8 X Single Molecule Detection [SMD]). Imaging was done with a 63X 1.10 water immersion or 10X objective. mNG was excited with a pulsed white light laser (WLL) at 507 nm and detected with hybrid detectors (HyD) with 520–550 nm spectral windows. The delivery efficiency was quantified by counting the cells showing mNG nuclear localization in the field of view of 10× microscope (100 ± 30 cells). In *Penium* strains, efficiency was quantified using the cells obtained from 3 independent electroporation experiments.

FLIM measurements of cells electroporated with and without mNG were performed using the above-mentioned WLL excitation settings with a 63× 1.10 water immersion objective. A HyD detector recorded photons of excited samples with acquisition times of 90 s, average count rates of around 3×10^4 photons s^{-1} and image resolution of 256×256 pixels. The output of the detector was coupled to a Becker & Hickl single photon counting module (SPC 830), and 2D intensity images were generated by the SPC 830 software compiling the collected photons. From the generated intensity images, the decay curves were calculated per pixel and fitted to a two-component decay model (in mNG-containing cells) and a single component (cell without mNG) without fixing any other parameters in the SPCImage 8.8 software (Becker & Hickl). The lifetimes of 33 cells of both *Penium* strains were determined from three experiments with three technical replicates, and 21 cells in *Mesotaenium* were determined from two independent experiments with three technical replicates.

3 | RESULTS

3.1 | Delivery of fluorescent proteins in *Penium margaritaceum*

We considered the unicellular desmid *Penium margaritaceum* as a possible genetic model for multiple reasons: its genome has been sequenced (Jiao et al., 2020), it has been employed in multiple studies

to dissect cell wall biology in a land plant evolutionary context and a method for transformation using *Agrobacterium tumefaciens* and for gene silencing using RNA interference has been reported (Sørensen et al., 2013). Unfortunately, despite repeated and systematic attempts, we have not been able to obtain transformants showing DNA integration or transient expression of exogenous proteins using *Agrobacterium* (not shown). Therefore, we aimed to develop a DNA-free alternative platform using recombinant protein delivery.

We based the protocol (See methods section) on a plasmid-based transformation method developed for the *Closterium peracerosum-strigosum-littorale* complex (hereafter *Closterium*) using a square electric pulse-generating electroporator (Kawai et al., 2022). To examine if the electroporation settings used for *Closterium* were suitable for intact cells of *Penium*, we performed exploratory experiments to test efficiency and cell viability by varying the poring pulse conditions (Figure S1). With these data, we selected the settings that gave us the best trade-off between both parameters for later experiments (Poring pulse: 400 V, 5 ms, transfer pulses at 50 ms pulse duration, 15 V, 50 ms pulse interval, 10 pulses for both (+) and (−) polarity) (Figure S1A). Pilot experiments also demonstrated that the electroporation buffer OPTI-MEM (20% v/v) positively contributed to cell viability (not shown) as previously reported (Furuhata et al., 2019; Kawai et al., 2022).

Penium is a ‘placoderm desmid’ (Hess et al., 2022) that has a cylindrical shape and contains two symmetrical semi-cells with one or two chloroplasts (Figure 1) (Domozych et al. 2009; Ochs et al. 2014). The compartments are separated by an isthmus at the cell center, wherein the nucleus is located (Figure S1A–L (Ochs et al., 2014)). The nucleus is free of autofluorescence under the confocal microscope, unlike the rest of the cell, which contains large chloroplasts showing autofluorescence due to the chlorophyll contained in those (Figure 1B, E, H) (Müller et al., 2013). Thus, we aimed to deliver a fluorescent protein cargo to the nucleus to avoid background fluorescence and validate the results using confocal microscopy. We used a 72 kDa Maltose Binding Protein (MBP) mNeonGreen (MBP-mNG) protein fused to two SV40 nuclear localization signals (NLS) at the amino- and carboxy-terminus (SV40^{NLS}-MBP-mNG-SV40^{NLS}).

Two days after electroporation, we observed mNG localized to the nucleus of both strains (Figure 1A, D, C, F) and uniformly distributed in the cytoplasm, especially of PmSkd (Figure 1A). We quantified the efficiency of delivery as well as the cell viability and observed in PmSkd 82% and 95% respectively and in PmNIES217 65% and 92%. In control experiments using cells electroporated without mNG, we also observed minimal fluorescence localized to the nucleus. Altogether, this indicates that the electroporation-based delivery method is highly efficient (>65%), shows high cell viability after two days of electroporation (>92%), and can be used to deliver proteins to the nucleus of two strains of this desmid species.

Cells electroporated without fluorescent protein showed minimal nuclear fluorescence (Figure 2A, F). Therefore, we further verified the intracellular delivery of mNG by Fluorescence Lifetime Imaging (FLIM.) Fluorescence lifetime is an intrinsic property of any fluorescent molecule (Borst and Visser, 2010), and should therefore help to

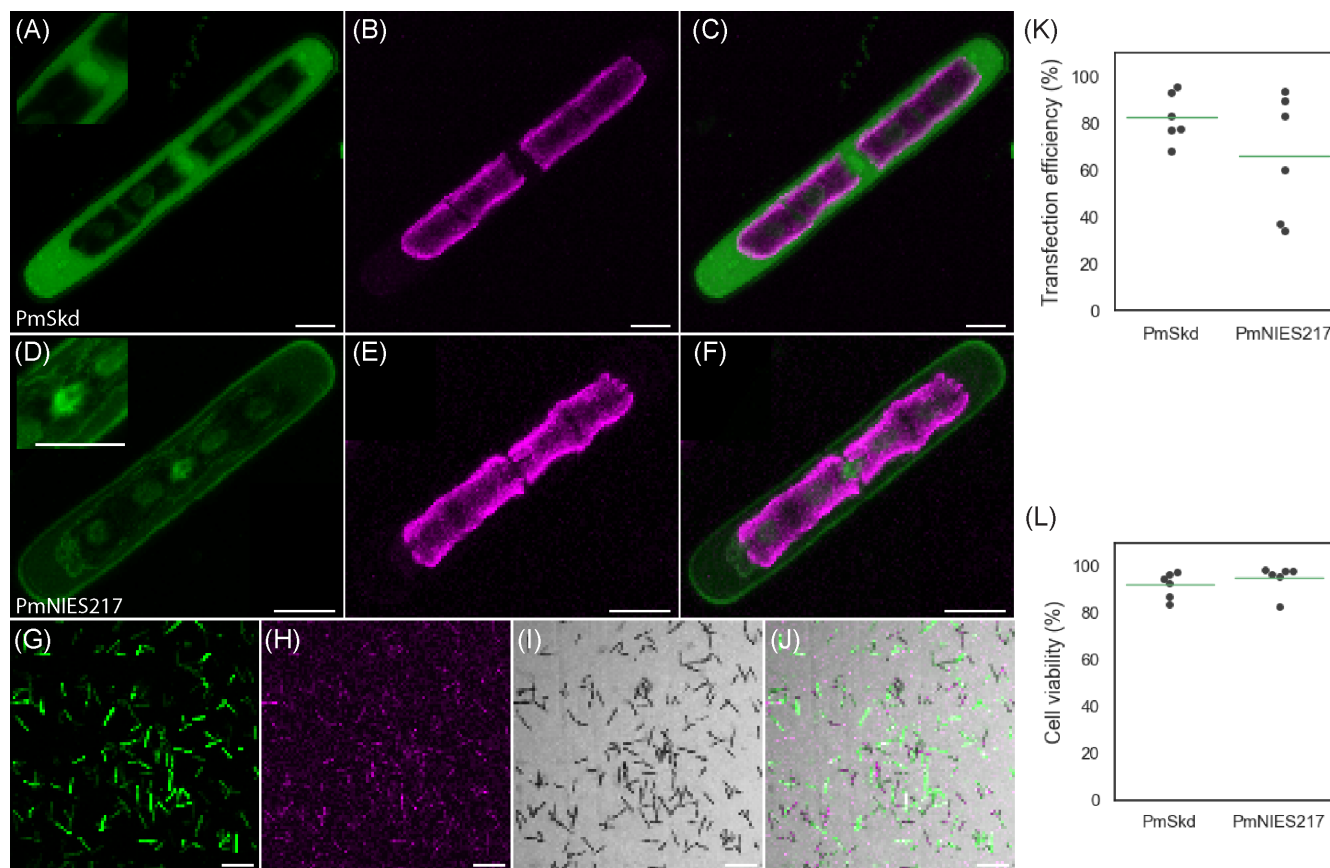


FIGURE 1 Protein transfection into *Penium margaritaceum* cells. A–C) *Penium* Skd strain transfected with mNG showing mNG fluorescence in green (A), chlorophyll autofluorescence in magenta (B) and an overlay of both (C). D–F) *Penium* NIES217 strain showing green mNG fluorescence mainly localised to the nucleus (D). E) Autofluorescence of the chloroplasts and (F) merged channels. Insets in (A,D): close-up view of the nuclear compartment of the same cell. G–J) Overview of *PmNIES217* electroporated cells taken with a 10X objective. G) in green cells transfected with mNG, (H) in magenta chloroplasts autofluorescence, (I) bright field channel and merged images (J). This field of view was used to quantify mNG transfection efficiency (K) and cell viability (L). Each dot of the swarm plots represents the percentage of transfection efficiency (K) or cell viability (L) in an image containing 100 ± 30 cells obtained from 3 independent experiments (*Pm Skd* = 559 cells, *Pm NIES217* = 562 cells). The efficiency of delivery was quantified by considering only the cells showing mNG localized to the nuclei as positive. Scale bar is 15 μm for figures (A–F) and 200 μm for (G–J).

uniquely identify the fluorescent signals derived from mNG even in the presence of other fluorescent molecules that spectrally overlap with it. We performed *in vivo* fluorescent lifetime measurements in cells electroporated with and without mNG under identical experimental and imaging conditions.

Lifetime imaging of control cells (Figure 2A–B, F–G) was analysed with a single-component exponential decay fit. The measurements showed lifetimes of 0.6 ± 0.3 ns in *PmSkd* and 0.2 ± 0.1 ns in *PmNIES217* (Figure 2E, J). Lifetime imaging of electroporated cells with mNG was analyzed with a two-component exponential decay fit (Shaner et al., 2013). The measurements showed two populations (Figure 2E, J). First, a short lifetime component $\tau_1: 1.5 \pm 0.2$ ns in *PmSkd* and $\tau_1: 1.7 \pm 0.2$ ns in *PmNIES217*. Second, a long life component $\tau_2: 3.3 \pm 0.2$ ns in *PmSkd* and 3.1 ± 0.1 ns in *PmNIES217* (Figure 2E, J), which is nearly identical to the reported 3.1 ns long lifetime component of recombinant mNG (Shaner et al., 2013). Hence, the difference in fluorescence lifetime measurements of electroporated cells containing and non-containing mNG shows that the

observed green fluorescence corresponds to the presence of intracellular mNG.

3.2 | Delivery of fluorescent protein in multiple zygnematophycean species

To further extend the zygnematophycean toolbox, we tested the protocol in other zygnematophycean members. First, we examined *Closterium* as it is closely related to *Penium* species, and both organisms belong to the order Desmidiaceae (desmids) (Hess et al., 2022). In addition, its genome is assembled and transient and stable transformation systems are established for reverse genetic studies (Abe et al., 2008; Kawai et al., 2022; Sekimoto et al., 2023). After electroporating cells with mNG, we observed fluorescence specially localized to the nucleus (Figure 3A–C) but also distributed in the cytoplasm, similar to the observations of mNG localization in *Penium* (Figure 1A–F).

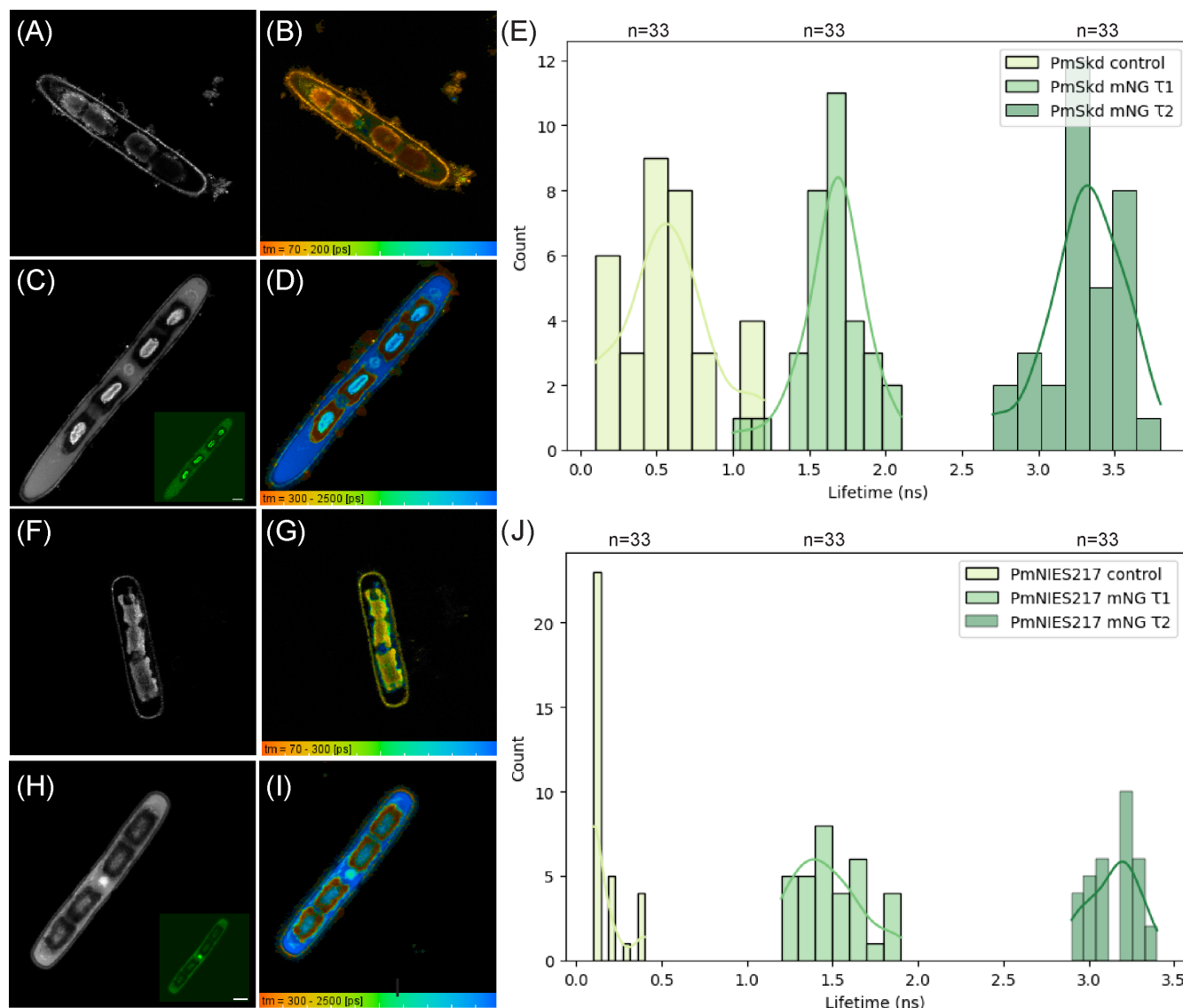


FIGURE 2 FLIM analysis of transfected cells. A, F) Fluorescence intensity images of a control cells of *PmSkd* (A) or *PmNIES217* (F) showing background fluorescence. B, G) FLIM images showing lifetime distributions (color scale: 70–200 ps range). C, H) Fluorescence intensity images of mNG containing cells *PmSkd* (C) or *PmNIES217* (H), on the right corner of (C) and (H) there are confocal images depicting mNG fluorescence (Scale bar 15 μ m). D, I) FLIM images showing lifetime distributions (colors) of *PmSkd* (D) or *PmNIES217* (I). Color scale range 300–2500 ps. E, J) Histograms representing lifetime distributions of the single component of control (light green) electroporated cells and short and long components of intracellular mNG of *PmSkd* (dark green) I or *PmNIES217* cells. J) The longer lifetime components of mNG-containing compared with the control cells depicted in FLIM images and summarized in the histograms corroborate the presence of intracellular mNG. Data was gathered from 3 independent experiments and 33 electroporated cells with and without mNG.

Next, we focused on “*Mesotaenium endlicherianum*” (strain SAG 12.97, hereafter *Mesotaenium*) as a representative of the order Serrietaeniales (Hess et al., 2022). *Mesotaenium* is a saccoderm desmid with an elongated shape, truncated ends, no isthmus, and a single chloroplast (Figure 3 D–F) (Busch and Hess, 2022). Confocal observations of electroporated cells with mNG showed green fluorescence localized to microcompartments within the chloroplasts (Figure 3D–I) and not to the nucleus (Figure S2D,E) as observed in *Penium* and *Closterium* species (Figure 1A–F, Figure 3A–C). We quantified the efficiency of delivery and cell viability and observed on average 7% efficiency of delivery (Figure 3J) and 94% cell viability after two days of electroporation.

To verify that the fluorescence signal is due to intracellular mNG, we performed *in vivo* FLIM measurements and compared electroporated cells with and without mNG (Figure 3K–N). We employed a single-component exponential decay fit for control cells and a two-component fit for the image analysis as we did with *Penium*. The measurements of short and long lifetime components of mNG-containing cells ($\tau_1 = 0.6 \pm 0.2$ ns, $\tau_2 = 2.5 \pm 0.3$ ns) were 25 times higher on average than the narrowly distributed lifetimes of control cells (0.1 ± 0.01 ns) (Figure 3O, Table S1). Nevertheless, the lifetime values are shorter than the reported lifetime values of recombinant mNG (Shaner et al., 2013). We conclude that we can use the same electroporation method adjusted for *Penium* to deliver

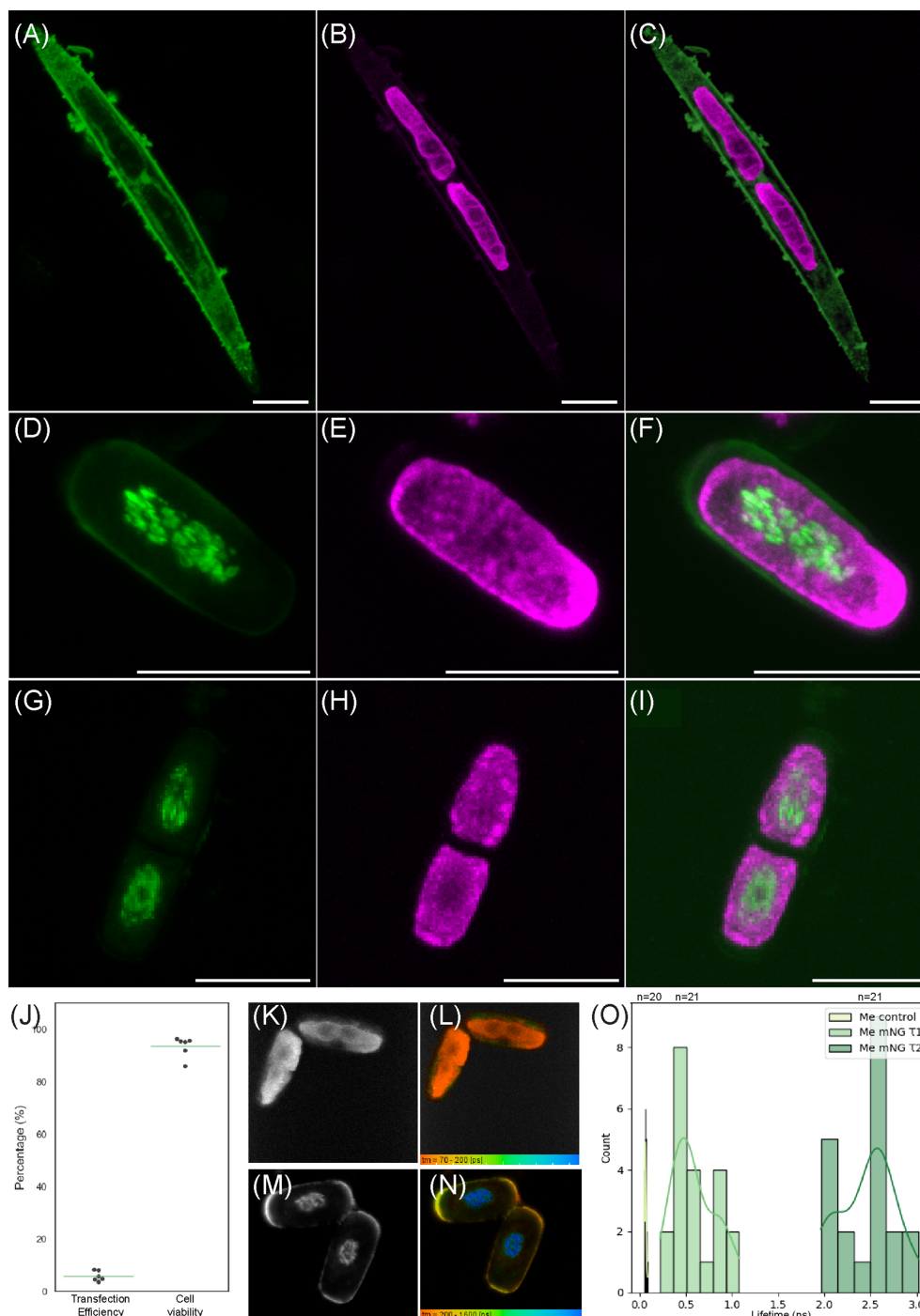


FIGURE 3 Protein delivery into *Closterium* and *Mesotaenium*. A) *Closterium* cell showing mNG fluorescence in green. B) In magenta, autofluorescence of the chlorophyll of the chloroplasts. C) merged channels. D-I) *Mesotaenium* cells showing in green mNG fluorescence localised within the chloroplasts (D, G), in magenta autofluorescence of chloroplasts (E, H) merged channels (F, I). J) Quantification of mNG transfection efficiency and cell viability (N = 297 cells). K) Fluorescence intensity images of a control *Mesotaenium* cells showing background fluorescence and (L) FLIM image showing the lifetime distribution (colors) (scale 70–200 ps range). M) Fluorescence intensity image of a mNG containing *Mesotaenium* and (N) FLIM image showing the lifetime distribution, with the highest lifetime distribution localized to the center of the cells (colors) (scale 200–1600 ps range). O) Histograms representing the lifetime distributions of the single component of control (light green) electroporated cells and short and long components (dark green) of cells showing intracellular mNG fluorescence. Data was gathered from 2 independent experiments containing 20 (control) and 21 (mNG) electroporated cells. Scale bar is 15 μ m.

proteins in zygnemathophycean species with different efficiencies. Further optimization, such as protein localization, protein concentration and efficiency of delivery, could be considered for species-specific studies.

4 | DISCUSSION AND CONCLUSIONS

For over a decade, there has been a growing interest in establishing zygnemathophycean algae as genetic model systems for land plant

evolutionary studies (Zhou and von Schwartzberg, 2020). However, a general protocol for genetic transformation that can be used in several ‘model’ species is still lacking. As an alternative to DNA-based methods, we developed an electroporation method to deliver recombinant proteins in intact cells of three zygnemathophycean species: *Penium margaritaceum* (strains Skd#8 and NIES217) (Figure 1, 2), *Closterium peracerosum-strigosum-littorale* complex NIES-67 (Figure 3A–C) and *Mesotaenium endlicherianum* SAG 12.97 (Figure 3D–O). We believe this proof-of-concept method would pave the way for developing protein-based applications such as

genome editing with CRISPR systems using ribonucleoprotein complexes or other recombinant protein-based functional studies.

The delivery of proteins for genome editing purposes offers an alternative to DNA-dependent genetic transformation. An advantage of such a system is the elimination of specific expression systems such as promoters, terminators, or codon usage. For example, a common genome engineering strategy used in various plants/microalgae is the delivery of ribonucleoproteins containing the two components of CRISPR systems (Cas proteins and guide RNAs) preassembled *in vitro* inside protoplasts or intact cells (Naduthodi et al., 2019; Park and Choe, 2019). Physical methods for RNP delivery in green organisms vary depending on the species; examples include transfection using PEG, particle bombardment, microinjection, or electroporation. Among the essential considerations when choosing the most suitable method are delivery efficiency, mutation rate, viability, and cell wall/plant regeneration. Here, we chose electroporation of intact cells as it was previously shown that this strategy leads to high transformation efficiency and cell recovery in *Closterium* (Kawai et al., 2022). We show that this approach can be optimized to obtain high efficiency of protein delivery (>65%) and cell viability (>92%) (Figure 1G–L) without the need for cell wall regeneration in *Penium* species. During the electroporation experiments, we observed that the extracellular polymeric substance (EPS) surrounding *Penium* and *Closterium* retains the protein cargo and thus negatively influences intracellular delivery. To prevent this issue, the EPS can be washed out with growth media before electroporation (See Methods section).

It is known that eukaryotic cells use NLSs to actively transport large macromolecules (>60 kDa) from the cytoplasm to the nucleus (Kalderon et al., 1984) through nuclear pore complexes (NPCs) (Panté and Aebi, 1996). Here, we used two SV40^{NLS} for a 72 kDa protein cargo to emulate a 'large macromolecule' delivered to the nucleus. Using confocal microscopy and FLIM we could observe that the fluorescent protein cargo (mNG fusion) indeed localizes to the nucleus of two desmid species (Figure 1, Figure 2, Figure 3A–C, Figure S2A–C). Instead, it appears that in *Mesotaenium*, the protein is retained in the pyrenoids localized inside the chloroplasts (Figure 3D–I). The fluorescent structures observed in mNG electroporated cells match the lentiform morphological description and localization of pyrenoids in *Mesotaenium* SAG 12.97. Fluorescent staining of the nucleus (Figure S2E) shows the organelle displaced by the chloroplast as previously reported (Busch and Hess, 2022). Thus, from these observations, we judge that mNG localises to the pyrenoids. Nevertheless, we cannot exclude that these structures represent nuclei undergoing positional rearrangements immediately after cell division and thus appear embedded in the chloroplasts (Figure 3 L,M)(Jarman and Pickett-Heaps, 1990). For future applications, detailed morphological experiments following the cell cycle and nuclear behaviour of *Mesotaenium* during cell division may be necessary, as well as tests of other nuclear localization signals (Abe et al., 2011).

As previously mentioned, we observed background autofluorescence in control *Penium* cells electroporated without fluorescent protein (Figure 2A, F). Similarly, minimal fluorescence in the form of yellowish speckles has been reported in *Agrobacterium*-mediated transformation experiments (Sørensen et al., 2013). This

autofluorescence can often be mistaken for actual protein fluorescence. Therefore, we used a quantitative tool (FLIM) to explore fluorescence in control and mNG electroporated cells in more detail under identical experimental conditions. The control measurements revealed lifetimes with a broad distribution that were 5.5 and 15.5 times lower compared to the long-lived component of mNG-containing cells in both strains of *Penium* (Skd and NIES217, respectively) and 25 times lower in *Mesotaenium* (Table S1). In *Mesotaenium*, the mNG long-lived average lifetime component was lower than the reference value of recombinant mNG; 2.5 ± 0.3 ns versus 3.1 ns, respectively (Shaner et al., 2013). This drop in the lifetime may be due to the mNG fluorescence signal being mixed with the endogenous chlorophyll autofluorescence signal contained in the chloroplasts. In addition, the fluorescence lifetime of a fluorophore is sensitive to the microenvironment, such as pH, ion or oxygen concentration, or binding or other molecules (Lakowicz, 2006). As the mNG signal in *Mesotaenium* localises in what seems to be a different cellular compartment in comparison with the tested desmids (mostly nuclear), this could partly explain the difference in fluorescence lifetime among species. Hence, FLIM allowed us to confirm the obvious presence of intracellular mNG and hypothesize that the yellowish fluorescence detected in control cells is an intracellular artefact that can be observed with the excitation and emission wavelength of mNG. The data gathered with confocal microscopy suggest that fluorescent proteins (such as mNeonGreen) that offer a low signal-to-noise ratio shall be used for localization studies in zygnematophycean algae. This will help to separate protein fluorescence from endogenous autofluorescence that may be caused by endogenous molecules (such as chlorophyll) or stress responses to experimental manipulation (Donaldson, 2020).

Here, we use the fluorescent protein mNeonGreen as a proof-of-concept method for protein delivery studies in various Zygnematophycean species. This does not imply that these exact experimental conditions will be successful in delivering and functionally test proteins of different properties such as size, concentration, solubility, or charge. However, we trust that this protocol will serve as a good starting point for establishing similar procedures for other zygnematophycean algae.

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CONFLICT OF INTEREST STATEMENT

None of the authors have a conflict of interest to disclose.

DATA AVAILABILITY STATEMENT

All primary data reported in this study is available upon request to the corresponding authors.

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REFERENCES

- Abe, J., Hiwatashi, Y., Ito, M., Hasebe, M., Sekimoto, H., 2008. Expression of Exogenous Genes Under the Control of Endogenous HSP70 and CAB Promoters in the *Closterium peracerosum-strigosum-littorale* complex. *Plant Cell Physiol.* 49, 625–632. <https://doi.org/10.1093/pcp/pcn039>
- Abe, J., Hori, S., Tsuchikane, Y., Kitao, N., Kato, M., Sekimoto, H., 2011. Stable Nuclear Transformation of the *Closterium peracerosum-strigosum-littorale* Complex. *Plant Cell Physiol.* 52, 1676–1685. <https://doi.org/10.1093/pcp/pcr103>
- Badis, Y., Scornet, D., Harada, M., Caillard, C., Godfroy, O., Raphalen, M., Gachon, C.M.M., Coelho, S.M., Motomura, T., Nagasato, C., Cock, J.M., 2021. Targeted CRISPR-Cas9-based gene knockouts in the model brown alga *Ectocarpus*. *New Phytol.* 231, 2077–2091. <https://doi.org/10.1111/nph.17525>
- Baek, K., Kim, D.H., Jeong, J., Sim, S.J., Melis, A., Kim, J.-S., Jin, E., Bae, S., 2016. DNA-free two-gene knockout in *Chlamydomonas reinhardtii* via CRISPR-Cas9 ribonucleoproteins. *Sci. Rep.* 6, 30620. <https://doi.org/10.1038/srep30620>
- Blázquez, M.A., Nelson, D.C., Weijers, D., 2020. Evolution of Plant Hormone Response Pathways. *Annu. Rev. Plant Biol.* 71, 327–353. <https://doi.org/10.1146/annurev-arplant-050718-100309>
- Bonucci, A., Ouari, O., Guigliarelli, B., Belle, V., Mileo, E., 2020. In-Cell EPR: Progress towards Structural Studies Inside Cells. *ChemBioChem* 21, 451–460. <https://doi.org/10.1002/cbic.201900291>
- Borst, J.W., Visser, A.J.W.G., 2010. Fluorescence lifetime imaging microscopy in life sciences. *Meas. Sci. Technol.* 21, 102002. <https://doi.org/10.1088/0957-0233/21/10/102002>
- Bowman, J.L., Briginshaw, L.N., Fisher, T.J., Flores-Sandoval, E., 2019. Something ancient and something neofunctionalized—evolution of land plant hormone signaling pathways. *Curr. Opin. Plant Biol., Growth and development* 47, 64–72. <https://doi.org/10.1016/j.pbi.2018.09.009>
- Busch, A., Hess, S., 2022. A diverse group of underappreciated zygnemato-phytes deserves in-depth exploration. *Appl. Phycol.* 3, 306–323. <https://doi.org/10.1080/26388081.2022.2081819>
- Cheng, S., Xian, W., Fu, Y., Marin, B., Keller, J., Wu, T., Sun, W., Li, X., Xu, Y., Zhang, Y., Wittek, S., Reder, T., Günther, G., Gontcharov, A., Wang, S., Li, L., Liu, X., Wang, J., Yang, H., Xu, X., Delaux, P.-M., Melkonian, B., Wong, G.K.-S., Melkonian, M., 2019. Genomes of Sub-aerial Zygnematophyceae Provide Insights into Land Plant Evolution. *Cell* 179, 1057–1067.e14. <https://doi.org/10.1016/j.cell.2019.10.019>
- Dadras, A., Fürst-Jansen, J.M.R., Darienko, T., Krone, D., Scholz, P., Sun, S., Herrfurth, C., Rieseberg, T.P., Irisarri, I., Steinkamp, R., Hansen, M., Buschmann, H., Valerius, O., Braus, G.H., Hoecker, U., Feussner, I., Mutwil, M., Ischebeck, T., de Vries, S., Lorenz, M., de Vries, J., 2023. Environmental gradients reveal stress hubs pre-dating plant terrestri- alization. *Nat. Plants* 9, 1419–1438. <https://doi.org/10.1038/s41477-023-01491-0>
- Domozych, D.S., Lambiasse, L., Kiemle, S.N., Gretz, M.R., 2009. Cell-Wall Development and Bipolar Growth in the Desmid *Penium margarita-ceum* (zygnematophyceae, Streptophyta). Asymmetry in a Symmetric World1. *J. Phycol.* 45, 879–893. <https://doi.org/10.1111/j.1529-8817.2009.00713.x>
- Donaldson, L., 2020. Autofluorescence in Plants. *Molecules* 25, 2393. <https://doi.org/10.3390/molecules25102393>
- Feng, X., Zheng, J., Irisarri, I., Yu, H., Zheng, B., Ali, Z., de Vries, S., Keller, J., Fürst-Jansen, J.M.R., Dadras, A., Zegers, J.M.S., Rieseberg, T.P., Ashok, A.D., Darienko, T., Bierenbroodspot, M.J., Gramzow, L., Petroll, R., Haas, F.B., Fernandez-Pozo, N., Nousias, O., Li, T., Fitzek, E., Grayburn, W.S., Rittmeier, N., Permann, C., Rümpler, F., Archibald, J.M., Theißen, G., Mower, J.P., Lorenz, M., Buschmann, H., von Schwanzenberg, K., Boston, L., Hayes, R.D., Daum, C., Barry, K., Grigoriev, I.V., Wang, X., Li, F.-W., Rensing, S.A., Ari, J.B., Keren, N., Mosquana, A., Holzinger, A., Delaux, P.-M., Zhang, C., Huang, J., Mutwil, M., de Vries, J., Yin, Y., 2023. Chromosome-level genomes of multicellular algal sisters to land plants illuminate signaling network evolution. *BioRxiv Prepr. Serv. Biol.* 2023.01.31.526407. <https://doi.org/10.1101/2023.01.31.526407>
- Furuhata, Y., Sakai, A., Murakami, T., Morikawa, M., Nakamura, C., Yoshizumi, T., Fujikura, U., Nishida, K., Kato, Y., 2019. A method using electroporation for the protein delivery of Cre recombinase into cul- tured Arabidopsis cells with an intact cell wall. *Sci. Rep.* 9, 2163. <https://doi.org/10.1038/s41598-018-38119-9>
- Hao, Z., Su, X., 2019. Fast gene disruption in *Trichoderma reesei* using in vitro assembled Cas9/gRNA complex. *BMC Biotechnol.* 19, 2. <https://doi.org/10.1186/s12896-018-0498-y>
- Hess, S., Williams, S.K., Busch, A., Irisarri, I., Delwiche, C.F., de Vries, S., Darienko, T., Roger, A.J., Archibald, J.M., Buschmann, H., von Schwanzenberg, K., de Vries, J., 2022. A phylogenomically informed five-order system for the closest relatives of land plants. *Curr. Biol.* 32, 4473–4482.e7. <https://doi.org/10.1016/j.cub.2022.08.022>
- Jarman, M., Pickett-Heaps, J., 1990. Cell division and nuclear movement in the saccoderm desmid *Netrium interruptus*. *Protoplasma* 157, 136–143. <https://doi.org/10.1007/BF01322646>
- Jiao, C., Sørensen, I., Sun, X., Sun, H., Behar, H., Alseekh, S., Philippe, G., Palacio Lopez, K., Sun, L., Reed, R., Jeon, S., Kiyonami, R., Zhang, S., Fernie, A.R., Brumer, H., Domozych, D.S., Fei, Z., Rose, J.K.C., 2020. The *Penium margaritaceum* Genome: Hallmarks of the Origins of Land Plants. *Cell* 181, 1097–1111.e12. <https://doi.org/10.1016/j.cell.2020.04.019>
- Kalderon, D., Roberts, B.L., Richardson, W.D., Smith, A.E., 1984. A short amino acid sequence able to specify nuclear location. *Cell* 39, 499–509. [https://doi.org/10.1016/0092-8674\(84\)90457-4](https://doi.org/10.1016/0092-8674(84)90457-4)
- Kanda, N., Ichikawa, M., Ono, A., Toyoda, A., Fujiyama, A., Abe, J., Tsuchikane, Y., Nishiyama, T., Sekimoto, H., 2017. CRISPR/Cas9-based knockouts reveal that CpRLP1 is a negative regulator of the sex pheromone PR-IP in the *Closterium peracerosum-strigosum-littorale* complex. *Sci. Rep.* 7, 17873. <https://doi.org/10.1038/s41598-017-18251-8>
- Kawai, J., Kanazawa, M., Suzuki, R., Kikuchi, N., Hayakawa, Y., Sekimoto, H., 2022. Highly efficient transformation of the model zyg- nematophycean alga *Closterium peracerosum-strigosum-littorale* com- plex by square-pulse electroporation. *New Phytol.* 233, 569–578. <https://doi.org/10.1111/nph.17763>
- Kim, H., Kim, S.-T., Ryu, J., Kang, B.-C., Kim, J.-S., Kim, S.-G., 2017. CRISPR/Cpf1-mediated DNA-free plant genome editing. *Nat. Com- mun.* 8, 14406. <https://doi.org/10.1038/ncomms14406>
- Lakowicz, J.R., 2006. *Principles of fluorescence spectroscopy*, 3rd ed. ed. Springer, New York.
- Leebens-Mack, J.H., Barker, M.S., Carpenter, E.J., Deyholos, M.K., Gitzendanner, M.A., Graham, S.W., Grosse, I., Li, Z., Melkonian, M., Mirarab, S., Porsch, M., Quint, M., Rensing, S.A., Soltis, D.E., Soltis, P.S., Stevenson, D.W., Ullrich, K.K., Wickett, N.J., DeGironimo, L., Edger, P.P., Jordon-Thaden, I.E., Joya, S., Liu, T., Melkonian, B., Miles, N.W., Pokorny, L., Quigley, C., Thomas, P., Villarreal, J.C., Augustin, M.M., Barrett, M.D., Baucom, R.S., Beerling, D.J., Benstein, R.M., Biffin, E., Brockington, S.F., Burge, D.O., Burris, J.N., Burris, K.P., Burtet-Sarramegna, V., Caicedo, A.L., Cannon, S.B., Çebi, Z., Chang, Y., Chater, C., Cheeseman, J.M., Chen, T., Clarke, N.D., Clayton, H., Covshoff, S., Crandall-Stotler, B.J., Cross, H., dePamphilis, C.W., Der, J.P., Determann, R., Dickson, R.C., Di

- Stilio, V.S., Ellis, S., Fast, E., Feja, N., Field, K.J., Filatov, D.A., Finnegan, P.M., Floyd, S.K., Fogliani, B., García, N., Gâteblé, G., Godden, G.T., Goh, F. (Qi Y., Greiner, S., Harkess, A., Heaney, J.M., Helliwell, K.E., Heyduk, K., Hibberd, J.M., Hodel, R.G.J., Hollingsworth, P.M., Johnson, M.T.J., Jost, R., Joyce, B., Kapralov, M.V., Kazamia, E., Kellogg, E.A., Koch, M.A., Von Konrat, M., Könyves, K., Kutchan, T.M., Lam, V., Larsson, A., Leitch, A.R., Lentz, R., Li, F.-W., Lowe, A.J., Ludwig, M., Manos, P.S., Mavrodiev, E., McCormick, M.K., McKain, M., McLellan, T., McNeal, J.R., Miller, R.E., Nelson, M.N., Peng, Y., Ralph, P., Real, D., Riggins, C.W., Ruhsam, M., Sage, R.F., Sakai, A.K., Scascitella, M., Schilling, E.E., Schlösser, E.-M., Sederoff, H., Servick, S., Sessa, E.B., Shaw, A.J., Shaw, S.W., Sigel, E.M., Skema, C., Smith, A.G., Smithson, A., Stewart, C.N., Stinchcombe, J.R., Szövényi, P., Tate, J.A., Tiebel, H., Trapnell, D., Villegente, M., Wang, C.-N., Weller, S.G., Wenzel, M., Weststrand, S., Westwood, J.H., Whigham, D.F., Wu, S., Wulff, A.S., Yang, Y., Zhu, D., Zhuang, C., Zuidof, J., Chase, M.W., Pires, J.C., Rothfels, C.J., Yu, J., Chen, C., Chen, L., Cheng, S., Li, J., Li, R., Li, X., Lu, H., Ou, Y., Sun, X., Tan, X., Tang, J., Tian, Z., Wang, F., Wang, J., Wei, X., Xu, X., Yan, Z., Yang, F., Zhong, X., Zhou, F., Zhu, Y., Zhang, Y., Ayyampalayam, S., Barkman, T.J., Nguyen, N., Matasci, N., Nelson, D.R., Sayyari, E., Wafula, E.K., Walls, R.L., Warnow, T., An, H., Arrigo, N., Baniaga, A.E., Galuska, S., Jorgensen, S.A., Kidder, T.I., Kong, H., Lu-Irving, P., Marx, H.E., Qi, X., Reardon, C.R., Sutherland, B.L., Tiley, G.P., Welles, S.R., Yu, R., Zhan, S., Gramzow, L., Theißen, G., Wong, G.K.-S., One Thousand Plant Transcriptomes Initiative, 2019. One thousand plant transcriptomes and the phylogenomics of green plants. *Nature* 574, 679–685. <https://doi.org/10.1038/s41586-019-1693-2>
- Morris, J.L., Puttick, M.N., Clark, J.W., Edwards, D., Kenrick, P., Pressel, S., Wellman, C.H., Yang, Z., Schneider, H., Donoghue, P.C.J., 2018. The timescale of early land plant evolution. *Proc. Natl. Acad. Sci.* 115, E2274–E2283. <https://doi.org/10.1073/pnas.1719588115>
- Müller, S.M., Galliardt, H., Schneider, J., Barisas, B.G., Seidel, T., 2013. Quantification of Förster resonance energy transfer by monitoring sensitized emission in living plant cells. *Front. Plant Sci.* 4, 413. <https://doi.org/10.3389/fpls.2013.00413>
- Naduthodi, M.I.S., Mohanraju, P., Südfeld, C., D'Adamo, S., Barbosa, M.J., van der Oost, J., 2019. CRISPR–Cas ribonucleoprotein mediated homology-directed repair for efficient targeted genome editing in microalgae *Nannochloropsis oceanica* IMET1. *Biotechnol. Biofuels* 12, 66. <https://doi.org/10.1186/s13068-019-1401-3>
- Nomura, T., Yoshikawa, M., Suzuki, K., Mochida, K., 2020. Highly Efficient CRISPR-Associated Protein 9 Ribonucleoprotein-Based Genome Editing in *Euglena gracilis*. *STAR Protoc.* 1, 100023. <https://doi.org/10.1016/j.xpro.2020.100023>
- Ochs, J., LaRue, T., Tinaz, B., Yongue, C., Domozych, D.S., 2014. The cortical cytoskeletal network and cell-wall dynamics in the unicellular charophycean green alga *Penium margaritaceum*. *Ann. Bot.* 114, 1237–1249. <https://doi.org/10.1093/aob/mcu013>
- Panté, N., Aebi, U., 1996. Sequential Binding of Import Ligands to Distinct Nucleopore Regions During Their Nuclear Import. *Science* 273, 1729–1732. <https://doi.org/10.1126/science.273.5282.1729>
- Park, J., Choe, S., 2019. DNA-free genome editing with preassembled CRISPR/Cas9 ribonucleoproteins in plants. *Transgenic Res.* 28, 61–64. <https://doi.org/10.1007/s11248-019-00136-3>
- Regensdorff, M., Deckena, M., Stein, M., Borchers, A., Scherer, G., Lammers, M., Hänsch, R., Zachgo, S., Buschmann, H., 2018. Transient genetic transformation of *Mougeotia scalaris* (Zygnematophyceae) mediated by the endogenous α -tubulin1 promoter. *J. Phycol.* 54, 840–849. <https://doi.org/10.1111/jpy.12781>
- Rieseberg, T.P., Dadras, A., Fürst-Jansen, J.M.R., Dhabalia Ashok, A., Darienko, T., de Vries, S., Irisarri, I., de Vries, J., 2023. Crossroads in the evolution of plant specialized metabolism. *Semin. Cell Dev. Biol., Special Issue: Algal model organisms by Susana Coelho and Olivier de Clerck* 134, 37–58. <https://doi.org/10.1016/j.semcdb.2022.03.004>
- Sekimoto, H., Komiya, A., Tsuyuki, N., Kawai, J., Kanda, N., Ootsuki, R., Suzuki, Y., Toyoda, A., Fujiyama, A., Kasahara, M., Abe, J., Tsuchikane, Y., Nishiyama, T., 2023. A divergent RWP-RK transcription factor determines mating type in heterothallic *Closterium*. *New Phytol.* 237, 1636–1651. <https://doi.org/10.1111/nph.18662>
- Shaner, N.C., Lambert, G.G., Chammas, A., Ni, Y., Cranfill, P.J., Baird, M.A., Sell, B.R., Allen, J.R., Day, R.N., Israelsson, M., Davidson, M.W., Wang, J., 2013. A bright monomeric green fluorescent protein derived from *Branchiostoma lanceolatum*. *Nat. Methods* 10, 407–409. <https://doi.org/10.1038/nmeth.2413>
- Sørensen, I., Fei, Z., Andreas, A., Willats, W.G.T., Domozych, D.S., Rose, J.K.C., 2013. Stable transformation and reverse genetic analysis of *Penium margaritaceum*: a platform for studies of charophyte green algae, the immediate ancestors of land plants. *Plant J.* 77, 339–351. <https://doi.org/10.1111/tpj.12375>
- Torricella, F., Barbieri, L., Bazzurro, V., Diaspro, A., Banci, L., 2022. Protein delivery to living cells by thermal stimulation for biophysical investigation. *Sci. Rep.* 12, 17190. <https://doi.org/10.1038/s41598-022-21103-9>
- Vannerum, K., Abe, J., Sekimoto, H., Inzé, D., Vyverman, W., 2010. Intracellular Localization of an Endogenous Cellulose Synthase of *Micrastrias Denticulata* (desmidiaceae, Chlorophyta) by Means of Transient Genetic Transformation1. *J. Phycol.* 46, 839–845. <https://doi.org/10.1111/j.1529-8817.2010.00867.x>
- Wang, J.W., Squire, H.J., Goh, N.S., Ni, H.M., Lien, E., Wong, C., González-Grandío, E., Landry, M.P., 2023. Delivered complementation in planta (DCIP) enables measurement of peptide-mediated protein delivery efficiency in plants. *Commun. Biol.* 6, 1–17. <https://doi.org/10.1038/s42003-023-05191-5>
- Wickett, N.J., Mirarab, S., Nguyen, N., Warnow, T., Carpenter, E., Matasci, N., Ayyampalayam, S., Barker, M.S., Burleigh, J.G., Gitzendanner, M.A., Ruhfel, B.R., Wafula, E., Der, J.P., Graham, S.W., Mathews, S., Melkonian, M., Soltis, D.E., Soltis, P.S., Miles, N.W., Rothfels, C.J., Pokorny, L., Shaw, A.J., DeGironimo, L., Stevenson, D.W., Surek, B., Villarreal, J.C., Roure, B., Philippe, H., dePamphilis, C.W., Chen, T., Deyholos, M.K., Baucom, R.S., Kutchan, T.M., Augustin, M.M., Wang, J., Zhang, Y., Tian, Z., Yan, Z., Wu, X., Sun, X., Wong, G.K.-S., Leebens-Mack, J., 2014. Phylotranscriptomic analysis of the origin and early diversification of land plants. *Proc. Natl. Acad. Sci.* 111, E4859–E4868. <https://doi.org/10.1073/pnas.1323926111>
- Woo, J.W., Kim, J., Kwon, S.I., Corvalán, C., Cho, S.W., Kim, H., Kim, S.-G., Kim, S.-T., Choe, S., Kim, J.-S., 2015. DNA-free genome editing in plants with preassembled CRISPR-Cas9 ribonucleoproteins. *Nat. Biotechnol.* 33, 1162–1164. <https://doi.org/10.1038/nbt.3389>
- Zhou, H., von Schwanzenberg, K., 2020. Zygnematophyceae: from living algae collections to the establishment of future models. *J. Exp. Bot.* 71, 3296–3304. <https://doi.org/10.1093/jxb/eraa091>

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