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PROTOCOL NOTE

Applications in Plant Sciences

Step-by-step protocol for the isolation and transient transformation of hornwort protoplasts

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Abstract

Premise: A detailed protocol for the protoplast transformation of hornwort tissue is not yet available, limiting molecular biological investigations of these plants and comparative analyses with other bryophytes, which display a gametophyte-dominant life cycle and are critical to understanding the evolution of key land plant traits.

Methods and Results: We describe a detailed protocol to isolate and transiently transform protoplasts of the model hornwort *Anthoceros agrestis*. The digestion of liquid cultures with Driselase yields a high number of viable protoplasts suitable for polyethylene glycol (PEG)-mediated transformation. We also report early signs of protoplast regeneration, such as chloroplast division and cell wall reconstitution.

Conclusions: This protocol represents a straightforward method for isolating and transforming *A. agrestis* protoplasts that is less laborious than previously described approaches. In combination with the recently developed stable genome transformation technique, this work further expands the prospects of functional studies in this model hornwort.

KEYWORDS

Anthoceros, hornworts, model organism, protoplasts, transient transformation

The first protoplasts were obtained by digesting the tips of tomato (*Solanum lycopersicum* L.) seedlings using a fungal cellulase (Cocking, 1960). These protoplasts were unstable and underwent cell lysis, releasing intact vacuoles and other cellular contents. Today, more than 60 years later, plant protoplasts are commonly used to determine the localization of proteins, assess their interaction and function with other cellular components/proteins in vivo, and create somatic hybrids using cell fusion, an important tool of strain improvement in plants, fungi, and prokaryotes (Eeckhaut et al., 2013). In addition, transiently transforming protoplasts enables genome editing using CRISPR/Cas9 without the integration of plasmid DNA into the target genome.

While protocols for protoplast isolation have been established for many plant species, they should be adapted and fine-tuned for every model organism. Hornworts (Anthocerotophyta) represent one of the three monophyletic groups of bryophytes (liverworts, hornworts, and mosses), and are often resolved as being sister to the clade of mosses and liverworts (Setaphytes) (Puttick et al., 2018; de Sousa et al., 2019; Li et al., 2020). The model organisms *Marchantia polymorpha* L. and *Physcomitrium* (*Physcomitrella*) *patens* (Hedw.) Mitt. have

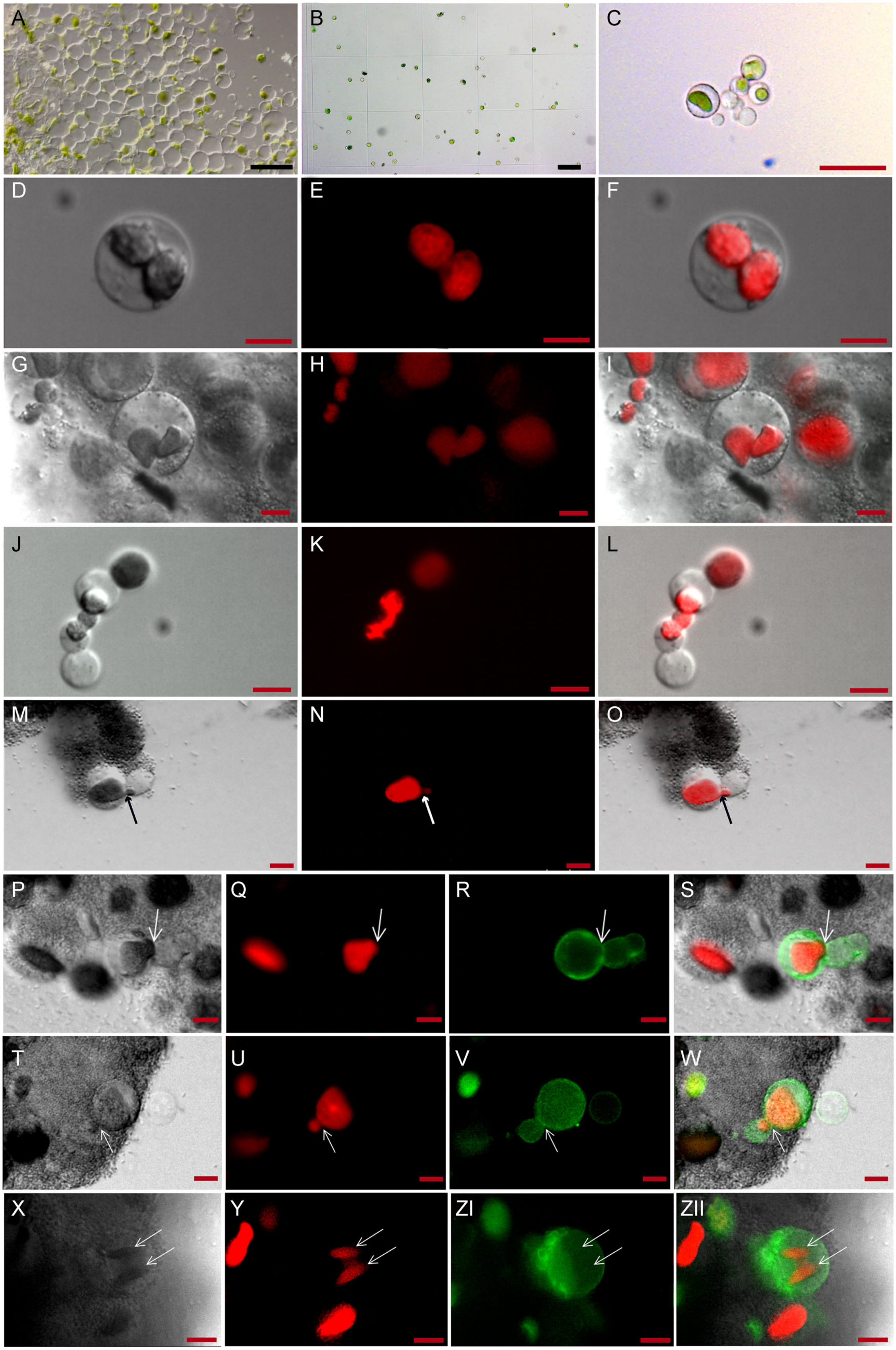

FIGURE 1 (See caption on next page)

greatly benefited the study of liverwort (Marchantiophyta) and moss (Bryophyta) biology, respectively, for years; however, model species resources for the hornworts have only become available relatively recently (Szövényi et al., 2015). The model hornwort *Anthoceros agrestis* Paton can routinely be grown axenically under laboratory conditions, be propagated sexually or vegetatively, and its genome has been sequenced (Li et al., 2020; available from https://www.hornworts.uzh.ch/en/hornwort-genomes.html). A range of molecular techniques have also been adapted for the hornworts (Frangedakis et al., 2021b). This establishment of *A. agrestis* as a tractable model organism is beginning to fill a crucial phylogenetic gap, enabling comparative analyses across all three bryophyte clades. The introduction of a tractable hornwort experimental system is envisioned to provide more accurate insights into fundamental questions of bryophyte and land plant evolution (Rensing, 2017; Szövényi et al., 2021).

Bryophytes differ from vascular plants by having a gametophyte-dominant life cycle, and all cells of the haploid gametophyte can potentially regenerate into a fully functional new plant (Frangedakis et al., 2021a; McDaniel, 2021). Due to the exceptional regeneration ability of the gametophyte phase, bryophytes can be manipulated without the use of additional phytohormones (Frangedakis et al., 2021b); thus, protoplasts are an attractive platform for bryophyte genome transformation. For the model moss *P. patens*, several efficient protocols for the isolation and regeneration of protoplasts have been developed since the 1980s and continuously optimized (Grimsley et al., 1977; Jenkins and Cove, 1983; Schween et al., 2003; Ermert et al., 2019; Sugita, 2021); however, the isolation and regeneration of protoplasts in the model liverwort *M. polymorpha* have scarcely been studied (Ono et al., 1979; Shibaya and Sugawara, 2007). Even less is known about the isolation, transformation, and regeneration of hornwort protoplasts. To date, only three studies have described a method for hornwort protoplast isolation and regeneration in two different species: *A. punctatus* L. (Takami et al., 1988; Ono et al., 1992) and *A. crispulus* (Mont.) Douin (Binding and Mordhorst, 1991). Ono et al. (1992) and Binding and Mordhorst (1991) both reported the first chloroplast divisions two days after protoplast isolation, and all three studies reported callus formation and thalli regeneration after approximately 10–14 days and two months, respectively. Nevertheless, all three protocols lack a detailed description of the conditions used, which makes the replication of the described procedures difficult. Furthermore, none of the above-mentioned studies attempted a transient transformation of the protoplast. We therefore aimed to establish a protocol for the extraction of a considerable number of protoplasts from gametophyte tissues of the model hornwort *A. agrestis* and their transient transformation with plasmid DNA. Our method not only provides an easy and time-efficient approach to test the subcellular localization of proteins, but also opens up the possibility of developing several additional techniques that require the transient or stable transformation of protoplasts.

METHODS AND RESULTS

Protoplastation and transformation

Here, we provide a step-by-step protocol for the extraction and transformation of hornwort protoplasts. In this publication, we use the word protoplastation, which is sometimes also referred to as enzymolysis. Aiming to develop a simple method, we used a protoplastation protocol available for *P. patens* as a starting point (Hohe et al., 2004). We used a liquid culture of *A. agrestis* (Bonn strain) thallus fragments as the starting material, which is the most promising procedure for most plants (Eeckhaut et al., 2013), and achieved a yield of 35,000–65,000 protoplasts$\cdot$mg^{-1} of tissue (dry weight; see Appendix 1). By changing several parameters (e.g., using more tissue, increasing the fragment size, and prolonging the duration of the digestion with Driselase from 45 min [described for *P. patens*] to 12–13 h), we were able to obtain three to six times more viable protoplasts than was previously reported for the

FIGURE 1 Protoplast isolation and regeneration. (A) Thallus fragment after 13 h of digestion with Driselase. (B) Protoplasts in a counting chamber at a concentration of $2.6 \times 10^6 \cdot \text{mL}^{-1}$. (C) *Anthoceros agrestis* protoplast with a typical single chloroplast. (D–F) A protoplast at 4 d post-digestion (dpd) that contains two chloroplasts, potentially entering symmetric cell division. (D) Differential interference contrast (DIC) microscopy image. (E) Red autofluorescence of the two chloroplasts. (F) Merged image of D and E. (G–I) Protoplast containing two chloroplasts at 23 dpd. (G) DIC image. (H) Red autofluorescence of the two chloroplasts. (I) Merged image of G and H. (J–L) A chain of protoplasts, chloroplasts, and potential buds or vesicles at 4 dpd. From the top-right to the bottom-left object, these appear to be an extracellular chloroplast, an intact protoplast, an extracellular or potentially shared chloroplast, an intact protoplast, and a putative vesicle or bud. (J) DIC image. (K) Red autofluorescence of the chloroplasts, with the extracellular chloroplast displaying a reduced autofluorescence. (L) Merged image of J and K. (M–O) Budding protoplast potentially undergoing asymmetric cell division at 23 dpd. The arrow indicates the transition of the protoplast to the bud. (M) DIC image. (N) Red autofluorescence of the shared chloroplast. (O) Merged image of M and N. (P–S) Budding of a protoplast transformed with the L2-MW-AA42-CsA plasmid at 10 d post-transformation. The budding cells share a cell membrane. The arrows indicate the transition to the first bud. (P) DIC image. (Q) Red autofluorescence of the chloroplast located close to the bud. (R) Enhanced green fluorescent protein (eGFP) membrane-localized signal (*AaTip1;1* promoter–driven eGFP fused to the membrane-localization signal Lti6b). (S) Merged image of P, Q, and R. (T–W) Transformed protoplast (plasmid L2-MW-AA42-CsA) and budding cell sharing a chloroplast at 10 d post-transformation. The transition zone is indicated with an arrow. (T) DIC image. (U) Red autofluorescence of the potentially shared chloroplast. (V) eGFP membrane-localized signal. (W) Merged image of T, U, and V. (X–ZII) Protoplast with two chloroplasts (arrows) at 23 d post-transformation with the L2-MW-AA42-CsA plasmid. (X) DIC image. (Y) Red autofluorescence of the two chloroplasts. (ZI) eGFP membrane-localized signal. (ZII) Merged image of X, Y, and ZI. The red autofluorescence was detected using a Leica DM6000B Tx2 filter (excitation 520–600 nm, emission 570–720 nm), and the green fluorescence was detected using an L5 filter (excitation 440–520 nm, emission 505 nm). Black scale bar = 100 μm, red scale bar = 10 μm

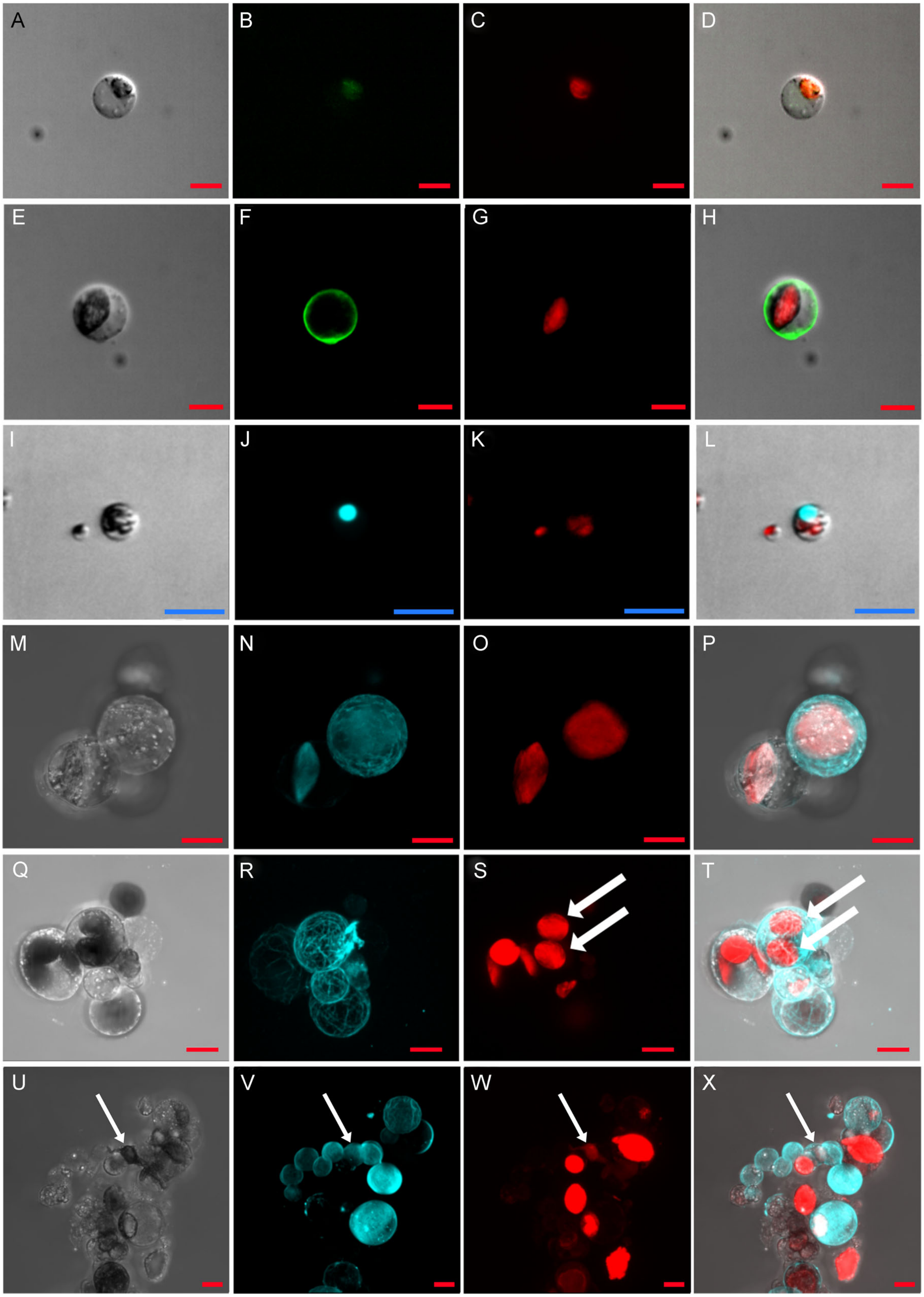

FIGURE 2 (See caption on next page)

hornwort *A. crispulus* (10,000 protoplasts·mg^{-1}) (Binding and Mordhorst, 1991). A detailed protocol for the protoplastation and transient transformation can be found in Appendix 1.

Protoplast regeneration

A major challenge is the regeneration of a whole plant from totipotent protoplasts. Even though gametophytic cells of the hornwort *A. agrestis* typically feature a single chloroplast, protoplasts containing two chloroplasts were present throughout our experiments, from right after the digestion to more than 50 d post-digestion (dpd) (Figure 1). This is an indication of active cell division because chloroplast divisions in hornworts occur right before nuclear, and thus cell, division (Vaughn et al., 1992). Generally, we would expect symmetric cell divisions in the undifferentiated protoplasts, as compared with the regeneration of other plant protoplasts or callus tissue. Although we could only observe evidence of the lead up to symmetric cell division, asymmetric cell division was detected as early as 4 dpd. Asymmetric cell divisions have been observed during regeneration in other plant protoplasts (Cove et al., 1996; Wiszniewska and Piwowarczyk, 2014). We could also observe two chloroplasts per cell, as well as budding, in transiently transformed protoplasts (Figure 1). The regeneration of a functional cell wall could be observed starting at 48 h post-digestion (Figure 2). We tested for the presence of a cell wall by staining protoplasts that were 5, 24, and 48 h old with Calcofluor white (CFW) which was only successful for the 48-h-old protoplasts. The observed regeneration is slower than the 12 h stated for *A. punctatus* (Ono et al., 1992), and might be the result of the 3 dpd incubation in the dark used in this protocol. The functionality of the cell wall was tested by staining for several components of the cell wall. Furthermore, we observed that protoplasts regenerating cellulose microfibrils (visualized using CFW) did not let the membrane stain FM 1-43 pass through. This was not the case for shriveled and potentially dead protoplasts (data not shown).

TABLE 1 Survival rate of *Anthoceros agrestis* (Bonn strain) protoplasts under various regeneration conditions. The relatively low survival rate is partly due to protoplasts that did not survive the isolation procedure. The initial survival rate right after the isolation was not estimated

Treatment	Survival rate after 9 d (SD)[a]
Control, Ø 6 cm Petri dish	48% (6.1%)
2 mM 2,4-D, Ø 6 cm Petri dish	65% (6.1%)
6 mM 2,4-D, Ø 6 cm Petri dish	58% (11.1%)
10 mM 2,4-D, Ø 6 cm Petri dish	60% (7%)
Micropore tape Ø 2 cm Petri dish	52% (9.2%)
Parafilm, Ø 2 cm Petri dish	56% (7.6%)

Note: 2,4-D = 2,4-dichlorophenoxyacetic acid.

[a] Survival rate is reported as the mean (standard deviation) estimated by counting three biological replicates with ≥100 protoplasts, respectively.

When the protoplasts were left undisturbed in glass tubes for ~50 d, clusters of protoplasts with cell walls could be observed (Figure 2). We tested several conditions and treatments, such as different light conditions after digestion, several protoplast densities in both liquid culture and solid media (BCD medium [Cove et al., 2009] or regeneration medium, solidified with 1% Gelrite [ref. G1101.0500; Duchefa Biochemie, Haarlem, the Netherlands]), as well as supplementing the regeneration medium with the auxin analog 2,4-dichlorophenoxyacetic acid to enhance protoplast regeneration, with the results summarized in Table 1. Although slight improvement could be achieved, none of these treatments considerably increased the protoplast regeneration rates.

It must be noted that the regeneration of protoplasts is difficult not only in this model plant, but is a challenge in most plant species (Eeckhaut et al., 2013). Further assessment of the regeneration parameters will improve protoplast viability and thus lead to the regeneration of fully functional plants. Multiple studies have assessed the

FIGURE 2 Transformed and untransformed protoplasts regenerating their cell walls. (A–D) Non-transformed protoplast 4 d post-transformation. (A) Differential interference contrast (DIC) microscopy image. (B) Weak autofluorescence of the chloroplast detected in the filter for eGFP fluorescence. (C) Red autofluorescence of the chloroplast. (D) Merged image of A, B, and C. (E–H) Protoplast at 4 d after transformation with the L1-AA026-Ck2 plasmid. (E) DIC image. (F) *AaTip1;1* promoter–driven eGFP fused to the membrane localization signal Lti6b. (G) Red autofluorescence of the chloroplast. (H) Merged image of E, F, and G. (I–L) Protoplast at 5 d after transformation with the L1-AA016-Ck3 plasmid. (I) DIC image. (J) *AaEf1α* promoter–driven mTurquoise2 fluorescent protein fused to the nuclear localization signal N7. (K) Red autofluorescence of the chloroplasts. (L) Merged image of I, J, and K. (M–P) Regeneration of the hornwort protoplast cell wall at 2 d post-digestion (dpd). The protoplast on the right shows a cellulose layer stained by Calcofluor white (CFW), while the protoplast on the left does not have a cell wall. (M) DIC image. (N) Cellulose in the cell wall stained with CFW. The protoplast to the left is degrading and its chloroplast is emitting a signal indicating the presence of chlorophyll products. (O) Red autofluorescence of the chloroplasts. (P) Merged image of M, N, and O. (Q–T) Different stages of the regeneration of the cell wall component cellulose at 2 dpd. The large protoplast in the center has two chloroplasts. (Q) DIC image. (R) Cellulose in the cell wall stained with CFW (shown in turquoise). (S) Red autofluorescence of the chloroplasts (arrows). (T) Merged image of Q, R, and S. (U–X) Cell wall regeneration at 52 dpd. In this image, at least four protoplasts share a chloroplast (indicated by the arrows). (U) DIC image. (V) Cellulose in the cell wall stained with CFW. (W) Red autofluorescence of the chloroplasts with the arrows marking the shared chloroplast. (X) Merged image of U, V, and W. The red fluorescence in C and G was detected using a Leica DM6000B Tx2 filter (excitation 520–600 nm, emission 570–720 nm) and the green fluorescence in B and F was detected using an L5 filter (excitation 440–520 nm, emission 505 nm). The red fluorescence in K was detected using a DSR ET filter (excitation 530–560 nm, emission 590–650 nm) and the green fluorescence in J was detected using an ET GFP filter (excitation 450–490 nm, emission 500–550 nm). The images of the cell wall regeneration were taken using a Leica TCS SPE. Blue scale bar = 50 µm, red scale bar = 10 µm conditions for successful protoplast regeneration, e.g., phytohormone supplements, adjusting protoplast densities, or immobilizing the protoplasts in agar beads or layers of agar (Schween et al., 2003; Pati et al., 2005; Wiszniewska and Piwowarczyk, 2014). We note that not all forms of regeneration treatment have been tested or evaluated thoroughly in the present study, and should be explored in future studies.

Transient transformation

As a high number of protoplasts were achieved, a transient transformation can be performed successfully. We tested the DNA delivery into *A. agrestis* protoplasts using both polyethylene glycol (PEG) with gentle mixing over a longer period (30 min) and PEG combined with a heat-shock treatment (10 min incubation with PEG at room temperature followed by 3 min at 45°C). Our results suggest that PEG-mediated DNA delivery alone is preferable over the frequently used heat shock–mediated approach, which drastically decreased the number of viable protoplasts (results of this comparison not shown). Using the 13,407-bp L2-MW-AA42-CsA plasmid (Appendix 1), we achieved a 10% protoplast-transformation rate using the PEG-mediated approach.

CONCLUSIONS

We established an easy-to-use method for isolating and transiently transforming *A. agrestis* hornwort protoplasts that is simpler than earlier laborious approaches and yields a high number of viable protoplasts. In addition to being the first report on hornwort protoplasts in 30 years, this study provides new insight into the cell wall regeneration of hornwort protoplasts, by showing that the cell wall regenerates between 24 and 48 h post-digestion, and that the cellulose rebuilds as a tight mesh of microfibrils that eventually becomes impenetrable for the cell membrane staining color FM 1-43 after 48 h. Furthermore, this protocol opens up new avenues for simple assays of protein–protein interaction, protein localization, protein function, and for CRISPR/Cas9 editing in the model organism *A. agrestis*. We are currently focusing on developing an efficient method for the regeneration of *A. agrestis* protoplasts, which is crucial for CRISPR/Cas9 editing.

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AUTHOR CONTRIBUTIONS

P.S. coordinated the project. A.N. wrote the manuscript. P.S., E.F., A.B., S.I.N., and S.W. revised the manuscript. F.-W.L. and P.S. carried out preliminary protoplastation trials. A.N., S.R., and M.W. carried out the isolation and transformation of protoplasts. A.N. obtained the microscopy images. A.B. and A.N. produced the cell wall staining images. E.F. and M.W. developed the plasmids. All authors approved the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The plasmid files can be found in the supplementary material.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

Appendix S1. L1_AA016-Ck3 plasmid sequence.

Appendix S2. L1_AA026-Ck2 plasmid sequence.

Appendix S3. L2-MW_AA42-Csa plasmid sequence.

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Appendix 1. Detailed protocol to isolate and transiently transform protoplasts of the model hornwort *Anthoceros agrestis*

General plant culture

Approximately 150 mg (fresh weight) of *Anthoceros agrestis* (Bonn strain) tissue was collected from gametophytes grown on a Petri dish, fragmented into 4-mm^2 pieces using tweezers, then cultivated in 200 mL of liquid medium (BCD medium; Cove et al., 2009) in 500-mL Erlenmeyer flasks sealed with a sponge plug (Type C-40, Silicosen silicone sponge plugs; Hirschmann Laborgeräte, Eberstadt, Germany). The plant cultures were placed on an orbital shaker (150 rpm; SHKE2000-1CE; Thermo Fisher Scientific, Waltham, Massachusetts, USA) and grown at 23°C under continuous light (17–20 μmol·s^{-1}·m^{-2}, as measured using an LM2-1137 light meter with a Quantum Q37008 sensor [LI-COR Biosciences, Lincoln, Nebraska, USA]). The liquid culture medium was replaced one week after the start of the culture and subsequently every 2–4 weeks depending on the tissue density; the more plant material per flask, the more often the medium was changed. Before protoplastation, the plants were grown in the liquid cultures for a minimum of two weeks and at a density of 40 mg of tissue (dry weight; mean of four samples, air-dried for two days, which corresponds to ~1.1 g fresh weight) per Erlenmeyer flask.

Detailed protoplast isolation and transformation protocol

In this section, we provide a detailed description of the protocol for general use. Unless otherwise stated, all steps should be performed at room temperature (22°C) under sterile conditions in a laminar flow hood (Safe 2020 Biological Class II Safety Cabinets; ref. 51027655; Thermo Fisher Scientific).

Preculture

The pH and density of the *A. agrestis* liquid culture should be adjusted 7 d prior to tissue collection and protoplastation. The tissue is fragmented into approximately 4-mm^2 fragments until ~3/4 of a cell strainer (ref. 93100; Bioswisstec ECO Cell Strainer 100 µm; Bioswisstec, Schaffhaussen, Switzerland) is filled (equivalent to 1.0–1.2 g fresh weight or 30–40 mg dry weight) and transferred into 200 mL of BCD medium (pH 4.0) in a 500-mL flask sealed with a sponge plug. After 4 d, the tissue is transferred into fresh medium (pH 4.0). All transformation media should be prepared fresh as the efficiency decreases when using media more than 1–2 weeks old.

Protoplastation media preparation

All media were filter-sterilized using a vacuum-driven filtration system (Stericup Quick Release, S2GPU05RE; Merck, Darmstadt, Germany).

0.5 M mannitol (91.2 g·L^{-1} mannitol [ᴅ-mannitol]; ref. M1902-1KG; Sigma-Aldrich, St. Louis, Missouri, USA), pH 5.6–5.8; add 7.6 g mannitol to adjust the osmolarity to 560 mOsm).

3 M medium (3.04 g·L^{-1} $MgCl_2$ [15 mM]; 1 g·L^{-1} MES [0.1% w/v; ref. M2933-500G; Sigma-Aldrich], 87.4 g·L^{-1} mannitol [pH 5.6]; add 7 g mannitol to adjust the osmolarity to 580 mOsm).

Regeneration medium (50 g·L^{-1} glucose; 30 g·L^{-1} mannitol, in BCD medium [pH 5.8]; add 28 g mannitol to adjust the osmolarity to 540 mOsm).

Digestion

For the digestion, incubate the tissue of one flask in 12 mL of 0.5 M mannitol in a Petri dish on an orbital shaker (model MSR 8270349; Adolf Kuehner, Birsfelden, Switzerland) at 100 rpm for 30 min. In the meantime, prepare the Driselase (0.04 g·mL^{-1} Driselase; ref. D9515-5G; Sigma-Aldrich) in 0.5 M mannitol solution, incubate for 30 min on a rocking shaker (model M33120; Barnstead/Thermolyne, Dubuque, Iowa, USA), centrifuge at 3500 rpm for 1 min, and filter-sterilize. Add a 4-mL aliquot of the Driselase solution (end concentration 1% w/v) to the hornwort tissue, and cover the Petri dishes to prevent exposure to light during incubation on the orbital shaker for 10–12 h (Figure A1).

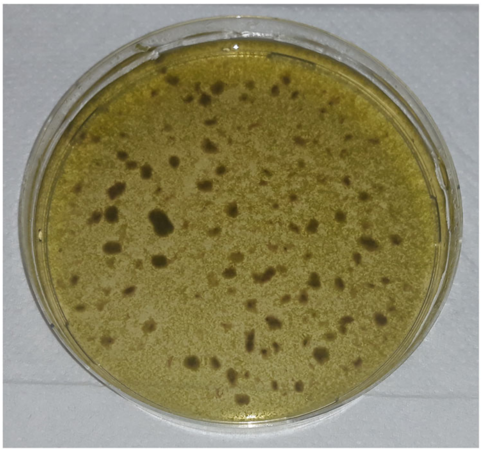

FIGURE A1 Petri dish filled with *Anthoceros agrestis* tissue after a 12-h incubation with Driselase

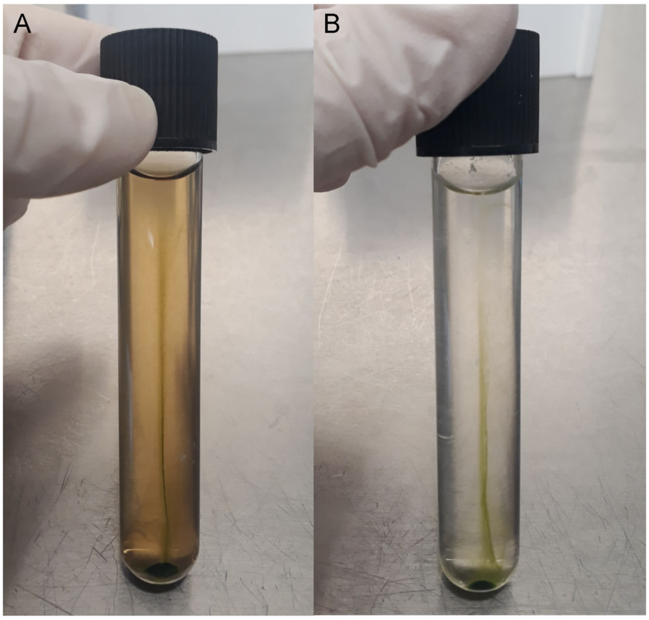

FIGURE A2 Protoplast yield before (left) and after (right) the first washing step

Harvesting protoplasts

The protoplasts must be handled carefully during the following steps, and pipetting should be performed slowly as the protoplasts are sensitive to mechanical damage. We used a 1-mL pipette tip with the tip removed (ca. 2 mm cut off from the end) or a wide-neck glass pipette. Pipette the protoplast solution through a 70-µm sieve into a 50-mL Falcon tube by placing the pipette tip on the mesh rather than shooting the solution through. Wash the Petri dish twice with 3 mL of 0.5 M mannitol by tilting the Petri dish and rinsing it 3–4 times for each wash (Figure A2). Divide the filtrate (ca. 10 mL each, topped up with 0.5 M mannitol if needed) into two glass tubes with screw caps (ref. HM-1000-1; Hopfen und Mehr, Neukirch, Germany) then centrifuge at low speed for 15 min using the lowest ramp up and down speeds (600 rpm, acceleration 3, brake 3; Mikro 220 R Centrifuge; ref. 2205; Andreas Hettich, Tuttlingen, Germany) to avoid damaging the protoplasts.

Discard the supernatant and carefully resuspend each pellet in 10 mL of 0.5 M mannitol by gently running the solution down the side of the tube. Slowly tilt the tube to a 40° angle, then gently roll the tube in the palm of your hands until all the protoplasts are resuspended and no longer form clumps. Centrifuge the tubes at 600 rpm for 15 min and discard the supernatant, then carefully resuspend each pellet in 5 mL of 0.5 M mannitol and combine the samples from both tubes into one, either using cut pipette tips or by slowly allowing the sample to flow from one tube into the other. Estimate the number of protoplasts using a hematocytometer (Neubauer advanced counting chamber; ref. 0640110; Paul Marienfeld, Lauda-Königshofen, Germany), and then centrifuge at 600 rpm for 15 min. Discard the supernatant and resuspend the protoplasts in 3 M medium to a concentration of 1.2×10^6 protoplasts·mL^{-1}.

Transformation

Dissolve an aliquot of 2.5 g PEG 4000 (ref. 81242-1KG; Sigma-Aldrich) in 3.5 mL of 3 M medium (sufficient for 10 transformations) and store at 30°C. The solution should be filter-sterilized before use (MS sterile syringe filter, PES 0.22 μm, SFPES030022S; Membrane Solutions, Auburn, Washington, USA). For each reaction, prepare the plasmid DNA in H_2O (to a concentration of 35 μg/100 μL to 50 μg/300 μL, depending on the plasmid) in a glass tube, and add 250 μL of the adjusted protoplast solution. Add a 350-μL aliquot of PEG solution and mix by rolling the tube carefully at a 45° angle; then incubate the samples for 30 min, rolling the tubes every 5 min for at least 1 min. The transformation mix should be diluted every 5 min by adding first 1 mL, then 2 mL, then 3 mL, and finally 4 mL of 3 M medium, and each time the tube should be rolled for about 1 min to mix. Centrifuge the samples at 800 rpm for 15 min (acceleration 3, brake 3), then discard the supernatant and resuspend the protoplasts in 4 mL of regeneration medium (mix by rolling the tube). Distribute a 2-mL aliquot of the protoplast solution into two wells of a six-well cell culture plate (ref. 657 160; Greiner Bio-One, Kremsmünster, Austria) or four wells of a 12-well cell culture plate (ref. 665 180; Greiner Bio-One). Wrap the plates with Parafilm (PM-996, Bemis Parafilm M Laboratory Wrapping Film; Bemis Company, Neenah, Wisconsin, USA) or with 3 M micropore tape (PZN-01319732; 3 M Science, Saint Paul, Minnesota, USA).

Regeneration

Incubate the obtained protoplasts in solution (regeneration medium) for 72 h in darkness and then place back into standard light conditions (8–9 μmol·s^{-1}·m^{-2}) or into a growth chamber (Versatile Environmental Test Chamber; MLR-351H; Sanyo Electric, Osaka, Japan; 13–18 μmol·s^{-1}·m^{-2}, 60% humidity, 21°C).

List of plasmids tested

The tested plasmids are listed in Table A1. The constructs were generated using DNA parts and vectors from the OpenPlant kit (Sauret-Güeto et al., 2020) and the *AaEf1α* and *AaTip1;1* promoter L0 parts (Frangedakis et al., 2021b).

Plasmid maps

The maps (Figure A3) were created using SnapGene Viewer (version 5.3.2; GSL Biotech, Chicago, Illinois, USA).

Cell wall staining

Calcofluor white M2R (Sigma-Aldrich; 1 mg·mL^{-1} in water) was mixed with the protoplasts in a 1:10 ratio, either directly on a microscopy slide or in a 96-well plate.

Microscopy information

Microscopy images were acquired using a Leica DM6000 B microscope (Leica Microsystems, Wetzlar, Germany), a Leica Thunder M205 FCA (Leica Microsystems), and a Leica TCS SPE DM5500Q confocal microscope (Leica Microsystems). The images were processed using LAS X (version 3.3.0.16799; Leica Microsystems), ICY (de Chaumont et al., 2012), and Affinity Photo (version 1.9.2.1035; Serif, West Bridgford, United Kingdom).

TABLE A1 Details of the plasmids used

Plasmid name	Promoter	Localization tag	Fluorescence marker
L2-MW-AA42-CsA	*AaTip1;1*	Membrane	eGFP
L1-AA026-Ck2	*AaTip1;1*	Membrane	eGFP
L1-AA016-Ck3	*AaEf1α*	Nucleus	mTurquoise2

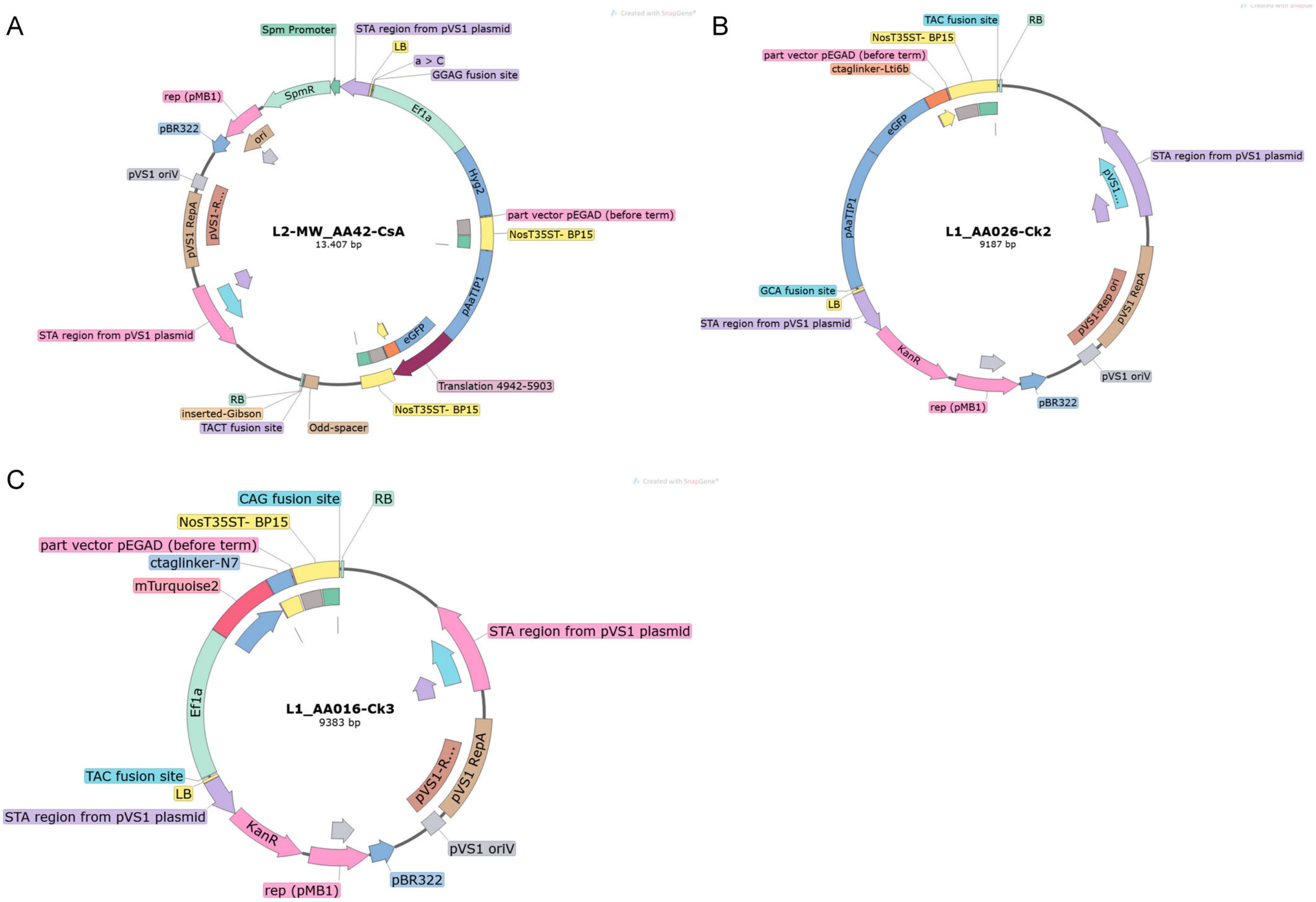

FIGURE A3 Plasmid maps of the plasmids used to transiently transform the hornwort protoplasts and report a fluorescent signal. The plasmid L2-MW_AA42-CsA (A) and L1-AA026-Ck2 (B) contain a membrane-localized eGFP marker that is expressed by the *AaTip1;1* promoter. (C) The plasmid L1-AA016-Ck3 contains a AaEf1α-driven mTurquoise2 reporter fused to a nuclear localization signal