


# Subcellular Localization of the D27 Protein in Sugarcane (*Saccharum* spp. Hybrids) Using an Optimized Protoplast Isolation, Purification, and Transient Gene Expression Protocol

Zhuan-di Wu<sup>1</sup> · Xin Hu<sup>1</sup> · Feng-gang Zan<sup>1,2</sup> · Yong-bao Pan<sup>2</sup> · David M. Burner<sup>1</sup> · Zheng-ying Luo<sup>1</sup> · Jia-yong Liu<sup>1</sup> · Li-ping Zhao<sup>1</sup> · Li Yao<sup>1</sup> · Yong Zhao<sup>1</sup> · Xin-long Liu<sup>1</sup> · Hong-ming Xia<sup>1</sup> · Kun Yang<sup>1</sup> · Jun Zhao<sup>1</sup> · Pei-fang Zhao<sup>1</sup> · Wei Qin<sup>1</sup> · Xue-kuan Chen<sup>1,2</sup> · Cai-wen Wu<sup>1,2</sup> 

Received: 14 April 2020 / Accepted: 31 July 2020 / Published online: 3 October 2020  
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**Abstract** The transient gene expression of protoplasts in plant is a considerable tool for gene functional research that has been widely used in gene analysis and functional characterization. Therefore, the objectives of this study were to develop a protocol for the isolation and purification of sugarcane protoplasts (*Saccharum* spp. hybrids), conduct transient PEG-mediated protoplast transfection with D27, and localize the D27 protein in sugarcane protoplasts. Total yield and viability of protoplasts were optimized for enzyme combination, mannitol concentration, and duration and temperature of enzymatic hydrolysis. High production of intact protoplasts ( $10.94 \times 10^6$  protoplasts  $g^{-1}$  FW) and a survival rate of  $> 80.0\%$  was achieved through enzymatic hydrolysis at constant temperature of  $28\text{ }^\circ\text{C}$ ,  $60\text{--}70\text{ rpm min}^{-1}$  for 8 h in a solution containing 2.0% cellulase R-10, 0.5% macerozyme R-10, 0.6% pectolyase Y-23, 20 mM 2-(N-morpholine) ethanesulfonic acid (MES), 20 mM KCl, and 400 mM mannitol (pH 5.7). Using GFP as the reporter gene, the protoplasts were transformed most efficiently with 25% PEG 4000 for

25 min and the ScD27 protein was localized in the chloroplasts. The localization of ScD27 protein in sugarcane protoplast demonstrated that the newly developed protocol was functionally effective. This optimized sugarcane protoplast isolation, purification, and transient expression protocol lays a foundation for future molecular biology research in sugarcane.

**Keywords**  $\beta$ -Carotene isomerase · Protoplasts · *Saccharum* · Strigolactones · Subcellular localization · Transformation

## Introduction

Sugarcane is a high yield sugar crop in the world that widely grows in tropical area. Due to its complex polyploid genome, there has been little genetic research on this crop. The yield formation of sugarcane is a complex process influenced by physiological, biochemical, and genetic factors as well as growth environment. Strigolactones (SLs) are a group of terpenoid lactone hormones produced in the plant roots that have diverse regulatory effects on plant growth and development, including inhibiting tillering or branching, shaping root morphology, promoting leaf senescence, and regulating secondary growth (Flematti et al. 2016; Gomez-Roldan et al. 2008; Umehara et al. 2008).

*DWARF27* (*D27*) was previously proven to be involved in SL biosynthesis. The D27 protein is the first enzyme involved in the biosynthesis of strigolactone by converting all-*trans*-  $\beta$ -carotene into 9-*cis*- $\beta$ -carotene in a reversible isomerization reaction (Vogel et al. 2010). The *D27* gene encodes an iron-binding protein in rice, which catalyzes the isomerization of 9-*cis*/all-*trans* isomerization (Lin et al.

Zhuan-di Wu, Xin Hu and Feng-gang Zan have contributed equally to this work.

✉ Xue-kuan Chen  
chxk\_ysri@163.com

✉ Cai-wen Wu  
gksky\_wcw@163.com

<sup>1</sup> Sugarcane Research Institute, Yunnan Academy of Agricultural Sciences/Yunnan Key Laboratory of Sugarcane Genetic Improvement, Kaiyuan 661699, People's Republic of China

<sup>2</sup> United States Department of Agriculture, Agricultural Research Service, Sugarcane Research Unit, Houma, LA 70360, USA

2009). In rice, expression of *D27* may depend on auxin signal (Waters et al. 2012) and affect the regulation of the biosynthesis of SL (Waters et al. 2017). The expression of *DgD27* in *Chrysanthemum morifolium* is inhibited by decapitation, but is induced by auxin. *DgD27* is also sensitive to light, *P* deficiency, and low N (Wen et al. 2016). We reported earlier that the 1379 bp full-length sequence of  $\beta$ -carotene isomerase *D27* (*KP987221.1*) from sugarcane (*ScD27*) contained a complete open reading frame of 867 bp encoding for 288 amino acids. *ScD27* expression was tissue specific, higher in sugarcane shoot tips than axillary buds, and could be induced by drought, *P* deficiency, or salt stress (Wu et al. 2017a, b). We proposed that the *ScD27* protein might locate in the chloroplast (Wu et al. 2017a, b).

Plant protoplasts are often used to study somatic hybridization, genome editing, transient gene expression, and subcellular protein localization, as they are totipotent and able to receive exogenous nucleic acid through transformation (Shen et al. 2014). Polyethylene glycol (PEG)-mediated protoplast transformation has been successful in a variety of plants to achieve high recombination frequency and homozygosity (Yu et al. 2017). Transient gene expression in protoplasts is a primary method to study the function of transgenes in genetic and physiological processes, such as cell wall regeneration, cell division, differentiation, cell genetic transformation, and somatic cell hybridization (Nanjareddy et al. 2016). Plant tissue, enzymatic hydrolysis, enzymolysis time, and temperature can affect the yield and viability of protoplasts (Wu et al. 2017a, b). Methods of protoplast isolation and transient gene expression have been established in many plants, such as *Arabidopsis* (Yoo et al. 2007), *Zea mays* (Chen et al. 2015), *Manihot esculenta* Crantz (Wu et al. 2017a, b), *Brassica oleracea* var. *alboglabra* Bailey (Wu et al. 2017a, b), and *Cucumis sativus* L (Sun et al. 2018). Sugarcane protoplasts have been isolated and cultured to obtain embryogenic calli (Sahab et al. 2019). However, until now, an efficient transient gene expression system has not been optimized for use on sugarcane protoplasts.

The objectives of the present study, therefore, were to optimize the isolation, purification, and PEG-mediated transient transfection of sugarcane protoplasts and to determine the subcellular location of the *ScD27* protein.

## Materials and Methods

### Plant Materials and Growth Conditions

Young leaves were obtained from the stem apex of ROC22, the most popular sugarcane cultivar in China that can be readily cultured in vitro. The leaves were disinfected, cut

into 1-mm slices, and placed on MS culture media (pH 5.8.) containing 1 mg L<sup>-1</sup> 2,4-D, 3% sucrose, and 0.8% carrageenan. After 4 to 5 weeks, embryogenic calli were formed and seedlings were produced by transferring to differentiation and rooting media. Tissue culture was conducted in an artificial climate incubator at 25 ± 1 °C and photosynthetic active radiation (PAR) of 120  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> under a 16 h light/8 h dark cycle.

### Protoplast Isolation

About 1 g of leaf tissue was collected from tissue culture seedlings, cut into 1-mm slices, and placed in a sterile 100 × 25 mm Petri dish containing 10 mL of enzyme mixture solution. A modified method of Yoo et al. (2007) and Huang et al. (2013) was used to prepare the enzyme mix: 1% or 2% (W/V) cellulase R-10 (Yakult, Japan), 0.3% or 0.5% macerozyme R-10 (Yakult, Japan), 0% or 0.6% pectolyase Y-23 (Yakult, Japan), 20 mM 2-(N-morpholine) ethanesulfonic acid (MES) (pH 5.7, preheating at 70 °C for 5 min before use), 20 mM potassium chloride (KCl), and 0.2, 0.4, 0.6, or 0.8 M mannitol, and ddH<sub>2</sub>O (Table 1). The enzyme solution was warmed to 55 °C for 10 min to improve enzyme solubility and allowed to cool to 25 °C, then 0.1% BSA, 10 mM CaCl<sub>2</sub>, 3–5  $\mu$ l of  $\beta$ -mercaptoethanol were added and the pH was adjusted to 5.8 with 1 M KOH. The enzyme solution was filter sterilized through a 45  $\mu$ m nylon membrane filter and was promptly used. The Petri dishes were incubated in darkness with gentle shaking at 50 rpm.

Enzymolysis was tested at 4, 6, 8, 12, and 16 h to determine the optimal time for protoplast isolation. Optimum culture temperature was tested at 25, 28, 30, and 35 °C. At each time and temperature point, a 10  $\mu$ l sample of the enzyme-protoplast extract solution was taken for

**Table 1** Enzymatic compositions used for the isolation of sugarcane mesophyll protoplasts

Composition	Cellulase R-10 (W/V) (%)	Macerozyme R-10 (W/V) (%)	Pectolyase Y-23 (W/V) (%)
1	1.00	0.30	0.00
2	2.00	0.30	0.00
3	1.00	0.50	0.00
4	2.00	0.50	0.00
5	1.00	0.30	0.60
6	2.00	0.30	0.60
7	1.00	0.50	0.60
8	2.00	0.50	0.60

microscopic examination with an Olympus CX31 optical microscope (CX 31 Olympus, Japan).

### Protoplast Purification

After enzymolysis, the enzyme-protoplast mixture was filtered through a 70  $\mu\text{M}$  (200 mesh) nylon membrane to remove large tissue debris. The filtrate was collected in a sterile test tube and, centrifuged at 200 rpm at 4 °C for 5 min to collect protoplasts. The supernatant was eluted and an equal volume of pre-cooled sterile W5 buffer solution (154 mM NaCl, 125 mM  $\text{CaCl}_2$ , 5 mM KCl, 2 mM MES, pH 5.7) was added with gentle shaking to wash the protoplasts. The collection and washing process was repeated once more and the protoplasts were stored in W5 solution in darkness at 22 °C. Number of protoplasts was determined using a double-chamber hemocytometer with an optical microscope (Olympus, Japan). Protoplast viability was measured using fluorescein diacetate (FDA) staining (Larkin 1976). The stain solution was prepared by dissolving 2 mg of FDA in 1 mL acetone and stored in a 4 °C refrigerator. Five mL of the FDA stain was added to 100  $\mu\text{L}$  of protoplast suspension [final FDA concentration 0.01% (w/v)] and incubated for 10 min in darkness. Protoplast viability was assessed by examining an aliquot of stained protoplast suspension under a fluorescence microscope (Zeiss AxioImager A2m Carl Zeiss, Germany).

Protoplast yield was calculated as: protoplast  $\text{g}^{-1}$  fresh weight (FW) = the number of protoplasts in enzyme solution/weight of plant leaves used for enzymolysis. Protoplast viability was calculated as: Protoplast viability (%) = (the number of fluorescing protoplasts/total number of protoplasts)  $\times$  100.

### Plasmid Construction and the ScD27 Protein Subcellular Localization

Competent cells of *Escherichia coli* strain DH5 $\alpha$  were purchased from Beijing TransGen Biotech Co., Ltd. (Beijing, China). The sugarcane SL biosynthetic gene  $\beta$ -carotene isomerase, *ScD27* was studied to evaluate the feasibility and practicability of this optimized transient expression system.

The Takara MiniBEST Universal RNA Extraction Kit (Takara, Japan) was used to extract RNA and PrimeScript<sup>®</sup> 1st Strand cDNA Synthesis Kit (Takara, Japan) was used to synthesize cDNA following the manufacturer's instructions. Based on the entire coding sequence of *ScD27* (Wu et al. 2017a, b), two primers, each containing *NcoI* or *BglII* enzyme restriction site at its 5' end, were designed and synthesized by Shenggong Biotech Co., Ltd (Shanghai, China). The nucleotide sequence (5' to 3') of Primer 1302D27-forward was CATGCCATGGATGCTCCTTGC

TCACGCT and the nucleotide sequence (5' to 3') of Primer 1302D27-reverse was GAAGATCTTCAAATAGAGCA ATTCACTTGACG. The *ScD27* gene was amplified by PCR using pMD18-T-ScD27 plasmid as the template. The reaction mixture contained 5  $\mu\text{L}$  1 pmol of each primer, 40  $\mu\text{M}$  dNTP, 0.1U Taq DNA polymerase (Takara, Japan), 1.5 mM  $\text{MgCl}_2$ , and 1  $\times$  buffer supplied with the enzyme. The thermal cycling program was 94 °C, 3 min; 30 cycles of (94 °C, 30 s; 60 °C, 30 s; 72 °C, 1 min); and 72 °C, 10 min. The PCR product 927 bp and *Nco I* and *Bgl II*-double digested pCMBIA1302 vector (1302 bp, a kind gift of Shu-Zhen Zhang, the Institute of Tropical Bioscience and Biotechnology Chinese Academy of Tropical Agricultural Sciences, Haikou, Hainan, China) were gel purified using T4 DNA ligase. The ligation mixture was used to transform *E. coli* DH5 $\alpha$  cells. Recombinant colonies were cultured to amplify the recombinant pCMBIA1302-ScD27 plasmids, which were verified by restriction enzyme digestion and DNA sequencing by Sango Biotech Co., Ltd (Shanghai, China). Sequencing results showed that the *ScD27* fragment was inserted between the 35S promoter and the *GFP* gene. The subcellular localization of the ScD27 protein was predicted using the website WoLF PSORT (<http://www.genscript.com/wolf.psорт.html>) and determined using laser scanning confocal microscopy.

### Protoplast Transformation and PEG-Mediated Gene Transient Expression

The methods of Yoo et al. (2007) and Sun et al. (2018) were used in PEG-mediated transient expression experiments. About 1–5  $\times$  10<sup>4</sup> protoplasts/mL 100  $\mu\text{L}$  solution in MMG buffer (0.4 M mannitol, 15 mM  $\text{MgCl}_2$ , and 4.0 mM MES, pH 5.7) were mixed with 110  $\mu\text{L}$  PEG solution [40% PEG 4000 (W/V), 2.0 M mannitol, and 0.1 M  $\text{CaCl}_2$ ,] and transformed with recombinant pCMBIA1302-35S-GFP plasmids.

Protoplast transformation was conducted at room temperature in darkness by mixing 10  $\mu\text{L}$  of pCMBIA1302-35S-GFP (1  $\mu\text{g}/\mu\text{L}$ ) with 100  $\mu\text{L}$  of protoplast solution and 110  $\mu\text{L}$  of PEG 4000 solution. The PEG concentrations tested were 10%, 15%, 20%, 25%, 30%, and 35% (w/v). Transformation times were tested at 10, 15, 20, 25, 30, and 35 min. Transformation was terminated by adding 800  $\mu\text{L}$  W5 solution. After termination, transformed solutions were centrifuged at 500 rpm for 2 min. The supernatant was discarded. The protoplast pellet was resuspended in 1 mL W5 solution, which was then transferred to a sterile BSA-coated Petri dish and incubated in the dark at 25  $\pm$  1 °C for 16–24 h.

The expression of the GFP fusion protein was observed using fluorescence microscope. Transformation efficiency

was calculated as: transformation efficiency (%) = (number of fluorescent protoplasts/total protoplasts)  $\times$  100.

## Data Analysis

Experiments were repeated three times, and analysis of variance was conducted using SPSS software Version 18.0 (SPSS Inc. Chicago, IL, USA). The least significant difference (LSD) was  $P \leq 0.05$ .

## Results

### Isolation of Sugarcane Protoplasts

#### Enzyme Composition

Yield of isolated protoplasts ranged from 0.05 to  $10.94 \times 10^6$  protoplasts/g FW depending on enzyme composition (Fig. 1). Enzyme composition 8 (2.0% cellulase R-10, 0.5% macerozyme R-10, and 0.6% pectolyase Y-23) yielded significantly more protoplasts ( $10.94 \times 10^6$  g<sup>-1</sup> FW) than the other enzyme compositions (Table 1; Fig. 1). However, there was no predictable change in viability of protoplasts due to enzyme composition, which ranged from 46.96 to 85.6% (Fig. 1). Enzyme compositions 5 to 8, all contained 0.6% pectolyase Y-23, and they yielded significantly more sugarcane protoplasts than compositions 1 to 4 that contained no pectolyase (Fig. 1).

#### Mannitol Concentration and Enzymolysis Time

To determine the optimal enzymatic osmotic pressures, mannitol at different concentration was set. Mannitol at 0.4 M resulted in significantly higher protoplast yields ( $6.30 \times 10^6$  protoplasts/g FW,  $P \leq 0.05$ ) with 86%

viability (Fig. 2a). Compared to that of 0.4 mM mannitol, however, yield and viability of protoplasts were all significantly lower at 0.2 M, 0.6 M, or 0.8 M mannitol concentration, with  $3.76 \times 10^6$ ,  $1.47 \times 10^6$ , or  $1.37 \times 10^6$  protoplasts/g FW and 58%, 50%, or 32% viability, respectively. Thus, 0.4 M mannitol was the optimal concentration for enzymolytic protoplast production.

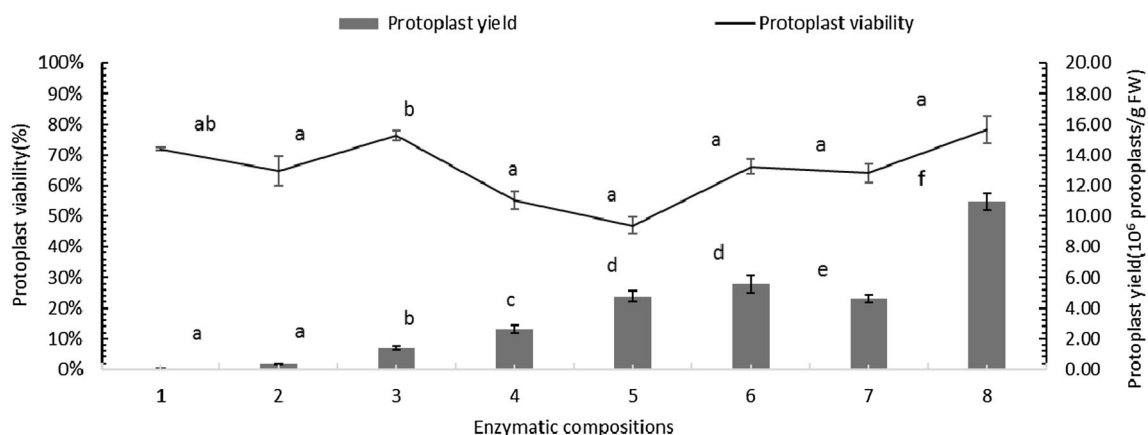
Protoplast viability was significantly higher at 4, 6, or 8 h enzymolysis (69%, 76%, or 67%) compared to 12 or 16 h enzymolysis (56% or 35%) (Fig. 2b). Significantly, higher protoplast yield of  $4.52 \times 10^6$  protoplasts/g FW was obtained at 12 h enzymolysis (Fig. 3). Similarly, 12 h enzymolysis significantly increased protoplast yield (Fig. 2b) with less debris (data not shown).

#### Influence of Temperature

Enzymolysis temperature (25, 28, 30, or 35 °C) affected protoplast yield and viability (Fig. 4). The highest mean yield of protoplasts ( $4.86 \times 10^6$  protoplasts/g FW) was obtained when enzymolysis was conducted at 28 °C (Fig. 4). Viability did not differ significantly among temperature treatments, although that at 28 °C (75.23%) tended to be greater than at 25, 30, or 35 °C. The FDA dye test further demonstrated the protoplast yield and viability were sufficiently optimized to attempt transformation studies (Fig. 5). We achieved these results by using 0.4 M mannitol concentration, 12 h enzymolysis at 28 °C, and enzyme composition 8 (2% cellulase R-10, 0.5% macerozyme R-10, and 0.6% pectolyase Y-23).

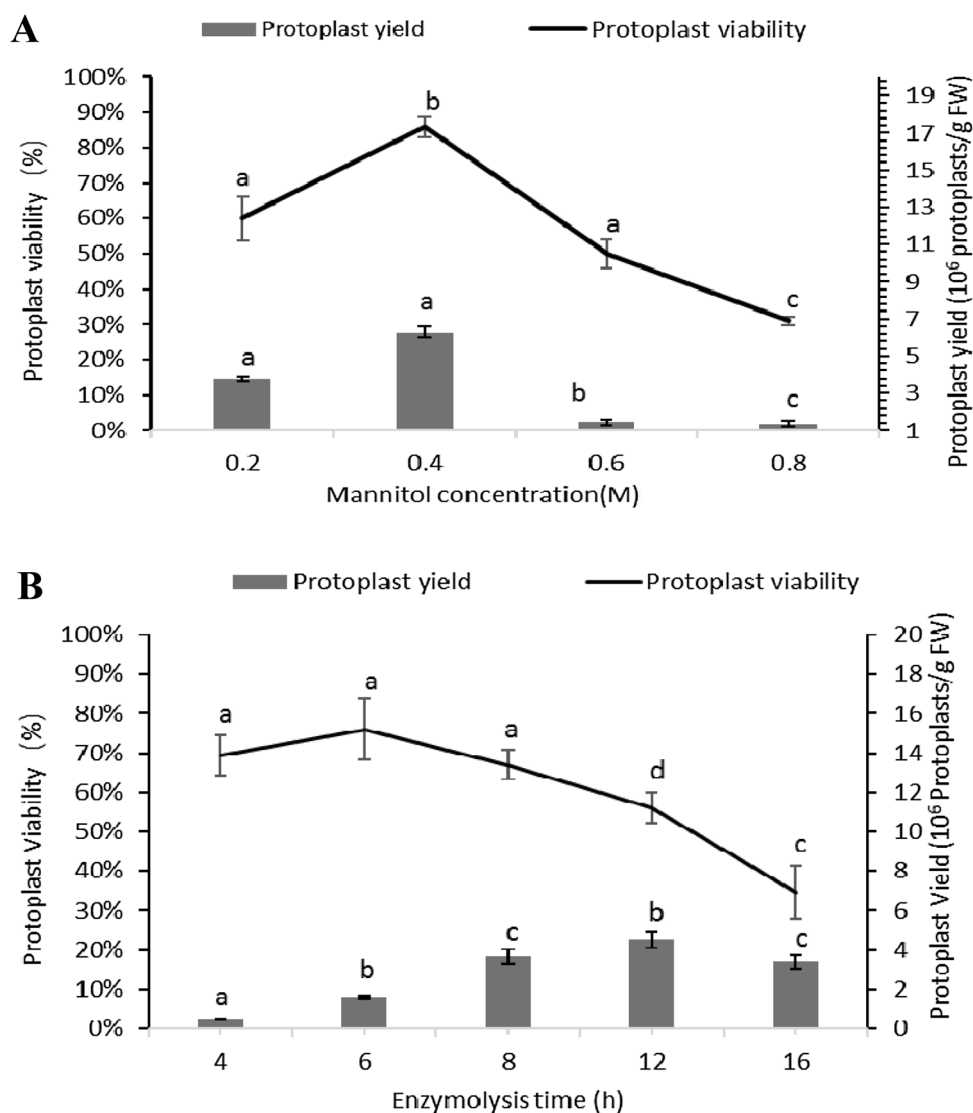
#### Transformation Efficiency of Sugarcane Protoplasts

The transformation efficiency of sugarcane protoplasts was significantly greater at 25% PEG4000 concentration compared to other higher (30%, 35%) or lower concentrations



**Fig. 1** Effect of enzymatic composition on yield (bars) and viability (line) of ROC 22 protoplasts. Different letters represent a statistically significant difference at  $P \leq 0.05$ , and bars represent standard errors (SEs)

**Fig. 2** Effects of mannitol concentration and enzymolysis time on the total protoplast yield ( $10^6$  protoplasts/g FW) and protoplast viability (%) from sugarcane. Different letters represent a statistically significant difference at  $P \leq 0.05$ , and bars represent standard errors (SEs)



(10%, 15%, or 20%) (Fig. 6). In terms of duration of transformation, a significantly greater efficiency was achieved at 25 min (41.0%) in comparison to other duration, i.e., 10(23.6%), 15(30.1%), 20(36.1%), 30(30.4%), or 35(19.1%) min (Fig. 7).

#### Subcellular Localization of GFP-Fused ScD27 Protein in Sugarcane Protoplasts

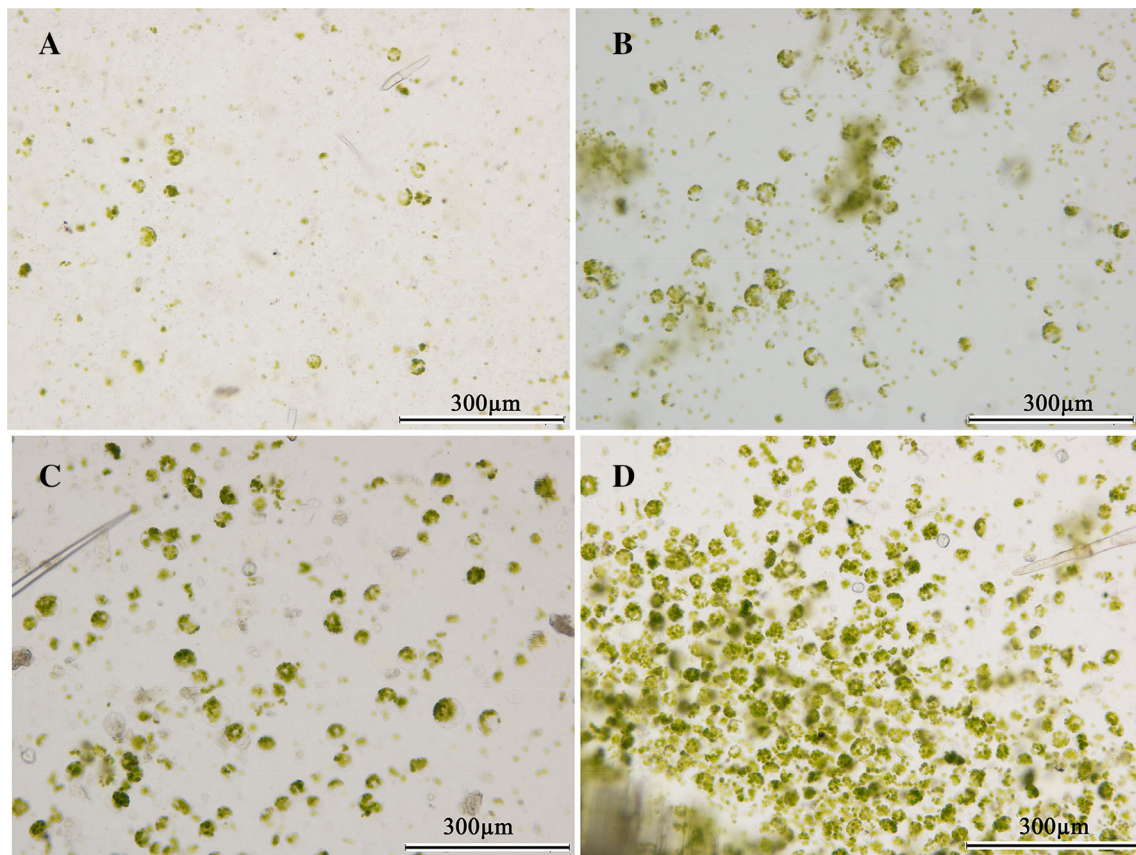
Using our optimized protocol, the pCAMBIA1302-35S-GFP recombinant plasmids were able to express transiently in transformed sugarcane protoplasts (Fig. 8). Furthermore, the ScD27-GFP also detected the co-expression of specific GFP fusion protein in the sugarcane protoplasts. In contrast, the expression of reporter gene GFP alone was detected in all organelles, indicating that sugarcane protoplasts had been transfected. This success will greatly facilitate molecular biology studies in sugarcane.

#### Discussion

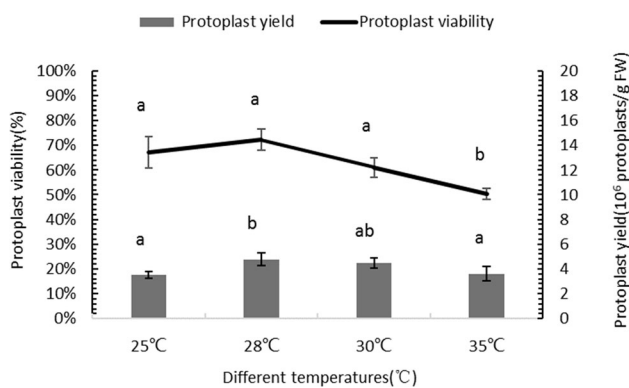
Transient gene expression is an efficient technique for assessing gene function in eukaryotes, such as calcium signaling, protein–protein interactions, cellular interaction analyses (Zhang et al. 2011a), signal transduction and regulation, ion channel regulation through light, stress, and hormone responses (Im and Yoo 2014; Rose 2019; Xiong et al. 2019), and even further, in high throughput CRISPR/Cas9 screening application (Andersson et al. 2017).

Protocols for protoplasts isolation and transient gene expression have been established in various monocots, such as *Oryza sativa* (Kim et al. 2015), *Zea mays* (Chen et al. 2015). However, the efficiency of protoplast isolation and transformation has been low in sugarcane (Chen et al. 1987, 1988; Smith et al. 1992) and few report is available on gene transient expression in sugarcane protoplasts in the recent 10 years (Wang et al. 2019). Disadvantages such as





**Fig. 3** Photomicrographs of isolated sugarcane protoplasts. **a, b, c, d** show protoplasts isolated after 4, 6, 8, and 12 h, respectively



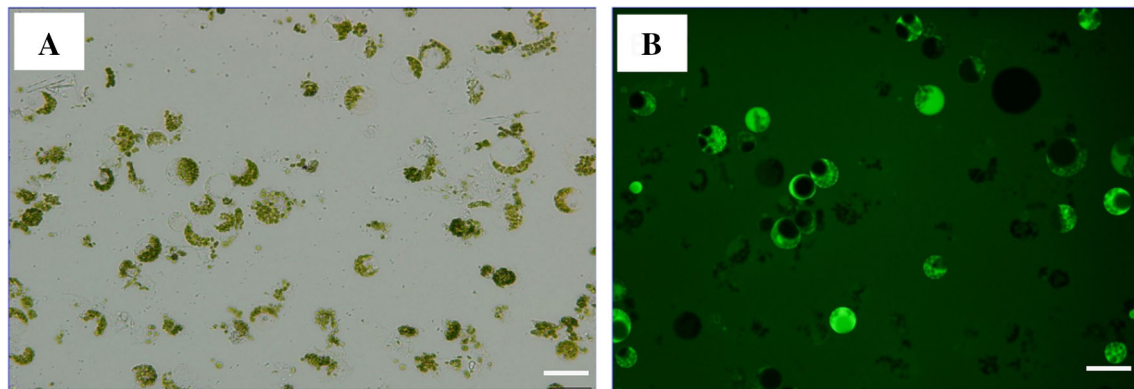
**Fig. 4** Effect of enzymolysis temperature on the yield and viability of ROC22 protoplasts. Different letters represent a statistically significant difference at  $P \leq 0.05$ , and bars represent standard errors (SEs)

abnormal protein expression in a heterogeneous system or false positive localization often exist in sugarcane gene functional studies that use tobacco (*Nicotiana tabacum* L.) or *Arabidopsis thaliana*'s protoplast transient expression system (Figueiredo et al. 2011). There is a need to develop an optimized protocol for the isolation and purification of sugarcane protoplasts, and to use these protoplasts for gene localization was essential.

A technical protocol for highly efficient isolation and purification of sugarcane protoplast has been established in this study, which was also used to study the transient expression and the subcellular localization of sugarcane D27 gene.

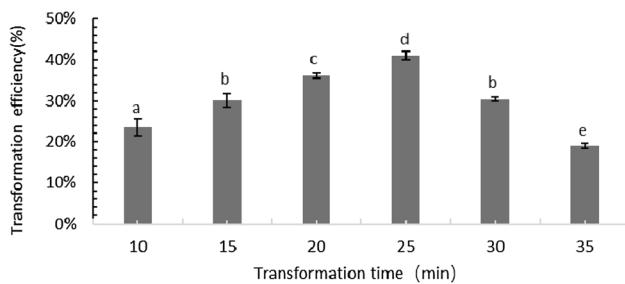
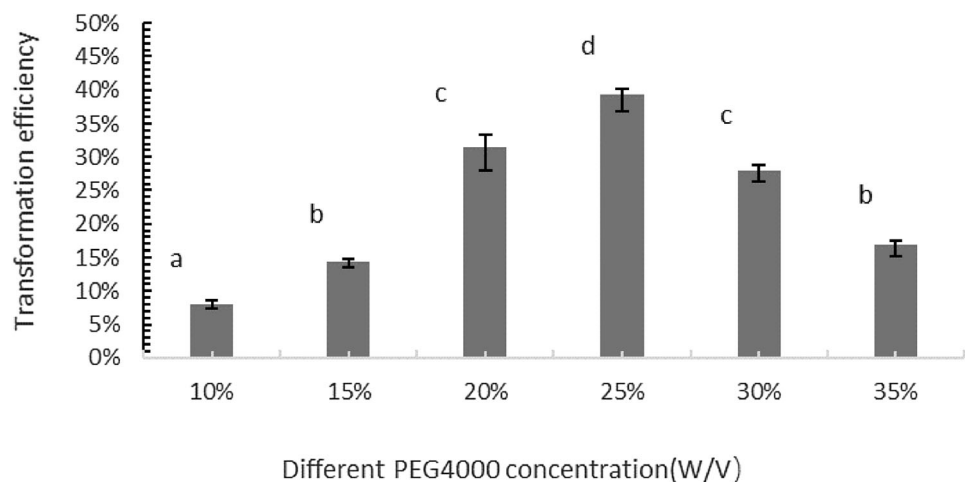
Protoplasts of high quality are very critical for transient expression studies. Several factors can impact the generation process of plant protoplasts, while the universal transient gene expression system used in model plants do not work well directly on sugarcane protoplasts, therefore, the universal transient gene expression system was optimized in this study and large numbers of viable sugarcane protoplasts were isolated.

Compared to the well-established protoplast isolation techniques of *Arabidopsis* (Yoo et al. 2007), relatively higher concentrations of cellulase R-10 (2.0%) and macerozyme R-10 (0.5%) with an additional digestion enzyme pectolyase Y-23(0.6%) were required for obtaining the highest yield of sugarcane protoplasts (Table 1). Different organs and tissues can be used as the starting materials for protoplast isolation in plants (He et al. 2016), including leaves (Shu et al. 2017; Shen et al. 2017; Zhang et al. 2011b), roots (Lin 1980), callus (Titouh et al. 2017), and shoots (Ortiz-Ramirez et al. 2018). However, in



**Fig. 5** The viability of protoplasts isolated from sugarcane mesophyll cells using bright-field (a) and ultraviolet light (b). Scale bar indicates 50  $\mu\text{m}$

**Fig. 6** Effect of PEG4000 concentration on protoplast transformation efficiency. Different letters represent a statistically significant difference at  $P \leq 0.05$ , and bars represent standard errors (SEs). Transformation efficiency was also significantly greater at 25 min compared to the other times, range 8.0 to 39.4%



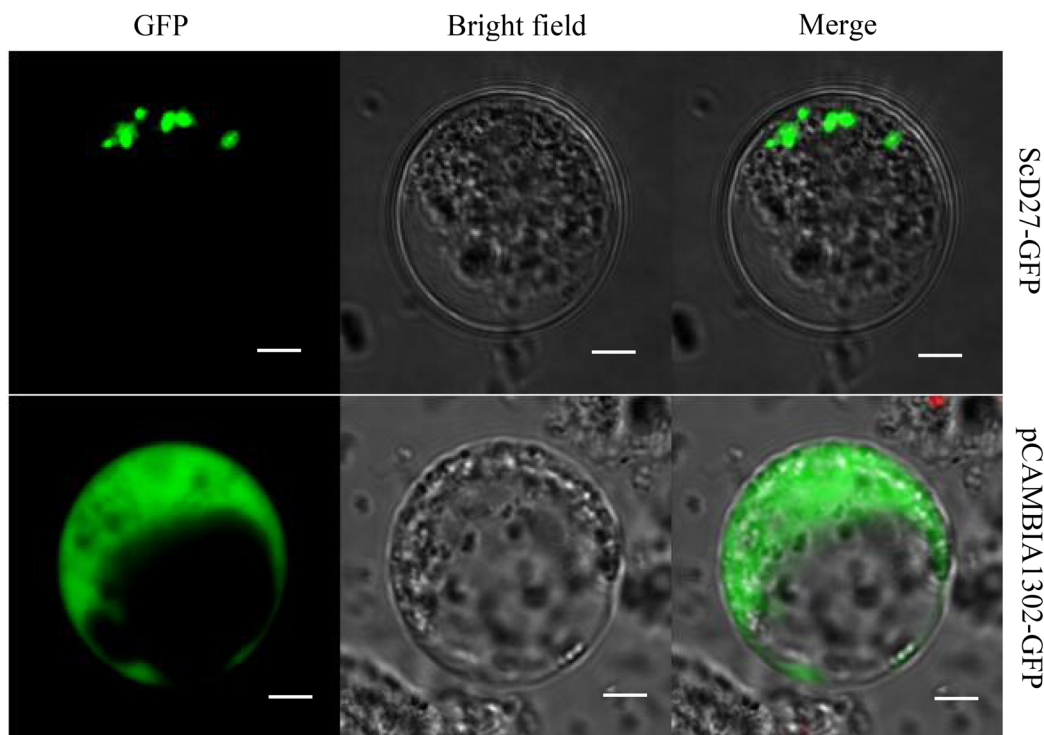
**Fig. 7** Effect of transformation time on protoplast transformation efficiency. Different letters represent a statistically significant difference at  $P \leq 0.05$ , and bars represent standard errors (SEs)

sugarcane, leaves or shoots are usually used as a reliable source for the isolation of high yield and relatively uniform protoplasts. The quality of protoplasts from the leaf mesophyll cells was better than that from the shoots (data are not shown).

Plant protoplasts remain intact only at a proper osmotic pressure. Different concentrations of mannitol have been used to maintain optimal osmotic pressure and high

protoplast viability for the study of gene transient expression (Sun et al. 2018; Sahab et al. 2019; Wu et al. 2017a, b). The study found that when mannitol concentration was  $0.4 \text{ mol L}^{-1}$ , the sugarcane protoplasts did not absorb water and there were few cell fragments. If the mannitol concentration was less than  $0.4 \text{ mol L}^{-1}$ , the viability of the sugarcane protoplasts decreased.

The study demonstrated that the *ScD27* gene protein was localized in the chloroplast. PEG-mediated protoplast transformation method is a mature, widely applied technology for studying transient expression in plant cells (Sahab et al. 2019). It has been established that PEG concentration, plasmid DNA concentration, and mass and viability of protoplasts affect the efficiency of protoplast transformation (Cao et al. 2016). Another key factor is the stability of the cell osmotic environment because the cytoplasm is fragile and uneven in concentration and the protoplasts are easy to break. In this study, the transformation efficiency and the number of transformed protoplasts increased gradually up to 25% PEG400 concentration. Other experimental factors might be



**Fig. 8** Subcellular localization of ScD27-GFP fusion. The empty pCAMBIA1302 vector with GFP and the pCAMBIA1302 vector with ScD27-GFP fusion were transiently expressed in sugarcane mesophyll protoplasts. The upper part is the intracellular location of ScD27-GFP fusion, and the lower part is the intracellular location of

GFP. Individual and merged images of GFP (green), and bright field in transformed protoplasts shown. The length of the scale bars is indicated. The scale bar is 5  $\mu$ m (color figure online)

investigated to further optimize the gene transient expression system in sugarcane.

## Conclusion

An optimized protocol for sugarcane mesophyll protoplast isolation, purification, and transient gene expression was developed in this study using 2% cellulase R-10, 0.5% macerozyme R-10, and 0.6% pectolyase Y-23, enzymolysis for 12 h at 28 °C in the dark with constant oscillation at 60–70 rpm/min. Protoplast yield was as high as  $10.94 \times 10^6$  protoplasts/g FW, and protoplast viability was 85.12%. Sugarcane protoplasts were transformed overnight at 28 °C with 40% PEG-4000 and 5  $\mu$ g of plasmid DNA. The ScD27 protein was localized in the chloroplasts. This simple method using protoplasts will be very useful for future research to elucidate sugarcane gene function.

**Acknowledgments** This study was supported by Fund for the National Natural Science Foundation of China (31860405); Earmarked Fund for China Agriculture Research System (CARS-170101); Provincial Innovation Team of Sugarcane Germplasm Innovation and New Variety Breeding of Yunnan Academy of Agricultural Sciences (2019HC013); Central Public-interest Scientific Institution Basal Research Fund for Chinese Academy of Tropical

Agricultural Sciences (No. 1630052017020-4); The Applied Basic Research Projects in Yunnan Province (2016FB071); Applied Basic Research Projects of Yunnan Academy of Agricultural Sciences (YJM201705); Overseas Top Talents Project “Sugarcane genetic improvement and extension”; Yunnan Provincial Science and Technology Cooperation Program, China (Yunnan)–Sri Lanka Sugarcane International Joint Research Center (2018IA076); The Key Research and Development Program of Yunnan Province, the Joint Research and Development Center of Sugarcane Variety Improvement in South and Southeast Asia (2019IB008).

**Author Contributions** CWW and FGZ, ZDW, XKC conceived and designed the experiments. ZDW, FGZ, XH, and ZYL performed the experiments. FGZ, ZDW, and XKC result analysis, and manuscript drafting. YBP, XKC, and DMB revised the manuscript. All authors have read and approved the final manuscript. XKC, JYL, LPZ, LY, YZ, XLL, HMX, KY, JZ, PFZ, and WQ participated in experimental work.

## Compliance with Ethical Standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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