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TECHNICAL REPORT

High-Efficient and Transient Transformation of Moso Bamboo (*Phyllostachys edulis*) and Ma Bamboo (*Dendrocalamus latiflorus* Munro)

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Abstract

Moso bamboo (*Phyllostachys edulis*) and Ma bamboo (*Dendrocalamus latiflorus* Munro) are two major species, which have significant economic and research application value. At present, methods for interrogation of gene function in bamboo are limited and time consuming. Therefore, establishing an efficient transient expression system for bamboo is very useful for dissecting the function of candidate genes. In this study, we developed protocols for the transient transformation of protoplasts from different tissues in Moso bamboo and Ma bamboo and established the optimized protocol and materials for improving the efficiency of transient transformation. The results indicated that the transformation efficiency of protoplasts from 15-day-old etiolated seedlings was higher than other tissues, which were 44.7% in Moso bamboo and 35.2% in Ma bamboo. In addition, we also established an experimental approach for optimizing overexpression of external proteins using *Agrobacterium*-mediated transient transformation of bamboo leaves and whole seedlings. Finally, we utilized the *RUBY* reporter for monitoring successful transformation samples with red color for downstream experiments in Moso bamboo. In summary, this study provides optimized protocols for the over-expression of candidate genes in protoplasts or bamboo leaves and whole seedlings.

Keywords *Phyllostachys edulis* · *Dendrocalamus latiflorus* munro · Protoplasts · Transient transformation · β-glucuronidase (GUS) · *RUBY* reporter system

Introduction

Moso Bamboo (*Phyllostachys edulis*) and Ma bamboo (*Dendrocalamus latiflorus* Munro) are species of Gramineae, which widely grow in tropical and subtropical regions and have important values from economic (Choudhury et al. 2012) and ecological perspectives (Song et al. 2013). Moso Bamboo and Ma bamboo are often used for medicines (Sarangthem and Singh 2010), building materials (Fumio et al. 1995), papermaking (Shinjiro and Ogita 2005), xylitol production (Gurbuz et al. 2013) and bamboo biochar (Wang et al. 2019c). At the same time bamboo presents many interesting biological questions concerning long intervals for flower formation(Lin et al. 2010; Ge et al. 2017), and fast growing shoots (Peng et al. 2013a, b; Wei et al. 2017; Wang et al. 2019b). Thus, investigation of the unique characteristics will provide a valuable perspective and applications for other Poaceae species, such as rice (*Oryza sativa*).

At present, the cold-tolerance in Ma bamboo can be improved by *Agrobacterium*-mediated transformation of a *CodA* gene encoding choline oxidase (Qiao et al. 2014). The anthocyanin over-accumulation phenotype also has been reported through the overexpression of the maize Leaf color (*Lc*) using Ma bamboo young shoots as explants (Ye et al. 2017). Although the stable genetic transformation of Ma bambo has been reported for *CodA* and *Lc*, the system is time-consuming. At the same time stable genetic transformation systems for Moso bamboo have never been reported due to low regeneration and transformation efficiency. Thus,

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it will be advantageous to investigate transient transformation for both Moso bamboo and Ma bamboo. Plant transient expression technology refers to high-level expression of a target gene into host cells in a relatively short period of time (Shen et al. 2014). Compared with stable genetic transformation technologies, transient expression techniques have the advantages of being simple, rapid, low cost, safe and effective, and providing high expression level (Li et al. 2017).

Plant protoplasts are spherical cells enclosed by the plasma membrane, lacking the cell wall and are highly versatile for transient transformation systems (Eeckhaut et al. 2013). In 1960 Cooking first isolated protoplasts from root tips of *Solanum lycopersicum* by enzymatically removing cell walls in a hydrolytic step (Cocking 1960). Since then, protoplast have been isolated from various species such as *Pisum sativum* stem (Long and Iino 2001), *Liriodendron tulipifera* (Huo et al. 2017) and *Zea mays* calli (Vasil and Vasil 1987). Especially, Aoki and Takebe successfully transformed *Nicotiana tabacum* protoplasts with tobacco mosaic virus RNA in 1969 (Aoki and Takebe 1969). In 1971, Takebe et al. isolated protoplasts from *Nicotiana tabacum* leaves and obtained regenerated plants by protoplast culture, which indicated that the research and application of protoplasts entered a new era (Takebe et al. 1971). Polyethylene glycol (PEG)-mediated transformation of protoplasts have been reported in the investigation of programmed cell death-related processes (Chen et al. 2015), protein–protein interactions (Chen et al. 2010), signal transduction (Cao et al. 2014), subcellular localization (Wu and Hanzawa 2018), transient gene expression (Yoo et al. 2007) etc.

In addition to PEG-mediated protoplast transformation, *Agrobacterium*-mediated infection is an alternative transient system to investigate gene function (Nanjareddy et al. 2016). At present, the *Agrobacterium*-mediated transient transformation system has been widely used in *Arabidopsis* (Tsuda et al. 2012), *Glycine max* (Li et al. 2017), *Rosa rugosa* Thunb (Lu et al. 2017) and *Gossypium* spp. (Li et al. 2018). Though the method provides an efficient way to study gene function in vivo, a highly efficient transient transformation approach is lacking in bamboo due to the high levels of epidermal waxes and silicon, which prevent *Agrobacterium* from penetrating the bamboo leaves. Therefore, it is necessary to generate a method of transient transformation mediated by *Agrobacterium* in Moso bamboo and Ma bamboo for the comprehensive investigation of gene function.

Green fluorescent protein (GFP) (Li et al. 1998) and β-glucuronidase (GUS) (Jefferson et al. 1987) are useful reporters for gene expression. However, fluorescent protein presents limitations due to autofluorescence in plant cells. GUS-staining is utilized as invasive method. Thus, the sample after GUS histochemical staining cannot be used for additional molecular biology experiments. Recently, a new *RUBY* reporter system has been reported as visible marker to track the expression of target genes by expression of all the enzymes of betalain biosynthesis, which displayed strong red color (He et al. 2020). This *RUBY* reporter system solved the limitation of the fluorescent or GUS-based methods.

In this study, we compared the protoplast yield, viability and transformation efficiency from different tissues of Moso bamboo and Ma bamboo. We also present a high-efficiency method for isolating protoplasts from different tissues and an improved PEG-mediated protoplast transformation scheme in bamboo. In addition, we developed a protocol for *Agrobacterium*-mediated overexpression in leaves and whole seedlings. We used non-invasive *RUBY* reporter system (He et al. 2020) to solve the issue of autofluorescence in Moso Bamboo. In summary, this study provided two transient transformation methods to investigate the function of candidate genes in bamboo.

Results

Protoplast Isolation from Different Developmental Stages and Tissues

To establish a highly efficient protocol for protoplast isolation, we collected materials from above ground and below ground (roots) portions from 15-day-old etiolated seedlings for Moso bamboo and Ma bamboo (Figs. 1a1, 9). We also collected 15-day-old above ground parts from light-grown seedlings (Figs. 1a2, 10) and the tip part from 30-day-old light-grown seedlings (Figs. 1a4, 12), respectively. In previous reports, longitudinal cutting allowed efficient digestion of cell walls by enzyme digestion solution (Zhang et al. 2014). Thus, the fresh materials were cut into longitudinal strips, which were transferred into enzyme digestion solution and gently shaken at 60 rpm in dark conditions at room temperature for 4 h.

Protoplast Viability and Yield at Different Developmental Stages and Tissues

We compared the protoplast yield isolated from four different materials, the result indicated that high yields of Moso bamboo protoplasts could be isolated from above ground portion from seedlings and stem tips but not from roots (Fig. 1b). Protoplast yields of Moso bamboo ranged from 2.51×10^5 to 1.42×10^7 protoplasts g^{-1} [fresh weight (FW)]. From stem tips of one-month-old seedlings about 1.42×10^7 protoplast cells were obtained, which was about 51-fold higher than the yield of protoplasts (2.51×10^5) isolated from roots. In Ma bamboo, the yield of protoplasts was 8.95×10^6 and 1.41×10^7 for stem tips and above ground parts from light-grown seedlings, respectively. In total, 9.6×10^6 protoplasts g^{-1} could be isolated from above ground etiolated

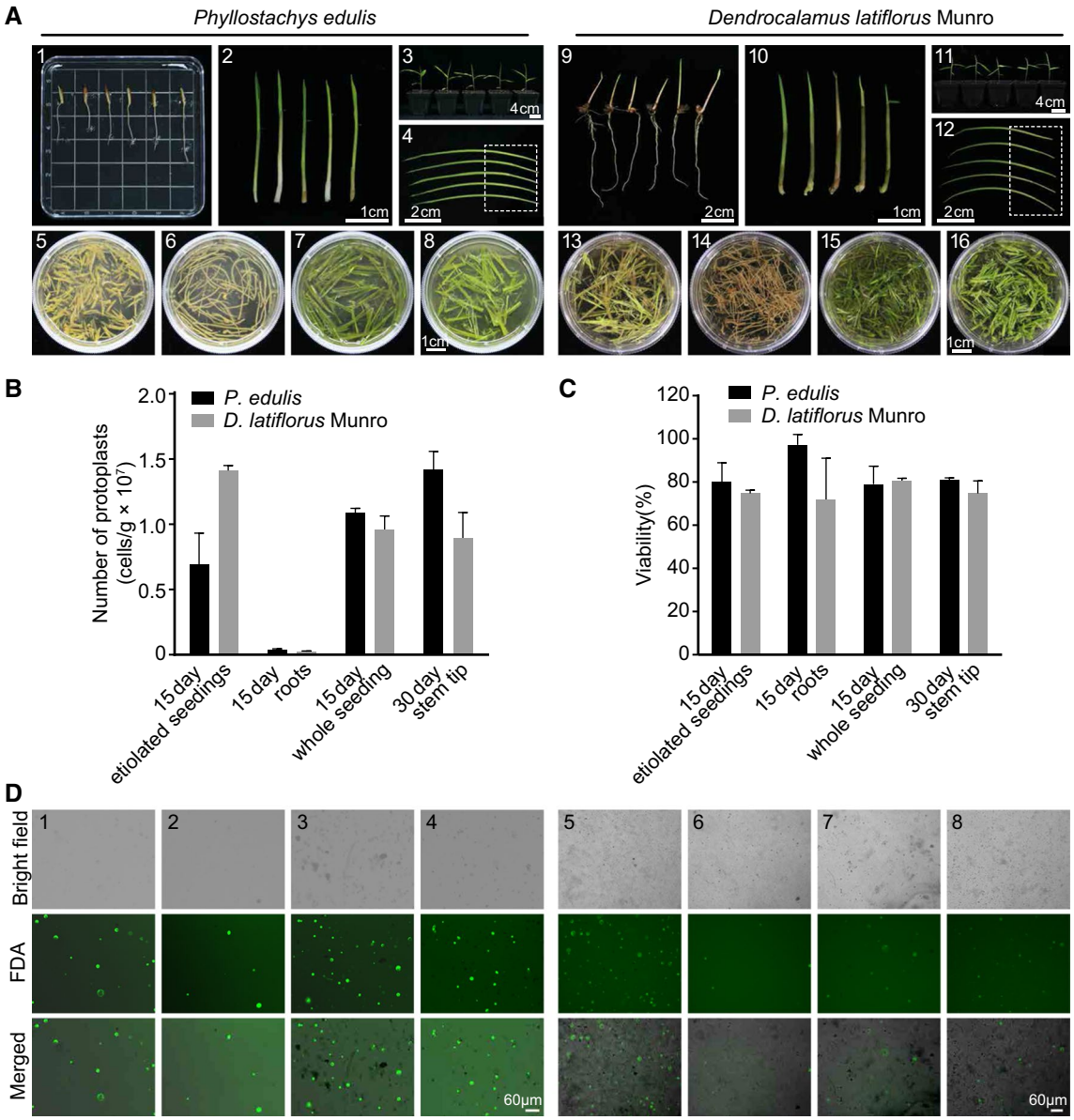

Fig. 1 Materials for protoplast isolation, protoplast viability and yield determination. **a1** 15-day-old etiolated seedlings and roots from Moso bamboo. **a2** 15-day-old whole seedlings from Moso bamboo. **a3, 4** 30-day-old seedlings and stem tips from Moso bamboo. **a5–8** 15-day-old etiolated seedlings, roots, 15-day-old whole seedlings and 30-day-old stem tips from Moso bamboo were cut into filaments and transferred to a Petri dish with enzyme solution. **a9** 15-day-old etiolated seedlings and roots from Ma bamboo. **a10** 15-day-old whole seedlings from Ma bamboo. **a11, 12** 30-day-old seedlings and stem tips from Ma bamboo. **a13–16** 15-day-old etiolated seedlings, roots, 15-day-old whole seedlings and 30-day-old stem tips from Ma bamboo were cut into filaments and transferred to a Petri dish with enzyme solution. **b** Comparison of protoplast yield from different tissues of Moso bamboo and Ma bamboo. **c** Comparison of protoplast viability. **d** Viability of Moso bamboo (**d1–d4**) and Ma bamboo (**d5–d8**) protoplasts derived from 15-day-old etiolated seedlings (**d1, d5**), 15-day-old roots (**d2, d6**), 15-day-old whole seedlings (**d3, d7**) and 30-day-old stem tips (**d4, d8**). Protoplast viability images were obtained using a confocal fluorescence microscopy (Leica TCS SP8X DLS) with a 10 × magnification

seedlings in Ma bamboo. However, it was difficult to isolate protoplasts from roots in both Moso bamboo and Ma bamboo, which might be associated with root structure.

The viability was detected after purification of isolated protoplasts and incubation with fluorescein diacetate (FDA) for 5 min. By comparing the protoplast viability from different materials, we found the viabilities of 79.9% for above ground portion from etiolated seedlings, 97.2% for roots from etiolated seedlings, 79.1% for above ground part from light-grown seedlings, and 80.9% stem tip part from light-grown seedlings (Fig. 1c). Overall, the viability of protoplasts showed little difference, and the average viability

was about 80%. In Ma Bamboo, protoplast viability of different materials ranged from 71.9% to 80.7% (Fig. 1c). Under blue fluorescence light, most protoplasts showed an intense green fluorescence (Fig. 1d).

Protoplast Transformation Efficiency

High efficiency transformation of protoplasts are the basic for gain-of-function and loss-of-function experiments. Since the protoplast transformation efficiency depends on the chosen tissue type and developmental stage, it is especially important to select the optimal material to obtain high transformation efficiency. To reveal variations in transformation efficiency from different materials, we transformed protoplasts derived from above ground portion of 15-day-old etiolated or light-grown seedlings as well as the shoot tips from 30-day-old light-grown seedlings. We did not use roots for the transformation experiment, because the protoplast yield in roots was very low. The protoplasts were cultured in the dark for 18 h after PEG-mediated transformation to measure transformation efficiency (Fig. 2a). In Moso bamboo, it was found that the transformation efficiency was 44.7% for the above ground portion from 15-day-old etiolated seedlings, 38.5% for stem tips of 30-day-old light-grown seedlings, and 34.9% for above ground portion from 15-day-old light-grown seedlings (Fig. 2b). In Ma bamboo, the transformation efficiency of protoplasts for above materials was 35.2% for the above ground portion from 15-day-old etiolated seedlings, 26.0% for stem tips of 30-day-old light-grown seedlings, and 19.3% for above ground portion from 15-day-old light-grown seedling (Fig. 2b). In summary, these studies indicated that a high transformation efficiency was achieved in protoplasts from etiolated seedlings, which was consistent with the result of etiolated seedlings in maize (*Zea mays* L.) (Xie et al. 2014).

Agrobacterium-Mediated Transient Transformation of Leaves

Agrobacterium-mediated transient transformation provides a simple, quick and effective transient gene expression system (Zheng et al. 2012). To establish an *Agrobacterium*-mediated transient transformation method in Moso bamboo and Ma bamboo, we used *Agrobacterium* (EHA105) carrying pCambia1301 vectors as well as different infection media (Hiei et al. 1994; Verma et al. 2008; Andrieu et al. 2012; Zeng et al. 2019), temperatures and concentrations of acetosyringone (AS) to infect leaves and evaluated the transformation efficiency by GUS staining. We used a scalpel to slice the leaves and immediately immersion in the *Agrobacterium* suspension. We revealed that using only the AAM medium to resuspend agrobacteria could induce GUS gene expression in infection leaves. Temperature also

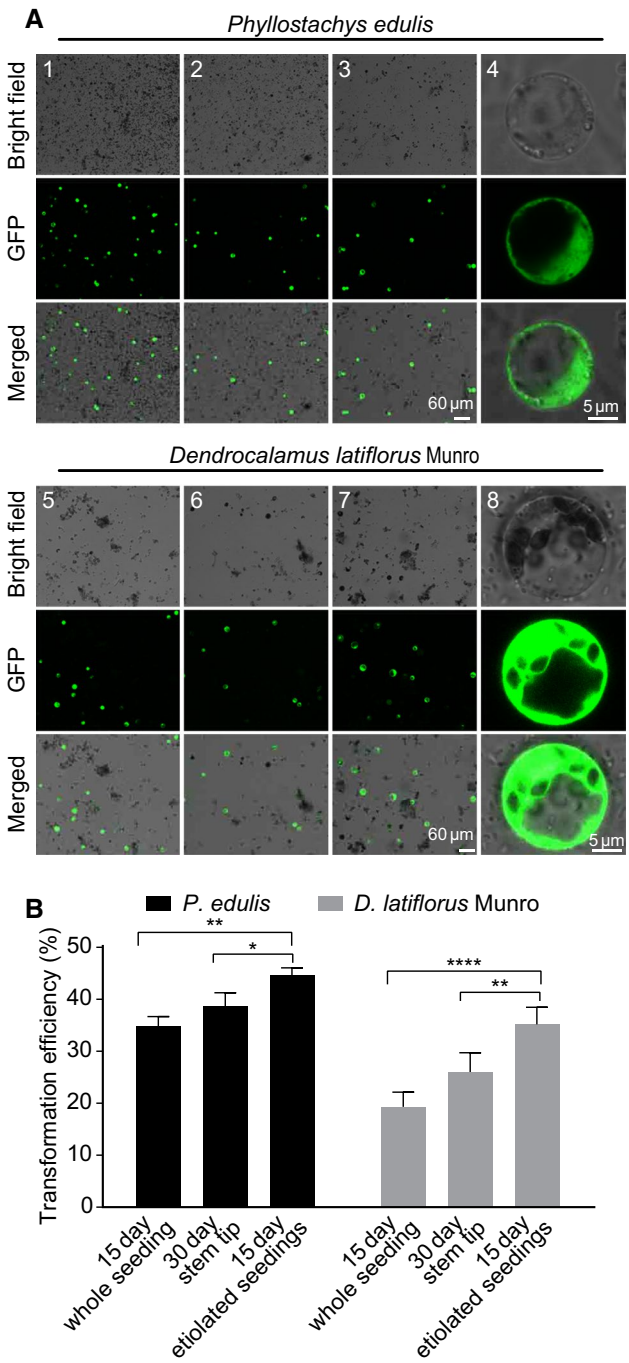

Fig. 2 Detection of protoplast transformation efficiency of Moso bamboo and Ma bamboo by vector GFP-pUC22. Protoplasts derived from 15-day-old etiolated seedlings (**a1, a5**), 15-day-old whole seedlings (**a2, a6**), 30-day-old stem tips (**a3, a7**). **a4, a8** Close-up view of the expression pattern of pUC22-35s-sGFP with a Leica TCS SP8X DLS fluorescent microscope. **b** Comparison of protoplast transformation efficiencies of different tissues of Moso bamboo and Ma bamboo

plays a significant role in *Agrobacterium*-mediated transient transformation. Our results showed that the appropriate temperatures were 21 °C for Moso bamboo and 26 °C for Ma

bamboo. In addition, we attempted to improve the leaf transfection efficiency by changing the concentration of the AS (100 μM, 150 μM, 200 μM) in the Moso bamboo and Ma bamboo *Agrobacterium* culture medium. GUS proteins were found in both Moso bamboo and Ma bamboo only when the concentrations of AS were 100 μM and 200 μM, respectively. GUS staining could be detected from 2-day post inoculation (dpi) to 5 dpi (Fig. 3b). GUS staining showed that the transformation efficiency for Moso bamboo and Ma bamboo was 20–30%. We also further confirmed GUS expression by

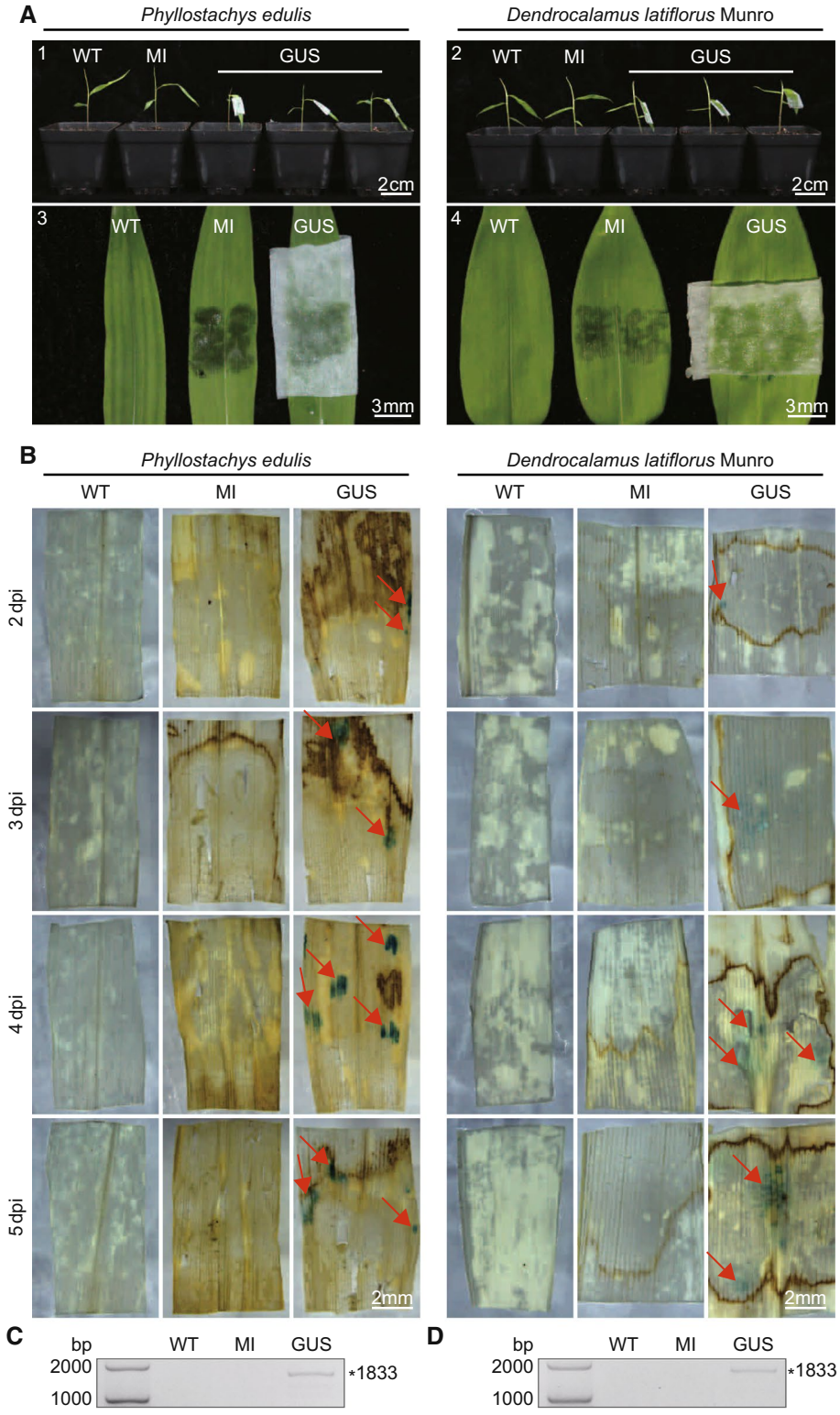

Fig. 3 Agroinfection of Moso bamboo and Ma bamboo leaf tissues with *Agrobacterium* (EHA105) suspension. **a1**, **a2** 30-day-old Moso bamboo and Ma bamboo grown in an artificial climate chamber for the transient transformation. **a3**, **a4** Magnified of (**a1**, **a2**) showing wild type (WT), only mechanical injury (MI) and *Agrobacterium*-mediated GUS transformation (GUS). **b** GUS staining was observed after 2-, 3-, 4- and 5-day post inoculation (dpi) in Moso bamboo and Ma bamboo infected leaves for WT, MI without inoculation treatment and GUS transient transformation. **c**, **d** GUS transcripts were detected by PCR, which showed GUS expression in *Agrobacterium*-infected leaves, but not in the control group

PCR, which showed that GUS expression in *Agrobacterium*-infected leaves could be detected by PCR in Moso bamboo (Fig. 3c) and Ma bamboo (Fig. 3d). All results confirmed that the *Agrobacterium*-mediated transient transformation system has been successfully expression.

Agrobacterium-Mediated Transient Transformation of Whole Seedlings

The transient transformation of leaves only presented GUS expression in the wounded region of leaf. To overcome the disadvantage, we also applied the transformation system to whole seedlings in Moso bamboo. At first, we only cut off the root tip and immersed the whole root in *Agrobacterium* culture with OD_{600} values of 0.9 and a AS concentration of 100 µM. Using seedlings without any wounds (Fig. 4a1) and *Agrobacterium* re-suspension without vector (Fig. 4a2) as control, we only found GUS expression around the cut off root tip by GUS staining. To improve the transformation efficiency, we also longitudinally sliced the whole roots, which could improve GUS expression greatly (Fig. 4a3). The GUS expression was mainly located in the root, stem tip and the base of the stem of Moso bamboo (Fig. 4a3). After deducing the most effective transformation method, we also explored the effect of different AS concentrations (Figs. 4a4, 5). The transformation efficiency was 83.3% at 100 µM, 16.7% at 150 µM, and 66.7% at 200 µM concentrations of AS (Fig. 4b). We also applied the system to infect aseptic seedlings which also showed intense GUS staining (Fig. 4c). All the results indicate that this system works well for Moso bamboo transient transformation.

RUBY Reporter System in Bamboo

Moso bamboo presented a high autofluorescence background at different wavelength (Fig. 5a), which limited the application of fluorescent protein as a reporter in Moso Bamboo. We, therefore, we utilized the *RUBY* reporter system (He et al. 2020) as a non-invasive method to replace the GUS-reporter system. The *RUBY* reporter system was transformed into *Agrobacterium* and resuspended in AAM culture medium to infect 20-day-old Moso bamboo. We observed strong red coloration of bamboo roots after 3 days of transformation (Fig. 5b), which suggested that the *RUBY* reporter system was useful for monitoring transient expression in Moso bamboo. According to the reporter system, we could collect red colored samples with successful transformation for downstream experiments due to noninvasive treatment. In summary, the *RUBY* reporter system solved the problems of both invasive treatment of GUS-staining and inconvenient observation due to high autofluorescence background in Moso bamboo.

Discussion

A large number of gene models have been identified by whole-genome sequencing of Moso bamboo (Zhang et al. 2011; Zeng et al. 2015, 2017; Wang et al. 2019a; Zhao et al. 2019). Thus, the development of high transient transformation efficiency is important for subsequent investigations of gene functions. Transient expression systems have been extensively used in *Arabidopsis, Phaseolus vulgaris, Oryza sativa* (Verma et al. 2008; Andrieu et al. 2012; Mangano et al. 2014). However, the usage of PEG-mediated protoplasts and *Agrobacterium*-mediated transformation of plants were still at an initial stage in both Moso Bamboo and Ma bamboo. The option of *Agrobacterium*-mediated transformation is lacking in bamboo. We selected Moso Bamboo and Ma bamboo which belong to scattered bamboos and cluster bamboos, respectively, to establish highly efficient PEG-mediated protoplast and an *Agrobacterium*-mediated transient transformation systems.

In previous reports, growth environment, selection of different tissues, and the cutting method had certain effects on yield, viability, and transformation efficiency of protoplasts (Yoo et al. 2007; Wu et al. 2009; Nanjareddy et al. 2016). In this study, we comprehensively investigated the viability, yield and transformation efficiency of protoplasts separated from different stages and tissues in Moso Bamboo and Ma bamboo to identify the optimum material with the highest transformation efficiency for future studies. Our results indicated that the maximum yield of protoplasts in Moso bamboo and Ma bamboo were stem tips (1.42×10^7 protoplasts g^{-1}) and 15-day-old above ground parts from light-grown seedlings (1.41×10^7 protoplasts g^{-1}).

The yield of isolated protoplasts by our method was higher than the yield reported for *Triticum aestivum* (Jia et al. 2016) and *Manihot esculenta* Crantz calli (Sofiari 1998) and was comparable to *Populus trichocarpa* (Guo et al. 2012). The protoplast viability differs slightly between tissues which is mainly related to the growth period, the operation at the time of extraction and the composition of the enzyme digestion solution (Anthony et al. 1995). We further compared protoplasts derived from different organs and different ages to determine whether there is an effect on the transformation efficiency. Among the protoplasts from different transformation materials, above ground portions from 15-day-old etiolated seedlings were the best material for PEG-mediated protoplast transfection, which reached a transformation efficiency of 44.7% in Moso bamboo and 35.2% Ma bamboo, using 10 µl of plasmid DNA and 40% PEG. Therefore, we recommend that bamboo researchers begin using etiolated seedlings and further modify our optimized procedure according to their practical applications.

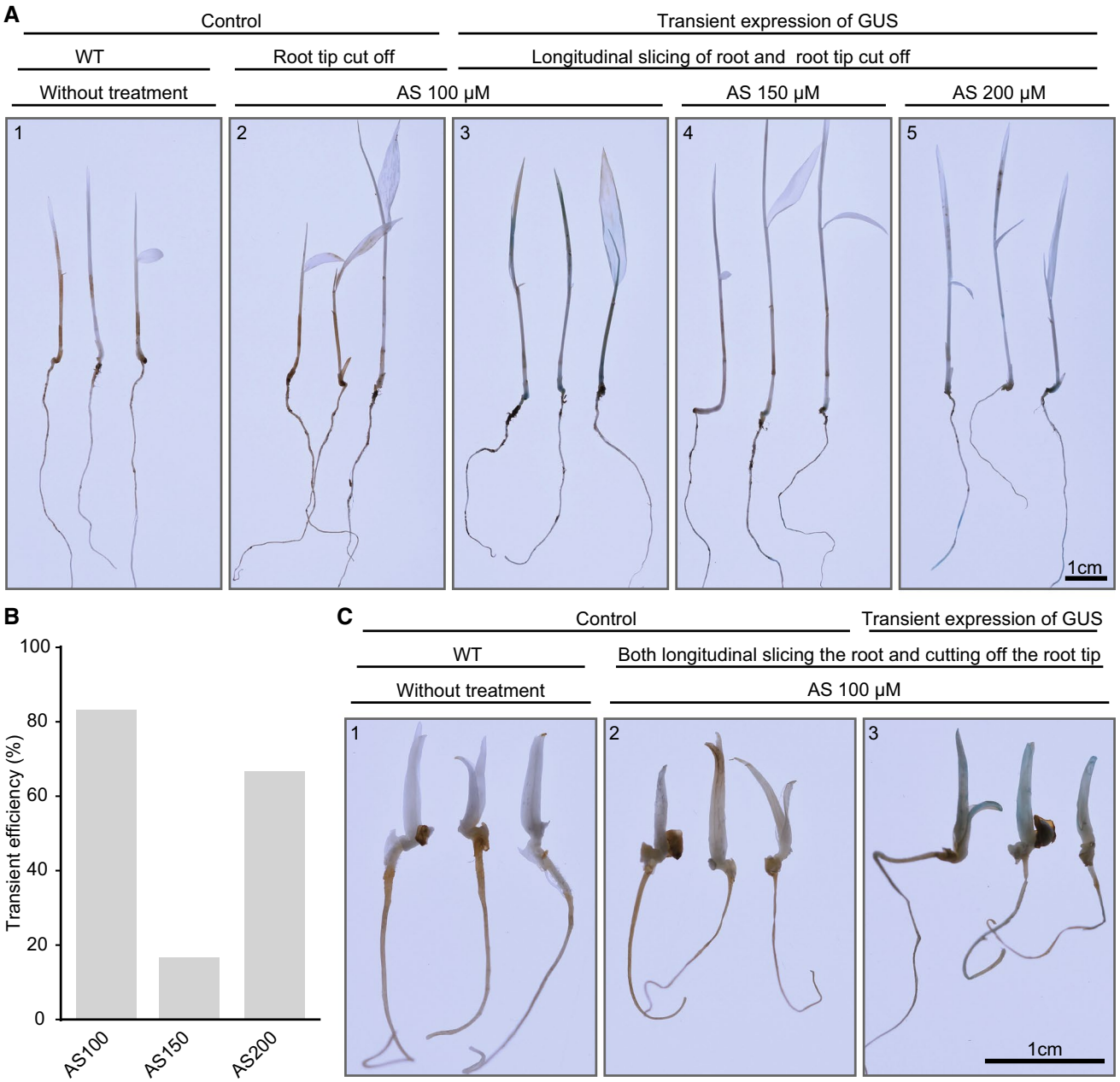

Fig. 4 GUS staining analyses of whole-seedling transient expression in Moso bamboo. **a1** Moso bamboo without any wound and *Agrobacterium* infection. **a2** the root tip was cut off and whole roots were immersed in *Agrobacterium* (EHA105) without vector. **a3–5** After the roots were longitudinally sliced and the root tip cut off, whole roots were immersed in *Agrobacterium* suspension with AS concentrations of 100 μM, 150 μM, 200 μM, respectively. **b** Transformation efficiency in the presence of 100 μM, 150 μM, and 200 μM AS. **c1** Aseptic, untreated WT seedlings. **c2** Aseptic seedlings treated with untransformed *Agrobacterium*. **c3** Aseptic seedlings treated with *Agrobacterium* transformed with the GUS vector. Bacteria suspension in (**c2**) and (**c3**) contained 100 μM AS

Agrobacterium-mediated transient transformation has been widely used in dicotyledonous species. However, this technology is lacking in bamboo. According to previous reports, AS concentrations played a significant role in the transient transformation efficiency (Aldemita and Hodges 1996; Manickavasagam et al. 2004; Verma et al. 2008). To determine the optimum concentration of AS, we tested different concentrations of AS (100 μM, 150 μM, 200 μM). Our results showed that the non-transformed control Moso bamboo and Ma bamboo were not stained blue, while transformed Moso bamboo and Ma bamboo exhibited blue color with AS concentrations of 100 μM and 200 μM, respectively. PCR results also indicated that *Agrobacterium*-mediated pCambia1301-GUS

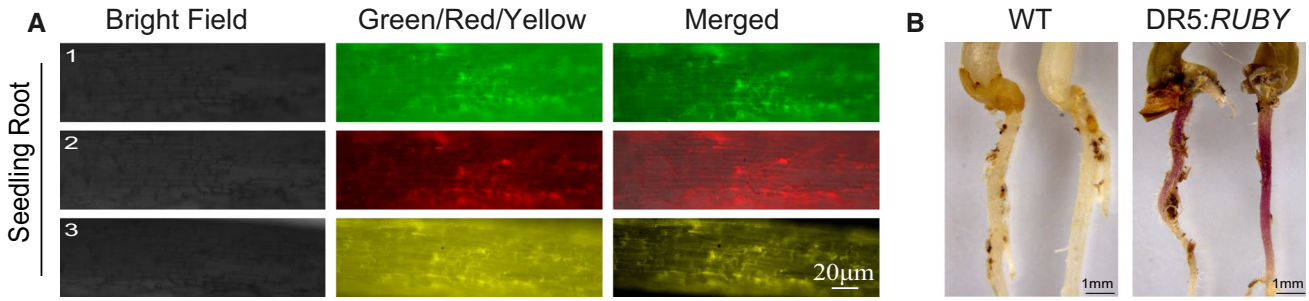

Fig. 5 Autofluorescence background of Moso bamboo was observed at different wavelengths. **a1–3** The images of autofluorescence in the root of 20-day-old Moso bamboo were presented at 495–535 nm (green wavelength), 648–709 nm (red wavelength) and 520–561 nm (yellow wavelength). **b** Comparison with wild type, 20-day-old seedlings of Moso bamboo displayed strong red color after 3 days of infection with *Agrobacterium* strain GV3101

could be successfully transformed and expressed in Moso bamboo and Ma bamboo leaves. The GUS staining only showed in the injured part, while the area distant from the injured part, which was consistent with the results in *Oryza sativa* (Andrieu et al. 2012).

During high-efficiency *Agrobacterium*-mediated transformation, only plants with wounds allow *Agrobacterium* to easily enter plant tissues and transform cells (Bakshi et al. 2011). In our study, we compared the method of cut-off root tips and longitudinal slicing of roots. Both methods were highly effective as the intense expression of GUS demonstrated. Our results revealed that injuring tissues was a critical step for high-efficiency expression, which was likely due to the plant cells being more accessible for *Agrobacteria.* By comparing different AS concentrations, we found that 100 μM was most suitable for Moso bamboo transient transformation. However, we only analyzed three concentrations and did. Thus, a more optimal concentration could possibly be revealed by examining the concentration gradient further. In addition to AS concentration, transformation efficiency might be related to the growth state of the plant. The greatest advantage of transforming whole seedlings was maintaining vital tissue, which did not turn yellow and form potentially necrotic areas. This provided a better chance to observe phenotypes at high transformation efficiency. We applied this to *Agrobacterium*-mediated transient transformation and utilized *RUBY* noninvasive treatment reporter system, which provides a solution for collecting samples with successful transformation based on a strong red color for downstream experiments in Moso bamboo. The establishment of the *Agrobacterium*-mediated transient transformation method in Moso bamboo and Ma bamboo reported here will not only provide an effective method for the over-expression of candidate genes but also a basis for the study of genotypes and phenotypes of Moso bamboo.

Materials and Methods

Efficient Protoplast Isolation from Moso Bamboo and Ma Bamboo

In this study, Moso bamboo and Ma bamboo were grown at 25 °C for 15 days and 30 days at room temperature (22 °C) and 60% relative humidity. Etiolated seedlings were grown in the dark at 25 °C for 15 days. Tissues from different developmental stages of Moso bamboo and Ma bamboo were collected, cut into longitudinal strips (Fig. 1a) and rapidly transferred to Petri dishes containing 10 mL of enzyme digestion solution (Zhang et al. 2011). This solution had been filtered with a 0.45 μm polyethersulfone syringe filer prior usage. Then, the immersed strips were gently shaken for 4 h at 60 rpm in constant darkness to release protoplast. The digested tissues were filtered through a double layer of nylon meshes and centrifuged at 1200 rpm for 5 min to collect protoplast, which were further purified with mannitol (CPW11M) for three times according to the protocol for soybeans (*Glycine max* (L.) Merr.) (Xiong et al. 2019) with minor modifications: 0.2 mmol L^{-1} KH_2 PO_4 (Sigma P5655), 1 mmol L^{-1} KNO_3 (CAS7757-79-1), 10 mmol L^{-1} $CaCl_2$ (Sigma C5670-500G), 1 mmol L^{-1} $MgSO_4$•$7H_2O$ (Sigma 63,138), 1×10^{-3} mmol L^{-1} KI (Sigma V900056), 1×10^{-4} mmol L^{-1} $CuSO_4$•$5H_2O$ (Sigma C3036). The pH of CPW was adjusted to 5.7. Finally, we added 11% (w/v) mannitol (Sigma M1902-500G) to generate CPW11 solution. Finally, the concentration of protoplasts was adjusted to $1.2 \sim 1.4 \times 10^6$ mL^{-1} by MMG solution [mannitol 400 mmol L^{-1}, $MgCl_2$ (MACKLIN M813763) 15 mmol L^{-1}, MES-KOH (pH 5.7) 4 mmol L^{-1} (Sigma V900336-100G)] as previously reported for soybean protoplasts (Xiong et al. 2019).

Comparison of Protoplast Yield and Viability from Different Materials

The yield of protoplasts was measured with a hemocytometer. The average protoplast yield was determined from three independent experiments with each experiment including three technical repeats and calculated as previously reported (Jia et al. 2016): protoplast yield (protoplast/g FW) = (protoplast density × protoplast suspension volume)/material weight (g FW).

Protoplast viability was determined by the FDA method. FDA was dissolved in dimethyl sulfoxide (DMSO) at 5 mg/mL and stored at − 20 °C. FDA was added to the protoplast suspension to a final concentration of 0.01% at room temperature. After five minutes, protoplast viability was examined with a Leica TCS SP8X DLS fluorescent microscope using a 488-nm excitation wavelength and 530 nm emission wavelength (Larkin 1976). Three independent experiments were performed to calculate average protoplast viability. We calculated the viability as previously reported (Huang et al. 2013; Wu et al. 2017) according to the following formula: protoplast viability (%) = (number of fluorescent protoplasts in view/total number of protoplasts in view) × 100%.

Statistics of protoplast yield and density were performed using a Nikon ECLIPSE E100 microscope. Transformation efficiency and protoplast viability were calculated under ZEISS AXIO OBSERVER A1. Images were taken with a Leica TCS SP8X DLS fluorescent microscope using 488 nm excitation wavelength and 530 nm emission wavelength.

PEG-Mediated Protoplast Transfection

In this study, pUC22-35s-sGFP was prepared using a Magen HiPure Plasmid EF Maxi Kit (P1156-02) and a final stock concentration of 1800 ng/μL was generated. PEG-mediated transfection was carried out as described (Xiong et al. 2019) with minor modifications. In brief, 100 μL protoplasts, 10 μL plasmid and 110 μL PEG solution [10 mmol L^{-1} $CaCl_2$, 200 mmol L^{-1} mannitol, and 40% (w/v) PEG (Sigma 81240-1KG)] were added into a 2 mL microfuge tube and quickly flicked for 1 min to ensure the mixture was homogeneous. After incubation for 25 min in dark at room temperature, the transfected protoplasts were cleaned with 500 μL CPW11M solution and centrifuged at 1200 rpm for 3 min to remove the supernatant. This process was repeated two times with 1 mL CPW11M solution. Then cleaned protoplasts were resuspended with 1 mL CPW11M solution and incubated for 18 h at room temperature in the dark.

Comparison of Protoplast Transformation Efficiency from Different Materials

The transformation efficiency of protoplasts was calculated using nine randomly selected microscope views. Three independent experiments were performed to calculate average transformation efficiencies using the following equation (Gomez Cano et al. 2019): number of protoplasts expressing GFP/total number of protoplasts.

Agrobacterium-Mediated Transient Transformation of Leaves

Leaves from 1-month-old Moso bamboo and Ma bamboo were used for the *Agrobacterium*-mediated transient transformation studies. We used the pCambia1301 vector encoding for an intron-containing GUS gene preventing reporter gene viability in *Agrobacterium* itself. The pCambia1301 vector was transformed into *Agrobacterium* EHA105 and incubated in liquid YEP medium (50 mg/L kanamycin and 50 mg/L rifampicin) at 28 °C and at 200 rpm for 24 h. The bacteria were centrifuged at 4000 rpm for 8 min and resuspended in AAM medium (Hiei et al. 1994) for Moso bamboo and infecting solution (MES-KOH pH 5.7 10 mM, $MgCl_2$ 20 mM, AS 200 μM) for Ma bamboo, respectively. The OD_{600} of *Agrobacterium* solution was adjusted to an OD_{600} of 0.7 and subsequently placed in dark for 30 min or 3 h for both Moso bamboo and Ma bamboo.

For tissue infiltration, the leaves were gently sliced with a scalpel on the abaxial side and immediately immersed in the bacterial liquid and vacuumed for 30 min. Then, sterilized absorbent paper permeated with *Agrobacterium* solution were adhered to the injured leaves and incubated for 48 h in dark at 21 °C and 26 °C for Moso bamboo or Ma bamboo, respectively. Finally, treated bamboo plants were grown in the greenhouse. Plants without any wounding and injured plants both without *Agrobacterium* infiltration treatment served as two control groups and were grown side by side with treated bamboo.

Agrobacterium-Mediated Transient Transformation of Whole Seedlings in Moso Bamboo

For the transient transformation of bamboo seedlings *Agrobacterium* cultures were centrifuged at 4000 rpm for 8 min and resuspended in AAM medium. The *Agrobacterium* suspensions were adjusted to an OD_{600} of 0.9 and placed in the dark for 30 min. Whole roots of 20-day-old seedlings were carefully sliced longitudinally, and the root tip was cut off with a scalpel. Finally, the wounded roots were immediately transferred into the bacteria solution and shaken at 150 rpm

at 21 °C. Plants without wound treatment and cut off root tips were immersed in *Agrobacterium* suspension and acted as the control group.

GUS Staining and Observation

GUS staining was performed as described by Chabaud et al. (2003) with minor modifications. In brief, the infected materials were incubated in GUS staining solution that contained 10% phosphate buffer solution (BOSTER AR0030), 0.1% Triton X-100 (Sigma v900502), 0.5 mM $K_3[Fe(CN)_6]$ (Sigma P9378-100G), 0.5 Mm $K_4[Fe(CN)_6]$ (Sigma 60299-100G-F), 0.5 M EDTA (Xiamen Tagene Amresco 0322), and 0.2 M X-Gluc (Yeasen Biotech 10904ES03) at 37 °C for 12 h. After GUS-staining, materials from Moso bamboo and Ma bamboo were immersed in 70% alcohol and 30% glacial acetic acid mixture to clear chlorophyll for GUS visualization.

RNA Extraction and PCR Analysis

Total RNA of Moso bamboo and Ma bamboo were extracted using TIAN GEN RNAprep Pure Plant RNA kit (DP441) according to the manufacturer's instructions. Thermo ReverAid First Strand cDNA Synthesis Kit (K1622) was used to generate cDNA. Transient expression of mRNA levels was detected by PCR using the forward (5′-AGATCTGAGGAA CCGACGAC-3′) and reverse primers (5′-TTGTTTGCCTCC CTGCTG-3′). The following PCR program was performed: (1) 98 °C for 2 min; (2) 98 °C for 10 s, 62 °C for 30 s, and 72 °C for 20 s for 38 cycles; and (3) 72 °C for 5 min.

Monitoring the Transient Transformation in Moso Bamboo using the *RUBY* Reporter System

The plasmid of *RUBY* reporter system was provided by He (2020). We transformed it into *Agrobacterium* GV3101 and incubated in liquid LB medium (50 mg L^{-1} kanamycin and 50 mg L^{-1} rifampicin) at 28 °C for 24 h at 200 rpm. *Agrobacterium* cultures were centrifuged at 4000 rpm for 8 min and resuspended in AAM medium. The *Agrobacterium* suspension was adjusted to an OD_{600} of 0.9 and placed in the dark for 30 min. We immersed the 20-day-old seedlings in *Agrobacterium* suspension for transformation and used wild type as the control group for Moso bamboo. The seedlings and roots of 20-day-old wild type were used for autofluorescence observation.

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Author Contributions LG conceived and designed the project. KC conducted the experiment and analyzed the data with the help from KH, FX, HW, PG, JL, WT, XL, and HZ. KC, MVK, and LG and wrote the manuscript. All the authors read and approved the final manuscript.

Compliance with Ethical Standards

Conflict of Interest The authors declare no conflict of interest.

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