

METHODOLOGY

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# An efficient protocol for perennial ryegrass mesophyll protoplast isolation and transformation, and its application on interaction study between LpNOL and LpNYC1

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## Abstract

**Background:** Perennial ryegrass (*Lolium perenne* L.) is an important temperate grass used for turf and forage purposes. With the increasing accumulation of genomic and transcriptomic data of perennial ryegrass, an efficient protoplast and transient gene expression protocol is highly desirable for in vivo gene functional studies in its homologous system.

**Results:** In this report, a highly efficient protoplast isolation ( $5.6 \times 10^7$  protoplasts per gram of leaf material) and transient expression (plasmid transformation efficiency at 55.2%) was developed and the detailed protocol presented. Using this protocol, the subcellular locations of two ryegrass proteins were visualized in chloroplasts and nuclei, respectively, and protein–protein interaction between two chlorophyll catabolic enzymes (LpNOL and LpNYC1) was recorded in its homologous system for the first time.

**Conclusion:** This efficient protoplast isolation and transformation protocol is sufficient for studies on protein subcellular localization and protein–protein interaction, and shall be suitable for many other molecular biology applications where the mesophyll protoplast system is desirable in perennial ryegrass.

**Keywords:** Ryegrass, *Lolium*, Protoplast, Transient gene expression

## Background

Perennial ryegrass (*Lolium perenne* L.) is the most widely distributed and cultivated turf and forage grass in temperate zones. World-wide concerted programs are working on the molecular genetics of this grass species. The draft genome of this perennial ryegrass was recently published [1] and the assembly of the genome dataset is undergoing. The accumulation of genomic and transcriptomic datasets provided unprecedented opportunity

to conduct functional genomic studies in perennial ryegrass. Stable genetic transformation systems have been established in perennial ryegrass, yet the whole transformation takes months to obtain rooted transgenic plants [2, 3].

The plant protoplast system provides a complementary or, sometimes, an alternative way to the stable genetic transformation system for gene functional analysis in many cases, such as protein subcellular localization, in vivo protein–protein and protein–DNA interactions, protein trafficking and signal transduction, etc. Currently, mesophyll protoplast-based transient expression assays are routinely used in biological studies in *Arabidopsis* (*Arabidopsis thaliana*) [4, 5], maize (*Zea mays*) [4], tobacco (*Nicotiana tabacum*) [6], rice (*Oryza sativa*)

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[7, 8], *Populus* [9, 10], and cucumber (*Cucumis sativus*) [11]. Successful ryegrass protoplast isolation from mesophyll cells has been reported previously and was used in the study on chloroplast photosynthetic and photorespiratory carbon metabolism [12]. However, its efficiency remains low and not sufficient enough for protoplast transformation studies according to our preliminary experimental results following the previously published one (data not shown). An efficient transient expression system based on ryegrass mesophyll protoplast transformation was not reported yet.

In this study, a highly repeatable and efficient protocol for mesophyll protoplast isolation and gene transient expression was developed using ryegrass leaves as starting materials. This protocol provides a facile tool for protein subcellular localization and bimolecular fluorescence complementation (BIFC) assays as shown in this study as well as the other in vivo molecular studies where this system is applicable.

## Methods

### Plant material and growth conditions

Perennial ryegrass (cv. Buena vista) was grown in vermiculite: perlite: peat moss (1:3:9) in a growth chamber with temperature set at 25/20 °C (day/night), photosynthetically active radiation (PAR) of 750  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  and 14 h of light per day. The potted plants were watered at about three-day intervals and fertilized with 1/2 MS minerals (Murashige and Skoog 1962) once a week. It is CRITICAL to water and fertilizer ryegrass plants regularly to obtain fine starting plant material.

### Reagents and solutions

Recipes for the enzyme solution, Modified W5 solution, MMg solution, PEG-Ca<sup>2+</sup> solution were listed in Table 1. Cellulase 'Onozuka' R-10 (Cat. No. BS197A) and macerozyme R-10 (Cat. No. YM-10-1 g) were purchased from Yakult Pharmaceutical Ind. Co., Ltd., Japan. Noting that the enzyme solution was thermally pretreated at 55 °C

for 10 min to inactivate nonspecific enzymes before the addition of BSA, CaCl<sub>2</sub> and KCl solutions.

PEG-4000 (Cat. No. 25322-68-3), D-Mannitol (Cat. No. DH190-2), and Bovine Serum Albumin (BSA) (Cat. No. 9048-46-8) were purchased from Bei Jing Ding Guo Chang Sheng Biotech Co., Ltd., China; MES (Cat. No. E169) was from Ameresco LLC, USA; CaCl<sub>2</sub>·2H<sub>2</sub>O (Cat. No. 10035-04-8) was from Xi long Chemical Co., Ltd., China; and KCl (Cat. No. 7447-40-7), NaCl (Cat. No. 7647-14-5), and MgCl<sub>2</sub>·6H<sub>2</sub>O (Cat. No. 7791-18-6) were from Sinopharm Chemical Reagent Co., Ltd., China.

### Protoplast isolation

Fully expanded leaves (about 10–12 days after leaf emergence) were collected from healthy ryegrass plants and only the middle sectioned leaves were used as the starting material (the tips and bases of leaf blades were removed). A total of 0.4–0.6 g of leaf sections were cut into 0.5 mm strips transversely with sharp razors wetted with the enzyme solution, and the leaf strips, once cut off, were immediately emerged into the 6 ml enzyme solution (Table 1). The leaf strips were then vacuumed (0.1 MPa) for 1 h under dark at room temperature. The enzymatic digestion was carried out in a thermal-stable shaker at 30 rpm, 28 °C in dark for about 6 h to the extent that the leaf strips were readily disassembled upon gentle touches with a pipette tip.

The suspended protoplasts were firstly filtered through a layer of cheese clothes and then through a nylon mesh (75  $\mu\text{m}$ ), and the residue was washed twice with 10 ml pre-chilled modified W5 solution (Table 1). The filtrate was centrifuged at 100 $\times$ g for 2 min with low acceleration and deceleration speed (BECKMAN, Model Allegra 64R, Hamburg, Germany). The pelleted protoplasts were re-suspended in 10 ml pre-chilled modified W5 solution, and set still for about one hr on ice (or overnight at 4 °C) to allow protoplast to sediment. The precipitated protoplasts were re-suspended in 3 ml MMg solution (Table 1). According to the counted number of viable protoplasts, the suspension was centrifuged down at 100 $\times$ g for 2 min

**Table 1** Solution recipes for protoplast isolation and transformation

Solution name	Solution composition	Storage	Usage
Enzyme solution (resuspension solution)	10 mM MES, 1.5% (wt/vol) cellulase R10, 0.75% (wt/vol) macerozyme R10, and 20 mM KCl. 10 mM CaCl <sub>2</sub> , 0.1% BSA, 1–5 mM $\beta$ -mercaptoethanol (optional) and mannitol (0.6 M), pH 5.7	Room temp. (freshly prepared)	Leaf strips lysis
W5 solution	2 mM MES, 154 mM NaCl, 125 mM CaCl <sub>2</sub> and 5 mM KCl, pH 5.7	4 °C	Release and wash protoplasts
MMg solution	4 mM MES, 0.4 M mannitol and 15 mM MgCl <sub>2</sub> , pH 5.7	4 °C	Resuspend protoplast pellet
PEG-Ca <sup>2+</sup> solution (resuspension solution)	20% (wt/vol) PEG4000, 100 mM CaCl <sub>2</sub> and mannitol (0.3 M)	Room temp. (freshly prepared)	Transform plasmids (10ug is used in this study) into protoplasts

and re-suspended in the MMg solution to a final concentration of  $\sim 5 \times 10^5$  cells  $\text{ml}^{-1}$  (depending on experimental purposes, the concentration can be readily achieved up to  $7.3 \times 10^6$  cells  $\text{ml}^{-1}$ ).

Noting that the pipette tips used in transferring protoplasts should be cut with scissors to minimize mechanical damage.

### Vector construction and plasmid preparation

One chloroplast-localized protein LpPPH [13] and one unnamed NAC transcription factor LpNACx (unpublished result) were used for the subcellular localization test. In brief, the *LpPPH* and *LpNACx* genes were inserted into the p2GWF7.0 vector [14] in fusion with a GFP tag at the C-terminal. For bimolecular fluorescence complementation (BiFC) test, putative *LpNOL* and *LpNYC1* genes were amplified from perennial ryegrass (unpublished), and cloned into a pair of split citrine vectors (pN-citrine-GW and pC-citrine-GW) to generate fusion proteins with citrine N-terminal or with citrine C-terminal tags. The pair of BiFC vectors were constructed in Dr. Bingyu Zhao's lab at Virginia Tech: the pN-citrine-GW vector was generated using the backbone of p2GW7.0 [15] with an insertion of citrine-N-terminal with a T7 tag in the C terminal of a GATEWAY ccdB cassette(B) which would result in a translational fusion of target protein and citrine-T7 after LR clonase recombination (Invitrogen, Carlsbad, CA); likewise, the pC-citrine-GW vector was constructed with the insertion of citrine-C-terminal with HA tag. The primers used in gene cloning were presented in Table 2. The constructed vectors were electro-transformed into *E. coli* T1 cells (Invitrogen) and the plasmids were extracted using a commercial midi-prep kit (TIANpure plasmid kit II, TianGen Co., Beijing, China) to yield quality DNAs ( $>300$  ng  $\text{ul}^{-1}$ ).

**Table 2 Primers used in gene cloning and vector construction**

Primer name	Sequence (5'–3')
<i>LpPPH</i> forward	ATCAGGAATTCATGGAAGTGGTTTCCTCCAG
<i>LpPPH</i> reverse	AACCGTCGACAGACTACCCGTATGTTGGAG
<i>LpNOL</i> forward	ATTAGGGATCCATGCCACCGTCGCCGCC
<i>LpNOL</i> reverse	TATTGAAGCTTATCCTCAGTAACATACTGTTCCTACG
<i>LpNYC1</i> forward	AACCAGGATCCATGCCGCCCGCGTCCGTC
<i>LpNYC1</i> reverse	AATGAAAGCTTTGTGCCAGGAAAGTCCAC
<i>NAC</i> forward	TTCATGGATCCATGCCACTGCCGCTTCC
<i>NAC</i> reverse	TATAGAAGCTTCTGCCGCGCTGCCGCCG
35S forward	AACGGATCCGGTACCCATGGAGTCAAAGATTCAAATAG
35S reverse	AACAAGCTTAGTCCCCGTTGTTCTCT

### PEG-mediated protoplast transformation

The transient gene expression system using ryegrass protoplasts was modified from a protocol reported by Yoo et al. [5]. The optimal amount of plasmid (from 1 to 10  $\mu\text{g}$ ) and concentration of mannitol (from 0.1 to 0.3 M) were tested. In brief, plasmids were mixed with 200  $\mu\text{l}$  protoplast stock at RT, adding equal volume of PEG- $\text{Ca}^{2+}$  medium (Table 1), gently mixed and setting still for 5 min at RT for plasmid transformation. Then, three ml W5 solution was added slowly and gently mixed with the protoplast suspension, followed by a centrifugation at  $100\times g$  for 2 min and the pellet was re-suspended gently in 0.5 ml of W5 solution. Finally, the protoplast suspension was transferred into 1.5 ml tubes that were pre-coated with 1% BSA to avoid protoplast attachment to the tube surface, and incubated for 10–24 h in dark at RT.

### Microscopy

The density and viability of isolated protoplasts were counted by using the standard hemocytometer and FDA staining assay [16] and observed under a light microscope (OLYMPUS Model BX53, Tokyo, Japan).

Transformed protoplasts were observed with a confocal laser scanning microscope (Zeiss LSM780 Exciter) for GFP, CFP (citrine), and chloroplast auto-fluorescence with the excitation wavelengths and emission filters set at 488 nm/band-pass 505–530 nm for GFP, 458 nm/band-pass 465–530 nm for CFP, and 488 nm/band-pass 650–710 nm for chloroplast auto-fluorescence. Image processing was performed using the Volocity software (Zeiss).

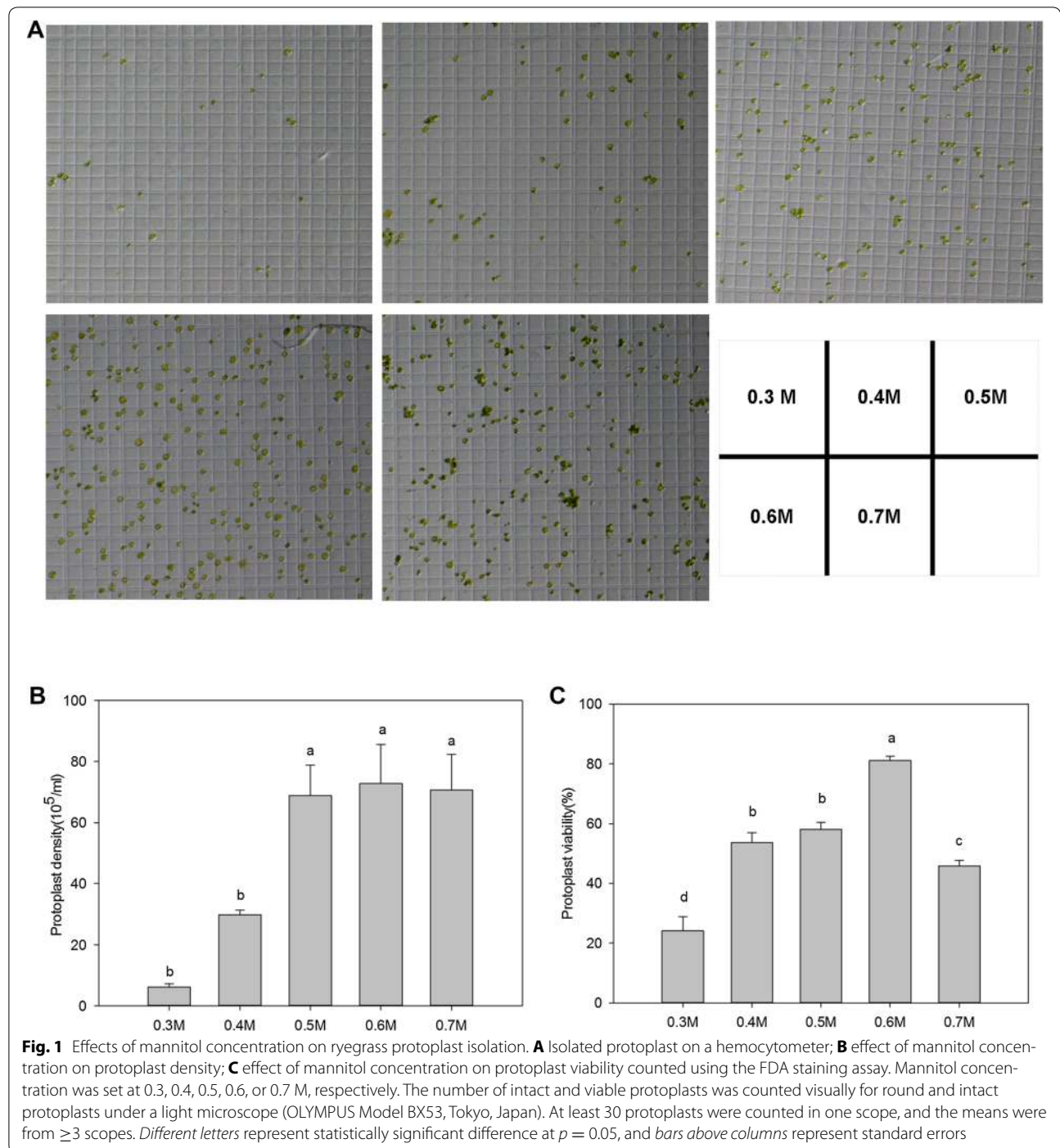
## Results and discussion

### Protoplast isolation from mesophyll cells of perennial ryegrass

Selecting the proper starting leaf material was critical for the whole protocol. Using unselective green leaves (the 1st to the 3rd leaves from the top) for the protoplast isolation and transient gene expression assay (e.g. for the detection of subcellular localization) yielded inconsistent results. While consistent result was only achieved with the newly and fully expanded leaves (the 2nd leaf from the top which is  $\sim 10$ – $12$  days after leaf emergence) from healthy plants. The optimum concentrations of cellulose R-10 and macerozyme R-10 used in this study on ryegrass were the same as those used in rice [8], maize [4] and wheat [17]. The concentration of mannitol in the enzyme solution was critical for integrity of isolated protoplasts [18]. After testing a series of mannitol concentrations in the enzyme solution (1.50% cellulose R-10 and 0.75% macerozyme R-10), we found

that mannitol at 0.6 M lead to the highly protoplast isolation efficiency ( $5.6 \times 10^7$  protoplasts per gram; Fig. 1) and protoplast viability (82.8%) after enzyme digestion (Fig. 1). After further removal of broken cell debris using centrifugation, the re-suspended protoplast

density can be readily achieved at  $7.3 \times 10^6$  protoplasts per ml (Fig. 1). Compared to the previous ryegrass protoplast isolation protocol [12], the current protocol used different digestive enzyme mix and mannitol concentration with optimized experimental procedures (see notes



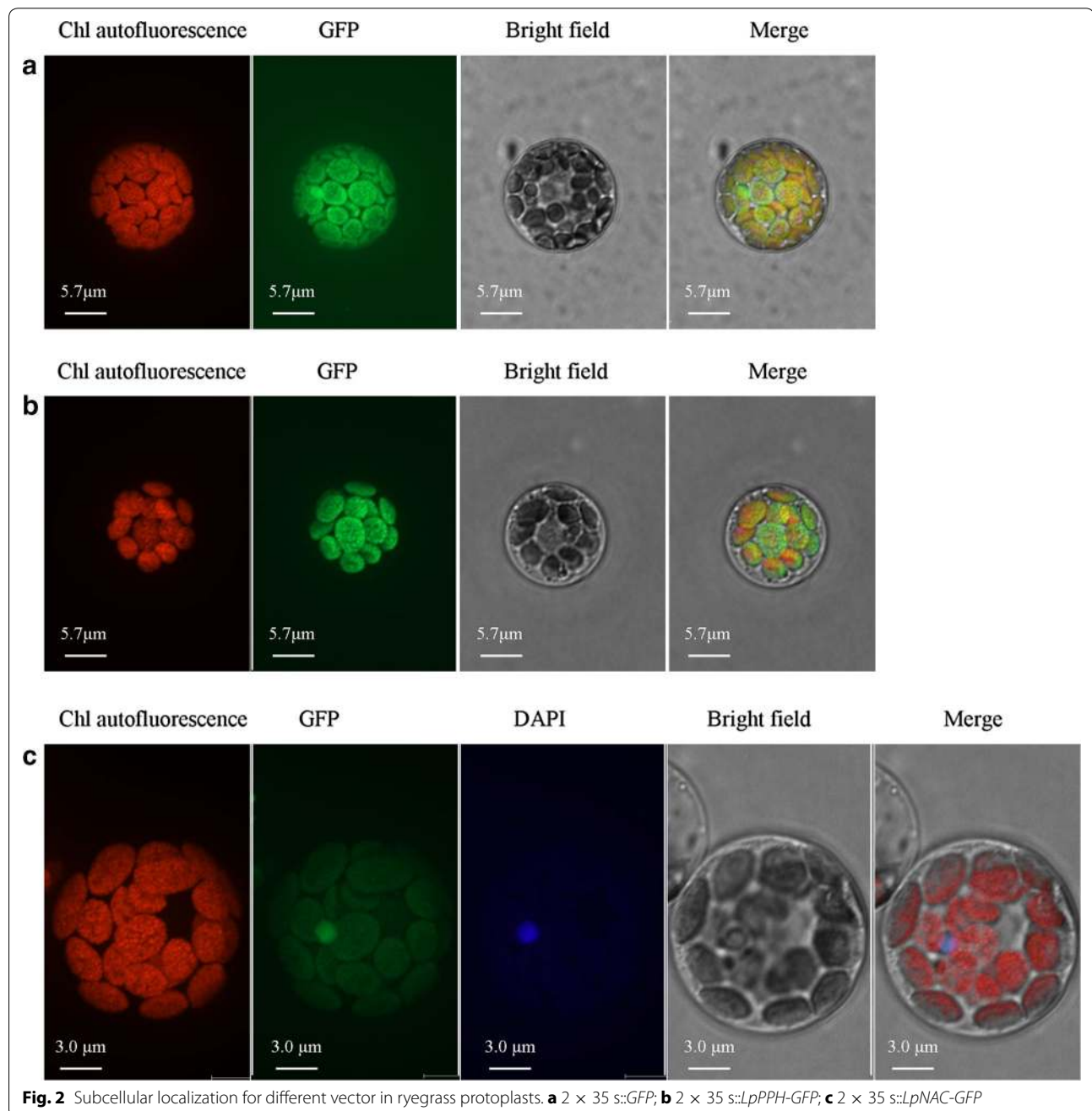
and details in “Methods” section), which were all indispensable factors for successful and efficient ryegrass protoplast isolation.

#### PEG-mediated protoplast transformation

The isolated protoplasts were subjected to PEG-mediated transformation. After adjusting variable parameters, we achieved up to 55.2% transformation efficiency with 10  $\mu\text{g}$  plasmid DNA (plasmid size  $\sim 10$  kb) prepared

with a regular plasmid midi-prep kit. Higher protoplast transformation efficiency could be achieved using higher amount or purity (e.g. with CsCl purified plasmid DNA) of plasmid DNA [5]. The mannitol concentration in the PEG- $\text{Ca}^{2+}$  solution was also critical for the transformation efficiency that the highest efficiency was achieved at 0.3 M mannitol (data not shown).

The developed protocol can be efficiently applied to protein subcellular localization and BiFC assays. For



examples, LpPPH, a ryegrass chlorophyll catabolic enzyme, was previously shown to localize in chloroplast using *Arabidopsis* protoplast [13]; using the current protocol, we were able to confirm its subcellular localization in its homologous system (Fig. 2). In another experiment, a putative ryegrass NAC transcription factor was shown localized in the nucleus in ryegrass protoplast (Fig. 2).

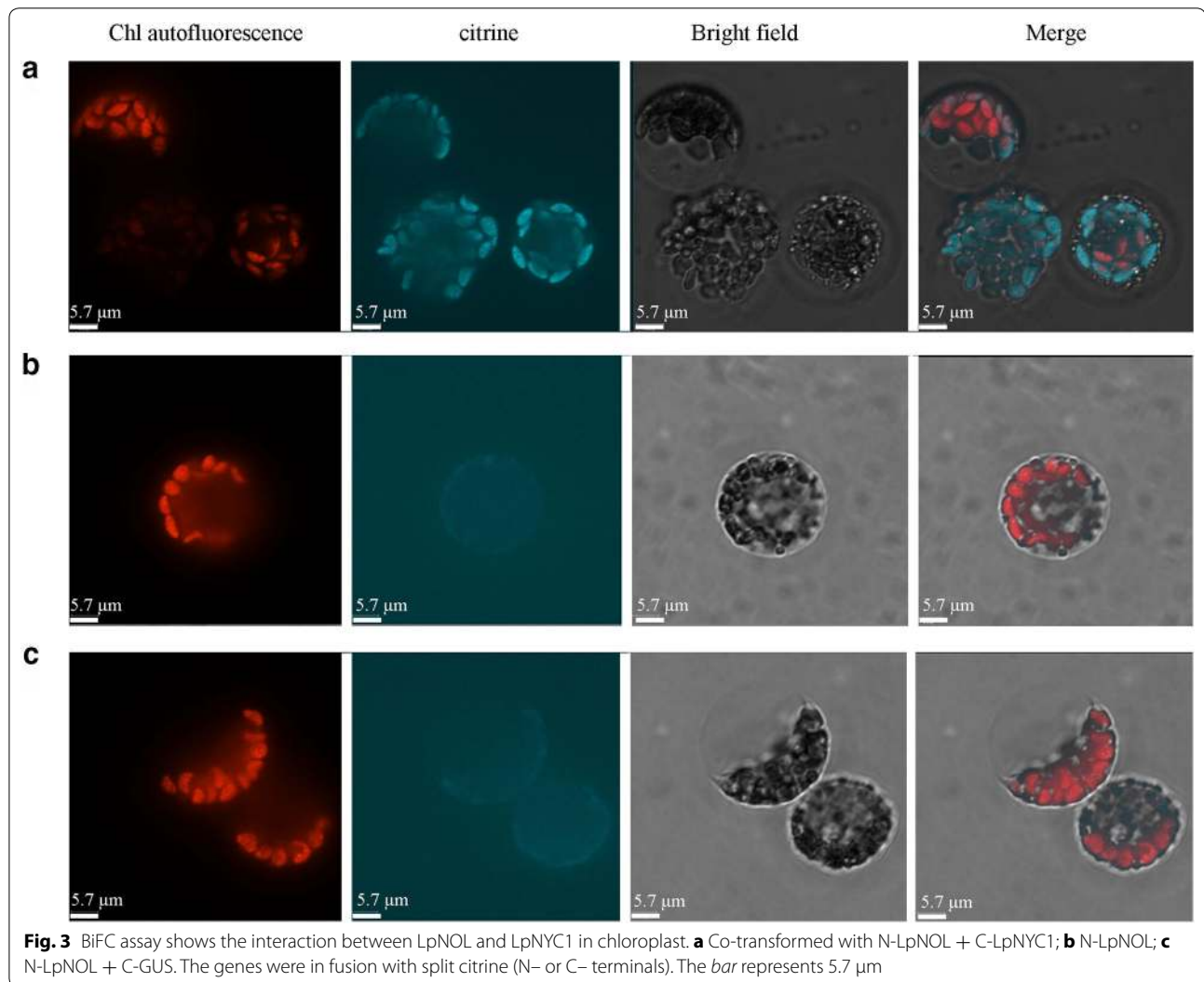
#### LpNOL and LpNYC1 interact in vivo using BIFC

The current protocol is also efficient enough for protein–protein interaction assay (e.g. BIFC). We cloned two putative chlorophyll catabolic enzyme-encoding genes, *LpNOL* & *LpNYC1*, from perennial ryegrass, and fused them with split N- and C- terminal citrine fragments, respectively. Orthologs of these two genes in model plant

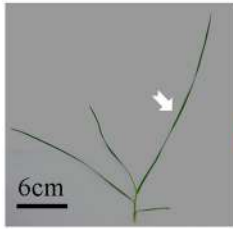
species *Arabidopsis* and rice encode key chlorophyll catabolic enzymes (CCEs), which physically interact with each other and cooperatively catalyze the degradation of Chl b [19, 20]. As shown in Fig. 3, the citrine signal was only visualized in the chloroplasts when *LpNOL* & *LpNYC1* were co-transformed, proving that these two proteins interact with each other in vivo in its homologous system.

#### Conclusions

In sum, a highly efficient mesophyll cell protoplast isolation and transformation protocol was developed for perennial ryegrass, which can be readily used for protein subcellular localization, and protein–protein interaction analysis. The protocol was illustrated in Fig. 4 with critical points pinpointed, and the recipes were shown in Table 1.



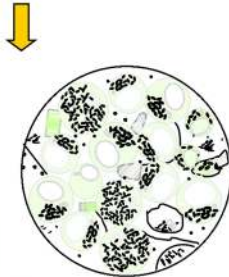
### 1. Leaf sample preparation



**Note 1:** Only use 2<sup>nd</sup> leaves from the top (~10–12 days after leaf emergence) from healthy and vividly growing plants; and only use the middle part of the leaf;

**Note 2:** Cut into 0.5mm sections w/ sharp razor; frequently dipping in enzyme solution to keep leaf sections moist.

### 2. Enzyme digestion



**Note 1:** Immerse leaf sections completely in enzyme solution by slight rotation; vacuum for 1 hr in dark, then incubate it in dark for ~6h with rotation (30rpm).

**Note 2:** The left figure is the appearance after enzyme digestion, and the right one is the cartoon illustration for digested material with integrate protoplasts and cell debris.

### 3. Protoplast purification



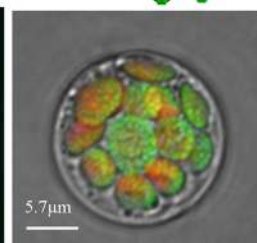
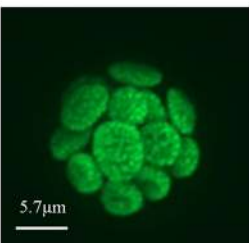
**Note 1:** Filter through 75µm nylon mesh and wash twice with pre-chilled W5 solution; centrifuge at 100 × g for 2 min and wash twice with pre-chilled W5 solution;

**Note 2:** Gentle operations in transferring protoplasts using pipette tips with tips cut off with scissors to minimize mechanical damage;

**Note 3:** At the step of pelleting protoplasts and re-suspending with MMg solution, it is suggested to remove as much supernatant (cell debris) as possible.

**Note 4:** The upper left figure represents the appearance of prepared protoplasts after resuspension; the upper right one is cartoon illustration for clean and intact protoplasts in the solution; the bottom figures are the ones stained with FDA to show the viability of isolated protoplasts.

### 4. PEG-Ca<sup>2+</sup> mediated protoplast transformation



**Note 1:** Dilute protoplasts to 2~5 × 10<sup>5</sup> per ml for transformation. Gently mixed the plasmid and protoplast solution and PEG-Ca<sup>2+</sup> solution by finger tapping. Incubation for 5-10min at room temperature (~25°C), slowly adding the pre-chilled W5 solution without vigorous mixing. Centrifuge at 100 × g for 2 min at 4 °C and wash once with pre-chilled W5 solution.

**Note 2:** Prepare pure tubes coated with 1% BSA and transferred the protoplasts mixed with W5 solution to avoid sticky tubes from attaching protoplasts.

**Note 3:** The figures represent successful plasmid transformation and transient expression of GFP-tagged LpPPH protein localized in chloroplast (left under UV light after GFP filter, right merged picture from those under UV and bright field.)

**Fig. 4** Outline of protoplast isolation and transformation

**Authors' contributions**

GY, QC and ZX performed vector construction, ryegrass protoplast isolation and transformation experiments, and participated in manuscript preparation; QC and BZ designed and constructed the pair of BiFC vectors; BX and BH conceived and designed the experiments, analyzed data and prepared the manuscript. All authors read and approved the final manuscript.

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**Competing interests**

The authors declare that they have no competing interests.

**Availability of data and materials**

The datasets supporting the conclusions and description of a complete protocol are included within the article.

**Consent for publication**

All authors agreed to publish this manuscript.

**Ethics approval and consent to participate**

All authors read and approved the manuscript.

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