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RESEARCH PAPER

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Agrobacterium tumefaciens Mediated Transformation of *Symbiodinium* spp.

Buntora Pasaribu[1,*], Pei-Luen Jiang[2,*]

[1]Rutgers University, Department of Plant biology, New Brunswick, USA
[2]National Formosa University, Department of Biotechnology, Taiwan

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Corresponding Author
Tel.: +88656315491
E-mail: buntora86@gmail.com
villy@nfu.edu.tw

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Abstract

Symbiodinium spp conducts symbiosis mutualism within a wide phyletic range of marine invertebrate hosts including corals and anemones. The present study investigates the transformation of foreign genes into the free living cultured *Symbiodinium* spp by co-cultured *Symbiodinium* cells with *A. tumefaciens*. Ti-plasmid-based binary vector harboring the GUS and GFP genes were transformed into *Symbiodinium* cells by co-cultivation. GUS histochemical assay was monitored in *Symbiodinium* cells under light microscopy. Putative GFP in transformed Symbiodinium cells was detected by immunoassaying with antibodies against GFP protein. These results suggest that *A. tumefaciens* could provide efficient tools for gene transformation of *Symbiodinium* cells.

Introduction

Symbiodinium is a photosynthetic dinoflagellate that conducts mutual relationship within marine organisms including coral, clams and sea anemone (Davy *et al*., 2012; Pasaribu *et al*., 2015). As the role contributor, the presence of *Symbiodinium* maintains the stability of the host to survive. The host provides the organic nutrients for *Symbiodinium* and in exchange *Symbiodinium* translocates the photosynthesis product to the host (Muscatine, 1990). The interactive machinery of translocation could lead the genetic regulation and expression during symbiotic partnership. Several molecular and cellular studies have reported new insights to the genetic regulation of *Symbiodinum* have been reported recently (Jiang *et al*., 2014; Peng *et al*., 2011, Mayfield *et al*., 2013). However, there are limited studies describing the genetic transformation of *Symbiodinium* (Lohuis & Miller, 1998). The development of genetic transformation technique could allow the exploitation of functional genetics in *Symbiodinium*.

In over a decade, the studies of gene transformation methods have been significantly developed in plants and algae (Wang *et al*., 2018; Mahto *et al*., 2018; Anila *et al*., 2011; Rathod *et al*., 2013). Several methods have been used for genetic transformation in algae including electroporation (Munoz *et al*., 2018), bombardment (Sharma *et al*., 2018), and glass beads (Ortiz *et al*., 2015). The unstable and low frequency transformation of these existing systems necessitate the development of alternative tools for gene transfer. Use of *Agrobacterium tumefaciens* (*A. tumefaciens)* results in efficient and stable transformation in plant cells (Wang *et al*., 2018;

Mahto *et al*., 2018). The presence of T-DNA as a part of Ti-plasmid in *Agrobacterium tumefaciens* enables integration at the target nuclear target chromosome (Bundock *et al*., 1995). It has been reported that the Agrobacterium mediated transformation was successfully achieved in microalgae such as *Chlamydomonas reinhardtii* (Kumar *et al*., 2004), *Chlorella* (Cha *et al*., 2012), *Haematococcus* (Kathiresan *et al*., 2009), and *Schizochytrium* (Cheng *et al*., 2012).

In this study, we developed an *Agrobacterium* mediated transformation system in *Symbiodinium*. Transformation attempted in *Symbiodinium* cells has been observed by treatment with biolistic, electroporation, glass bead, and polyethylene glycol of which the transformed cells were not capable of cell division (Ortiz-Matamoros *et al.,* 2015a). The transformation of free living Symbiodinium cells with plant pathogen Agrobacterium was resulted the failed of transformed cell to reproduce under herbicide selection (Ortiz-Matamoros *et al*., 2015b). However, transformation of free living *Symbiodinium* spp by combining electroporation and agrobacterium have never been understood. Studies the free living Symbiodinium spp transformation through these methods will be able to carry out the simple method to explore the possible genetic tools for Symbiodinium spp. We generated the transformation of β-glucuronidase (GUS) and green fluorescence protein (GFP) genes were transformed into *Symbiodinium* cells by *Agrobacterium tumefaciens* mediated transformation and stable transformation of the exogenous gene in the transgenic *Symbiodinium* was assessed using molecular and immunoblot assays. Transformed *Symbiodinium* presented the blue labelling (GUS) and green fluorescence protein (GFP) signal intracellularly in *Symbiodinium* cells. This result could facilitate an alternative system for efficient and in elucidating the stable transformation in *Symbiodinium*.

Material and Methods

Symbiodinium Culture

Symbiodinium spp. (clade B) maintained in the f/2 medium in filtered seawater (FSW) at room temperature under a photo-synthetically active radiation (PAR) of 40 mmol m_2 s_1 in a 12-h light/12-h dark (12L/12D) cycle.

Co-Cultivation of *Symbidinium* spp

Agrobacterium strains harboring plasmids 35s:GUS and ubi:GFP were cultivated in 5 ml of LB medium. The strains were supplemented with proper selective antibiotic and incubated at 28 for 48 h with shaking. Strains were collected by centrifugation at 5000 x g for 15 mins to an OD600 of 0.5. Then, *Symbiodinium* cells were submerged in agrobacterium suspension and an electroporator (BTX, San Diego) to assure the gene introduced into *Symbiodinium* cells was used. *Symbiodinium* cells were cocultured with *Agrobacterium* strains after Acetosyringone (AS) induction for two days. Afterward, the *Symbiodinium* cells were transferred to F/2 selection medium. The survival cells were cultured in the f/2 medium for two weeks. All steps of transformation were carried out at 25°C under the light of 40 mmol m^{-2} s^{-1} in a 12-h light/12-h dark (12L/12D) cycle.

GUS Expression Essay

Histochemical GUS staining was conducted as described by Ho *et al*. (2000). Symbiodinium wild type and transformed cells were cultured in 100ml ASW with F/2 medium. *Symbiodinium* cells were collected by centrifugation at 12000 x g for 5 min. The pellets were suspended in Histochemical buffer containing 1 mm 5-bromo-4-chloro-3-indolyl b-D-glucuronide (X-gluc) and then incubated at 37°C in darkness for 12 h. After incabtion, the *Symbiodinium* cells were suspended in 70% ethanol and rinsed with deionized water.

DNA Extraction and PCR Analysis

Total genomic DNA of *Symbiodinium* cells were extracted from wild type *Symbiodinium* as nontransformed control and transformed *Symbiodnium* cells using Plant genomic DNA extraction. The total genomic DNA from wild type and transformed Symbiodinium cells were detected for PCR analysis and GUS-specific primer were used. Primer used for GUS gene was GUS-F: 5'ATCAGCGCGAAGTCTTTATACC3' and GUS-R: 5'CAGTTGCAACCACCTGTTGA3'. PCR products were separated on a 1% agarose gel and observed by using UV machine.

Fluorescent Microscopy

Symbiodinium cells wild type and transformed were cultured for a week in F/2 medium. *Symbiodinium* cells were collected by centrifugation at 12000 x g for 5 min. The pellets were washed three times using ddH_2O to remove any micelles from *Symbiodinium* cells. *Symbiodinium* cells wild type and transformed were visualized by Axioskop 2 Plus microscope (Zeiss, Germany) equipped with a charge-coupled device (CCD) camera (Coolsnap-Prock, Photometrics Ltd., USA).

SDS-PAGE and Western Blotting

Total proteins in *Symbiodnium sp* wild type and transformants were extracted with an equal volume of 2x sample buffer according to the suggestions in the Bio-Rad (Bio-rad, USA) Trans-Blot instruction manual and resolved by SDS-PAGE using 12% (w/v) polyacrylamide in the separating gel and 4.75% polyacrylamide in the stacking gel (Schägger 2006). After electrophoresis, the gel was stained with Coomassie Blue R-250 and then destained. For Western blotting, proteins were

transferred from SDS-PAGE onto a nitrocellulose membrane in a Trans-Blot system (Bio-Rad, USA) according to the manufacturer's instructions. The membrane was subjected to immune-detection using a primary antibody against GFP (25 kDA) The membrane was washed and visualized by Super Signal West Pice, Chemiluminescent substrate kit (Thermo Fisher Scientific, Watham, MA)

Results

Pre-Treatment of *Symbiodinium* Cells

Several transformation techniques using *Agrobacterium* have been successfully reported for *Chlamydomonas* (Kumara *et al*., 2004). However, dinoflagellate is a unique organism that grows in both free living and symbiotic forms. Thus, we first isolated fresh *Symbiodinium* cells from *Exaiptasia pulchella* via percoll gradient and maintained the cells in culture for a few months (Pasaribu *et al*., 2015). Free living *Symbiodinium* cells were coccoid yellow brown in color and 5-6 mm in diameter. Generally, the free-living *Symbiodinium* are smaller than symbiotic *Symbiodinium* (Pasaribu *et al*., 2015). To produce sufficient biomass for transformation, *Symbiodinium* cells were cultured in F/2 medium and aerated. The cells were grown until reaching the log phase and then used for transformation (Figure 1).

Symbiodinium sp Transformation

Agrobacterium tumefaciens carrying one of two binary vectors was used in this study. We used the CaMV35S promoter to drive expression of the GUS reporter genes and the ubiquitin promoter to drive expression of the GFP reporter genes (Figure 2). CaMV35S and ubiquitin are two common promoters that have been successfully used in plant transformation systems. Additionally, the well-established electroporation method of transformation has been successfully applied to transform foreign genes in many eukaryotic species. Pre-treated *Symbiodinium* cells were co-incubated for 24 hours with *A. tumefaciens* carrying either GUS or GFP genes and transformed using electroporation. Then, the free-living transformed *Symbiodinium* cells were cultured in F/2 medium for hygromycin selection. After 7 days in selection medium, hygromycin resistant transgenic *Symbiodinium* cells were observed. Cells were stained using X-Gluc solution and monitored for GUS protein expression under light microscopy. For GFP expression, green fluorescence signal was observed in the transgenic *Symbiodinium* cells under fluorescence microscopy.

GUS Staining and GFP Detection

To determine the successful integration of the GUS gene into the genome of *Symbiodinium* cells, genomic

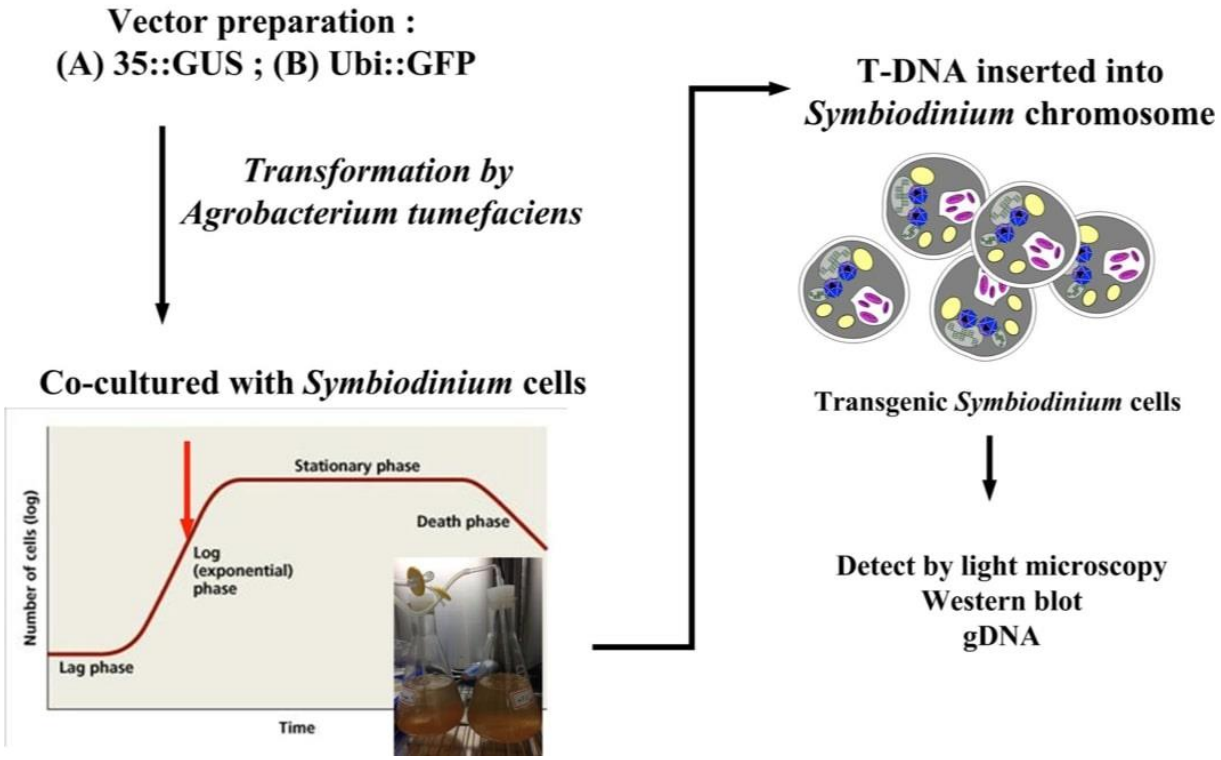

Figure 1. Overview of Agrobacterium-*Symbidinium* transformation. Vector transformed into *Symbiodinium* cells. T-DNA integrated into the *Symbiodinium* by co-culturing of Agrobcaterium and *Symbiodinium*. Inducible expression of reporter genes in transgenic *Symbiodinium* confirmed by microscopy and molecular approaches.

DNA was isolated from transgenic and wild type of *Symbiodinium* sp. and the GUS gene was screened for using a PCR assay. DNA electrophoresis gels showed one band at 700 bp representing the GUS gene that was detected in the transformants and agrobacterium sample (Figure 3). The GUS gene was clearly not detected in the wild type and used as negative control. This suggests that the transgenics line contained the GUS gene. To confirm whether the GUS gene was expressed in the *Symbiodinium* cells, we performed the GUS histochemical assay. GUS is commonly used as marker gene system for transgenic plants. With this approach, stained cells expressing the GUS gene would appear blue after a reaction with the substrate (X-gluc). Blue cells were observed under the microscope, thus confirming GUS gene expression in transgenic *Symbiodinium* sp. (Figure 4).

To examine green fluorescent protein (GFP) expression, the transgenic Symbiodinium cells were observed under the fluorescence microcope. Our result showed the green fluorescence signal observed in transgenic *Symbiodinium* sp but absent in wild type strains (Figure 5). We also confirmed the protein expression extracted from wild type and transgenic *Symbiodinium* cells. No band was observed in wild type *Symbiodinium* while one major protein band with a molecular weight of 27 kDA was detected in in transgenic *Symbiodinium* when cross-recognized with antibodies against green fluorescent protein (GFP) (Figure 5). This result indicates the exogenous of green fluorescent protein (GFP) genes was successfully integrated in *Symbiodinium* cells. Furthermore, Symbiodinium sp. could be potentially engineered from two main types of isolated Symbiodinium cells such as free living and symbiotic in cnidarians cells.

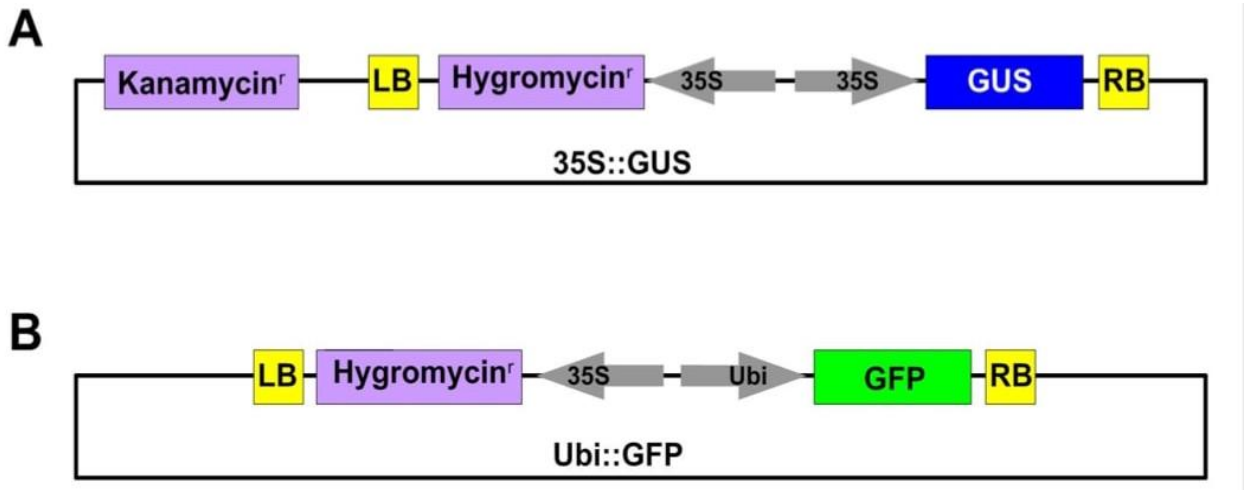

Figure 2. Schematic diagram of plasmids used for transformation in *Symbiodinium* sp. (A) CaMV35S promoter drives the reporter genes *GUS* (B) Ubiquitin promoter drives the reporter genes *GFP*.

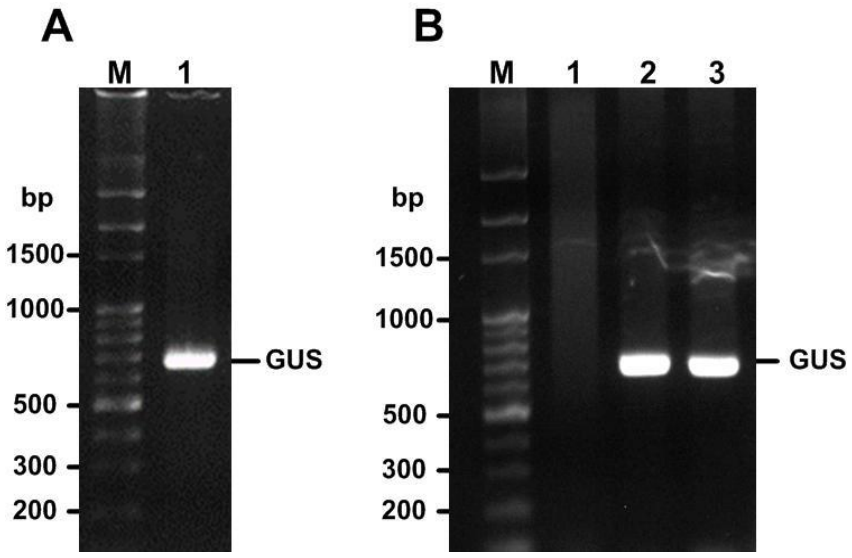

Figure 3. Agarose gel electrophoresis of GUS detection**.** (A) GUS confirmation for transformation plasmid of pCAMBIA to *Symbiodinium*. (B) PCR analysis using GUS gene detected in transgenic *Symbiodinium* (lane 2 and 3), no band detected in wild type *Symbiodinium* (lane 1). Positions of M.W. standards are indicated at left.

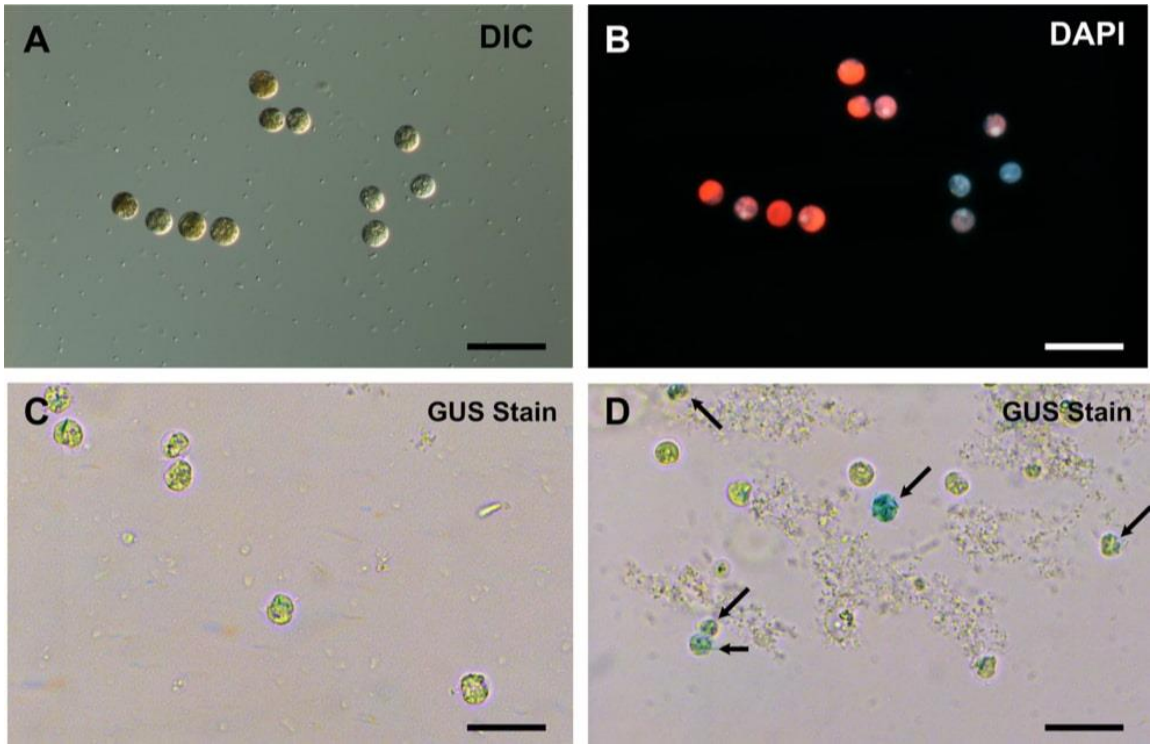

Figure 4. Microscopic observation of GUS staining in *Symbiodinium* cells. (A-B) Fresh isolated *Symbidinium* cells were brown in color. Red fluorescence represented the chlorophyll and blue color showed death cells of fresh isolated *Symbiodinium*. (C) Free living cultured *Symbiodinium* cells showed no staining at all. (D)Transgenic free living cultured *Symbiodinium* cells showing blue color due to activity of GUS. Positive staining indicated by the black-filled arrowheads. Scale bar, 10 µm.

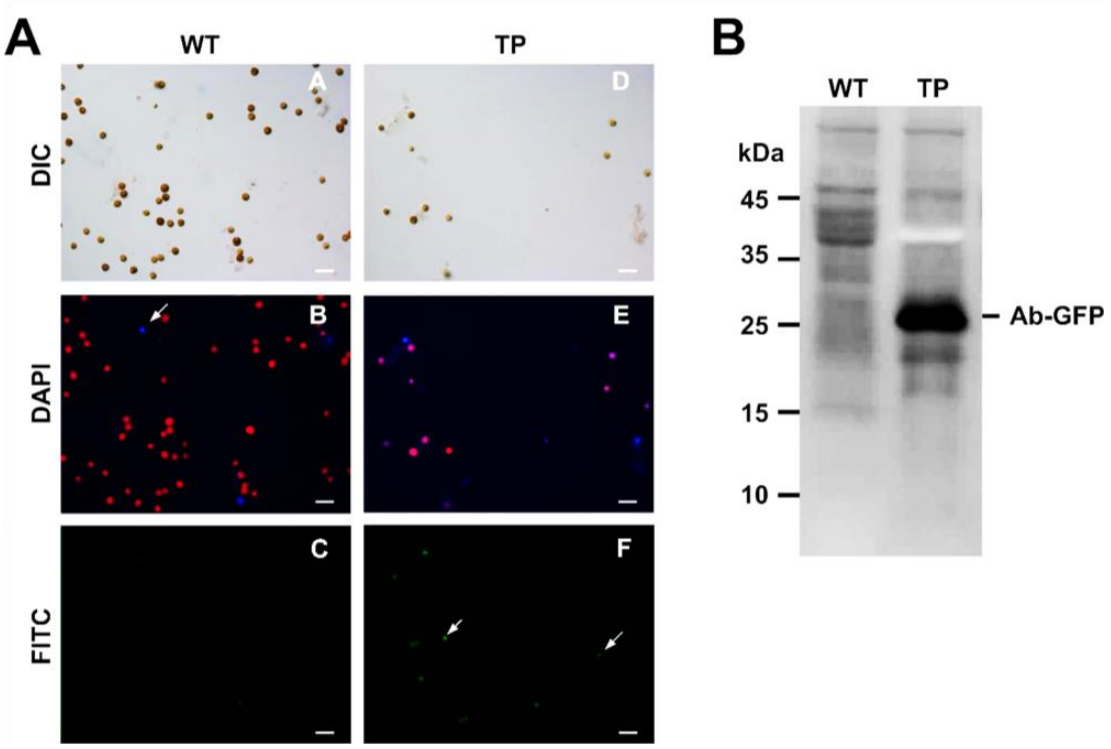

Figure 5. Microscopic and Western blot of Protein extracted from Symbiodinium cells. (A) Microscpic image of wild type (A,B,C) and transgenic *Symbiodinium* cells (D,E,F). Red fluorescence indicated chlorophyll (B,E) whereas the blue fluorescence represented death cells of *Symbiodinium* spp (B) indicated by the white-filled arrowheads. Green fluorescence (F) indicated by the white-filled arrowheads. Scale bar, 10 µm (B) Protein total extracted from wild type and transgenic *Symbiodinium* sp were resolved in SDS-Page and subjected to immunodetection gfp (27 kDa). The gfp detected by immunoassaying marked with black strip on the right of the protein band.

Discussion

Agrobacterium is an efficient tool to transfer foreign genetic material into the plant cells (Newell, 2000). Agrobacterium contains a unique transfer component that enables Ti-plasmid integration into the plant nuclear genome (Gelvin, 2003). GUS (β-glucuronidase) and GFP (green fluorescent protein) are commonly used reporter systems for visualizing gene expression in eukaryotic cells (Lorang *et al*., 2001). It has been reported that GFP and GUS reporters driven by 35S promoter were successfully used for agrobacterium transformation of algae (Cheng *et al*., 2012; Lohuis & miller, 1998). In the present study, two plasmids were used for assessing transformation of exogenous genes included reporter (GUS) driven by 35S promoter and reporter (GFP) driven by UBI promoter. In fact, the critical point for obtaining efficient transformation in *Symbiodinium* depends on the environment and growth response in each species. We demonstrated the transformation is based on the growth phase, therefore, the cells proliferation and survival could be optimized in transformed *Symbiodinium*. Accordingly, establishment of an efficient transformation *Agrobacterium*-mediated transformation in algae required the appropriate selectable period of growth and media to enable the successful transformation (Kumar *et al*., 2004; Rathod *et al*., 2013).

Transformation contacts between *A. tumifaciens* and the host are considered to play a key role in achieving proper transformation efficiencies of T-DNA transfer. It is necessary to generate the simple and reliable procedure to infiltrate *A. tumifaciens.* Various techniques have been applied for introducing *A. tumifaciens* strains such as pollen mediated, injection, and microprojectile bombardment in plant cells (Xu *et al*., 2014; Liu & Pijut, 2010; Manickavasagam *et al*., 2015). Considering *Symbodinium* cells size and morphology, Pasaribu *et al*. (2014) reported *Symbiodinium* cells could be disrupted by using beads. Therefore, we applied the electroporation method in *Symbiodinium* cells. It has been reported that electroporation method have been successfully applied to transform foreign genes in many eukaryotic species (Munoz *et al*., 2018). Electroporation is a simple method that enhances the access of A. *tumifaciens* to infiltrate the T-DNA transfer to cells the nuclear genome. The infiltration of Agrobacterium T-DNA is activated by inducer of vir region so called Acetosyringone (AS) at wounding site. Our results show GUS and GFP genes were expressed in *Symbiodinium* cells. We noted that the promoter selection is necessary for successful transformation of the exogenous gene. CaMV3 promoter is a frequently used promoter to drive the expression of transgenes in microalgae species such as *Chlamydomonas* (Kumar *et al*., 2004), *Dunaliella bardwill* (Anila *et al*., 2011), and *Parachlorella kessleri* (Rathod *et al*., 2103). In contrast, use of the Ubi promoter for *Agrobacterium*-mediated transformation in *Symbiodinium* cells is poorly understood. However, Ubi is a commonly used promotor in transformation studies to generate various transgenic monocot species (Wang *et al*., 2020; Xu *et al*., 2017; Koetle et al., 2017). Furthermore, our result demonstrated CaMV35S and UBI promoters could be employed in driving the expression of gene constructs for the functional gene analysis in *Symbiodinium*.

Insertion of agrobacterium T-DNA was observed in various ways that enabled to obtain the transformation efficiency. We could amplify genomic DNA of transgenic lines created with GUS:35S constructs by PCR method demostrating the successful insertion of T-DNA into the nuclear genomes of *Symbiodinium* cells. Previous studies described PCR strategies as a powerful tool to identify the mutations in plant cells (Muniz *et al*., 2014). Moreover, we maintained the transformant *Symbiodinium* in culture media for protein assesment. Immunoblot analysis showed the presence of expected size of gfp protein was observed. This is similar with previous reports of *Agrobacterium* mediated transformation with various constructs and reporter genes is driven by ubi promoter (Wang *et al*., 2020; Xu *et al*., 2017).

In conclusion, we reported a practical and simple technique Agrobacterium-mediated transformation in *Symbiodinium* cells. In recent study, fresh endosymbiotic *Symbiodinium* cells were isolated from *Exaiptasia pulchella* sample and subjected for *A. tumifaciens* mediated transformation. The plasmids integration was detected by PCR and immunoblot assay. The results showed the efficiency and simple approach that allows an efficient transformation for further understanding of genetic modification in *Symbiodinium* cells. This method will offer better utilization and exploitation of specifics gene functions and efficient manipulation of *Symbiodinium* clades.

Ethical Statement

Not applicable

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Author Contribution

BP carried out the major experimental work, analyzed data, and wrote the paper. PLJ designated research and wrote the paper.

Conflict of Interest

The authors declare that they have no conflict of interest.

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