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Master Thesis

大岩桐基因轉殖系統之優化

Optimization of the genetic transformation system for *Sinningia speciosa*

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中文摘要

大岩桐是十分著名的園藝植物，因其花色及花型上的多變而受到大眾喜愛。大岩桐基因體小(300 Mbp)、短的生活史、自交親和性高及組織培養上容易再生等優點，皆支持其發展成研究花部發育機制的模式物種，為此有必要以基因轉殖之技術，輔佐了解相關調控花發育基因之功能性，但目前仍沒有明確的轉殖流程可遵循，因此本研究之目的為優化農桿菌及基因槍的轉殖條件。農桿菌轉殖中 GUS 訊號顯示以 1 ppm BA 前處理大岩桐三週大幼苗三天並與農桿菌菌株 EHA 105 共配養五天的處理方式下可得到暫時性的轉殖率 78.3 %，同樣條件下可獲得 17.2 % 的再生率及 2.1 %的轉殖率。GUS 報導基因顯示大岩桐四週大幼苗在基因槍氦氣壓力 900 psi 下，6 公分及 9 公分的距離可分別獲得 58.1 % 與 21.6 % 的暫時性轉殖率。兩者轉殖的效率顯示農桿菌轉殖策略優於基因槍。另一方面，癒傷組織因生長快速容易再生適合做為轉殖的材料，本研究也成功從大岩桐葉片中以 0.1 ppm 2,4-D 和 1 或 2 ppm BA 搭配 25 mM 或 50 mM 的山梨糖醇誘導出胚性癒傷組織(embryogenic callus)。本研究優化農桿菌介導的轉殖條件將有助於大岩桐調控花部發育基因功能性分析研究。

關鍵字: 大岩桐、基因轉殖、農桿菌、基因槍、癒傷組織

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Abstract

Sinningia speciosa is a popular houseplant because of its big flower with a remarkable diversity in colors, patterns and shapes. *S. speciosa* has a small genome size (300 Mb), short life cycle, self-compatible, easily propagated in tissue culture therefore is emerging as a model plant for flower development studies. However, a reliable genetic transformation system is not available in *S. speciosa*. To this end, the *Agrobacterium*-mediated transformation and particle bombardment transformation were tested in this study. Transient GUS expression assay showed that 3 days pre-culture of three weeks old seedlings on medium supplied with 1 ppm BA and co-culture for 5 days with *Agrobacterium* strain EHA105 achieved an overall transient transformation rate of 78.3 %. Under these optimized conditions, the regeneration rate is 17.2 % and the transformation rate is up to 2.1 %. Another approach is particle bombardment transformation for optimizing genetic transformation system. In GUS transient assay, it was found that under helium pressure 900 psi, at distance 6 and 9 cm displayed the transient transformation rate of 58.1 % and 21.6 % respectively. The transformation efficiency of two approaches demonstrated that *Agrobacterium*-mediated transformation is better than particle bombardment transformation. Because callus grows rapidly and regenerate easily, it serves as a good material for transformation. I also successfully induced embryogenic callus with 0.1 ppm 2, 4-D and 2 ppm BA plus 25 or 50 mM sorbitol in the medium. Callus transformation rate will be tested further. This study optimized the transformation protocol for studying gene regulation and gene function in *S. speciosa*.

Key words: *Sinningia speciosa*, genetic transformation, *Agrobacterium*, particle bombardment, callus

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Abbreviations

BA	6-benzyladenine purine
cDNA	Complimentary DNA
CTAB	Hexadecyl trimethyl-ammonium bromide
DNA	Deoxyribonucleic acid
GUS	Beta-glucuronidase
NAA	Naphthalene acetic acid
RNA	Ribonucleic acid
RT	Reverse transcription
NPTII	Neomycin phosphotransferase II
TDZ	Thidiazuron
2, 4-D	2,4-dichlorophenoxyacetic acid
PGR	Plant growth regulator

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Introduction

1. Plant genetic transformation

Plant genetic transformation is a powerful tool for studying plant development and crop improvement. The introduced specific gene can be directly understood the gene function by observing phenotypic changes it conferred. In addition, useful traits with commercial value can be introduced to certain crops via genetic transformation of responsible genes especially when the traits are slowly available or unachievable by breeding (Birch, 1997). Although the plant genetic transformation shows high potential value, the system is difficult to develop. In order to establish a reliable system of plant genetic transformation, the *Agrobacterium* transformation and particle bombardment are most commonly used.

1.1. *Agrobacterium*-mediated transformation

Agrobacterium tumefaciens, a Gram-negative soil bacterium, is used widely in plant genetic engineering (Yuan and Williams, 2012). It was discovered first in grape crown gall disease presenting a tumor-like characteristic. This kind of tumor formation is caused by transferred DNA (T-DNA) insertion from *A. tumefaciens*, which resulted in uncontrollable cell division and opine synthesis to feed the infected *Agrobacterium*. T-DNA, a region of tumor-inducing (Ti) plasmid, is transferred to plant cell and

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incorporated into plant genome. This transferring process demonstrates that foreign gene can be introduced into plant genome.

To establish *Agrobacterium*-mediated transformation system in non-model plant species, there are two steps needed to optimize: *Agrobacterium* infection, and regeneration of transformed tissues. Previous studies indicated that the difficulty of *Agrobacterium* infection is mainly due to the huge size of Ti plasmid, which is more than 200 kb, and usually result in hard transformation and lower transformation rate. Therefore, the binary vector system has been established to resolve (Hoekema et al., 1983). The Ti plasmid is separated from two vectors, one contains *vir*-region which can help T-DNA delivery (help plasmid), while the other vector contains T-DNA region. The binary vector system has been proved working successfully in transformation of many species like tobacco, tomato, potato and Arabidopsis (An et al., 1986; Gleave, 1992). Besides the problem of huge Ti plasmid, several factors that influence *Agrobacterium* infection for successful transformation include *Agrobacterium* strain, *Agrobacterium* cell density, infection time, the developmental stage of seedlings selected for transformation, pre-culture time and co-culture time. Thus, these factors need to be optimized for efficient transformation.

On the other hand, the transgenic explant required suitable conditions to regenerate

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into whole plants from transformed cells. When the explants are placed on medium with requiring nutrients and appropriate hormone combinations, the plant cells can re-differentiate into stem cells and further regenerate by shooting into a whole plant. An efficient regeneration system is essential after these stem cells transformed with *Agrobacterium*. Therefore, a regeneration system needs to be established before optimizing conditions for genetic transformation to achieve the successfulness of a genetic transformation protocol.

1.2. Particle bombardment transformation

Particle bombardment/biolistic transformation has also been developed to overcome the limitation of *Agrobacterium*-mediated transformation like the genotype of plant, size of foreign DNA, single gene transformation and low transformation efficiency (Altpeter et al., 2005; Christou, 1992; Pawlowski and Somers, 1996). The limitation of genotype of plant means that the susceptibility of different genotype (cultivar) to the same *Agrobacterium* strain might be significantly various. Therefore, the optimal transformation system is suitable for specific genotype of same species. In contrast, the particle bombardment transformation introduces genes by appropriate hilum pressure and target distance, which can overcome the effect of different genotypes.

Foreign DNA is introduced into plant cells through gold particle which is DNA

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coated and accelerated by high speed hilum gas. In addition, the particle bombardment is also applied in transient expression system (Huang et al., 1998) or promotor assay (McCormick et al., 1991; Morikawa et al., 1989), for establishment of an optimized particle bombardment system of our target plant having highly economic value.

2. The high diversity in angiosperms (flowering plants)

The angiosperms (295,383) account for almost 80% of total number of plant species (374,000), and demonstrate much larger number and higher diversity than other plant groups like ferns (10,560) or gymnosperms (1,079) (Christenhusz and Byng, 2016). With highly diverse flower morphology in angiosperm, bilateral symmetry (zygomorphy) is considered a major trend for flower shape complexity (Busch and Zachgo, 2009). In addition, the species number of bilaterally symmetrical groups is significantly greater than their radially symmetrical sister groups. This evidence supports that the bilateral symmetry promotes a higher speciation rate and leads to more species number (Sargent, 2004). To understand the mystery in angiosperm diversification, knowledge of the molecular mechanism for regulating the floral symmetry will be helpful.

3. *Sinningia speciosa,* a good material to study floral symmetry and flower shape

The flowers in cultivars of *Sinningia speciosa* appear to be highly diverse, which have

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both bilateral symmetry and radial symmetry, together with various floral traits like color, shape and size. The flowers of wild *S. speciosa* are bilateral symmetry with purple color, rarely in white or pink (Fig. 1A and E). In contrast, the horticultural cultivar of *S. speciosa* shows radially symmetrical flowers with white, purple or red colors (Zaitlin, 2012) (Fig. 1B-D and 1F-H).

Among genus *Sinningia*, *S. speciosa* has a small genome size (300 Mb, about twice size of Arabidopsis) (Zaitlin and Pierce, 2010), short life cycle (about 4-6 months) and easily propagated characters. Moreover, the wild *S. speciosa* can interbreed with the horticultural cultivars or self-fertilize, which means that they can be interbreed to generate more varieties and self-compatible, a good model system to study inheritance of traits and their underlined genetic control. In our lab, two sets of transcriptome data, from F2 hybrids of selfed F1 between zygomorphic wild-type flowers and actinomorphic cultivar flowers have been analyzed to investigate candidate genes regulating floral symmetry and flower shape. Moreover, the genome of *S. speciosa* has also recently been sequenced by Dr. David Zaitlin (University of Kentucky), who provides the basic information of all genes in *S. speciosa*. With these data, *S. speciosa* holds the potential of becoming an excellent model species to study floral symmetry and flower shape.

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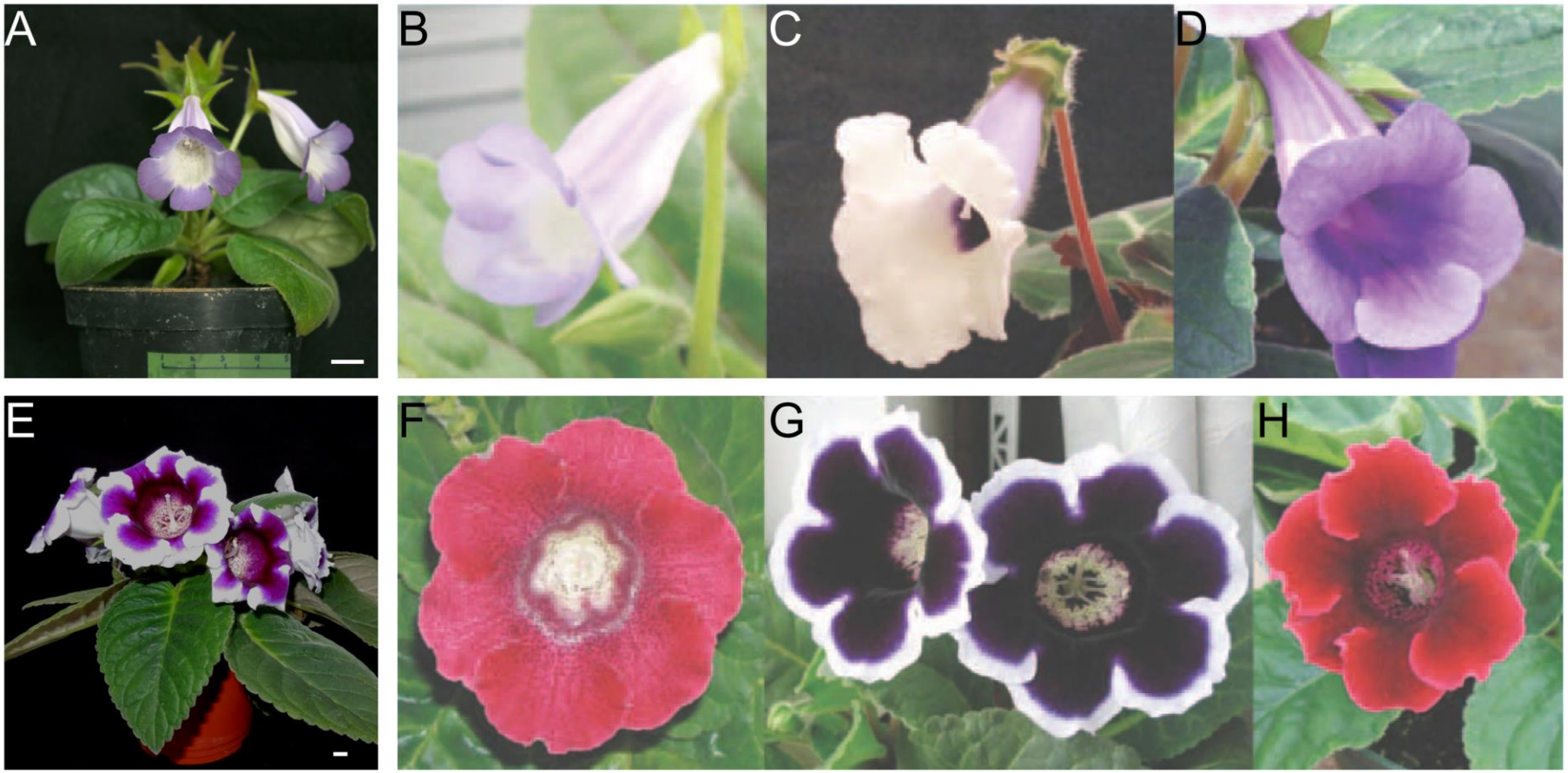

Figure 1 Floral diversity in *S. speciosa*.

A, wild *S. speciosa* 'Espirito Santo', the flower show bilateral symmetry (from Pan, Zhao-Jun, personal communication). B-D, wild *S. speciosa*, all the flowers show bilateral symmetry. E, horticultural *S. speciosa* 'Avanti', the major material the flower show radial symmetry. F-H, horticultural *S. speciosa*, all the flowers show the radial symmetry. Scale bar, 1 cm. B-D and F-H were adopted from Zaitlin (2012).

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4. Genetic transformation in *S. speciosa*

To evaluate how genes regulate floral symmetry and shape regulated in *S. speciosa*, identifying the function of these genes via genetic transformation system is necessary. In previous studies, the foreign gene has been introduced into *S. speciosa* (Li et al., 2013b; Zhang et al., 2008), but a reliable protocol of genetic transformation system is not available and the transformation rate is not reported. I and previous students in our lab repeated several times their protocol yet the transformation success is extremely low. For these reasons, the procedure of genetic transformation in *S. speciosa* need to be optimized. I propose to optimize both *Agrobacterium* transformation and particle bombardment systems in this study.

4.1. *Agrobacterium*-mediated transformation

The prerequisite for successful *Agrobacterium* transformation depends on establishment of an efficient shoot regeneration system. In *S. speciosa*, a regeneration system from leaf disc has been developed (Chae et al., 2012; Pang et al., 2006; Park et al., 2012; Scaramuzzi et al., 1999; T. et al., 2006; Xu et al., 2009). However, the response of the different cultivars of *S. speciosa* to hormone treatment are different, and this causes problem when applying the same protocol to different cultivars. Most of the previous studies mentioned above focused on establishment of regeneration system in zygomorphic cultivars of *S. speciosa*. Therefore, the regeneration system has been

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optimized in *S. speciosa* ‘Avanti’ (an actinomorphic cultivar) in our lab. When leaf discs of *S. speciosa* ‘Avanti’ cultured on MS medium with 0.1 ppm NAA and 1.0 ppm BA, the shoot regeneration rate is up to 90% with 2.5-3.0 shoots per explant (Kuo, 2014). With the support of high regeneration rate of *S. speciosa* ‘Avanti’, this species is potentially a good material to be optimized for *Agrobacterium*-mediated transformation system.

A Successful process of *Agrobacterium* infection involves *Agrobacterium* inoculation and T-DNA delivery. Several important factors influence this process including the genotype of target plants, plant tissue type and growth stage of explants used for infection, *Agrobacterium* strain, and infection procedure (Cheng et al., 2004; Opabode, 2006). To evaluate the efficiency of transformation, detection the introduced reporter gene, *β-glucuronidase* (*GUS*) activity, is usually conducted to visualize *Agrobacterium* infection rates and infected tissues (Jefferson et al., 1987).

4.1.1. Material source of *S. speciosa*

Different explant tissues may show diverse competence for *Agrobacterium* infection (Cheng et al., 1997; Jian et al., 2009). Leaf is the most commonly used explants. Similarly, cotyledon is a substitute for explant. In Arabidopsis, cotyledon work as excellent substitute due to the high shoot regeneration (Patton and Meinke, 1988; Tsai et al., 2012) and high transformation frequency than leaf discs (Renate and Lothar,

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1988). Some species with poor shoot regeneration rate, such as pepper, young seedling explants were a good substitute to leaf for *Agrobacterium*-mediated transformation (Javier et al., 2001). In the case of *S. speciosa*, preliminary data (Appendix Fig. 3) indicated that mature leaf explant is difficult to be infected by *Agrobacterium*. Therefore, the 2, 3 and 4 weeks old seedlings of *S. speciosa* were further investigated for the susceptibility of *Agrobacterium* infection. Three stages of seedlings contained one pair of opposite cotyledons, one pair of true leaves, and two pair of true leaves, respectively.

In addition to leaves and cotyledons, callus is also a potential substitute for *Agrobacterium*-mediated transformation and has been applied in several species (Komari, 1989) owing to high transformation rate. For example, in *Parthenium argentatum*, callus was a better material for transformation than leaf explants, because leaf explants usually turned brown after *Agrobacterium* infection (Turan et al., 2014). Such browning indicating explant necrosis after infection was also observed in *S. speciosa*, and brown tissues did no regenerate. Thus, callus was regarded as a substitute for *Agrobacterium*-mediated transformation.

In *Agrobacterium*-mediated transformation of callus, it was suggested that active growth and cell division of callus enhanced the regeneration and resulted in efficient transformation rate (An, 1985). According to this information, embryogenic callus with

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embryonic trait which is a very active growing tissue, should also be considered in *Agrobacterium*-mediated transformation.

4.1.2. Pre-culture time

Pre-culturing explants prior to inoculation increases the transformation efficiency due to the additional plant hormone (auxin) which induce cell activation and division (Sangwan et al., 1992). This idea was supported by experiments in Arabidopsis (Sangwan et al., 1992), *Linum usitatissimum* (McHughen et al., 1989), *Lotus corniculatus* (Jian et al., 2009) and *Chirita pumila* (Liu et al., 2014). However, in citrange and *Perilla frutescens*, additional pre-culture led to decreased transformation rate (Cervera et al., 1998; Kim et al., 2004). Based on these cases, the effect of pre-culture treatment on transformation rate varies in different species.

4.1.3. *Agrobacterium* strain

Previous studies demonstrates the virulence of *Agrobacterium* strains was different and this might lead to various transformation efficiency (Byrne et al., 1987; Owens and Cress, 1985; Sederoff et al., 1986). To increase the efficiency of *Agrobacterium* infection of *S. speciosa*, different *Agrobacterium* strains should be tested and compared. Among previous Gesneriaceae transformation studies, *Agrobacterium* strains including EHA 105 (supervirulent strain), LBA4404 (regular strain) and GV2260 were successfully applied in African violet, *Chirita pumila*, *Kohleria* and *Ramonda myconi*

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(Barth et al., 2009; Geier and Sangwan, 1996; Kushikawa et al., 2001; Liu et al., 2014; Mercuri et al., 2000; Toth et al., 2006). Therefore, these *Agrobacterium* strains were regarded as potential candidates for infecting *S. speciosa*. On the other hand, it was reported that *Agrobacterium* EHA 105 might be better than LBA4404 and GV2260 in *S. speciosa* (Li et al., 2013; Zhang et al., 2008). In this study, we have two genotypes (cultivars) of *S. speciosa*, Avanti and Espirito Santo, which have never been successfully transformed in previous studies. Therefore, testing the virulence of *Agrobacterium* strain for efficient infection in Avanti and Espirito Santo is necessary.

4.1.4. *Agrobacterium* cell density and inoculation time

For *Agrobacterium* inoculation, *Agrobacterium* cell density and inoculation time are also the factors that influence transformation efficiency. Higher *Agrobacterium* cell density shows the better transformation efficiency (Zhao et al., 2000), while lower *Agrobacterium* cell density needs longer inoculation time (Amoah et al., 2001). However, they were also reported to have no significant difference in affecting transformation efficient (Clough and Bent, 1998).

4.1.5. Co-culture time

The co-culture time is one of key factors affecting the transformation rate (Hiei et al., 1997), the *Agrobacterium* infect and transfer the T-DNA into host plant during co-culture period. A shorter co-culture time is insufficient for *Agrobacterium* proliferation,

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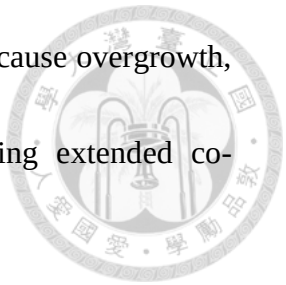
infection and T-DNA transfer. However, the *Agrobacterium* might cause overgrowth, resulting in damage of explant and difficulty to eliminate during extended co-cultivation time, leading to the lower transformation efficiency.



4.1.6. Temperature during co-culture

Previous studies demonstrate that low temperature appears beneficial for T-DNA delivery (Dillen et al., 1997; Jian et al., 2009; Kondo, 2000; Salas et al., 2001), while high temperature significantly decrease the level of virulence proteins and bacterial virulence (Baron et al., 2001). The effect of low temperature about 20 to 25ºC on T-DNA delivery was also evaluated in other studies (Jian et al., 2009; Kondo, 2000; Salas et al., 2001). In previous studies, the temperature requirement during co-culture has never been investigated in *S. speciosa*. Nonetheless, it showed the co-culturing temperature from 22 to 27ºC in other members of Gesneriaceae (Barth et al., 2009; Geier and Sangwan, 1996; Kushikawa et al., 2001; Li et al., 2013; Liu et al., 2014; Mercuri et al., 2000; Toth et al., 2006; Zhang et al., 2008).

4.2. Particle bombardment transformation

The ideal conditions for particle bombardment transformation have not been established for *S. speciosa*. Consequently, the transient transformation system of particle bombardment was established by GUS report assay in this study to examine whether the particle bombardment approach is more efficient or not.

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5. Callus as a novel substitute for genetic transformation in *S. speciosa*

In our previous data demonstrated that the regeneration form mature leaf was originated from a single vascular cell which is too inner to infect by *Agrobacterium* (Kou, 2014). Therefore, callus was used to overcome the difficult infection. To know whether callus is an excellent material for genetic transformation in *S. speciosa*, the optimal hormone condition was investigated to induce callus. It has been reported that callus can be regenerated with combination of auxin and cytokinin under certain ratio (White, 1939). Although 2, 4-D (a kind of auxin) is recorded to promote callus formation and differentiation from leaf explants, the callus grew small and time-consuming in *S. speciosa* (Wuttisit and Kanchanapoom, 1996). Based on this experiment, the different concentrations of 2, 4-D combining with BA or TDZ (two kinds of cytokinin) were applied to induce callus from leaf explants.

Due to the different tissue used (leaf vs. callus), the regeneration system might need to be optimized again for callus. According to the previous survey, different concentrations and combinations of NAA (a kind of auxin) and BA can induce shoot in *S. speciosa* (Kuo, 2014). This provides a starting point to establish an efficient regeneration system from callus.

To further confirm whether callus could be used for *Agrobacterium*-mediated transformation or particle bombardment transformation, several conditions were

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examined here. In *Agrobaterium*-mediated transformation, the co-culture time is considered the most important factor for transferring T-DNA into plant cells, thus it was tested first. At the same time, the physical conditions of particle bombardment were examined with factors such as hilum pressure and target distance by GUS report assay.

Aim of this study

The aim of this study is to optimize an efficient genetic transformation system for *S. speciosa*. To this end, the *Agrobacterium*-mediated transformation and particle bombardment transformation approaches were used. In *Agrobacterium*-mediated transformation, the factors including pre-culture time, *Agrobacterium* strain, *Agrobacterium* cell density, infection time, the developmental stage of seedlings selected and co-culture time we re optimized. In particle bombardment transformation, the hilum pressure and target distance were tested.

In addition, callus was investigated whether it is a better substitute to establish a more efficient genetic transformation system.

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Materials and Methods

1. Plant material and growth conditions

An actinomorphic cultivar of *Sinningia speciosa* 'Avanti', was purchased from a local seed distributer, Taiwan Horticultural Co., Ltd. Plants were grown in 3.5 inch diameter plots, cultivated in greenhouse at 25ºC before harvesting. The plotting medium for *Sinningia speciosa* was a mixture of peat, vermiculite and perlite in 2:1:1 ratio. Aseptic plants were grown in the incubator at 27ºC in long-day (16 hr light/ 8 hr dark), with 50 % relative humidity and under illumination with white fluorescent lamps at 50-60 μmol m^{-2} s^{-1}.

2. Transient transformation by *Agrobacterium*-mediated transformation for *Sinningia speciosa*

In order to know the best conditions of *Agrobacterium*-mediated transformation, GUS report gene as reporter system was used to optimize the transformation system. The testing for *Agrobacterium* infecting include the leaf developmental stage of *S. speciosa*, pre-culture time, *Agrobacterium* strains and co-culture time.

2.1. Plasmid and *Agrobacterium* strains

The *Agrobacterium* strains EHA 105 and GV3101 harboring pCambia 2301 were kindly obtained from Chwan-Yang Hong Lab (NTU). The vector contains the neomycin phosphotransferase II (*NPTII*) gene for transformed cell selection and *GUS*

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report gene with an intron (Appendix Fig. 1). Both of *NPTII* and *GUS* are driven by 35 S promoter. These two *Agrobacterium* strains were used to optimize the infection conditions for *Sinningia speciosa*.

2.2. Preparing aseptic seedlings for *Agrobacterium*-mediated transformation

For growing seedlings under aseptic conditions, seeds of *Sinningia speciosa* 'Avanti' were sterilized 30 seconds by 70% ethanol for surface cleaning. Then, 1% sodium hypochlorite solution with 0.2% tween 20 was used to sterilize for 3-5 min. After that, seeds were rinsed in sterilized water 3-5 times to made sure the sodium hypochlorite removing fully. The sterilized seeds were sowed on sowing medium in a 9 cm diameter, 2 cm high petri dish. The seeds germinated after culturing for 7 days. The seedlings were cultured on sowing medium in the petri dish 2, 3 and 4 weeks for *Agrobacterium*-mediated transformation. *Nicotiana benthamiana* was used as the positive control with the same method to sterilize, and cultured in the same medium for 7 days.

Sowing medium (1 L), pH 5.7

Regent/stock buffer	Adding weight/volume
MS medium (Murashige and Skoog, 1962)	2.15 g
Sucrose	10.0 g
1000× vitamin	1.0 mL
Phytagel ™ (P8169, Sigma)	3.2 g

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2.3. *Agrobacterium*-mediated transformation

2.3.1. Pre-culture treatment

Whole seedlings were treated with hormone in the pre-culture medium for 0, 3 and 4 days before *Agrobacterium* infection. Making sure the leaves of seedlings were touched the pre-culture medium.

2.3.2. Preparation of *Agrobacterium*

Before two days of infection, the *Agrobacterium* strains EHA 105 and GV3101 were grown in 4 mL YEP medium (1L, 10 g yeast extract, 10 g Bactopeptone, 5 g NaCl) with 50 ppm kanamycin, and incubated at 28 ºC with shaking at 200 rpm for 24 hr. 100 μL of *Agrobacterium* culture was sub-cultured to 50 mL AB medium with 20 μM acetosyringone, 0.5% glucose and 50 ppm kanamycin. The culture was incubated at the same condition described above for 14-16 hr until the OD_{600} = 0.8-1.0. Next step, the *Agrobacterium* culture was transferred to a 50 mL centrifuge tube, and centrifuged at 6000× g, 5 min at 4 ºC. The supernatant was discarded, then resuspended the *Agrobacterium* cells by 50 mL inoculation medium (OD_{600} = 0.8-1.0).

2.3.3. Seedling infection and co-culture with *Agrobacterium*

Agrobacterium resuspended by inoculation medium was transferred 7-10 ml into a 20 mL sample bottle. Then, the seedlings were put into the sample bottle and covered with *Agrobacterium* culture. The sample bottle containing seedlings was placed into a 50

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mL syringe on a rubber cork plug. Additional pressure was added by pushing the plunger until contacting the top of sample bottle 3-5 times. In this process, the air in leaves of seedling was crowed out from the edge and the leaves became crystal. After pressuring, the seedlings were dried on sterile filter papers fully and transferred to co-culture medium. Confirming the leaves were touching to the co-culture medium. The seedlings infected by *Agrobacterium* were cultured in the incubator at 27ºC with long-day photoperiod (16 hr light/ 8 hr dark), relative humidity of 50% and kept under illumination with white fluorescent lamps at 50-60 μmol m^{-2} s^{-1}. The culture time tested in this study was 3 days and 5 days.

The 7 days old seedlings of *Nicotiana benthamiana* as positive control were infected by *Agrobacterium* with the same method and cultured in same co-culture medium as described above.

2.3.4. GUS activity assay

After seedlings co-culturing with *Agrobacterium*, double distilled water was used to clean up *Agrobacterium* on the surface of seedlings completed 3-5 times. GUS staining solution was added for staining the transformed cells. The seedlings were cultured in dark at 37 ºC for 12-16 hr. Then, they were removed the chlorophyll by 70% ethanol at 60 ºC. 2% H_2O_2 was added into 70% ethanol after chlorophyll removing completely for decoloring the high background of seedlings.

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Pre-culture medium (1 L), pH 5.7

Regent/stock buffer	Adding weight/volume
MS medium	4.3 g
sucrose	30.0 g
1000× vitamin	1.0 mL
0.5 mg/mL BA	2.0 mL
phytagel	3.2 g

AB medium (50 mL)

Regent/stock buffer	Adding weight/volume
20× buffer A	2.5 mL
20× buffer B	2.5 mL
50% glucose	0.5 mL
0.5 M MES	5.0 mL
200 mM Acetosyringone	5.0 μL
50mg/mL kanamycin	50.0 μL

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20× buffer A (1 L)

Regent/stock buffer	Adding weight/volume
K_2HPO_4	60.0 g
NaH_2PO_4	20.0 g

20× buffer B (1 L)

Regent/stock buffer	Adding weight/volume
NH_4Cl	20.0 g
$MgSO_4•7H_2O$	6.0 g
KCl	3.0 g
$CaCl_2$	0.2 g
$FeSO_4•7H_2O$	0.05 g

Inoculation medium (1 L), **pH 5.2**

Regent/stock buffer	Adding weight/volume
MS medium	4.3 g
sucrose	30.0 g
200 mM acetosyringone	1.0 mL
50% glucose	10.0 mL
1000× vitamin	1.0 mL

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Co-culture medium (1 L), **pH 5.2**

Regent/stock buffer	Adding weight/volume
MS medium	4.3 g
sucrose	30.0 g
1000× vitamin	1.0 mL
200 mM acetosyringone	1.0 mL
50% glucose	10.0 mL
0.5 mg/mL BA	2.0 mL
0.5 mg/mL NAA	0.2 mL
phytagel	3.2 g

GUS staining solution (0.1 M Sodium phosphate buffer as solvent, pH 7.0)

Regent
10 mM EDTA (pH=8.0)
0.5 mM K-ferrocyanide
0.5 mM K-ferricyanide
1 mM X-Gluc
0.1% Triton X-100

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2.4. Paraffin section

Paraffin section was used to know the location of GUS signals in seedlings of *S. speciosa* infected by *Agrobacterium*. Seedlings with GUS signals were fixed in FAA fixative (50% ethanol: formalin: acetic acid = 90: 5: 5, v/v/v) for 6-8 hr. Vacuum infiltration (about 40 cmHg) was used for helping full fixation. During fixation process, shaking the sampling bottle was helpful for air bubbles releasing form seedlings. The fixation was finished after the seedlings precipitating in the bottom of sampling bottle. Then, the seedlings were dehydrated via *t*-butyl alcohol/ ethanol series for 2 hr at least each step. At last step in dehydration, seedlings were stood in pure *t*-butyl alcohol overnight. Then, the seedlings were infiltrated and embedded in paraffin. Continue paraffin sections were cut at a setting of 15-20 μm thick by a rotary microtome. Serial paraffin sections were spread out and mounted on a slide coating poly-lysine (P4707, Sigma). The sections were stained with 1% safranin-O for 30 min and mounted with DPX (44581, Sigma). The slides mounted were placed at 37 ºC overnight, and the GUS signals were observed by a light microscope (BX51, Olympus, Japan).

3. Transgenic plant selection

The optimized conditions for *Agrobacterium*-mediated transformation were used to get the most transformed cells. The transformed cells were induced to regenerate shoots, then selected the transgenic plants which are GUS expressed.

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3.1. *Agrobacterium* washing

The *Agrobacterium* were washed out and eliminated from the seedlings for avoiding relapse after co-culture step. Sterile water was used to clean up the seedlings 3-5 times first, then seedlings were dried on sterile filter paper, this step could remove *Agrobacterium* mostly. The seedlings were transferred into a 250 mL flask with 50 mL washing buffer and cultured at 27 ºC in dark with shaking 200 rpm for 2 hr. For full eliminating *Agrobacterium*, the washing step was repeated for 3 times with fresh washing buffer added additional 100 ppm cefotaxime each time. Finally, the washing buffer will look transparent.

Washing buffer (1 L), pH 5.7

Regent/stock buffer	Adding weight/volume
MS medium	2.15 g
sucrose	10.0 g
1000× vitamin	1.0 mL
cefotaxime	300/400/500 mg

3.2. Selection of transformed shoots

3.2.1. Selection and regeneration from transformed cells

After washing step, the cotyledons, the first and second pair of true leaves were cut

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from whole seedlings. Keeping the adaxial side of leaves were touched to selection medium and cultured at same conditions as growth environment in the incubator. These leaves were sub-cultured every 1-2 weeks. After 12-28 weeks of selection, the regenerated shoots appeared.

3.2.2. Regenerated shoots rooting

When the regenerated shoots grew up to 2 pairs of leaf, they were cut and transferred to the rooting medium. For 4-6 weeks culture, the roots of regenerated shoots were induced and ready to transfer into pots. The pots were covered with a plastic bags for 1 week keeping the moisture.

Selection medium (1 L), pH 5.7

Regent/stock buffer	Adding weight/volume
MS medium	4.3 g
sucrose	30.0 g
1000× vitamin	1.0 mL
0.5 mg/mL BA	2.0 mL
0.5 mg/mL NAA	0.2 mL
cefotaxime	200.0 mg
kanamycin	100.0 mg
phytagel	3.2 g

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Rooting medium (1 L), pH 5.7

Regent/stock buffer	Adding weight/volume
MS medium	4.3 g
sucrose	30.0 g
1000× vitamin	1.0 mL
0.5 mg/mL NAA	0.2 mL
cefotaxime	200.0 mg
phytagel	3.2 g

3.3. Calculation of regeneration rate

Each regenerated shoots were signed an ID number, for example: A1-1a[1]. The first latter shows that the days of pre-culture day in 3 weeks old seedlings. The 'A' stands the 0 days of pre-culture, 'B' stands 3 days of pre-culture, and 'C' stands 4 days of pre-culture. The 'D' stands 4 days of pre-culture in 4 weeks old seedlings. The number next to the first latter indicated that the repeat times under same treatment. The number next '-'demonstrates that the same seedling which has two pairs of true leaves and two cotyledons. The lowercase latter shows the same leaf of the same seedling, in this study, the lowercase latter might form 'a' to 'f'. The regenerated shoots obtaining form same leaf were donated a superscript number next to the lowercase latter.

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On the other hand, the 3 weeks seedling with 4 days of pre-culture were tested the effect of *Agrobacterium* cell density and infection time. The regenerated shoots were also signed an ID number descripted above. However, the first latter ‘a’, ‘b’, ‘c’ and ‘d’ stand the *Agrobacterium* cell density of OD_{600}= 0.4, 0.6, 0.8 and 1.0 respectively. The number next ‘-‘, a, b and c, demonstrates that the infection time for 5, 20 and 30 min respectively.

Only one regenerated shoot form one seedling was calculated to insure the independent transformation line.

Regeneration rate = (Numbers of regenerated shoots / numbers of total transformed seedlings) × 100%

3.4. Vitrification of transgenic plants

In order to know that whether regenerated shoots were inserted foreign gene: *GUS* and *NPTII* or not, three methods were used to detect as below:

3.4.1. GUS activity assay

The leaves were cut from regenerated shoots (juvenile and mature) and detected the GUS protein activity with the method descripted in 2.3.4.

3.4.2. RT-PCR of GUS and NPTII

Total RNA extraction and reverse transcription

The total RNA of regenerated shoots were extracted by Trizol® Reagent. The protocol

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were followed from the manufacturer's manual with some modifications. Before RNA precipitation, additional acid phenol chloroform (P1944, Sigma) and chloroform was added into aqueous phase involving total RNA with same volume. The two steps could remove all traces of DNA and protein contamination.

Total RNA of regenerated shoots was reverse transcribed into complementary DNA (cDNA) by M-MLV Reverse Transcriptase (28025013, Invitrogen). The protocol was followed by manufacturer's manual. After the reaction finishing, DEPC water was added to dilute the cDNA into 20 ng/ μL for RT-PCR.

RT-PCR (Reverse transcriptase-polymerase chain reaction)

For RT-PCR, GUS and NPTII were detected to confirm the mRNA expression or not and the housing keeping gene, *Actin* gene, was chosen as positive control. The general recipe and program are listed as below:

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Single reaction for RT-PCR

Regent/stock buffer	Adding weight/volume
Taq DNA Polymerase 2× Master Mix (Ampliqon)	10.0 μL
10 μM forward primer	1.0 μL
10 μM reverse primer	1.0 μL
DEPC water	7.0 μL
Template (cDNA, 20ng/μL)	1.0 μL

RT-PCR program

Step	Temperature	Time
1	94℃	5 min
2	94℃	30 s
3	Annealing temperature (GUS, 60℃, Actin and NPTII, 55℃)	30 s
4	72℃	1 kb/min
5	Go to step 2 and repeat 30-35 times depending on genes	
6	72℃	7-10 min
7	25℃	hold

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3.4.3. Southern blotting

To confirm the *GUS* insert the genome of regenerated shoots or not, Southern blotting was applied in this study. The protocol of was followed by Roche's DIG Application Manual. The probe was designed for length 551bp of *GUS* sequence from vector pCambia 2301, which is no restriction enzyme (EcoRI, HindIII, BamHI) cutting site in the probe and only one cutting site in vector. The probe was synthesized by PCR DIG Probe Synthesis Kit (Roche, 11636090910). Because of high CG content and short length of probe, the DIG-labeled was adjusted at 1: 6 ratio. The quantity of PCR product was measured by running electrophoresis gel. Before hybridization, probe was stored at -20 ºC.

For each lane, 10 μg Genomic DNA was digested with three restriction enzyme, EcoRI, HindIII and BamHI for 8 hr and separated on a 0.8% agarose gel with low voltage, about 1-2 V/ cm. DIG-labeled Molecular Weight Marker was load 5 ul per lane for confirming the size of hybridization product. Next step, the gDNA was transferred to a positive charge nylon membrane (Pall Corporation, 524342) and fixed by UV light (UV stratalinker^{TM} 1800) with 120 mJ. The probe was hybridized to the gDNA on nylon membrane with DIG Easy Hyb (Roche, 11603558001) for 16 hr at setting hybridization temperature 48 ºC for 80-100% homologous targeting. After hybridization, the probe-target hybrids was localized by Anti-Dig-AP (Roche, 11093274910) and visualized

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with NBT/BCIP (Roche, 11681451001) for 16 hr.

3.5. Calculation of transformation rate

The regenerated shoots were named transgenic shoots as all of the three detected methods described above showing positive results. Transformation rate = (the numbers of transgenic shoots/ the numbers of total transformed seedlings) × 100%

4. Callus induction and regeneration of *S. speciosa*

Because callus grows rapidly and regenerates easily, it serves a good material for transformation. The mature leaves and seeds were treated with auxin and cytokinin combination for inducing callus. The regeneration condition of callus was tested for finding the best shoot regeneration.

4.1. Callus induction from mature leaf

4.1.1. Preparation of aseptic seedlings for inducing callus

3 weeks old aseptic seedlings were transferred to a 9 cm diameter, 11 cm height glass cans with same sowing medium and cultured at same conditions for 2-2.5 months. Then, the top 2-3 pairs of leaves were ready for inducing callus.

4.1.2. Hormone conditions inducing callus from mature leaf

The callus induction media contained MS basal salt, vitamin and 3% sucrose supplemented different concentrations and combinations of 2, 4-D (0.1, 0.5, 1.0 and 2.0 ppm) with BA (0, 1.0 and 2.0 ppm) or 2, 4-D (0 and 0.1 ppm) with TDZ (0, 0.5 and 1.0

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ppm). The leaves of 3-3.5 months old aseptic seedlings were cut and punched to leaf discs which are 6 mm dimeter. The adaxial side of leaves were touched to callus induction medium and cultured at same conditions as growth environment in the incubator. The results were observed after culturing 30-35 days.

Callus induction medium (1 L), pH=5.7

Regent/stock buffer	Adding weight/volume
MS medium	4.3 g
Sucrose	30.0 g
1000× vitamin	1.0 mL
0.5 mg/mL BA	0/2000/4000 μL
0.5 mg/ mL 2, 4-D	200/1000/2000/4000 μL
Phytagel	3.2 g

Regent/stock buffer	Adding weight/volume
MS medium	4.3 g
Sucrose	30.0 g
1000× vitamin	1.0 mL
0.1 mg/mL TDZ (cytokinin)	1/5/10 mL
0.5 mg/ mL 2, 4-D	0/200 μL

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Phytagel	3.2 g

4.1.3 Prevent the vitrification of callus

The best callus induction media were found a problem of vitrification, so sorbitol as an osmotic regulator was added into callus induction media to prevent it. Three concentrations of sorbitol (0.025 M, 0.05 M and 0.1 M) were tested.

4.2. Callus induction from seeds

4.2.1 The best osmotic condition for inducing callus from seeds

The seeds were sowed in optimizing callus induction medium supplemented different concentrations of sorbitol (0 M, 0.01M, 0.05 M and 0.1 M) for finding the optimal osmotic condition. After culturing 30-35 days, the result showed that low sorbitol concentration induced the most callus with no vitrification. However, the high shooting rate was needed to resolve in the future test.

4.2.2. The best callus induction medium from seeds

The callus inducing from seeds showed the high shoot rate, the concentration of BA should decrease for full callus inducing without sorbitol treatment. The callus induction medium supplemented combinations of 2, 4-D (0.01, 0.025, 0.05 and 0.1 ppm) and BA (0, 0.1, 0.25, 0.5, 1.0 and 2.0 ppm) were tested.

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Callus induction medium for seeds (1 L), pH=5.7

Regent/stock buffer	Adding weight/volume
MS medium	4.3 g
Sucrose	30.0 g
1000× vitamin	1.0 mL
0.5 mg/mL BA	0/0.2/0.5/1/2/4 mL
0.5 mg/ mL 2, 4-D	20/50/100/200 μL
Phytagel	3.2 g

4.3. The best condition of callus regeneration

The callus was cut and transferred to the regeneration medium containing NAA (0, 0.05 and 0.1 ppm) supplying with BA (1, 2, 3 and 5 ppm).

Regeneration medium (1 L), pH= 5.7

Regent/stock buffer	Adding weight/volume
MS medium	4.3 g
Sucrose	30.0 g
1000× vitamin	1.0 mL
0.5 mg/mL BA	2/4/6/10 mL
0.5 mg/ mL NAA	0/100/200 μL
Phytagel	3.2 g

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5. Particle bombardment transformation

Particle bombardment transformation was another approach for optimizing genetic transformation system. The golden particles coating with DNA were shot into target leaves of *S. speciosa* by helium gas. The plasmid pCambia 2301 with GUS report gene was used for testing the parameters of gun settings involving rupture pressure and target distance. The protocol was followed by Gene Gun protocol of Dr. Hong-Hwa Chen Lab (NCKU), and the instrument, Biolistic® PDS-1000/He Particle Delivery System (Bio-Rad, 165-2257) in Dr. Wei-Chiang Shen Lab (NTU) was used.

5.1. Preparation of gold particle

Golden particles (size: 0.6 μm) 0.03 g and 70% ethanol 1 mL were added into 1.5 mL microcentrifuge tube, vortex vigorously for 3-5 min and settled for 5 min. The golden particles were spun for 5 seconds and discarded the supernatant. 1 mL sterilized water was added for washing, vortexed for 10 min and settled for 1 min, after that, spun for 5 seconds and discarded the supernatant. The washing step was repeated again. Finally, the 50% sterilized glycerol 500 μL was added and vortexed. The golden particles were stored at 4ºC before using.

5.2. Preparation of plant material - leaves of *S. speciosa*

The 1st and 2nd pair of true leaf from 4 weeks old seedlings were cut and transferred to the 0.7% agar plate, keeping the adaxial side contacting the agar. The edge of the leaf

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is curling easily, scarifying the surface of agar is helpful for attaching. Caution of the attaching side of leaves because the trichomes of the adaxial side are more than the abaxial side.

5.3. DNA coating (for 3 shoots)

The amount of golden particles for DNA coating was prepared for 3 shoots each tube, because of the aggregation of microparticles easily. The golden particles (60 mg/ mL) were vortexed vigorously for 5 min and pipetted 30 μL in 1.5 mL microcentrifuge tube. The plasmid DNA (1μg/ μL) 30 μL was added and vortexed slightly for a while. The 30 μL 2.5 M CaCl2 was added and vortexed for a while. Next, 12 μL 0.1 M spermidine was added and vortexed for 2.5 min. The golden particles coating with DNA were spun for 3 seconds and discarded the supernatant. The 140 μL 70% ethanol was added and vortexed slowly for 1 min. the particles were settled for 5 min, spun for 3 seconds and discarded the supernatant. The last step, the 30 μL 100% ethanol was added to the golden particles and pipetted for resuspending. 9 μL of coated golden particles were loaded on each macrocarrier for each shoot.

5.4. Operation of Biolistic® PDS-1000/He Particle Delivery System

The operation protocol was followed the instructions of Dr. Wei-Chiang Shen Lab.

6. Genomic DNA extraction

In order to confirm whether the foreign gene inserted into the genome of the transgenic

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plants or not, the genomic DNA was extracted by CTAB described by Rogers and Bendich (1985) with modifications. The putative transgenic plants of *S. speciosa* were homogenized by liquid nitrogen with mortars. The homogenized tissue was transferred to a 50 mL centrifuge tube with preheated 8 mL CTAB buffer. The mixture was incubated at 65ºC for 30 min with vigorously shaking the tube frequently. The 10 mL PCI (Phenol: Chloroform: isoamyl alcohol, 25: 24: 1, pH 8.0) was added to the mixture for isolating DNA form protein and cell lysate. Each sample was shaked gently (75 rpm) for 60-90 min, then the samples were centrifuged at 10000 rpm for 13 min. The aqueous layer was transferred to a new centrifuge tube, then 10 mL PCI was added and repeated the following step again. Next, 500 μL CI (Chloroform: isoamyl alcohol, 24: 1) was added for removing the remaining phenol and protein. The mixture was mixed fully for another 30 min and centrifuged at 10000 rpm for 13 min. After centrifugation, the aqueous layer was transferred to a new tube and added one-tenth volume of 3 M sodium acetate (pH 5.5) settling for 10 min. the three-fourth volume of isopropanol was added for precipitate DNA at room temperature for 15 min. After centrifugation at 10000 rpm for 15 min, the supernatant was discarded. Pellet was washed by 5 mL 70% ethanol and resuspended in 100-150 μL sterilized water depending on pellet size. Finally, the DNA was stored at -20 ºC.

The RNA can removed by adding 3-5 μL RNase A (10 mg/ mL) and incubated at

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37 ºC for 30 min before CI step.

CTAB buffer (100 mL)

Regent/stock buffer	Adding weight/volume
CTAB (Hexadecyl trimethyl-ammonium bromide)	2.0 g
1 M Tris, pH 8.0	10.0 mL
0.5 M EDTA, pH 8.0 (Ethylenediaminetetraacetic acid)	4.0 mL
5 M NaCl	28.0 mL
ddH_2O	56.0 mL

Adjust to pH 8.0 with NaOH and store at room temperature.

Add 5 μL β-Mercaptoethanol and 30 mg Polyvinylpolypyrrolidone (PVPP) into per 1 mL CTAB buffer and incubate at 65 ºC for 30 min before using.

7. Statistical analysis

All the statistical analysis was performed with R software (R Core Team, 2013). In this study, the transient transformation rate under different pre-culture treatment were analyzed by ANOVA for detecting if the significantly different from control and experimental groups.

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Results

1. Optimization of *Agrobacterium*-mediated transformation

Factors that influence *Agrobacterium* infection for successful transformation, include *Agrobacterium* strain, *Agrobacterium* cell density, infection time, the developmental stage of seedlings selected for transformation, pre-culture time and co-culture time. In this study, genetic transformation system of *Sinningia speciosa* was optimized by GUS gene as reporter system.

1.1. Optimization of co-culture time

Many studies demonstrate that the co-culture time is a key factor affecting transformation efficiency. To test the best co-culture time, 2 weeks old seedlings of *S. speciosa* were cultured on co-culture medium for 3 or 5 days, inoculated with *Agrobacterium* strains EHA 105 or GV3101 of OD_{600} = 0.8 and additional pressure exerting by 50 mL syringe.

To evaluate the better co-culture time for transformation, transient activity of GUS was measured to confirm the successful delivery of T-DNA into the seedlings. We calculated the number of individuals with GUS signals divided by total number of treated explants as transient transformation rate (Jian et al., 2009). The best condition was chosen according to the highest transient transformation rate and the broadest area of GUS signals.

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The efficiency of *Agrobacterium* infection might be influenced by temperature and photoperiod during co-culture duration. When extending the co-culture time to 5 days, the transient transformation rate were increased in both *S. speciosa* (54.6%) and *N. benthamiana* (90%) with inoculation of *Agrobacterium* EHA105 under 27ºC and long-day (16 hr light/ 8 hr dark) (Fig. 2C).

In contrast, seedlings with 3 days of co-culture resulted in low transient transformation rate both in *S. speciose* (<5%) and *N. benthamiana* (66.7%) no matter they were cultured at either low or high growth temperature (22 ºC and 27 ºC) and different photoperiod (long-day and all dark) (see appendix Fig. 2 and Table 2). This suggests that 3 days of co-culture might not be enough for infecting 2 weeks old seedling of *S. speciosa*.

Moreover, it was observed that seedlings were surrounded by visible *Agrobacterium* plaques after 5 days of co-culture, more obvious than that of 3 days (Fig. 2A and 2B). This indicates that longer co-culture time (5 days) is required and more efficient for infecting 2 weeks old seedling of *S. speciosa*.

On the other hand, when 2 weeks old seedlings inoculated with *Agrobacterium* GV3101, GUS transient activity was not detected either in 3 days (0%) or 5 days (0%) of co-culture. It implied that the *Agrobacterium* GV3101 might not be suitable for inoculating seedlings of *S. speciosa* (Fig. 2C).

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To sum up, 5 days of co-culture resulted in more efficient transformation than 3 days, and *Agrobacterium* strain EHA105 is better than GV3101. Therefore, 5 days of co-culture was applied in subsequent optimizations.

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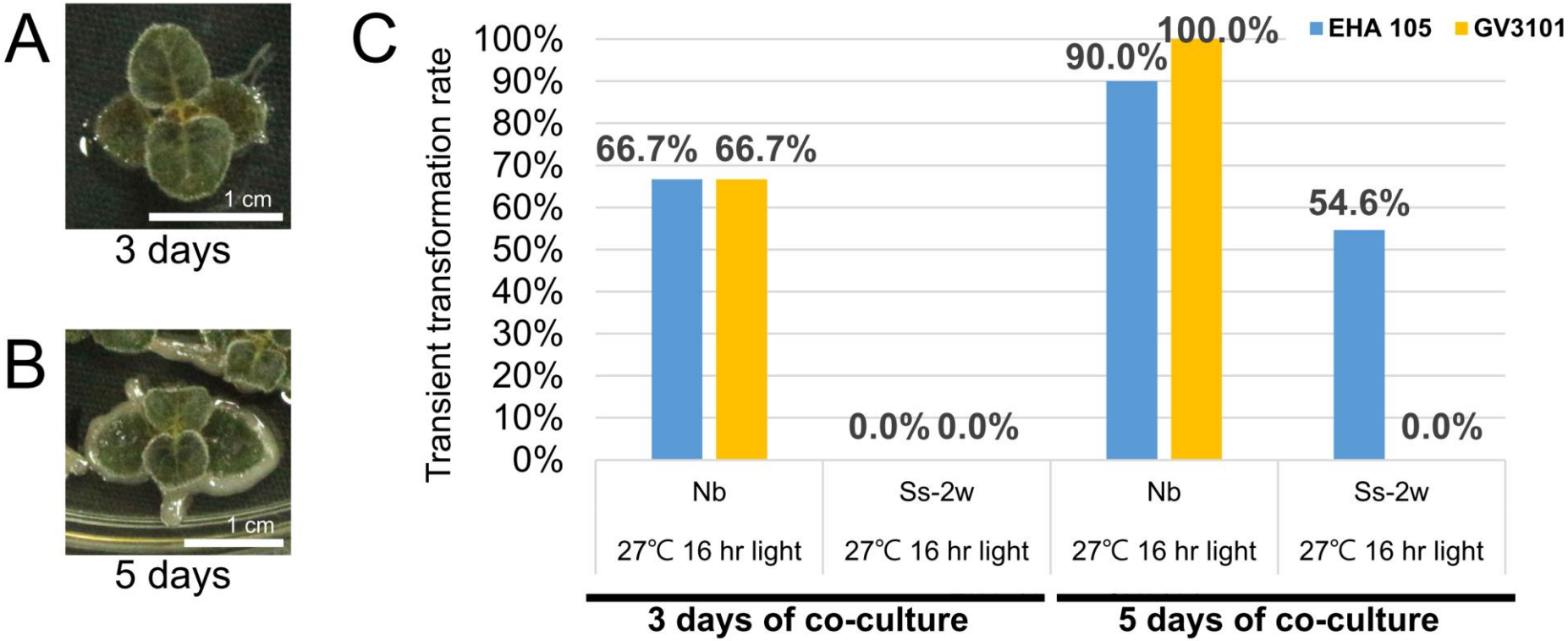

Figure 2 Co-culture time test for *Agrobacterium* infecting 2 weeks old seedlings of *S. speciosa*.

A, *S. speciosa* inoculated with *Agrobacterium* after 3 days of co-culture. B, *S. speciosa* inoculated with *Agrobacterium* after 5 days of co-culture. C, transient transformation rate of *S. speciosa* in combination with 3 or 5 days of co-culture by either *Agrobacterium* EHA105 or GV3101. Nb, *Nicotiana benthamiana*, n = 30. Ss_2w, 2 weeks old seedlings of *S. speciosa*. 3 days of co-culture, n = 20. 5 days of co-culture, n = 11.

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1.2. Optimization of developmental stage of *S. speciosa* and *Agrobacterium* strain

To evaluate whether the developmental stage of *S. speciosa* affects the efficiency of *Agrobacterium* infection, seedlings in three stages (2, 3 or 4 weeks old) and mature leaf in combination with *Agrobacterium* EHA 105 and GV3101 were tested. Seedlings were found easier to be infected than mature leaf. Low transient transformation rate was found in mature leaf inoculated with both *Agrobacterium* EHA 105 (13.0%) and GV3101 (10.0%) (Appendix Fig. 3). In contrast, after 5 days of co-culture, high transient transformation rate were observed when inoculated with *Agrobacterium* EHA 105 among 2 (54.6%), 3 (76.4%), and 4 (83.3%) weeks old seedlings. (Fig. 3). On the other hand, GUS signals can only be detected in 3 weeks old seedling inoculated with *Agrobacterium* GV3101 (1.7%) (Fig. 3). Thus, the *Agrobacterium* EHA 105 exhibited higher transformation efficiency than *Agrobacterium* GV3101 did in *S. speciosa*. Although four weeks old seedlings showed the highest transient transformation rate (83.3%) when infected with EHA 105, three weeks old seedlings possessed more and broader GUS signals, which means more cells can be transformed (Fig. 3). In this respect, 3 weeks old seedlings were selected due to the better competence of *Agrobacterium* EHA 105. Therefore, three weeks old seedlings of *S. speciosa* with inoculation of *Agrobacterium* EHA105 and 5 days of co-culture was applied in subsequent assays.

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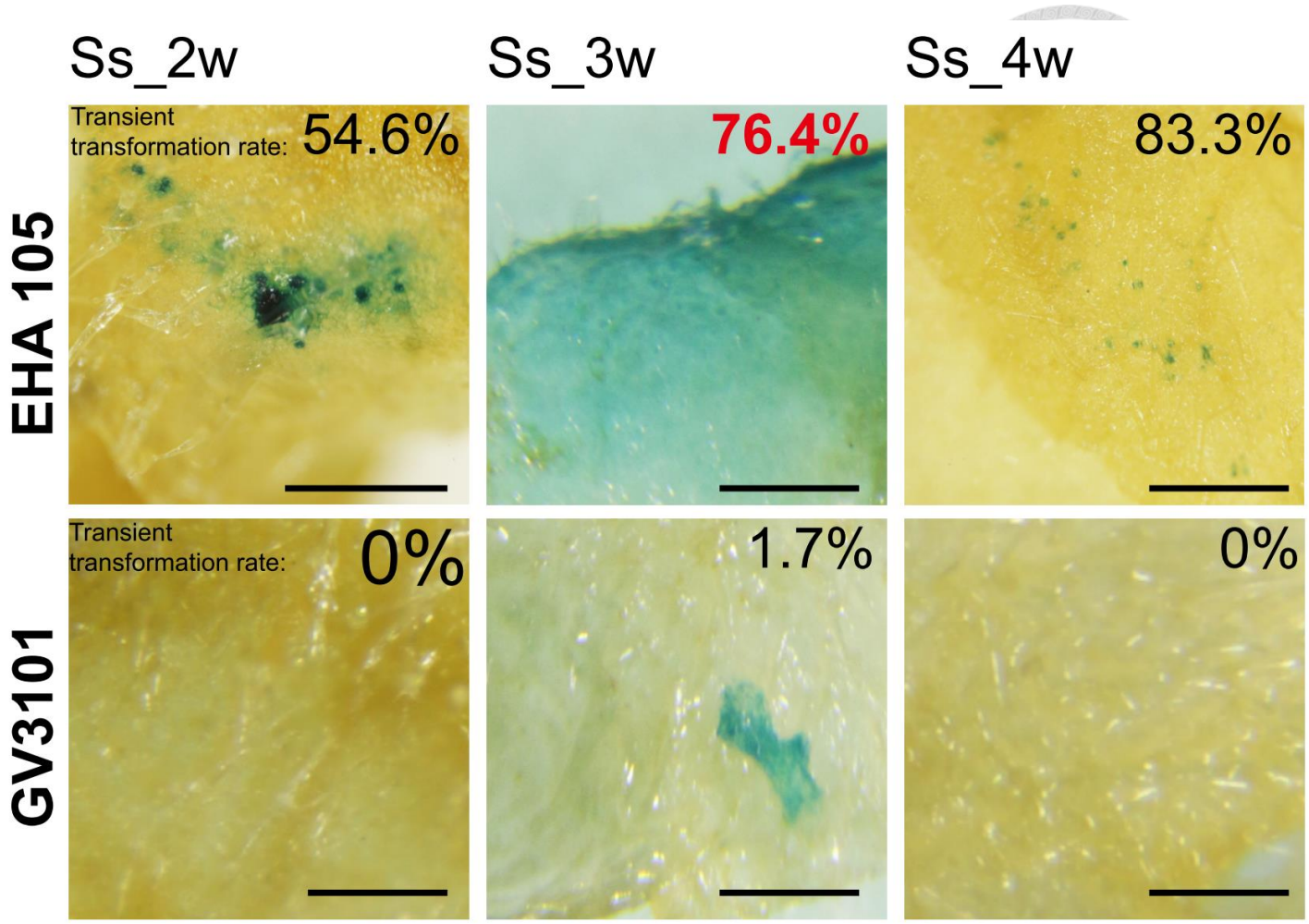

Figure 3 GUS signals in 3 stages of seedling of *S. speciosa* inoculating with *Agrobacterium* strain EHA 105 and GV3101.

The transformed region of seedlings can be observed by blue GUS signals. The transient transformation rate is calculated on upper right. Scale bar, 0.5 mm. Ss_2w, 2 weeks old seedlings of *S. speciosa*. Ss_3w, 3 weeks old seedlings of *S. speciosa*. Ss_4w, 4 weeks old seedlings of *S. speciosa*.

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1.3. Optimization of pre-culture time

In order to know whether the pre-culture procedure enhances the *Agrobacterium* infection, the 3 weeks old seedlings were pre-cultured on MS medium supplied with 1 ppm BA (pre-culture medium) for 3 and 4 days, and the 0 day of pre-culture showed as control group. The GUS signals indicated that the higher transient transformation rate can be achieved at 78.3 % and 100 % on 3 and 4 days of pre-culture respectively, and transient transformation rate in 0 day of pre-culture control is 62.8 % (Fig. 4). However, these data showed that no significant difference among these pre-culture times (0, 3 and 4 days) as revealed by ANOVA analysis ($p = 0.243$). The reason for no significant different might be due to the rate deviation among individuals in 0 and 3 days of pre-culture. Although the 4 days of pre-culture demonstrated the most stable and highest transient transformation rate, these pre-culture times were all followed in regeneration assay below.

The pre-culture time (0, 3 and 4 days) were continuously tested in order to evaluate whether it influences regeneration rate after *Agrobacterium* infection. Regeneration rate is calculated as the number of individuals with shoots divided by the total number of treated explants. Fig. 5 showed that the highest regeneration rate was found as 22.8% with 4 days of pre-culture, 17.2% with 3 days of pre-culture, and as 8.1% in 0 day of pre-culture, the lowest regeneration rate. These results demonstrate that the treatment

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of pre-culture can enhance the regeneration.

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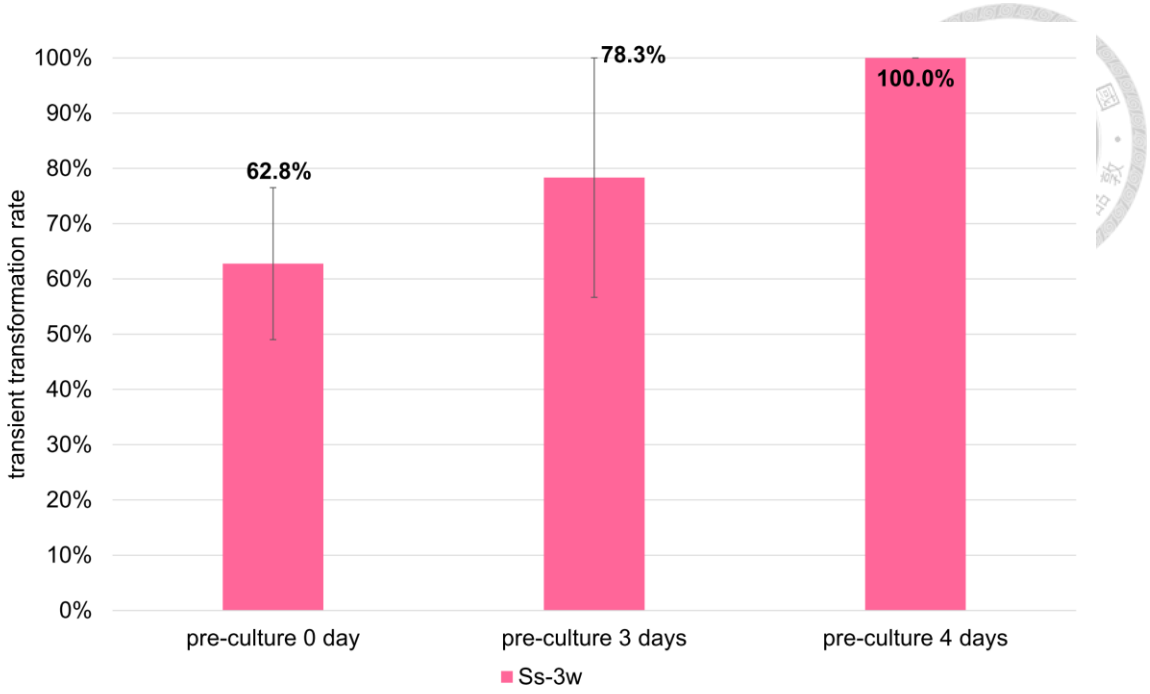

Figure 4 The effect of pre-culture time on transient transformation rate in *S. speciosa*.

The rate is presented as bar graph in mean ±standard error. The standard error are not presented in Ss_4w and Ss_5w as only one repeated test was done. Ss_3w, 3 weeks old seedlings of *S. speciosa*. Ss_4w, 4 weeks old seedlings of *S. speciosa*. Ss_5w, 5 weeks old seedlings of *S. speciosa*. All the experiments were repeated 3 times at least.

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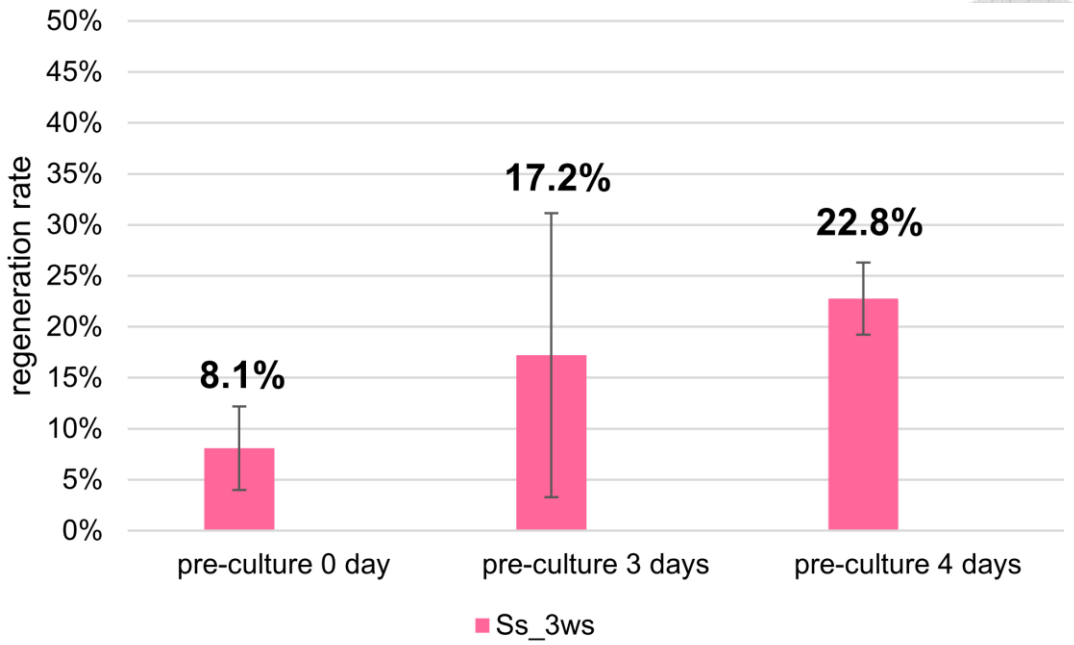

Figure 5 The effect of pre-culture time on regeneration rate in *S. speciosa*.

The rate is presented as mean ±standard error. Ss_3w, *S. speciosa* 3 weeks old seedlings. Ss_4w, *S. speciosa* 4 weeks old seedlings. Ss_5w, *S. speciosa* 5 weeks old seedlings. All the experiments were repeated 3 times at least.

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1.4. T-DNA insertion confirmation

All the regenerated shoots obtaining from optimal conditions were classified as putative transgenic plants, and they were subsequently examined by GUS activity assay and RT-PCR for confirmation of T-DNA insertion or not. Only seven of them showed at least one positive result among these two assays (Fig. 6), the negative results were given in Appendix Fig. 4.

The GUS activity was continuously checked throughout the developmental stages of both juvenile and mature leaf on these putative transgenic plants to exclude those individuals with transiently expressed GUS signals (false positive) at early regeneration stages. After that, four of 5 independent putative transgenic plants showed chimerically positive GUS signals under 3 days of pre-culture (displayed as B in first letter) (Fig. 6). After 3 months of culture, one of the 5 independent putative transgenic plants with positive GUS signals had died (B1-23a^{1}), and only two of them retained positive GUS signals in mature leaf, implying that T-DNA have been integrated into genome of these two individual plants. On the other hand, positive GUS signals were also expressed in the juvenile leaf of two independent putative transgenic plant under 4 days of pre-culture (displaying the first letter C) (Fig. 6). Thereafter, one of them presented the week GUS signals in mature leaf (C3-9a^{1}), the other one had died (C3-11a^{1}) (Fig. 6A) after 3 months culture.

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RT-PCR was conducted to reconfirm whether the T-DNA insertion is true or not among these putative transgenic plants. To ascertain the T-DNA insertions are permanently among these plants, *GUS* and *NPTII* expression were examined in mature leaf (Appendix Fig. 5). Positively exogenous *GUS* (421 bp) and *NPTII* (788 bp) expressions can be amplified in some or all of these five putative transgenic plants which presented positive GUS signals at the juvenile leaf once and *Actin* was amplified as an internal reference (Fig. 6B). *GUS* expression was not detected in wild type (data not shown) and other putative transgenic plants which have no GUS signal detected. The putative transgenic plants that showed at least one positive result of *GUS* or *NPTII* expression were further analyzed the copy number of introduced *GUS* gene by Southern blotting with *GUS* probe (Fig. 7A). The results showed that B1-17a^1 had only one copy of *GUS* gene, both B1-2a^1 and C3-9a^1 had two copy of *GUS* gene, whereas the B1-5a^1 and B1-22a^1 had no *GUS* insertion due to no hybridization signals detected (Fig. 7B). The above results demonstrated that *GUS* gene can be successfully introduced into some transformants of *S. speciosa*.

Only those putative transgenic plants detected with positive signals in GUS activity, RT-PCR and Southern blotting were classified as true transgenic plants. They were calculated the transformation rate as the number of transgenic plants divided by total number of treated explants. The transformation rate under 3 and 4 days of pre-

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culture were showed 2.1% and 0.7% respectively (Fig. 8).

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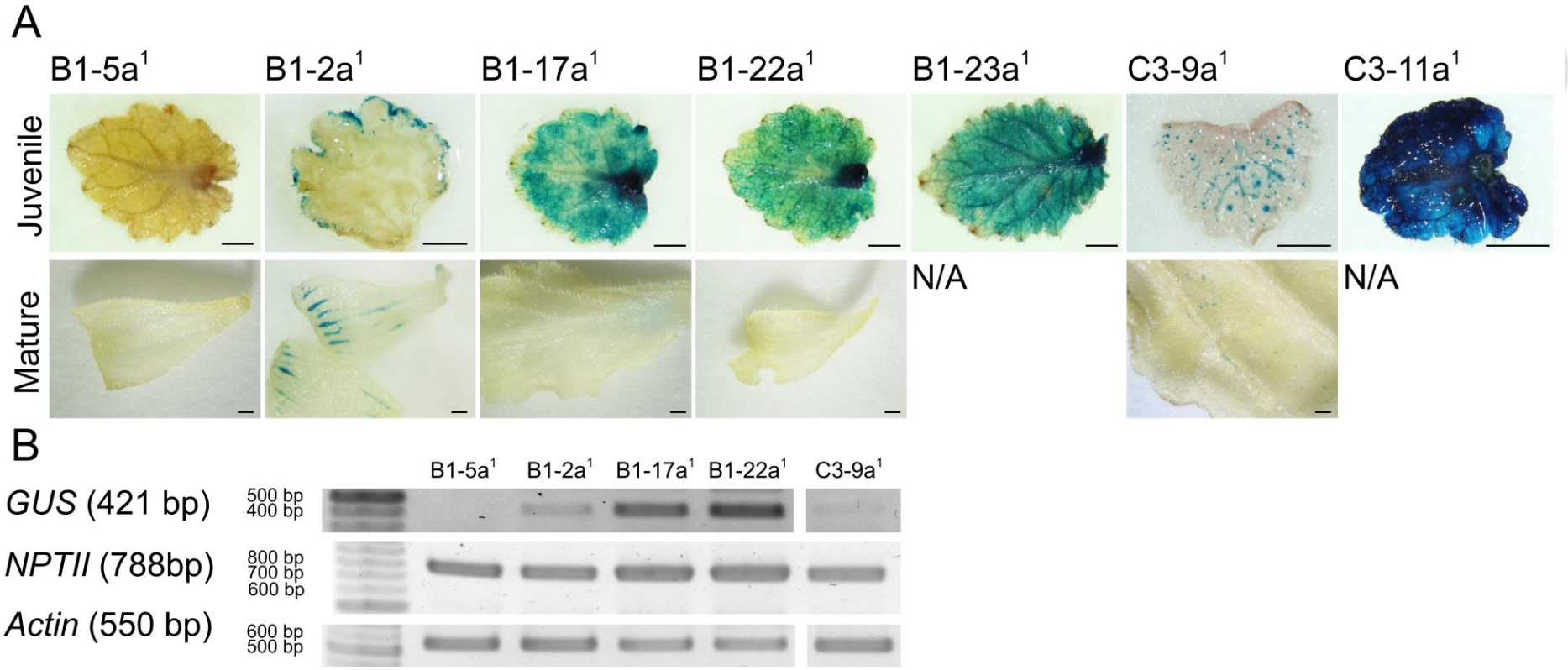

Figure 6 The detection of T-DNA insertion in regenerated shoots.

A, the GUS activity assay of juvenile and mature leaf among in 7 independent regenerated shoots/ individuals. Scale bar, 1mm. B, RT-PCR of *GUS* and *NPTII* among 5 independent putative transgenic plants.

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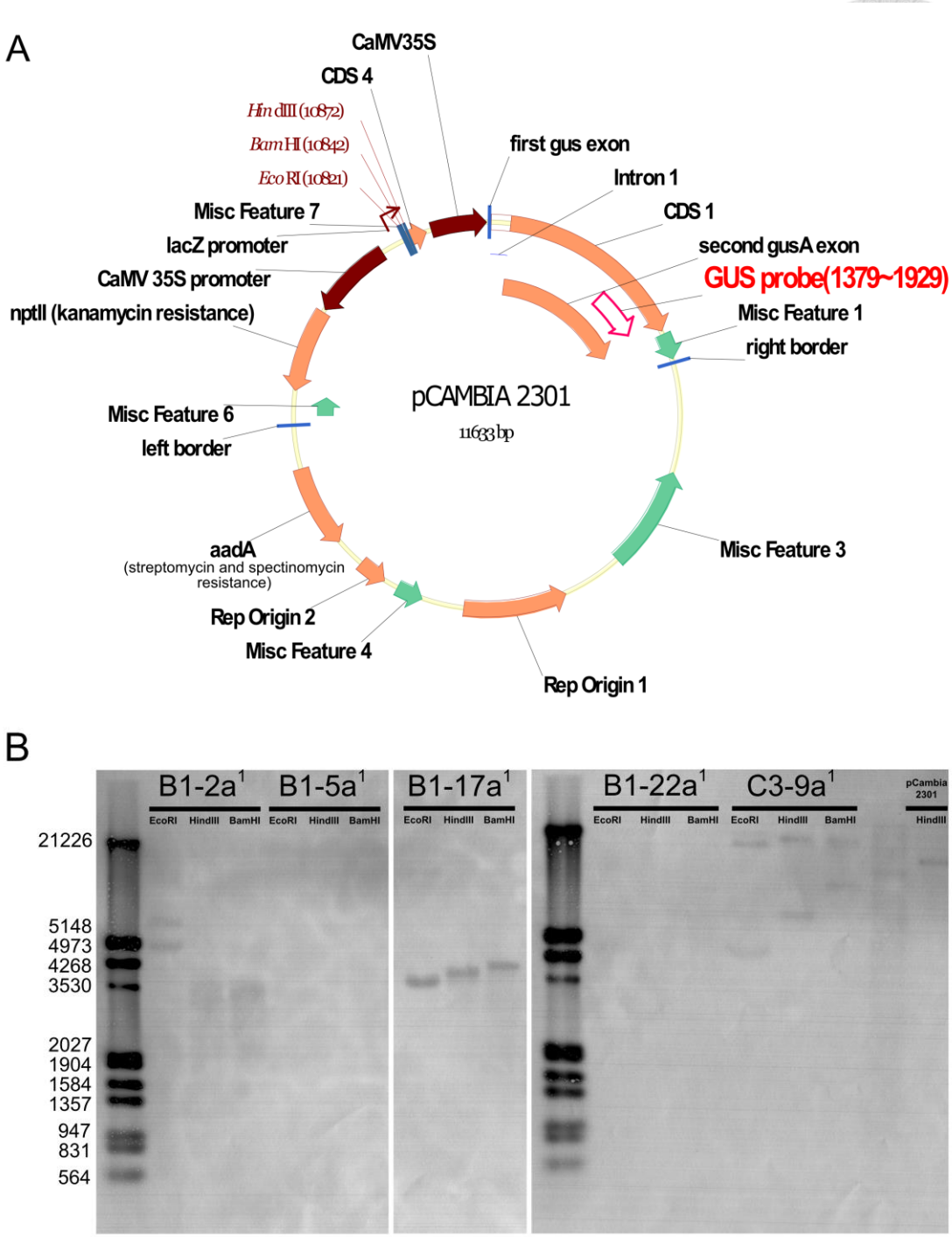

Figure 7 Southern blotting of regenerated shoots in *S. speciosa* for detecting the copy number of *GUS*.

A, *GUS* probe design. The hollow arrow indicates the probe position. B, the results of Southern blotting. The name of putative transgenic plants and restriction enzyme (EcoRI, HindIII and BamHI) used are indicated at the top of each lane. The most left lane is marker with annotated size (bp). The most right lane is plasmid as positive control.

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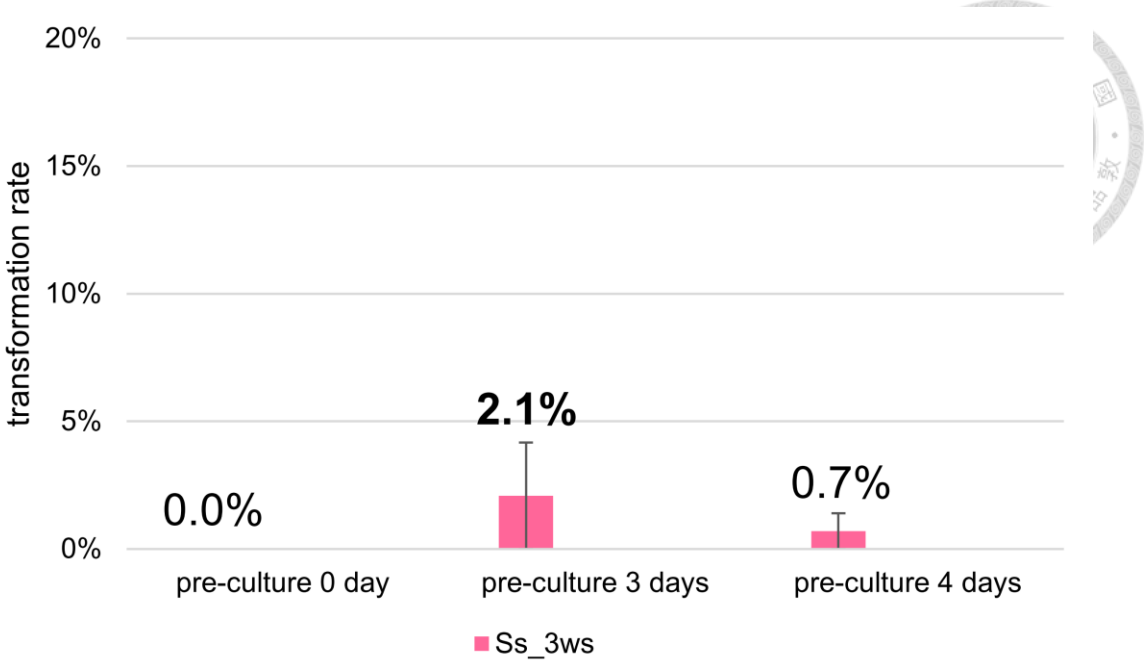

Figure 8 Transformation rate under 0, 3 and 4 days of pre-culture in *S. speciosa*.

The rate is presented as bar graph in mean ±standard error. Ss_3w, 3 weeks old seedlings of *S. speciosa*.

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1.5. The location of GUS signals

The paraffin section technique was used to detect the location of GUS signals inside leaf tissue of transient transformed seedling. Fig. 9 showed that the GUS signals were located in the mesophyll cells and epidermal cells of the 1st pair true leaf, indicating these cells could be successfully transformed.

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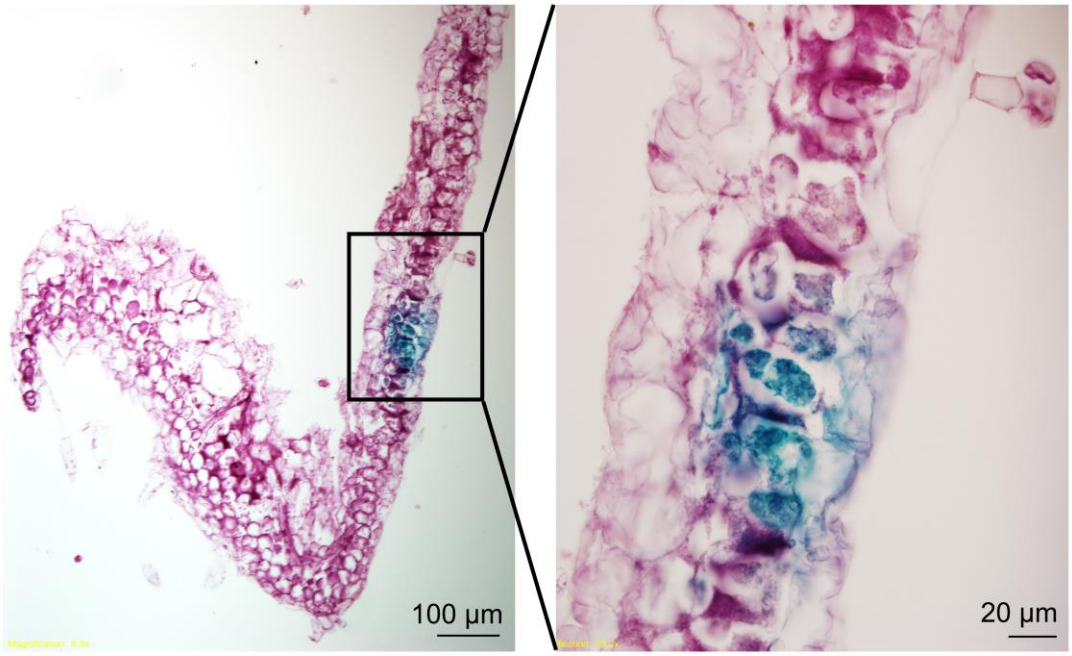

Figure 9 The locations of GUS signals in 1st pair true leaf in *S. speciosa* after *Agrobacterium* EHA 105 inoculation.

The 3 weeks old seedling was inoculated by *Agrobacterium* EHA 105 and cultured in co-culture medium for 5 days.

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2. Establishment of particle bombardment transformation for genetic transformation in *S. speciosa*:

To test whether particle bombardment is more efficient than *Agrobacterium*-mediated transformation, GUS reporter gene as a reporter system was used to optimize the conditions of particle bombardment transformation. Factors that influence the efficiency of successful particle bombardment transformation, include size and total amount of gold particle, hilum pressure, target distance, the process of DNA coating, etc. Each shot was fixed for the total amount of 0.6 mg gold particles with the size of 0.6 μm in diameter. The physical parameters were tested with combinations of hilum pressure as 900 psi and 1100 psi and target distance as 6, 9 and 12 cm.

2.1. Optimization of physical parameters of hilum pressure and target distance

The results showed that the GUS signals were presented at hilum pressure 1100 psi with 6 and 9 cm target distance, and 900 psi with all target distance (6, 9 and 12 cm) (Fig. 10C). GUS signals can be detected at any of three target working distances in hilum pressure 900 psi but not in 1100 psi, indicating that 900 psi is good for particle bombardment in *S. speciosa*.

At hilum pressure 900 psi, leaves in 6 cm target distance showed more GUS signals on the leaf and higher transient transformation rate than that in 9 cm (Fig 10A and B). Signals of these transformed cells mostly fall on trichome cells (black arrow)

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at 900 psi with 6 cm target distance (Fig. 10A). In contrast, GUS signals were mostly in trichome (black arrow) and vascular (red arrow) cells (Fig. 10B) at 900 psi with 9 cm target distance. Nevertheless, trichome cells are highly differentiated tissue which are difficult to regenerate comparing to vascular cells. This suggested that hilum pressure 900psi with 9 cm target distance is more suitable than 6 cm for regenerating because the gold particle can carry DNA into deeper vascular tissues, where the regenerated shoots usually initiated there as indicated in previous study (Kuo, 2014).

In comparison, the transient transformation rate of *Agrobacterium*-mediated transformation (Fig. 4) is higher than that of particle bombardment transformation (Fig. 10C), demonstrating that the *Agrobacterium*-mediated transformation is more efficient way for genetic transformation.

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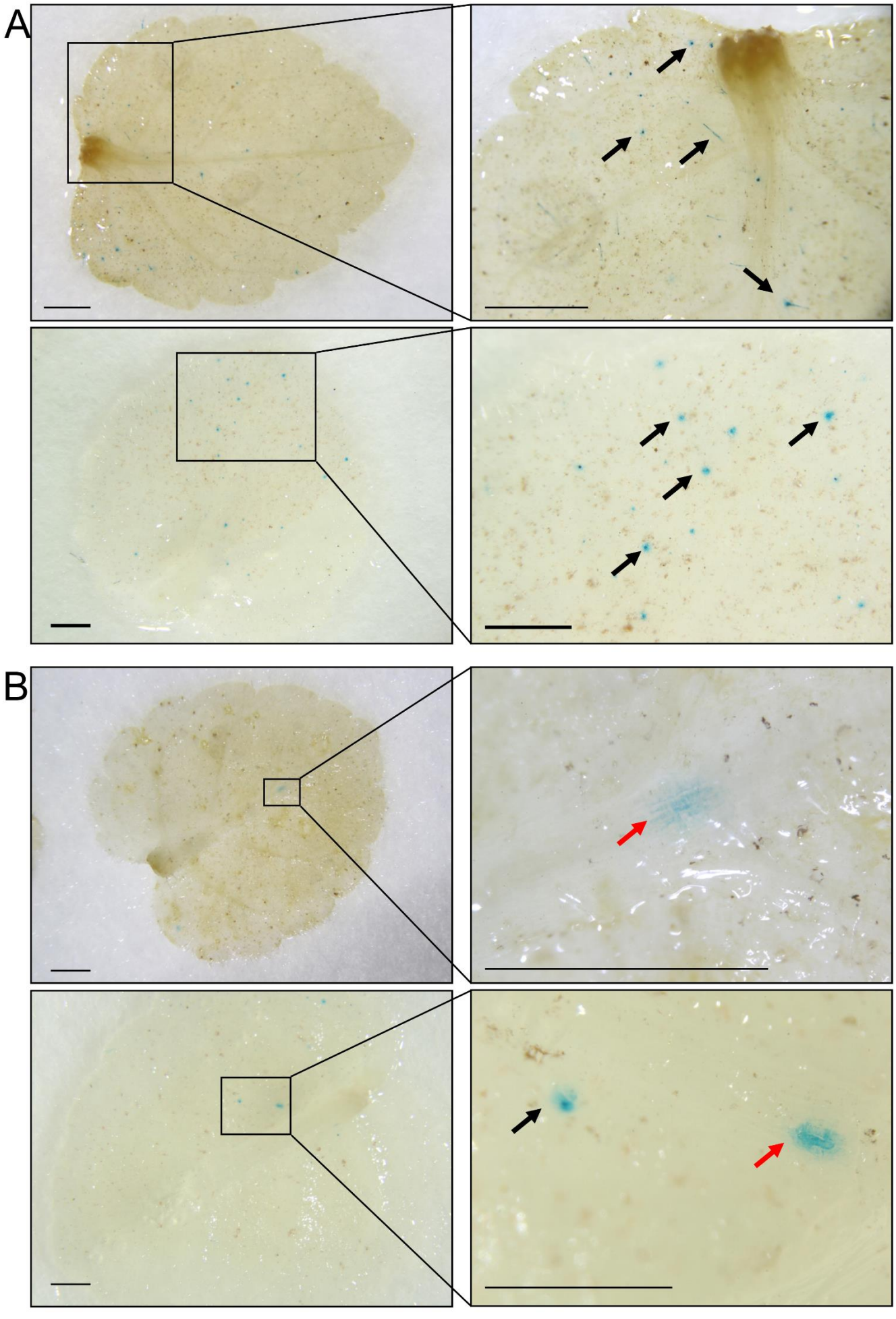

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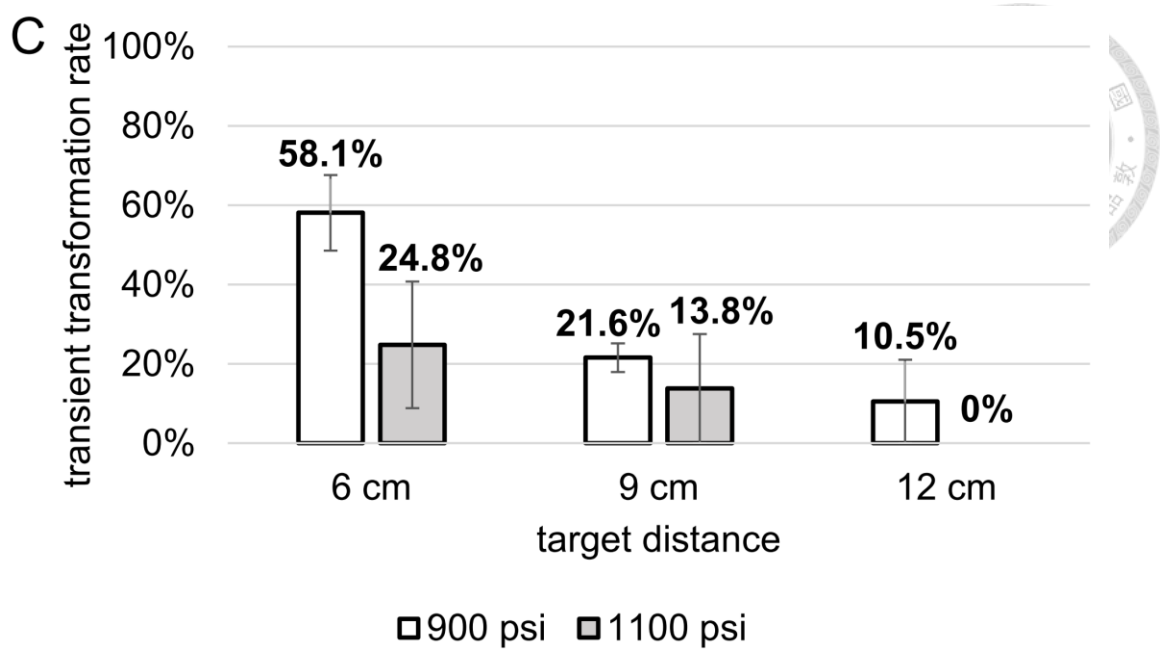

Figure 10 Particle bombardment conditions test by GUS report assay in 4 weeks old seedling of *S. speciosa*.

A, leaf is bombarded with hilum pressure 900 psi at 6 cm target distance. B, leaf is bombarded with hilum pressure 900 psi at 9 cm target distance. The black arrow shows the GUS signal locating in trichome cell. The red arrow shows the GUS signal locating in vascular cell. The upper row and lower row are biological repeats. Scale bar, 1 mm. C, the transient transformation rate in combinations of hilum pressure 900 and 1100 psi and 6, 9 and 12 cm target distance. The rate is presented as bar graph in mean ± standard error. The treatment with hilum pressure 900 psi at 6, 9 and 12 target distance were repeated 3 times, and the 1100 psi were repeated 2 times.

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3. Callus as an excellent alternative explant for genetic transformation

Callus is a cluster of unorganized cells with the ability of rapid cell division, which is often applied in *Agrobacterium*-mediated transformation because these cells with thin cell wall could be easily infected thus confers a high transformation efficiency (Delbreil et al., 1993). Due to the difficulties of mature leaf infection in *S. speciosa*, the callus might be a good substitute. For stable callus source, leaf explant and seed explant were used for inducing callus.

3.1. Callus induction

3.1.1. Callus induction - Leaf explants

Different concentrations of 2, 4- D and BA or TDZ were combined to investigate the callus inducing ability of leaf explants. The optimal hormone conditions were determined with combinations of high callus induction rate (>80%), and both low shoot (<20%) and root induction rate (<20%) at beginning, whereas callus size degree was compared next. 0.1 ppm 2, 4-D with 1 or 2 ppm BA was found the best hormone combination with highest callus induction rate without shoot and root induction (Table 1 and Fig. 11B and C). On the other hand, the combinations of 2, 4-D (0.5 and 1.0 ppm) and BA (0, 1.0 and 2.0 ppm) were also efficient in callus induction rate (>60%) without shoot and root formation, but the callus size was smaller and brown. Leaf explants culturing on MS medium with different concentrations of 2, 4-D (0 and 0.1 ppm) and

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TDZ (0.1, 0.5 and 1.0 ppm) in combinations gave no callus induction, or the induced callus is accompanied by high shoot and root induction rate. This indicated that TDZ is inappropriate to induce pure callus in *S. speciosa* (Table 1, Fig. 11D~L and Appendix Fig 6).

Although the callus induced by 0.1 ppm 2, 4-D with 1 or 2 ppm BA has the highest induction rate, the callus was vitrified which indicates a reduced mechanical strength and physiological disorders of regenerated tissues. In order to solve this problem, the 0, 0.025, 0.05 and 0.1M sorbitol was added to the medium as an osmotic regulator. After one month of culture, the embryogenic callus was induced with low shoot and root rate (< 20%) form leaf discs on MS medium supplemented 0.1 ppm 2, 4-D and 1 or 2 ppm BA with 0.025 or 0.05 M sorbitol (Table 2, Fig. 12C, D, E and F). In contrast, compact callus induced by treatment of 0.1 M sorbitol, 0.1 ppm 2, 4-D and 1 or 2 ppm BA showed the smaller size and a tight connection of cells (Fig. 11G and H).

Combinations of 2, 4-D and TDZ with 0.1 M sorbitol were also tried to induce callus, but the effect is not good (Table 2 and Appendix Fig. 7).

3.1.2. Callus induction – Seed explants

Seed was used to induce callus for saving time of the preparation of leaf explants (about 3-3.5 months). The best hormone condition was tested with 0, 0.01, 0.05 and 0.1 M sorbitol to regulate the osmotic pressure of medium at first. The results showed that

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callus induced without vitrification under low concentration or no sorbitol treatment (Table 3, Fig. 13A, blue star and Appendix Fig 8). However, the callus inducing from embryo was accompanied with high shooting rate (>60%) (Table 3 and Fig. 13B, red star), indicating the hormone condition for seed needs to further optimize.

In order to eliminate the high shooting rate from seed, hormone concentrations of 2, 4-D and BA were reduced. The seed sowed on MS medium containing the combination of 0.01, 0.025, 0.05 and 0.1 ppm 2, 4-D and 0, 0.1, 0.25, 0.5, 1 and 2 ppm BA, were tested for finding the best callus induction condition. Unfortunately, there is none hormone combination which confines both low shoot (<20%, showing pink color) and root rate (<20%, showing green color) (Table 4).

The above results demonstrate that leaf explants were better materials for callus induction.

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Table 1 Effect of plant growth hormone on callus induction from leaf explants of *S. speciosa*.

PGR (mg/l)		Number of explants	Morphology			
2, 4-D	BA	(leaf disc)	Callus (%)	Degree*	Shoot (%)	Root (%)
	0	48	100	H^{v}	0	100
0.1	**1**	**48**	**100**	**H^{v}**	**0**	**0**
	2	**47**	**97.9 ± 3.61**	**H^{v}**	**0**	**0**
	0	48	100	M^{b}	0	0
0.5	1	45	93.8 ± 10.8	M^{b}	0	0
	2	41	75.9 ± 1.6	M^{b}	0	0
	0	48	100	M^{b}	0	0
1	1	48	89.6 ± 18	M^{b}	0	0
	2	32	65.6 ± 13.2	L^{b}	0	0
	0	48	35.4 ± 13	M^{b}	0	0
2	1	48	33.3 ± 20.1	M^{b}	0	0
	2	48	43.6 ± 22.5	L^{b}	0	0
2, 4-D	TDZ					
	0.1	16	0		18.75	0
0	0.5	16	0		81.25	0
	1	15	0		100	0
	0.1	16	100	H^{v}	0	100
0.1	0.5	14	100	H^{v}	0.5	42.8
	1	16	93.75	M^{v}	31.25	0

Note: callus (%), the number of leaf discs inducing callus divided by total number of leaf discs. degree, the growth of callus size. H, high growth of callus. M, medium growth of callus. L, low growth of callus. *, the status of callus. ^{v}, vitrification. ^{b}, brown color. The hormone conditions showing in pink color were the best condition to induce callus formation. The results were recorded after 4 weeks culture. The data is presented as mean ± stand deviation.

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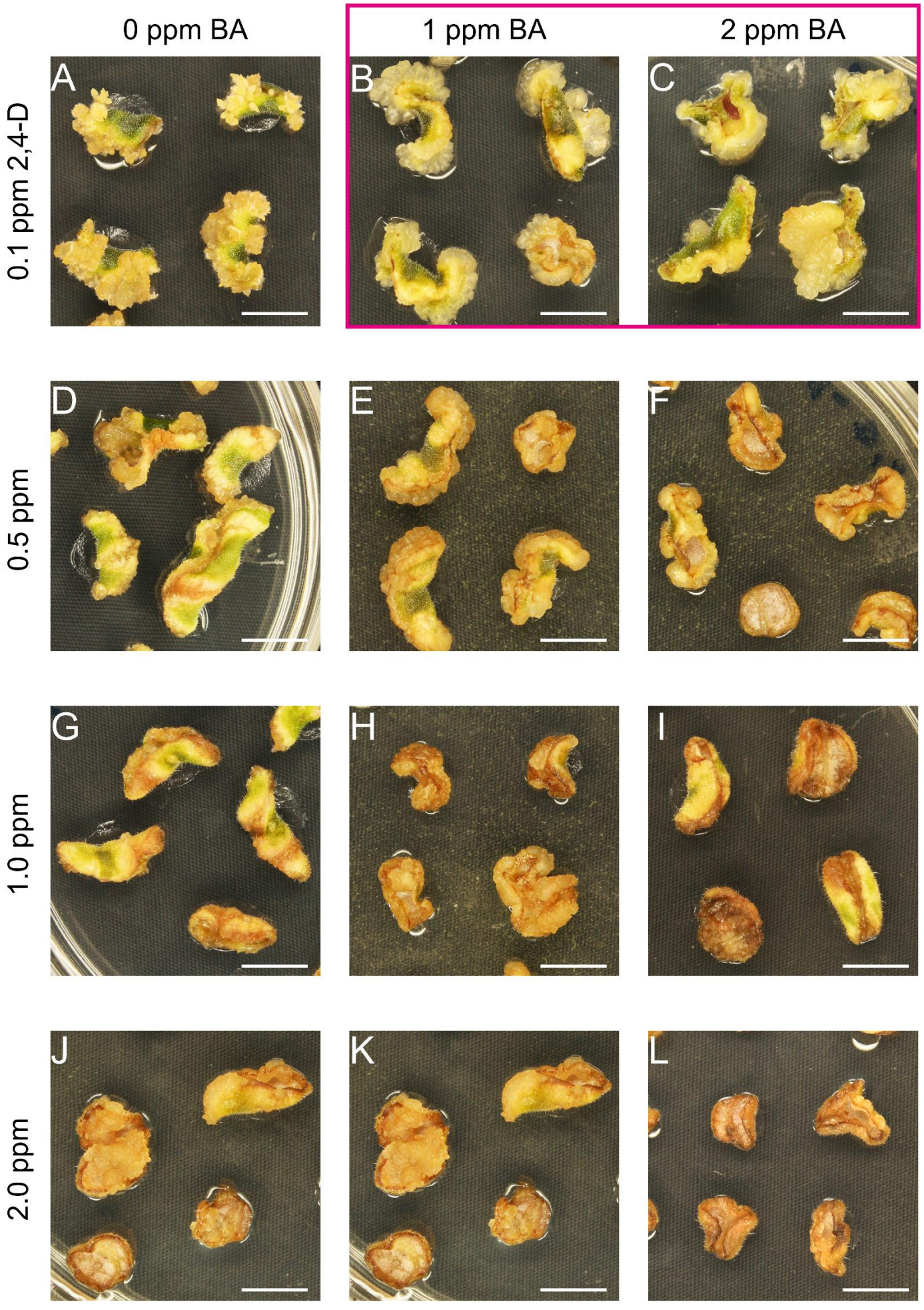

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Figure 11 The morphology of callus under auxin (2, 4-D) and cytokinin (BA) treatment.

The 12 different concentrations of 2, 4- D (0.1, 0.5, 1.0 and 2.0 ppm) and BA (0, 1 and 2 ppm) in combinations were tested for best callus inducing condition. The results were recorded after 4 weeks culture. Scale bar, 1 cm.

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Table 2 Effect of sorbitol on callus induction from leaf explants of *S. speciosa*.

PGR (mg/l)		Sorbitol (M)	Number of explants	Morphology			
2, 4-D	BA		leaf disc	Callus (%)	Degree*	Shoot (%)	Root (%)
0.1	0	0	48	100	H^{v}	0	100
	1		48	100	H^{v}	0	0
	2		47	97.9 ± 3.6	H^{v}	0	0
	1	0.025	139	95.3±0.7	M^{e}	1.1±1.5	0
	2		152	93.2±9.7	M^{e}	12.5±17.7	2.4±3.3
	1	0.05	287	96.8±2.8	M^{e}	7.1±8.6	5.5±9.6
	2		324	94.6±7.3	M^{e}	6.2±5.5	0.2±0.3
	0.1	0.1	48	100	M^{v}	0	100
	1		48	100	M^{c}	20.8 ± 3.6	18.8±6.3
	2		47	100	M^{c}	6.25 ± 6.3	0
2, 4-D	TDZ						
0.1	0.1	0.1	47	100	M^{c}		100
	0.5		48	100	M^{c}	4.2 ± 3.6	35.4±7.2
	1		32	100	M^{c}	9.4 ± 13.3	37.5±26.5

Note: Callus (%), the number of leaf discs inducing callus divided by total number of leaf discs. Degree, the growth of callus size. H, high growth of callus. M, medium growth of callus. L, low growth of callus. *, the status of callus. ^{v}, vitrification. ^{e}, embryogenic. ^{c}, compact. The hormone conditions showing in pink color were the best condition to induce embryogenic callus formation. The data is presented as mean ± stand deviation. The results were recorded after 4 weeks culture.

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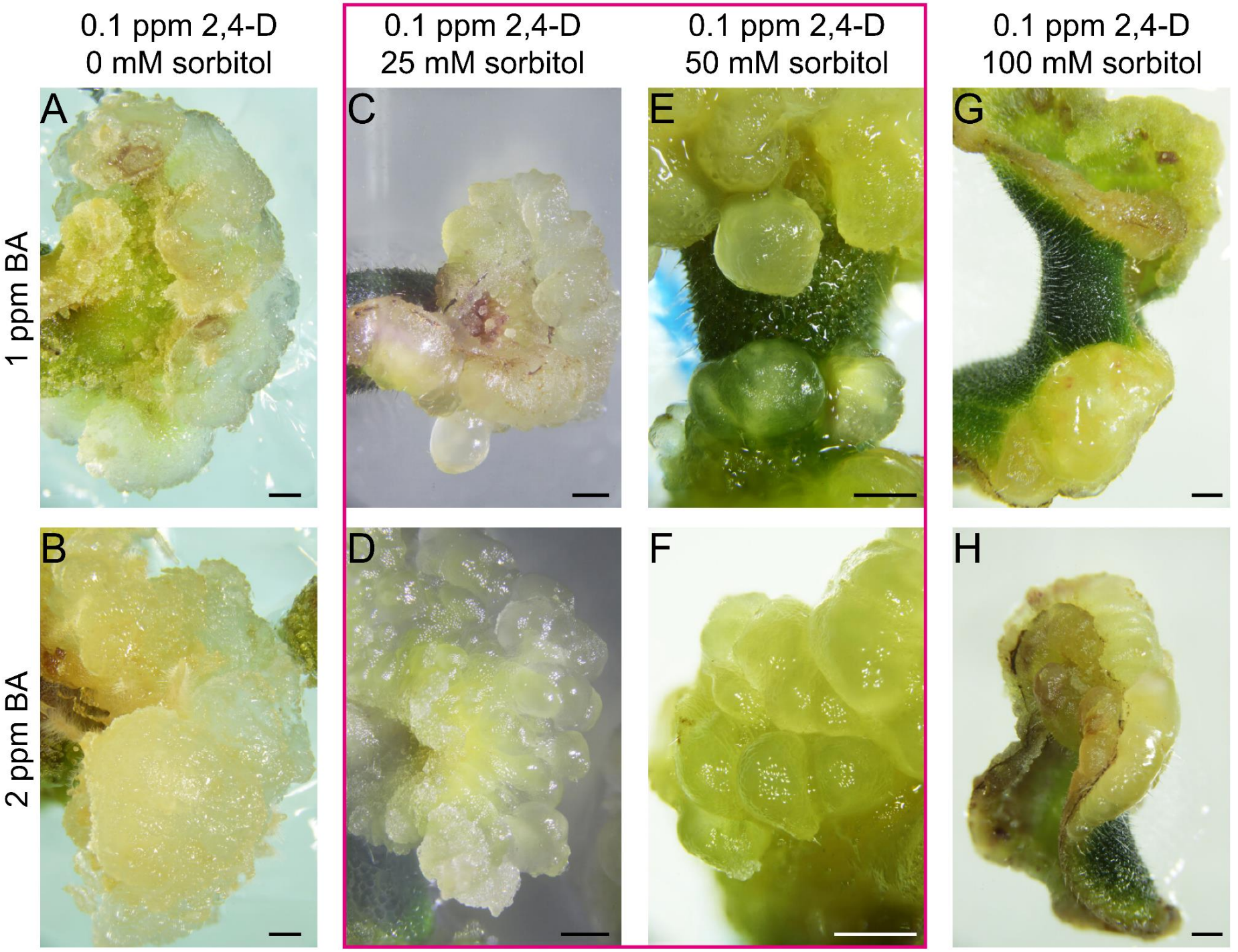
0.1 ppm 2,4-D
0 mM sorbitol
0.1 ppm 2,4-D
25 mM sorbitol
0.1 ppm 2,4-D
50 mM sorbitol
0.1 ppm 2,4-D
100 mM sorbitol
1 ppm BA
2 ppm BA
A
B
C
D
E
F
G
H

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Figure 12 The morphology of callus under different concentrations of sorbitol treatment.

The callus was induced by 0.1 ppm 2, 4-D and 1 or 2 ppm BA combined with different sorbitol concentrations. The embryogenic callus showed in frame. The results were recorded after 4 weeks culture. Scale bar, 1mm.

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Table 3 Effect of sorbitol on callus induction from seedling explants of *S. speciosa*.

PGR (mg/l)		Sorbitol (M)	Number of explants	Morphology			
2, 4-D	BA		(seedling)	Callus (%)	Degree*	Shoot (%)	Root (%)
0.1	1	**0**	80	100	**H**	75 ± 8.2	0
		0.01	71	100	**H**	77.5 ± 0.7	0
		0.05	76	100	M[c]	69.7 ± 4.1	0
		0.1	86	100	L[c]	84.9 ±7.4	0
	2	**0**	96	100	**H**	76 ± 9.9	0
		0.01	79	100	**H**	84.8 ± 4.5	0
		0.05	86	100	M[c]	82.6 ± 6	0
		0.1	71	100	L[c]	69 ± 15.1	0

Note: seedling, the number of geminated seed. Callus (%), the number of seedlings inducing callus divided by total number of seedlings. Degree, the growth of callus size. H, high growth of callus. M, medium growth of callus. L, low growth of callus. *, the status of callus. [c], compact. The sorbitol concentrations showing in pink color were selected for the high growth of callus without vitrification. The data is presented as mean ± stand deviation. The results were recorded after 4 weeks culture.

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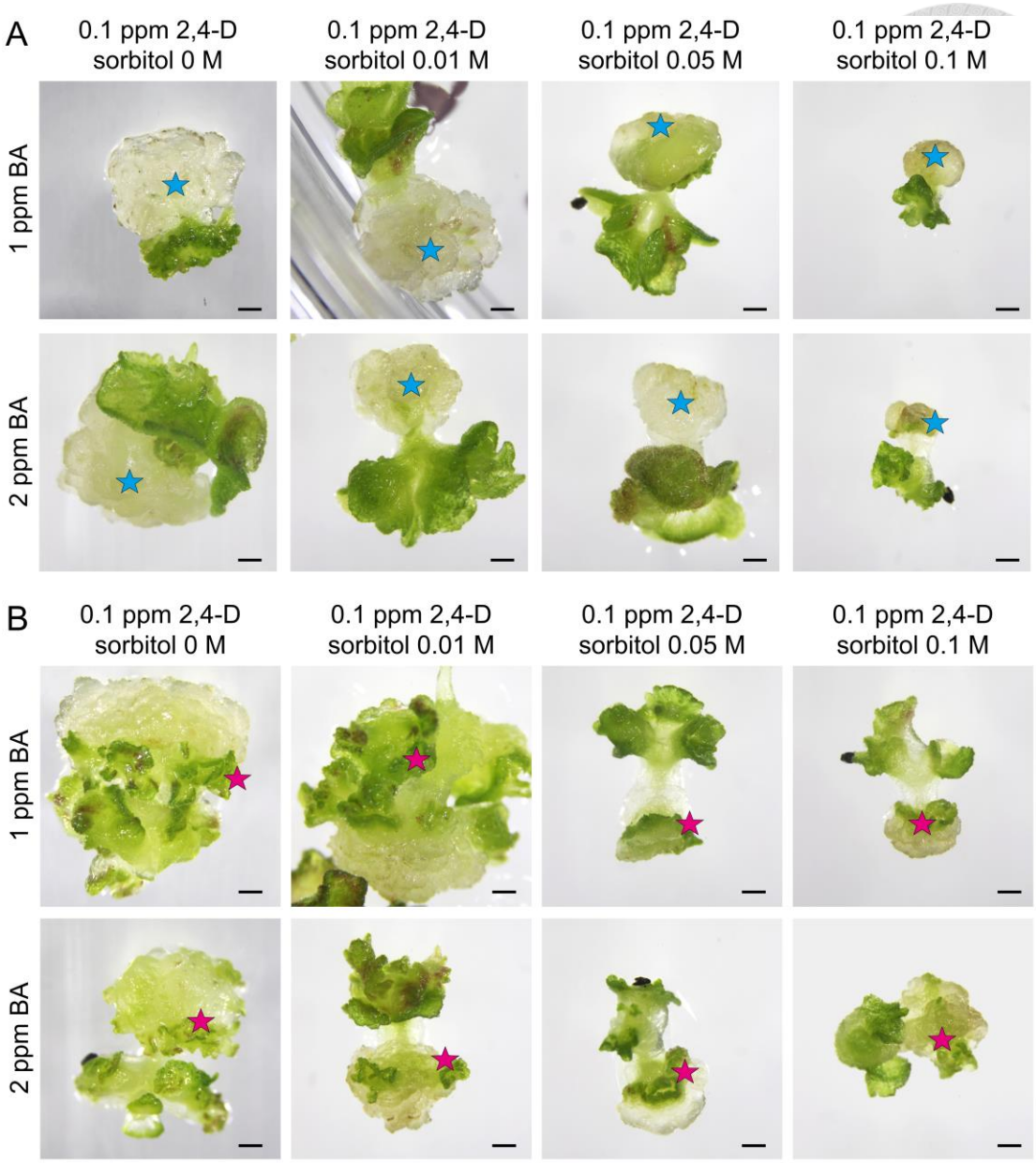

Figure 13 Effect of sorbitol on callus induction from seedling explants of *S. speciosa*.

A, callus inducing form hypocotyl of seedling without shoot. The blue star shows the callus. B, callus inducing form hypocotyl of seedling with shoot. The red star shows the shoot formation from callus. The results were recorded after 4 weeks culture. Scale bar, 1 mm.

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Table 4 Effect of plant growth hormone on callus induction from seedling explants of *S. speciosa*.

PGR(mg/l)		Number of explants	Morphological		
2, 4-D	BA	(seedling)	Callus(%)	Shoot(%)	Root(%)
0.01	0	82	9.0	0.0	41.0
	0.1	76	100.0	72.7	16.3
	0.25	75	100.0	84.8	27.8
	0.5	51	91.1	75.6	37.7
	1	84	100.0	58.2	34.0
	2	80	100.0	78.0	30.0
0.025	0	87	29.6	0.0	75.3
	0.1	73	100.0	64.4	57.0
	0.25	51	100.0	84.4	25.5
	0.5	78	100.0	97.1	8.1
	1	88	100.0	86.4	4.2
	2	85	100.0	83.8	0.0
0.05	0	75	100.0	0.0	97.3
	0.1	72	100.0	35.9	98.6
	0.25	70	100.0	61.4	75.6
	0.5	74	100.0	71.6	29.9
	1	79	100.0	75.6	1.4
	2	82	100.0	74.7	0.0
0.1	0	54	78.2	0.0	78.2
	0.1	81	100.0	14.5	92.1
	0.25	80	100.0	42.3	81.0
	0.5	30	100.0	76.7	0.0
	1	76	100.0	62.6	0.0
	2	79	99.1	72.4	0.0

Note: The shoot rate lower than 20% is color in gray. The root rate lower than 20% is color in gray. The hormone condition is selected with both shoot and root rate lower than 20%. The results were recorded after 4 weeks culture.

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3.2. Shoot regeneration from callus

The optimal hormone condition for callus to induce shoots must be established for efficient regeneration before genetic transformation implementing. Therefore, 4 kinds of embryogenic callus (inducing from 0.1 ppm 2, 4-D and 1 or 2 ppm BA with 0.025 or 0.05 M sorbitol) were respectively transferred to MS medium supplemented with 12 different concentrations and combinations of NAA (0, 0.05, 0.1 ppm) and BA (1, 2, 3 and 5 ppm) to find the best hormone conditions inducing shoot regeneration. The regeneration rate was recorded after 5 weeks (Appendix Fig 9) and 8 weeks of culture (Fig. 14). The hormone condition was selected with high (>50%) shoot rate and low (10%) root rate. Only two hormone combinations achieved this goal. Callus induced from 0.1 ppm 2, 4-D and 2 ppm BA with 25 mM sorbitol and subsequently treated with 0.05 ppm NAA and 1 ppm BA showed the best shooting rate (91%) and low rooting rate with about 3 shoots per responsive embryogenic callus (Fig. 14B and E), but the shoots were vitrificated mostly (Appendix Fig. 10B-b). embryogenic callus treated with 0.05 ppm NAA and 3 ppm BA exhibited high shooting rate (57%) and low rooting rate, in 2 shoots per responsive callus, in which the callus was induced by 0.1 ppm 2, 4-D and 2 ppm BA with 50 mM sorbitol (Fig. 14D and E, Appendix Fig 10A-h). Here, I concluded that MS medium supplied with 0.05 ppm NAA and 3 ppm BA is the best hormone condition for shoot regeneration from callus.

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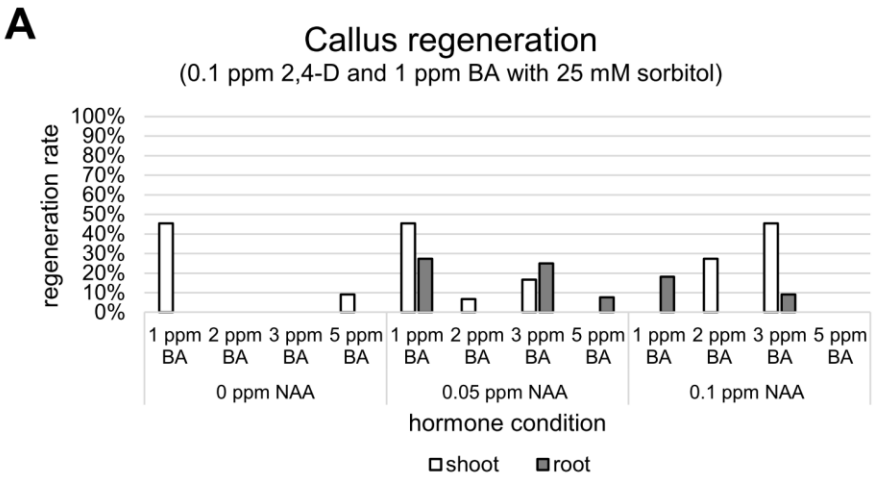

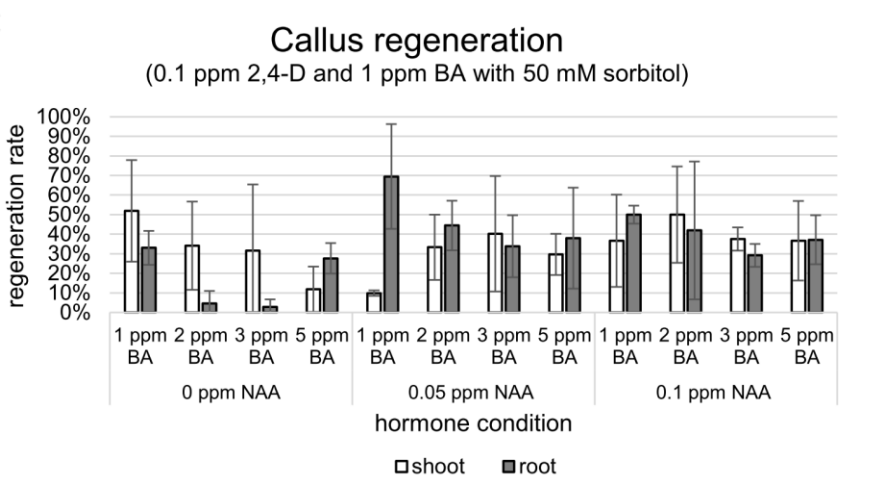

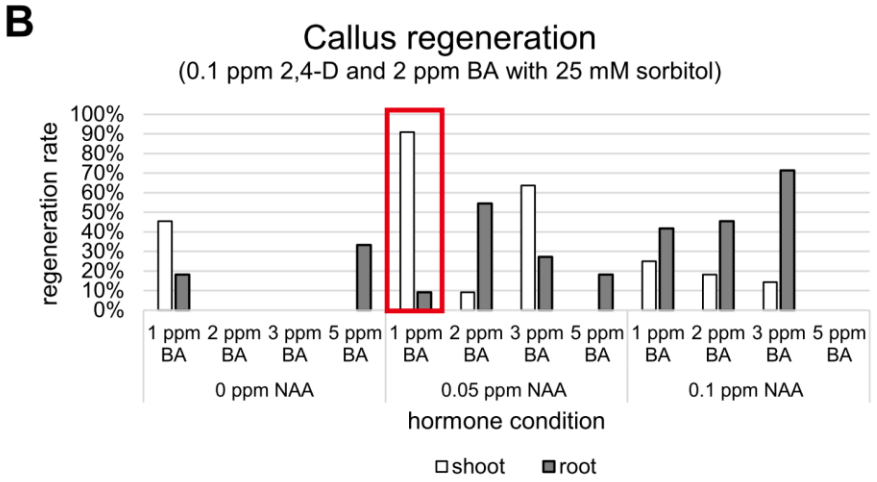

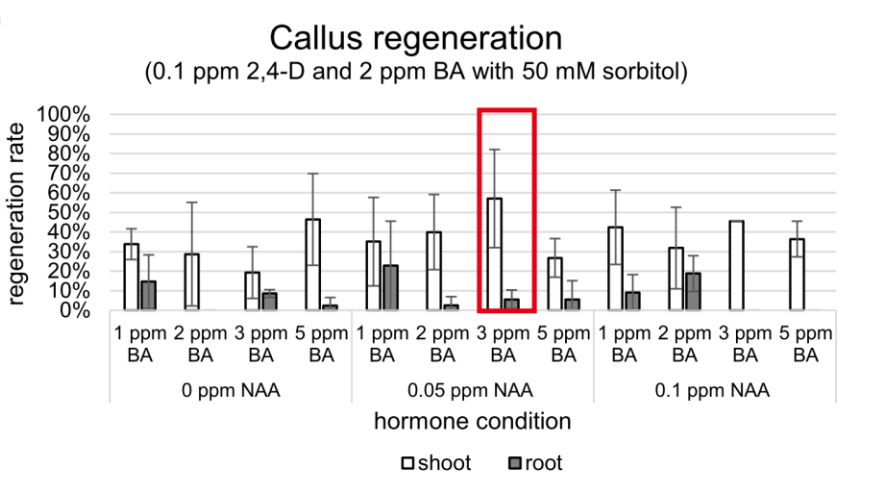

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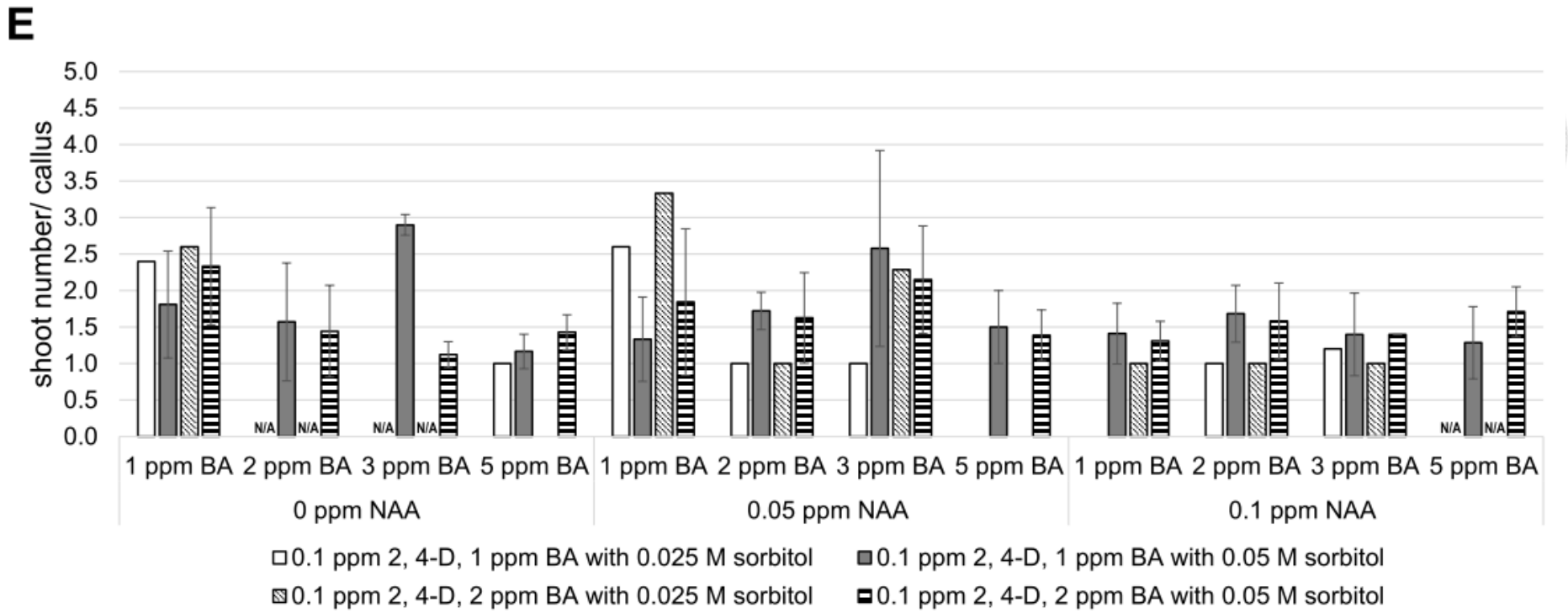

Figure 14 Effect of growth hormone on shoot regeneration from 4 kinds of callus.

The regeneration rate of shoot and root in combinations with different concentrations of BA and NAA on callus inducing from (A) 0.1 ppm 2, 4-D and 1 ppm BA with 25 mM sorbitol. (B), 0.1 ppm 2, 4-D and 2 ppm BA with 25 mM sorbitol. (C), 0.1 ppm 2, 4-D and 1 ppm BA with 50 mM sorbitol. (D), 0.1 ppm 2, 4-D and 2 ppm BA with 50 mM sorbitol. The red frame emphasizes the best hormone condition with high shoot rate (>50%) and low root rate (<10%). E, the shoot number pre callus of each combination of 12 hormone conditions on 4 kinds of callus. The results were recorded after 8 weeks culture. The rate is presented as bar graph in mean ±standard error.

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3.3. Callus transformation

Agrobacterium-mediated transformation and particle bombardment transformation were used to investigate whether callus was a satisfactory substitute in genetic transformation.

The co-culture time was examined first for 1-4 days of co-culture, inoculated with *Agrobacterium* EHA 105 harboring pCambia 2301 of OD_{600} = 0.8 for 20 min. The GUS activity assay showed that no GUS signals in all of conditions (Fig. 15), suggesting that the optimal co-culture time is not found.

In particle bombardment transformation, the physical parameters were tested with combinations of hilum pressure 900 and 1100 psi, and with 6, 9 and 12 target distance. The petal of *Phalaenopsis amabilis* was used as the positive control at hilum pressure 1100 psi with 9 cm target distance (Fig. 16A). Two types of callus (inducing from 0.1 ppm 2, 4-D and 1or 2 ppm BA with 0.05 M sorbitol) was mixed and bombarded. The GUS activity assay showed that no GUS signals at all of the six combinations of hilum pressure and target distance (Fig. 16B), indicating that the parameters of particle bombardment transformation need to be further optimized.

The preliminary results showed the optimal conditions of genetic transformation for callus have not been found, so it is not sure that whether callus is a good substitute to seedling.

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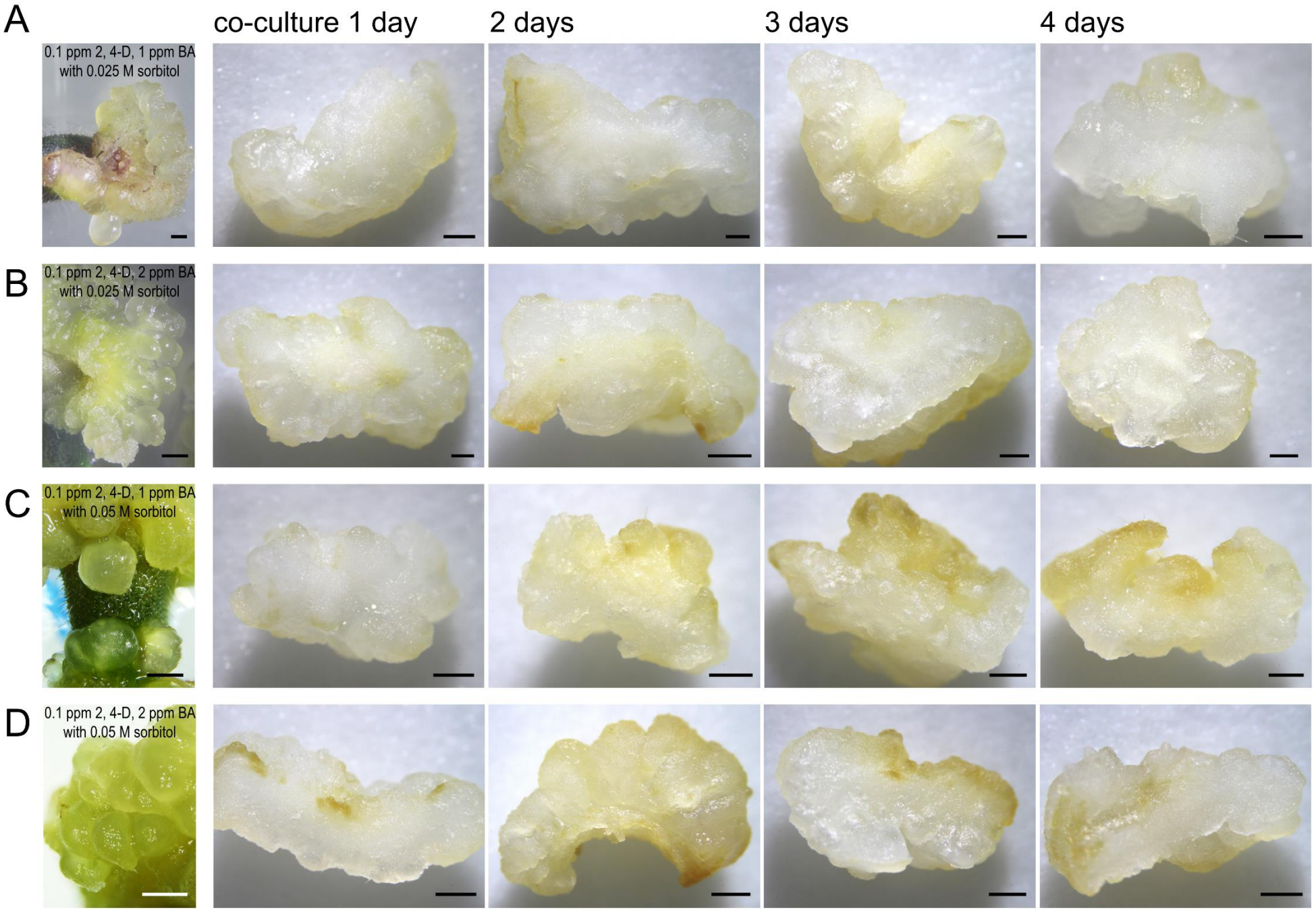

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Figure 15 The test of *Agrobacterium* EHA 105 infection form 4 day of co-culture on 4 kinds of callus.

A, the callus inducing by 0.1 ppm 2, 4-D and 1 ppm BA with 0.025 M sorbitol co-culture with *Agrobacterium* EHA 105 for 1, 2, 3 and 4 days.

B, the callus inducing by 0.1 ppm 2, 4-D and 2 ppm BA with 0.025 M sorbitol co-culture with *Agrobacterium* EHA 105 for 1, 2, 3 and 4 days.

C, the callus inducing by 0.1 ppm 2, 4-D and 1 ppm BA with 0.050 M sorbitol co-culture with *Agrobacterium* EHA 105 for 1, 2, 3 and 4 days.

D, the callus inducing by 0.1 ppm 2, 4-D and 2 ppm BA with 0.050 M sorbitol co-culture with *Agrobacterium* EHA 105 for 1, 2, 3 and 4 days.

The most left figure of each row shows the resource of callus. Scale bar, 1 mm.

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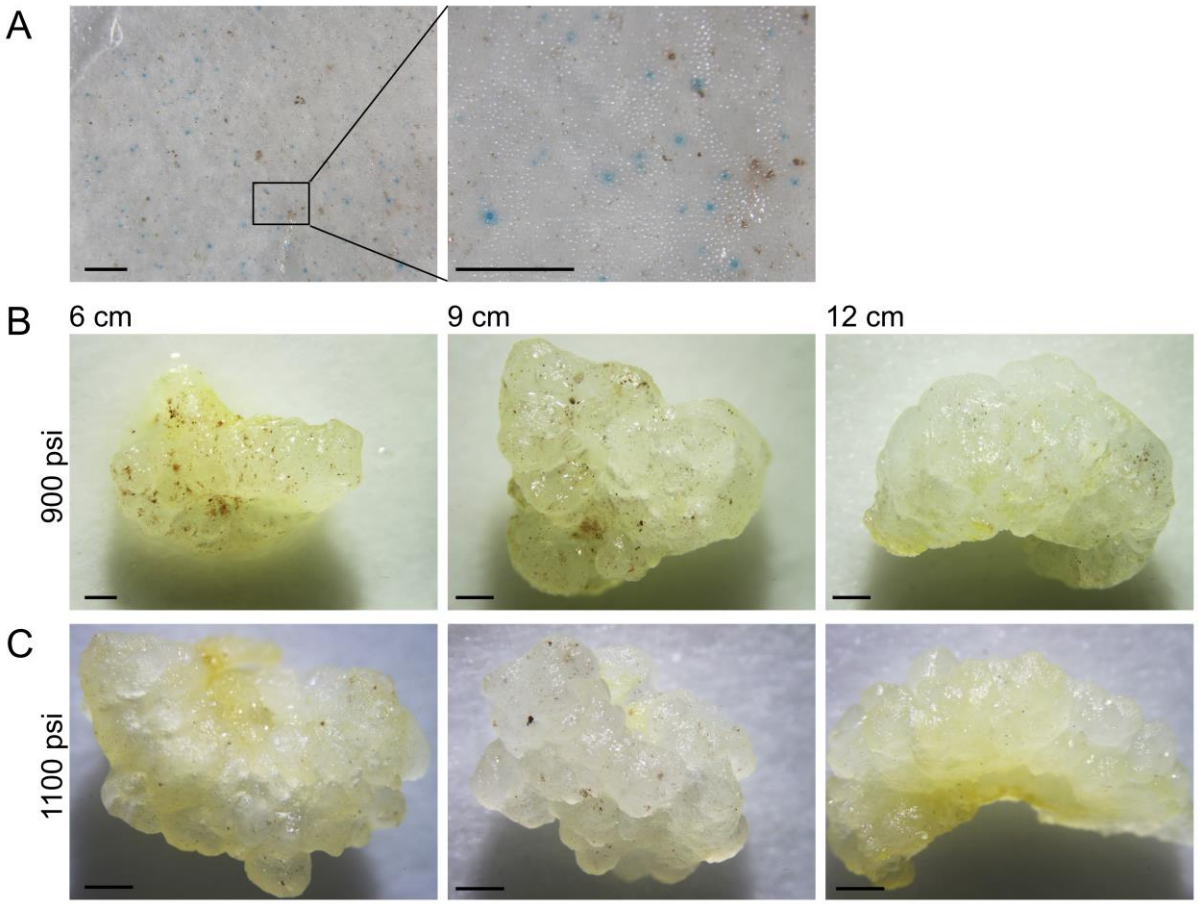

Figure 16 Particle bombardment conditions test by GUS report assay in 4 kinds of callus.

A, control group, *Phalaenopsis amabilis*. Petal was bombarded with hilum pressure 1100 psi at 9 cm target distance B, experimental group, callus is bombarded with hilum pressure hilum 900 psi at 6, 9 and 12 cm target distance, repeated for twice. C, experimental group, callus is bombarded with hilum pressure hilum 1100 psi at 6, 9 and 12 cm target distance. Scale bar, 1 mm.

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Discussion

1. The conditions of *Agrobacterium* infection in *S. speciosa*

1.1. The effects of photoperiod and temperature during co-culture on transformation

In *Agrobacterium*-mediated transformation, continuously dark culture with low temperature (about 20-25 ºC) is commonly used during co-culture duration because they influenced the T-DNA delivery during co-culture duration. Previous study presented that the lower temperature (22ºC) could increase the transient GUS activity in *Phaseolus acutifolium* callus and *Nicotiana tabacum* leaves (Dillen et al., 1997). Nevertheless, in some case, 25-28 ºC were used during co-culture in other members of Gesneriaceae (Geier and Sangwan, 1996a; Kushikawa et al., 2001; Toth et al., 2006). In our case, the transient transformation rate were low with either lower temperature (22 ºC) or higher temperature (27 ºC) under 3 days of co-culture (Appendix Fig. 2). This suggested that temperature might not participate in the process of T-DNA delivery. The effect of light on successful genetic transformation is less studied. In *Phaseolus acutifolius*, it shows that transient and stable transformation rate is enhanced by long day light treatment (both photoperiod of 16 hr light/ 8 hr darkness and continuous light) (Zambre et al., 2003). However, the effect of light shows no difference in garlic (Kondo,

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2000). The photoperiod of 16 hr light/ 8 hr darkness and continuous darkness were tested to examine light effect on transformation rate of *S. speciosa* during the 3 days of co-culture. The results showed that the transient transformation rate was not improved with the light treatment (Appendix Fig. 2).

It could be argued that the effect of light and temperature were investigated under 3 days of co-culture only, which the treatment time might not be enough. These two factors can be further examined with 5 days or more of co-culture time to ascertain their effect in further experiments.

1.2. The extended co-culture time needed for *S. speciosa* transformation.

An appropriate co-culture time have to be optimized for efficient transformation. During co-culture duration, T-DNA was transferred into plant cell, insufficient co-culture time might not be enough for T-DNA insertion and the prolonged co-culture time might make damages of explants. In general, the best transformation rate can be obtained with 2-3 days of co-culture, which is also applied in genetic transformation of *S. speciosa* in previous studies (Li et al., 2013b; Zhang et al., 2008), however, the transient transformation rate is low (almost 0 %) in our case (Fig. 2). In contrast, some studies demonstrate that longer co-culture (4-7 days) time can indeed improve the transformation rate (Barik et al., 2005; Mondal et al., 2001; Suzuki et al., 2001). Our

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results showed that the transient transformation rate is higher (54.6%) under 5 days of co-culture, in which bacteria colony could to be clearly observed around leaf discs (Fig. 2). Although the transformation rate has been reported that it would dramatically decreased due to the overgrowth *Agrobacterium* with more than 5 days of co-culture (Cervera et al., 1998), the overgrowing *Agrobacterium* led to more efficient transformation in our case. This might be the reason that extended co-culture time is needed for genetic transformation in *S. speciosa*.

1.3. The genotype-dependent susceptibility to *Agrobacterium* EHA 105

It has been reported that the genotype of focal plant influences the efficiency of transformation (Cheng et al., 2004; Opabode, 2006), but the underlying mechanism is not clearly understood. In this study, another genotype (different cultivar) with bilateral symmetry flower, *S. speciosa* 'Espirito Santo' (Fig. 1A), was also attempted to establish the transformation system. However, the GUS signals were detected in 'Avanti' (Fig. 2) but not in 'Espirito Santo' after co-culturing for 3 or 5 days without pre-culture treatment with *Agrobacterium* EHA 105 (data not shown).

On the other hand, the genotypic difficulty of transformation could be overcome by extended pre-culture period with additional plant growth hormone (Chateau et al., 2000). The pre-culture treatment helps to activate cell division and dedifferentiation.

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The new and thin cell wall produced during pre-culture could lead to easier infection by *Agrobacterium*. Therefore, the additional pre-culture treatment with plant growth hormone could be tested in 'Espirito Santo' to improve the transformation rate in the future.

2. Factors that cause long regeneration time in transformation of *S. speciosa*

Dating the time from *Agrobacterium* infection to shoot regeneration took about 3-7 months long (Appendix Fig. 11). This suggests that the stress of cefotaxime and kanamycin is the main factor responsible for that.

2.1. The effect of cefotaxime on callus formation and shoot regeneration

Cefotaxime is used for eliminating *Agrobacterium* regrowth after co-culture, and it might influence the callus formation and shoot regeneration. Cefotaxime has been reported that it improved the embryogenic callus formation, callus induction and growth in wheat, barley and sweet sorghum (Mathias, 1987; Mathias and Boyd, 1986; Rao et al., 1995). It also enhanced the morphogenesis of embryogenic callus in maize (Danilova and Dolgikh, 2004). The formation of embryogenic callus was defined to increase the shoot regeneration due to the active-growing trait. In contrast, the callus formation is not affected by cefotaxime but the shoot regeneration decreased by treating cefotaxime in tomato (Ling et al., 1998). These demonstrate that cefotaxime

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participates in the pathway of callus formation and shoot regeneration, but the effect depends from species to species. In my case, the leaf discs cultured on regeneration medium with additional 200 ppm cefotaxime for 30 days, the callus formatted and no shoots regenerated (Appendix Fig. 12B). Comparing to control group without cefotaxime, the shoots formatted directly at the edge without callus phase after 30 days of culture (Appendix Fig. 12A). This indicated that cefotaxime induced callus formation and delayed shoot regeneration in *S. speciosa*.

The delayed shoot regeneration might extend the regeneration period and lead to the longer regeneration time of transformed cells.

2.2. Dosage effect of kanamycin on transformation success

Kanamycin, an antibiotic widely used to select successfully transformed cells, however, can strongly inhibit the regeneration at low dose (Fiola et al., 1990; Zhang et al., 2001). Consequently, the minimal dosage of kanamycin has to be determined for efficient regeneration in selection medium. After one month of culture, the untransformed explants was turned brown and no shoot formation on selection medium with 75 ppm kanamycin but no cefotaxime (by Kuo, Wen-His, personal communication). To ensure complete elimination of non-transformed cells, the concentration of kanamycin was added to 100 ppm in this study and *Agrobacterium*-infected explants were sub-cultured

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to fresh selection medium every week. Although at this high concentration of kanamycin, many false positive transgenic plants still appeared. This is a similar situation as in sweet orange, it suggested that kanamycin could not fully inhibit the regeneration of non-transformed cells (Peña et al., 1995). Due to many false positive transformants found in *S. speciosa*, continuously high concentration of kanamycin to eliminate non-transformed is inevitable; but this might inhibit regeneration or delay shoot regeneration time.

3. Factors contribute to the unstable transformation system

In spite of having the high transient transformation rate (Fig. 3), the genetic transformation system in *S. speciosa* is still unstable. The genetic transformation under 3 days of pre-culture was repeated 5 times, however, the transgenic plants were obtained only in one repeat. The transgenic plants under 4 days of pre-culture which repeated 4 times were obtained only in one repeat. These indicated our transformation system is unstable. The unstable transformation efficiency might be due to mainly two factors, the *Agrobaterium* inoculation method and the cell type of transformed cells.

3.1. Unstable pressurization process by syringe

In *S. speciosa*, in order to infect deeper vascular parenchyma cells where the regenerated shoots normally initiated (Kuo, 2014), I used pressure generated by

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syringes to push *Agrobacterium* into inner tissue cells. However, it is difficult to constantly control the pressure that the syringe provided and quantify the damage of explant (seedling) during this pressurization process. In addition, I realized that the transient transformation rate is correlated with the frequency of pressurization process. Nevertheless, the higher transient transformation rate is usually accompanied by more damage of explant, and the regeneration capacity might be lost. Therefore, quantifying and reducing the damage are important. Recently, the inoculation methods of sonication or vacuum infiltration have been used to enhance the transformation rate because of the micro wounds producing and deeper tissue transformation (de Oliveira et al., 2009; Subramanyam et al., 2011). These two methods could replace the syringe method for reducing the damage to explants in future tests.

3.2. The different cell type between transformed cell and regenerated cell

It has been reported that shoot regeneration is highly cell-specific (Geier and Sangwan, 1996b; Lowe et al., 1993). The transformed cells (indicated by GUS signals) of seedlings I found are mainly distributed in epidermal layer or sometimes the mesophyll cell (Fig. 7); however, these tissues were not the normal shoot regeneration sites, which should be vascular cells in inner sides of the leaves, as reported in *S. speciosa* 'Avanti' from Kuo (2014). This creates difficulties in regenerating shoots on exact positions of

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transformed cells of *S. speciosa*. This is less problematic in others Gesneriaceae species because their regenerated shoots can be initiated from *Agrobacterium* infected epidermal cells, such as in *Chirita longgangensis*, *Lysionotus serratus*, *Titanotrichum oldhamii* and African violet (Li et al., 2013a; Mithila et al., 2003; Takagi et al., 2011; Tang et al., 2007).

In addition, the indirect shoot regeneration occurred from callus which is initiated from mesophyll cells in *S. speciosa* (Scaramuzzi et al., 1999). One alternative solution might be to transfer the transformed explants on the callus induction medium we optimized. The transformed mesophyll cells might be induced to embryogenic callus and further regenerated. This shall help the transformation success as *Agrobacterium* infection might not be too difficult to penetrate into mesophyll layer.

4. Callus as explants to facilitate transformation

4.1. Optimization of other factors on callus transformation with *Agrobacterium*

Although the failed preliminary results were showed in callus transient transformation with *Agrobacterium* and 1 to 4 days of co-culture (Fig. 14), the characteristic of rapid cell division was believed that callus is a good material for genetic transformation. In our observation, the callus was surrounded by visible *Agrobacterium* after 1 day of co-culture (Appendix Fig. 13), and the callus was covered completed by *Agrobacterium*

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after 2 days of co-culture, indicating the longer co-culture time might make severe damages on callus and that was not suitable. However, the shortest co-culture time (1 day) still has no successful transformation, it suggested that co-culture time might not be the key factor affecting transformation rate. Previous studies suggested that the appropriate time of pre-culture, low *Agrobacterium* cell density (OD_{600} = 0.5), low temperature (19 ºC) during co-culture, short time (2 days) of co-culture, suitable concentration of acetosyringone and desiccation after *Agrobacterium* infection can improve the competence of callus for *Agrobacterium* (Cheng et al., 2003; Jin et al., 2005; Li et al., 2003). Furthermore, whether the factors affect the transformation efficiency or not must be further investigated.

4.2. Particle bombardment transformation

An optimized protocol of particle bombardment transformation could be simpler in operation than *Agrobacterium*-mediated transformation. However, my results indicated that the hilum pressure 900 psi and 1100 psi with 6, 9 and 12 cm target distance were inappropriate. This might be due to the high hilum pressure which increased callus damages. Therefore, the parameters might be regulated first to lower hilum pressure at different target distance.

To further optimize the parameters of particle bombardment, the osmotic

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treatment which provides the plasmolysis of cells and low amount of gold particles (30 μg/ shot) could be tested. It was demonstrated that these two factors can enhance the efficiency of transformation by decrease the damage of tissue (Altpeter et al., 1996; Vain et al., 1993). In addition, small size gold particle (0.6 μm) also helps to reduce the damage when it penetrated the cell of callus (Frame et al., 2000; Tee and Maziah, 2005). On the other hand, increasing the frequency of bombardment (for twice) also reported to improve the efficiency of transformation (Bower and Birch, 1992), but the effect is depended on tissue type and species. We have been applyied the small gold particle (0.6 μm) in our optimizing experiment, however, the effect was not detected due to the inappropriate conditions. Therefore, the osmotic treatment, amount of golden particles (we used 600 μg/ shot) and frequency of bombardment can be examined afer finding the optimizd hilum pressure and target distance.

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Conclusion and future prospects

In this study, the genetic transformation protocol of *Agrobacterium*-mediated transformation was successfully optimized. It was found that the 5 days of co-culture is the most important factor which influenced the efficiency of *Agrobacterium* infection. In addition, the 3 weeks old seedlings demonstrated the best susceptibility to *Agrobacterium* EHA 105. With above conditions, the additional pre-culture treatment improved the efficiency of *Agrobacterium* infection and enhanced the transformation rate up to 2.1 %. On the other hand, in particle bombardment transformation, the 900 psi hilum press at 6 and 9 cm target distance showed the transient transformation rate 58.1 % and 21.6 %, respectively.

For another potential substitute, callus, it was observed that leaf discs cultured on the MS medium supplemented with 0.1 ppm 2, 4-D, 1 or 2 ppm BA and 0.025 or 0.05 M sorbitol gave the most embryogenic callus regeneration. Transferring the embryogenic callus (inducing from 0.1 ppm 2, 4-D and 2 ppm BA with 50 mM sorbitol) to MS medium supplied with 0.05 ppm NAA and 3 ppm BA presented the highest shoot regeneration rate (57 %). In genetic transformation of callus, the suitable conditions were not found in *Agrobacterium*-medium transformation or particle bombardment transformation.

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The future work in this project may include: 1) Check the effect of *Agrobacterium* cell density and infection time to the transformation rate by GUS activity assay and RT-PCR (partial of this work has been done in Appendix Fig. 4). 2) Identify the GUS inheritance or not in progenies of transgenic plants. 3) Modify the *Agrobacterium* inoculation method for less damages of transformed explants and stable transformation system. 4) Optimize the transformation protocol for callus. 5) Study the function of the symmetry-related genes in *S. speciosa* by genetic transformation system.

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Appendixes

Appendix Table 1 Primer list in this study

Primer name	Sequence (5'-3')	Tm (ºC)	Note
NPTFo	TCA GAA GAA CTC GTC AAG AA	47.7	F primer for *NPTII* gene
NPTBaR	AAC AAG ATG GAT TGC ACG CA	49.7	R primer for *NPTII* gene
SsCYC_118_F	CAC CAT GAC ATG CTT TCC AGC	54.4	F primer for *SsCYC* gene PCR
SsCYC_760_R	AGT GGA GGG TAG GCT GAA TTT G	54.8	R primer for *SsCYC* gene PCR
35S-F'_11554	AAT CCC ACT ATC CTT CGC AAG AC	55.3	F primer for *GUS* gene RT-PCR
GUS-R'_531	CCA TCA CTT CCT GAT TAT TGA CC	43	R primer for *GUS* gene RT-PCR
sCDS4-F_10923	GTT ACC CAA CTT AAT CGC CTT G	53	F primer for checking pCambia 2301 in *Agrobacterium*
sGUS-F_intron_44	TTC TTG GTT AGG ACC CTT TTC TC	53.5	F primer for checking pCambia 2301 in *Agrobacterium*
sGUS-F_799	AAG ACT GTA ACC ACG CGT CTG	54.4	F primer for checking pCambia 2301 in *Agrobacterium*
sGUS-F_1535	GAT CAC CTG CGT CAA TGT AAT G	53	F primer for checking pCambia 2301 in *Agrobacterium*
sGUS-R_2120	AAG ACC GGC AAC AGG ATT CA	51.8	R primer for checking pCambia 2301 in *Agrobacterium*

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GUS_F_1379	CTT ACA GGC GAT TAA AGA GCT GA	53.5	F primer for Southern blotting probe
GUS_R_1929	TGA AGA TCC CTT TCT TGT TAC CG	53.5	R primer for Southern blotting probe
SsACT F	TCC AGC AGC TTC CAT TCC TAT C	54.8	F primer for *SsActin* gene RT-PCR
SsACT R	CCA TAA ATT GCG TGT TGC TCC TGA G	57.7	R primer for *SsActin* gene RT-PCR

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Appendix Table 2 The transient transformation rate and *Agrobacterium*-mediated transformation conditions in seedlings of *S. speciosa*.

Agrobacterium strain	EHA105						GV3101							EHA105				GV3101			
OD_{600}	0.7-0.8																				
co-culture time	**3 days**													**5 days**							
photoperiod	22 ℃ 16 hr light			22 ℃ dark			27 ℃ 16 hr light	22 ℃ 16 hr light			22 ℃ dark			27 ℃ 16 hr light	27 ℃ 16 hr light							
species	Nb	Ss_2w		Nb	Ss_2w		Ss_2w	Nb	Ss_2w		Nb	Ss_2w		Ss_2w	Nb	**Ss_2w**	**Ss_3w**	**Ss_4w**	Nb	Ss_2w	Ss_3w	Ss_4w
treat	press	press	suc	press	press	suc	press	press	press	suc	press	press	suc	press	press							
number	10	25	25	30	115	25	20	10	25	25	30	115	25	20	25	11	55	12	15	10	60	10
transformation rate (%)	0	0	0	66.7	1.1	4	0	0	0	0	66.7	0	0	0	90	**54.6**	**76.4**	**83.3**	100	0	1.7	0

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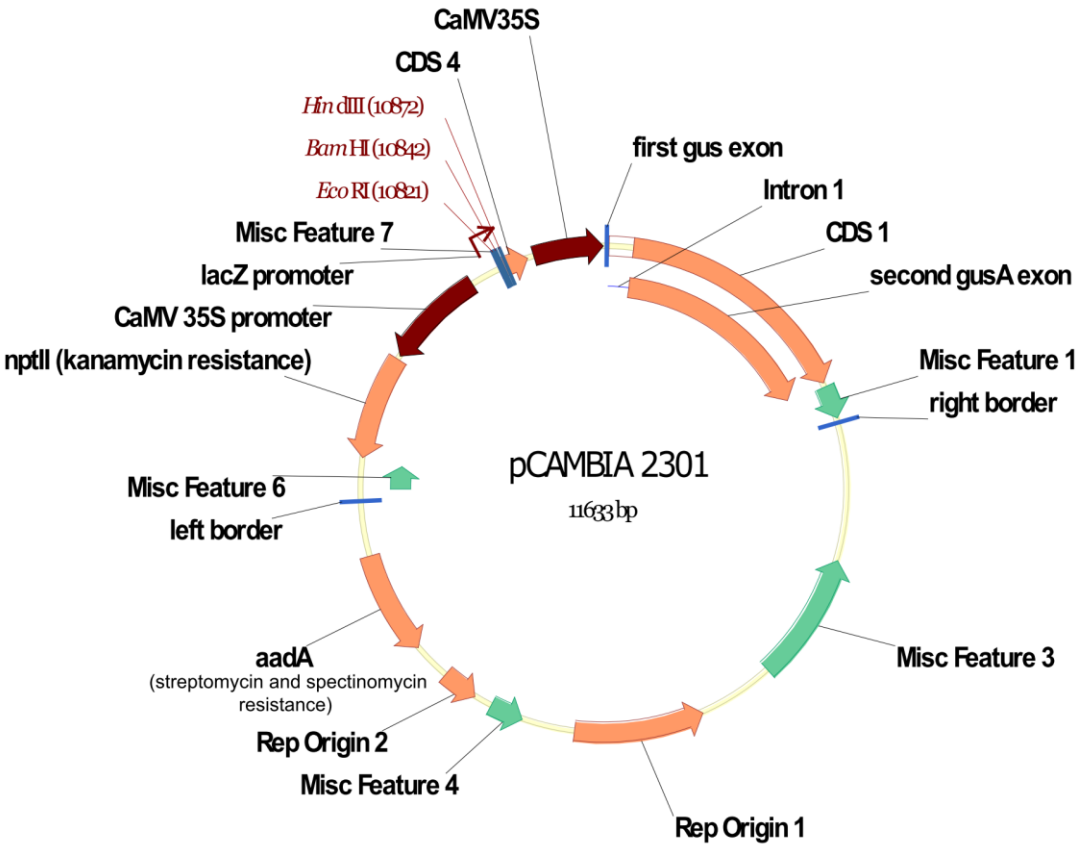

Appendix Figure 1 The vector pCambia2301 with GUS report gene used in *Agrobacterium*-mediated transformation.

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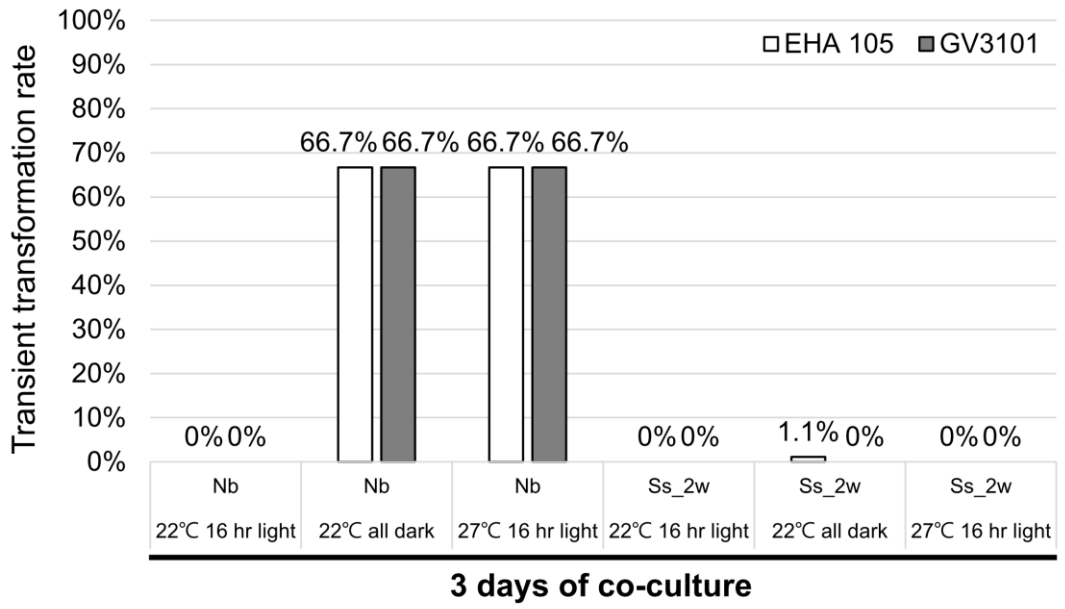

Appendix Figure 2 The transient transformation rate of *S. speciosa* inoculated by two *Agrobacterium* strains in different photoperiods (long-day and all dark) and temperatures (22 ºC and 27 ºC).

The Seedlings were inoculated by *Agrobacterium* EHA 105 and GV3101 and co-cultured for 3days. Nb, *Nicotiana Benthamiana*. Ss_2w, *S. speciosa* 2 weeks old seedling.

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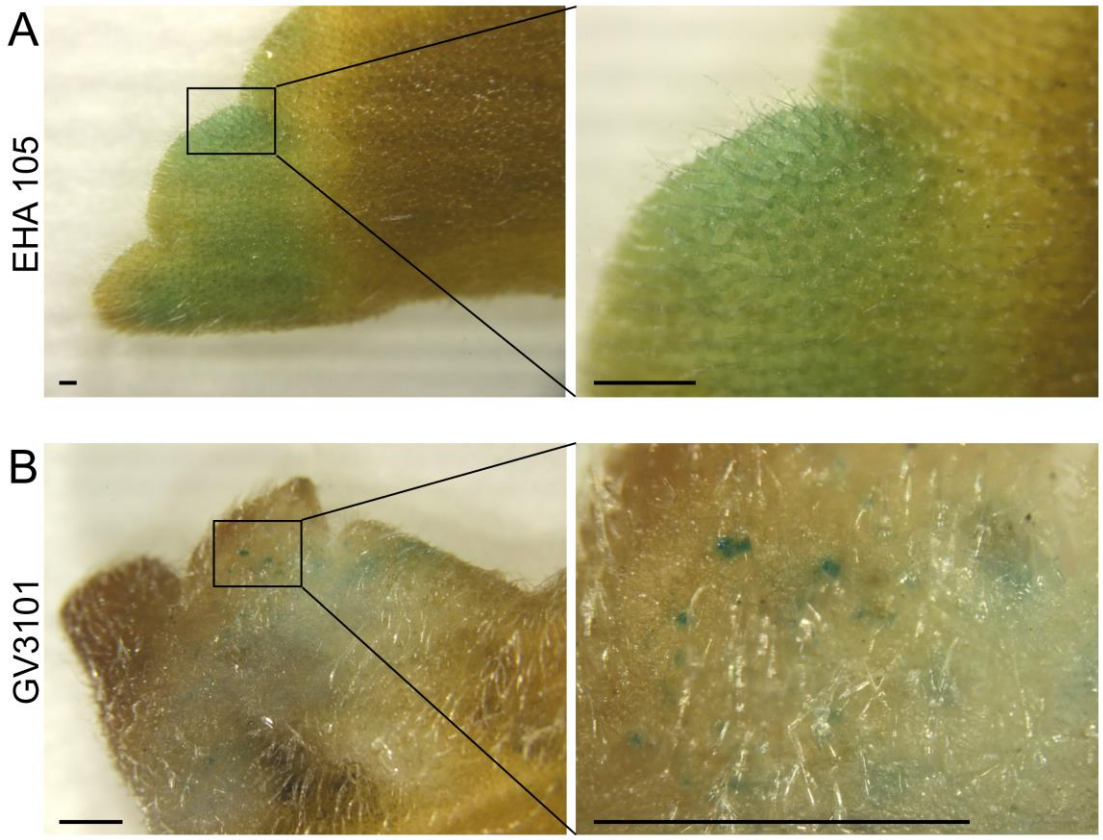

Appendix Figure 3 The GUS signals in mature leaf of *S. speciosa*.

A, *Agrobacterium* EHA 105 inoculated the mature leaf and co-cultured for 5 days. The GUS signals shows the transient transformation rate is 13.0%. B, *Agrobacterium* GV3101 inoculated the mature leaf and co-cultured for 5 days. The GUS signals shows the transient transformation rate is 10.0%. Scale bar, 0.5 mm.

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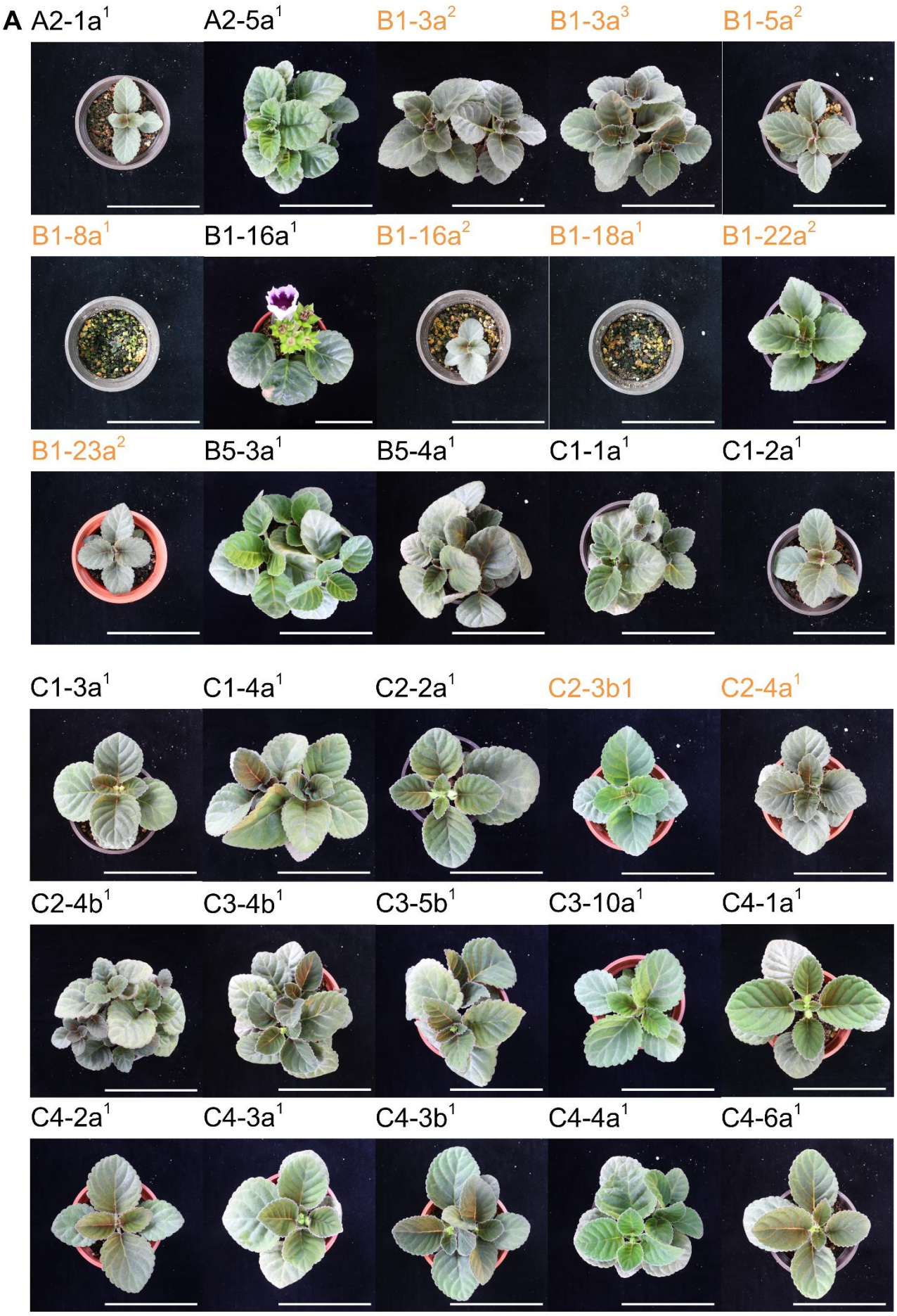
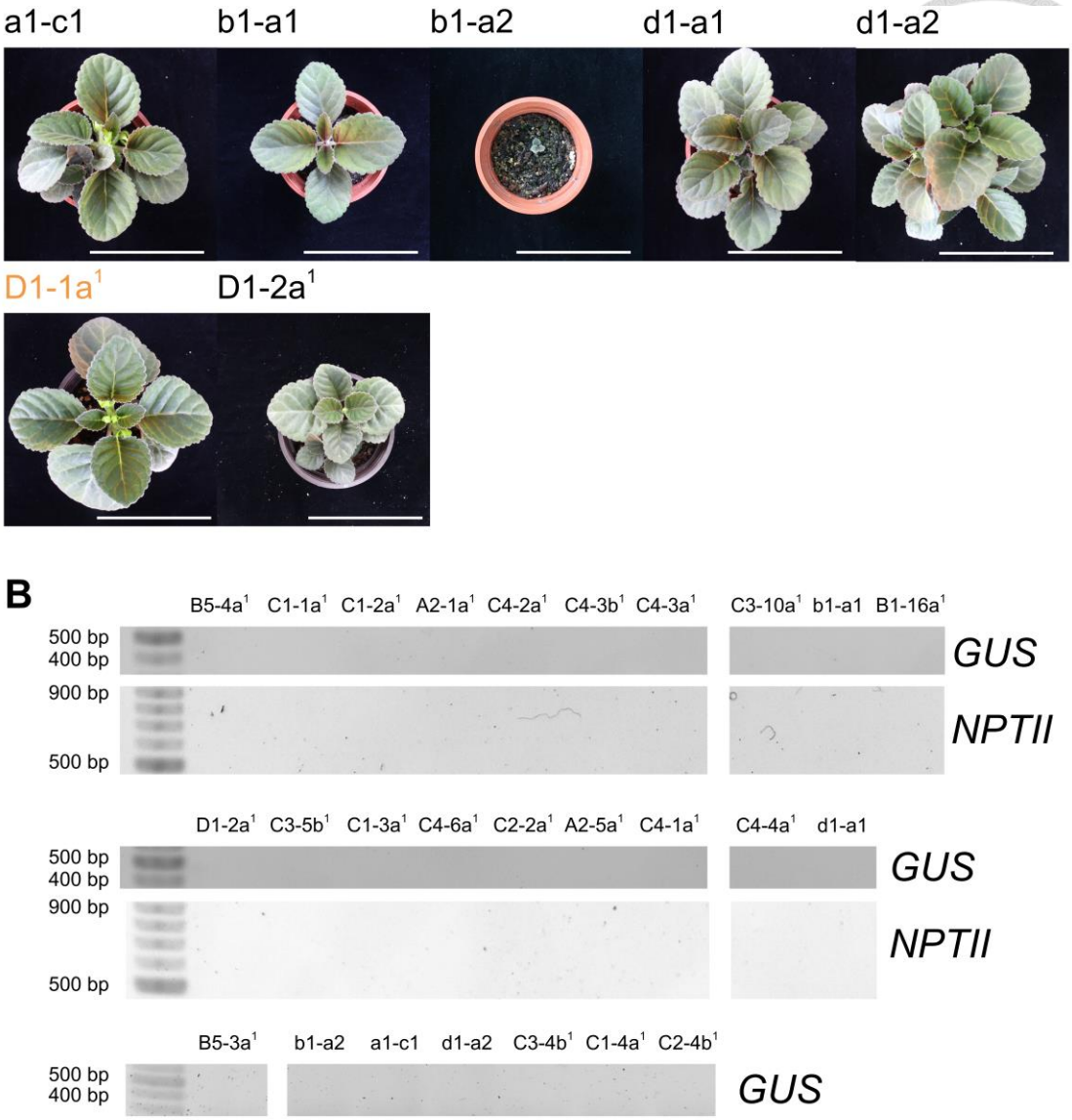


doi:10.6342/NTU201602983



Appendix Figure 4 The morphology of false-positive transgenic plants and expression of foreign transgenes.

A, the growth status of putative transgenic plants which have no GUS signals. The ID is showed at the top of putative transgenic plants, black indicates the RT-PCR has finished, the orange indicates not yet. B, the RT-PCR results of *GUS* and *NPTII* in these putative transgenic plants. Scale bar, 9 cm.

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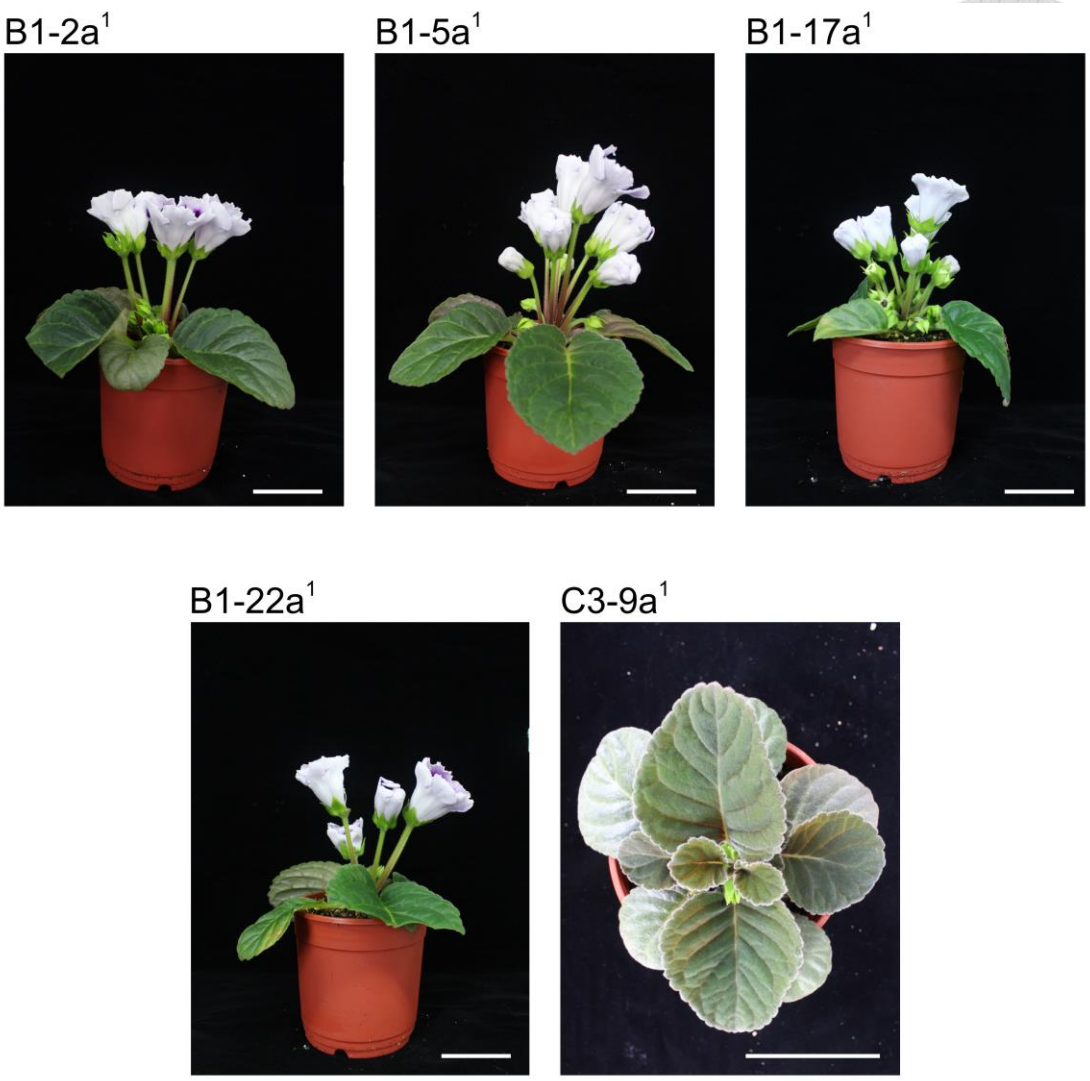

Appendix Figure 5 The morphology of candidate transgenic plants.

Scale bar, 6 cm.

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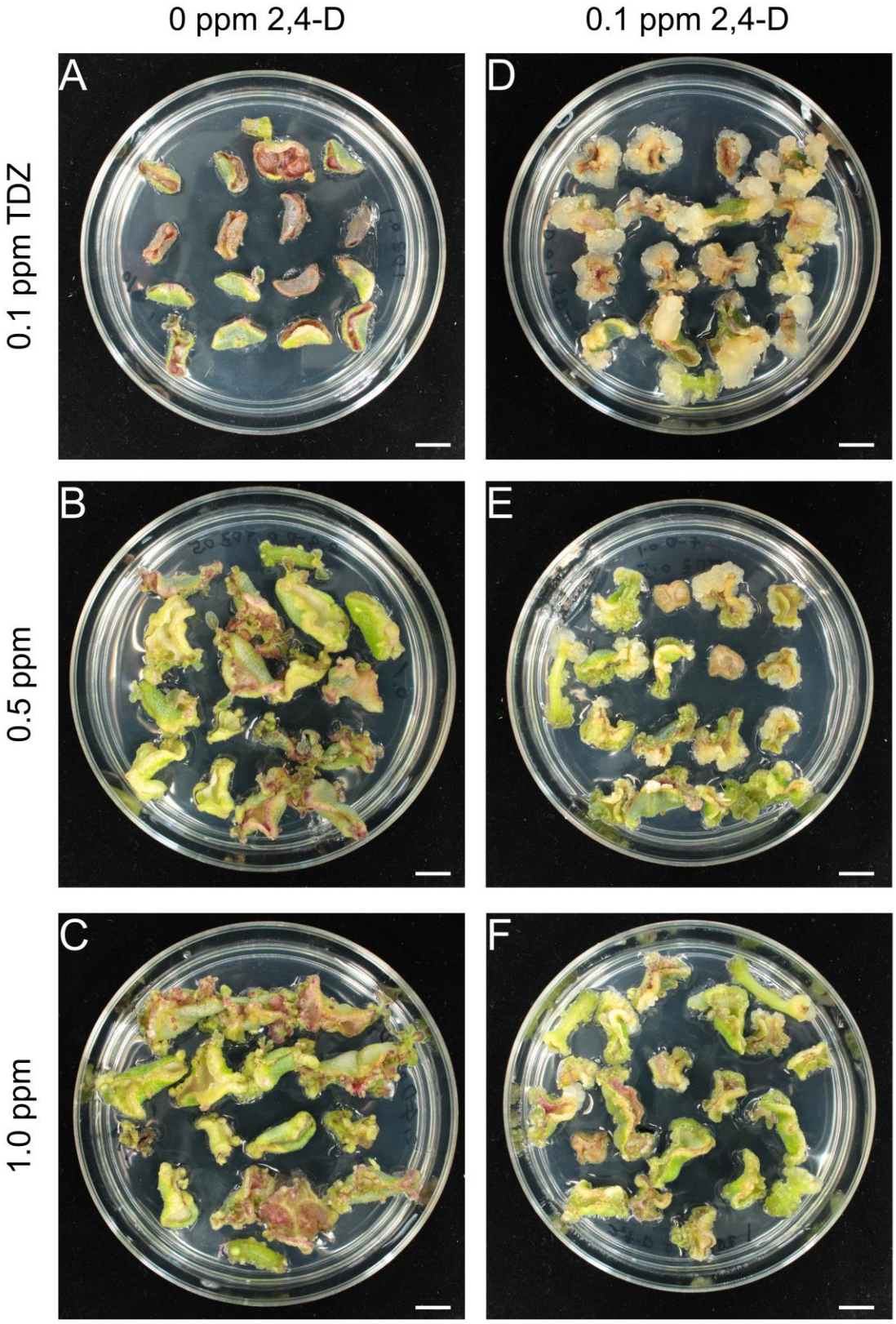

Appendix Figure 6 The morphology of callus under auxin (2, 4-D) and cytokinin (TDZ) treatment.

The results were recorded after 4 weeks culture. Scale bar, 1 cm.

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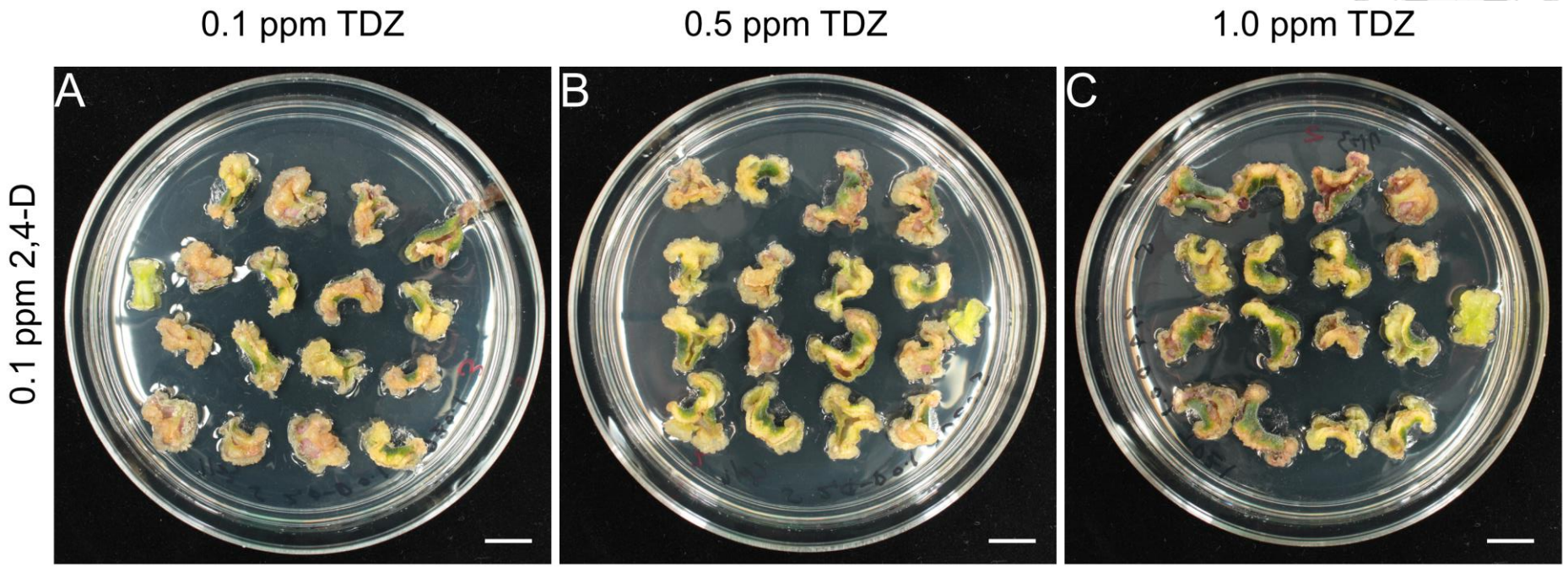
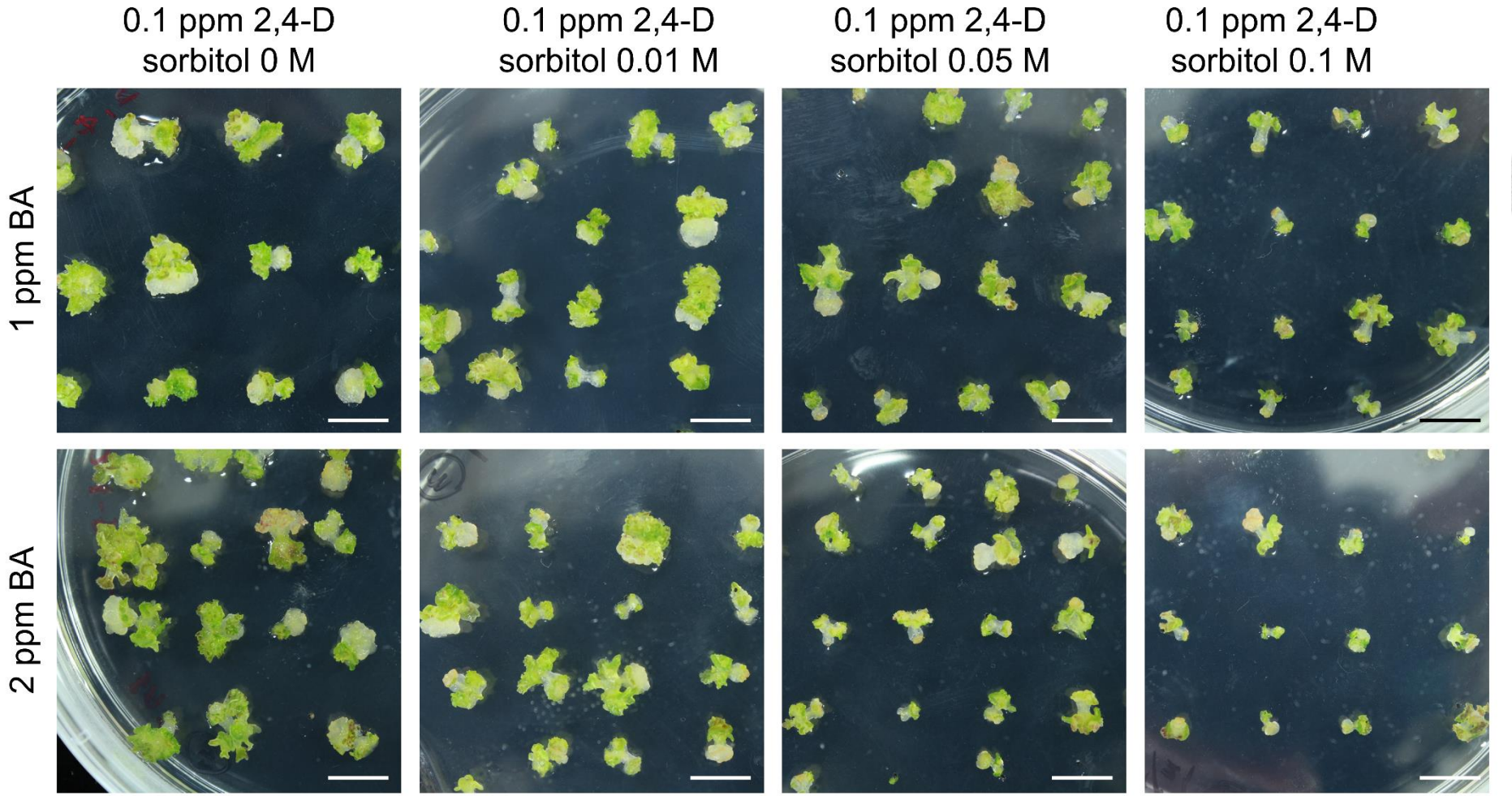


Appendix Figure 7 The morphology of callus induction with 0.1 M sorbitol form leaf explant.

The callus was cultured on MS medium supplemented 0.1 ppm 2, 4-D and 0.1, 0.5, or 1.0 ppm TDZ with 0.1 M sorbitol for 4 weeks. Scale bar, 1 cm

doi:10.6342/NTU201602983



Appendix Figure 8 The morphology of callus induction with different concentrations of sorbitol form seed.

The callus was cultured on MS medium supplemented 0.1 ppm 2, 4-D, 1 and 2 ppm BA with 0, 0.01, 0.05 and 0.1 M sorbitol for 4 weeks. Scale bar, 1 cm.

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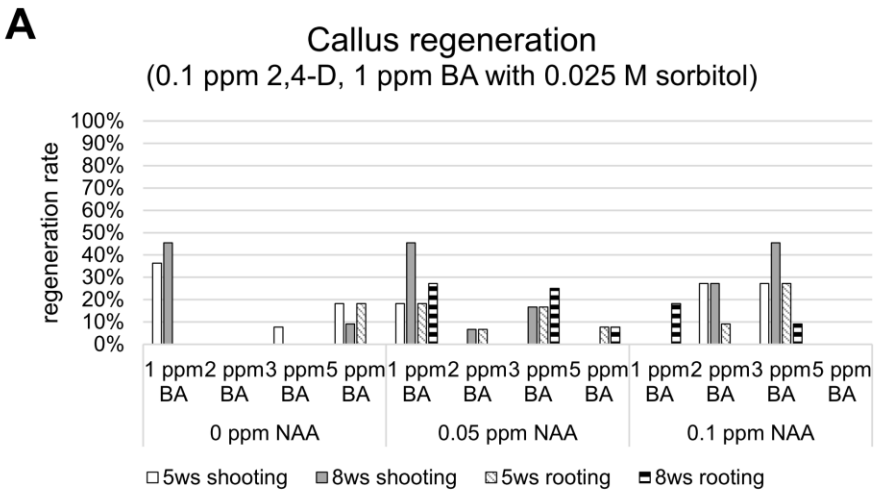

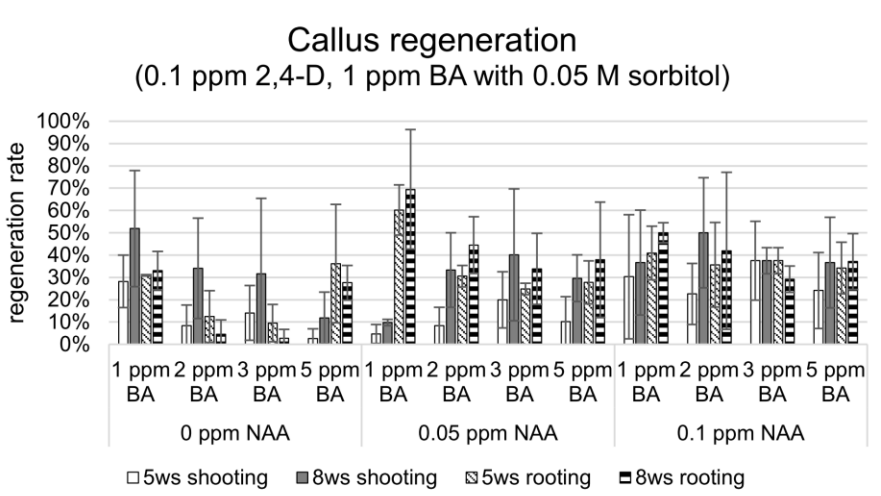

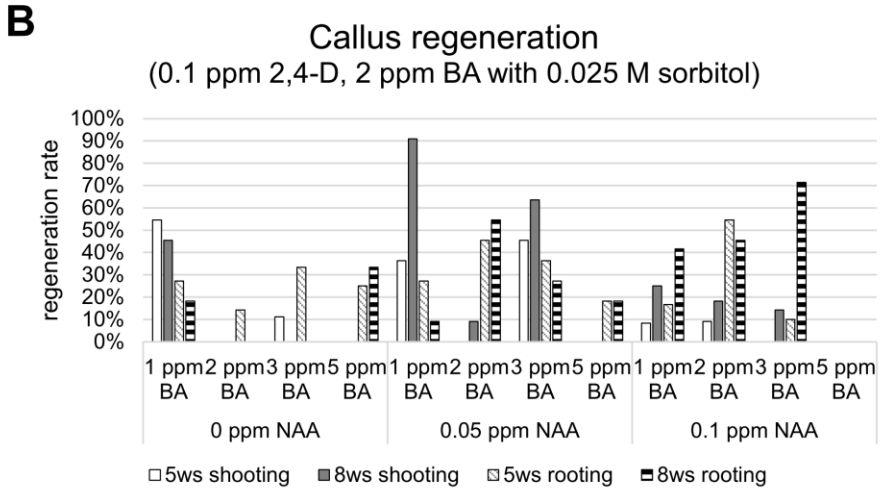

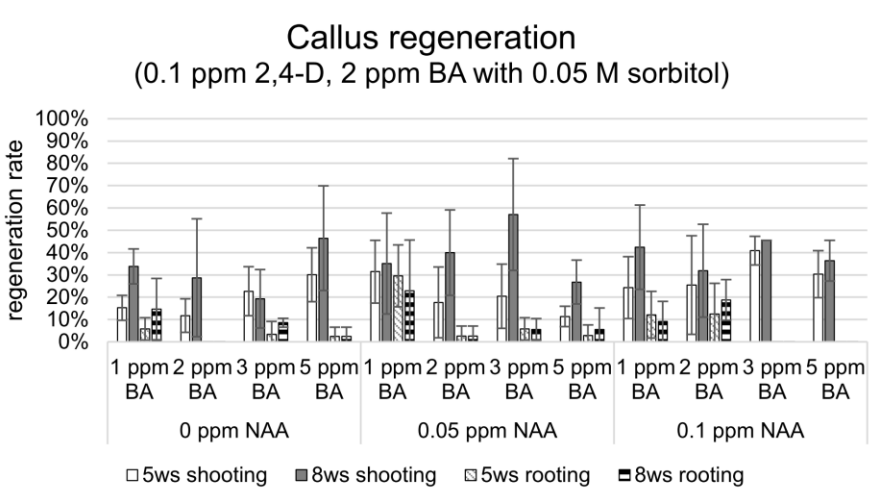

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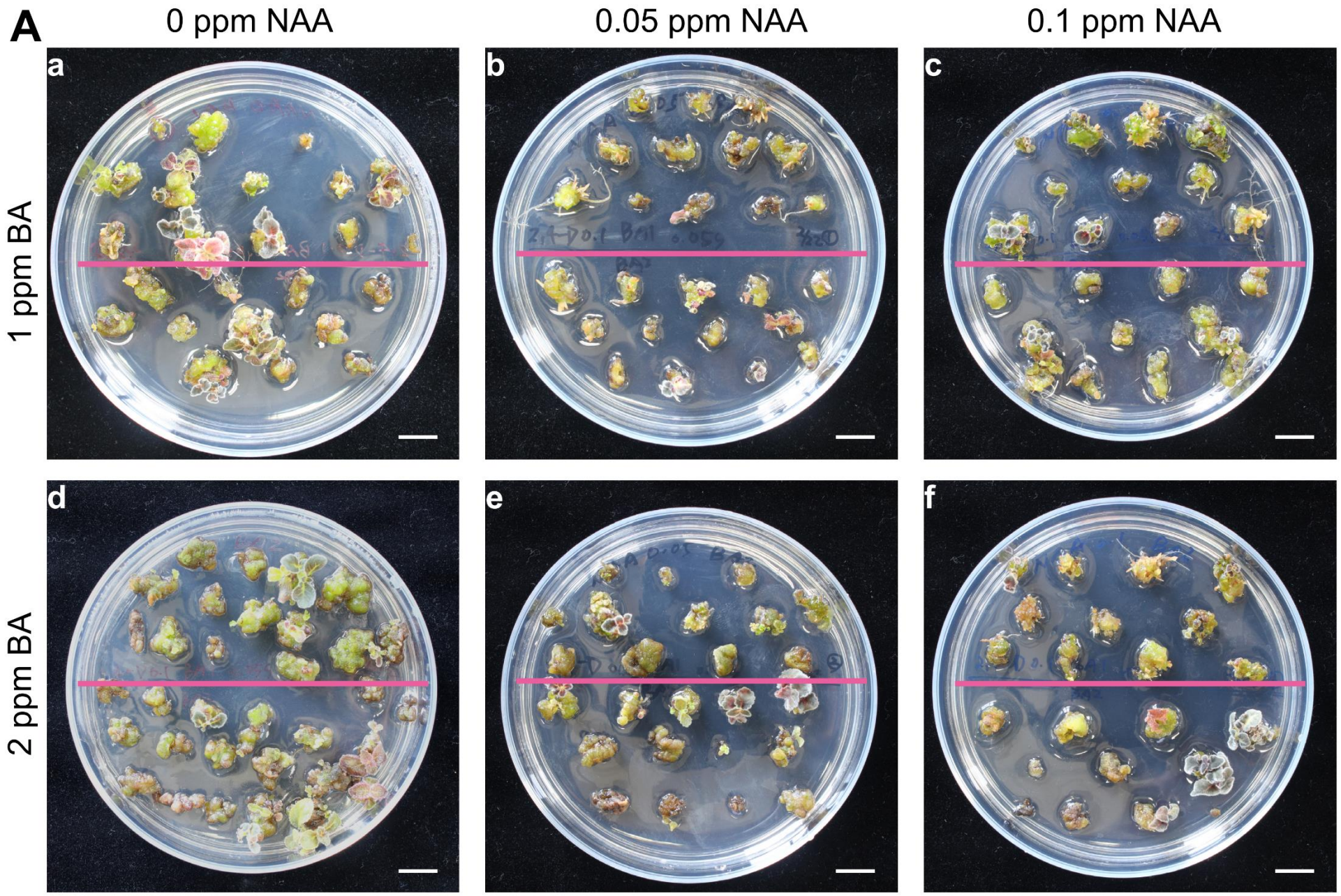
Appendix Figure 9 Effect of PGR on 4 kinds of callus after 5 and 8 weeks culture.

A, the regeneration of callus inducing from 0.1 ppm 2, 4-D and 1 ppm BA with 25 mM sorbitol. B, the regeneration of callus inducing from 0.1 ppm 2, 4-D and 2 ppm BA with 25 mM sorbitol. C, the regeneration of callus inducing from 0.1 ppm 2, 4-D and 1 ppm BA with 50 mM sorbitol. D, the regeneration of callus inducing from 0.1 ppm 2, 4-D and 2 ppm BA with 50 mM sorbitol. The data is presented as mean ± stand deviation.

doi:10.6342/NTU201602983



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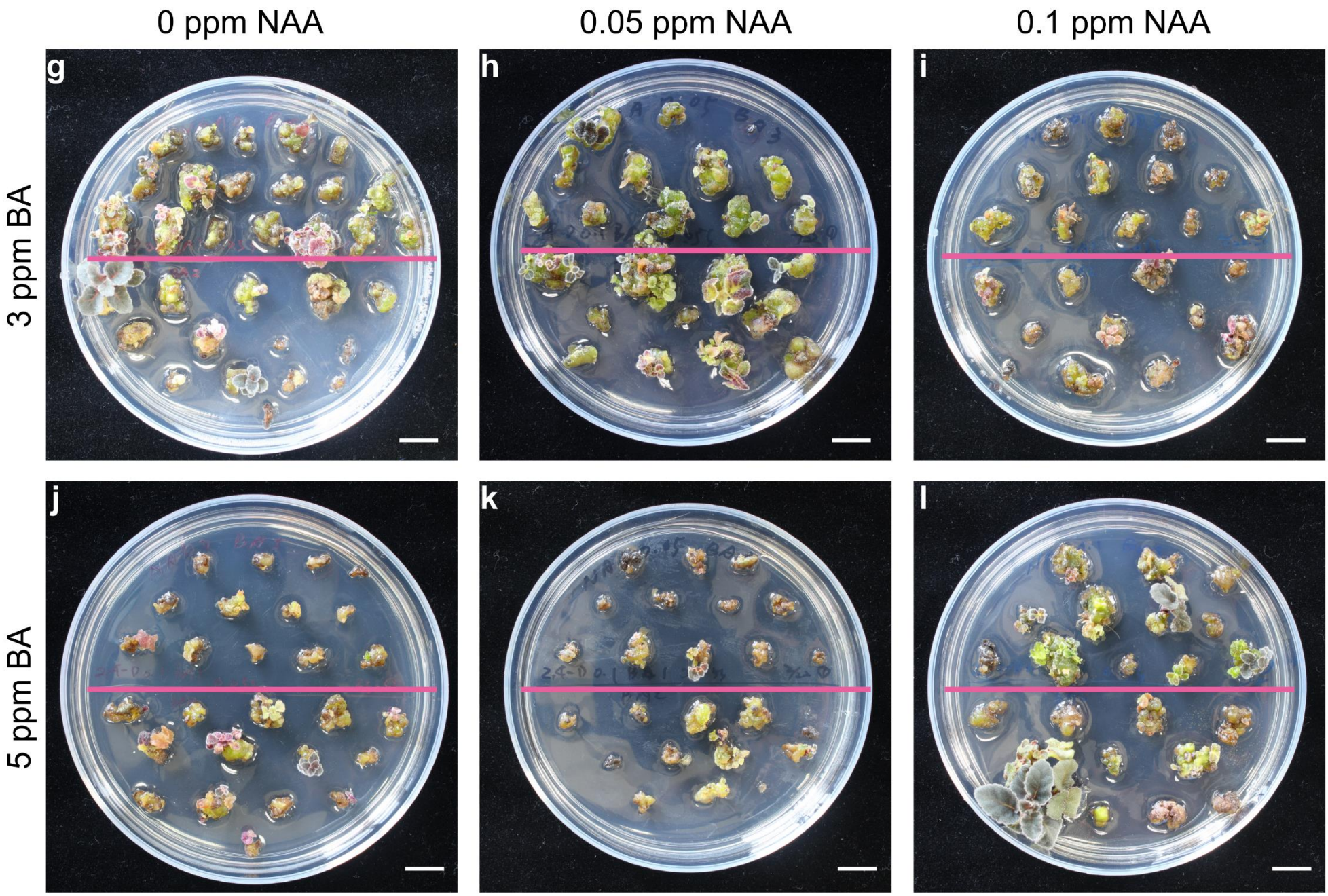

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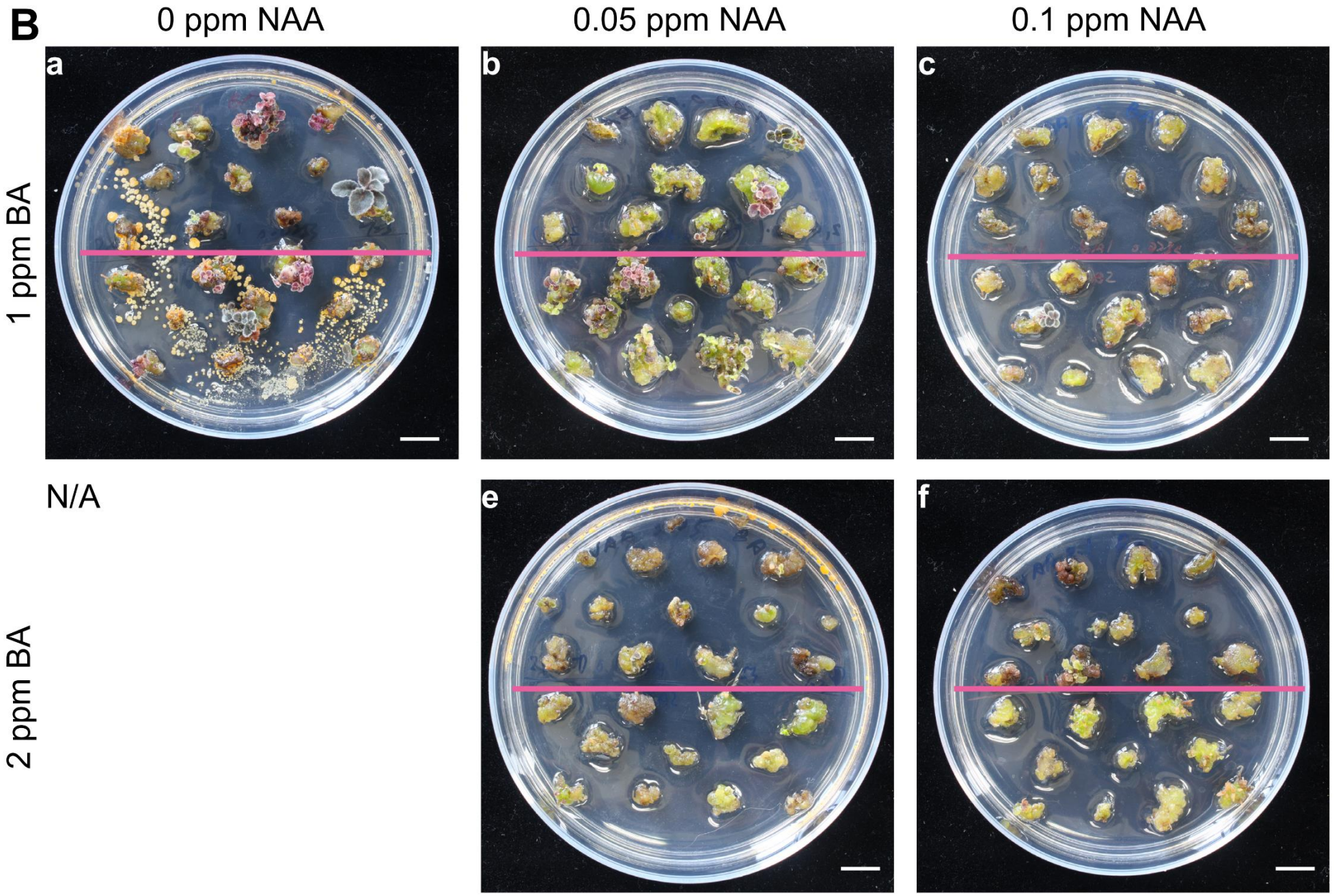

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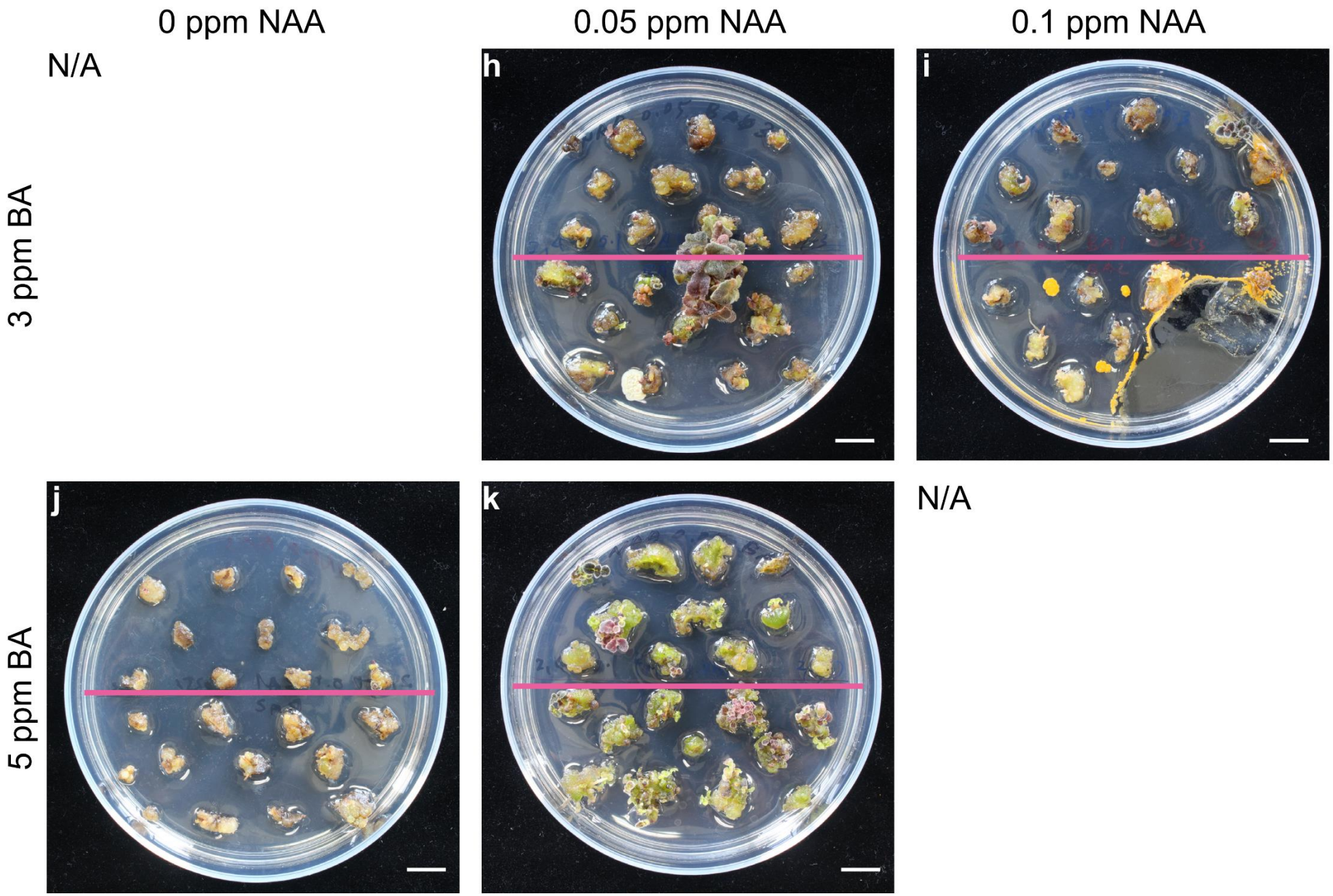
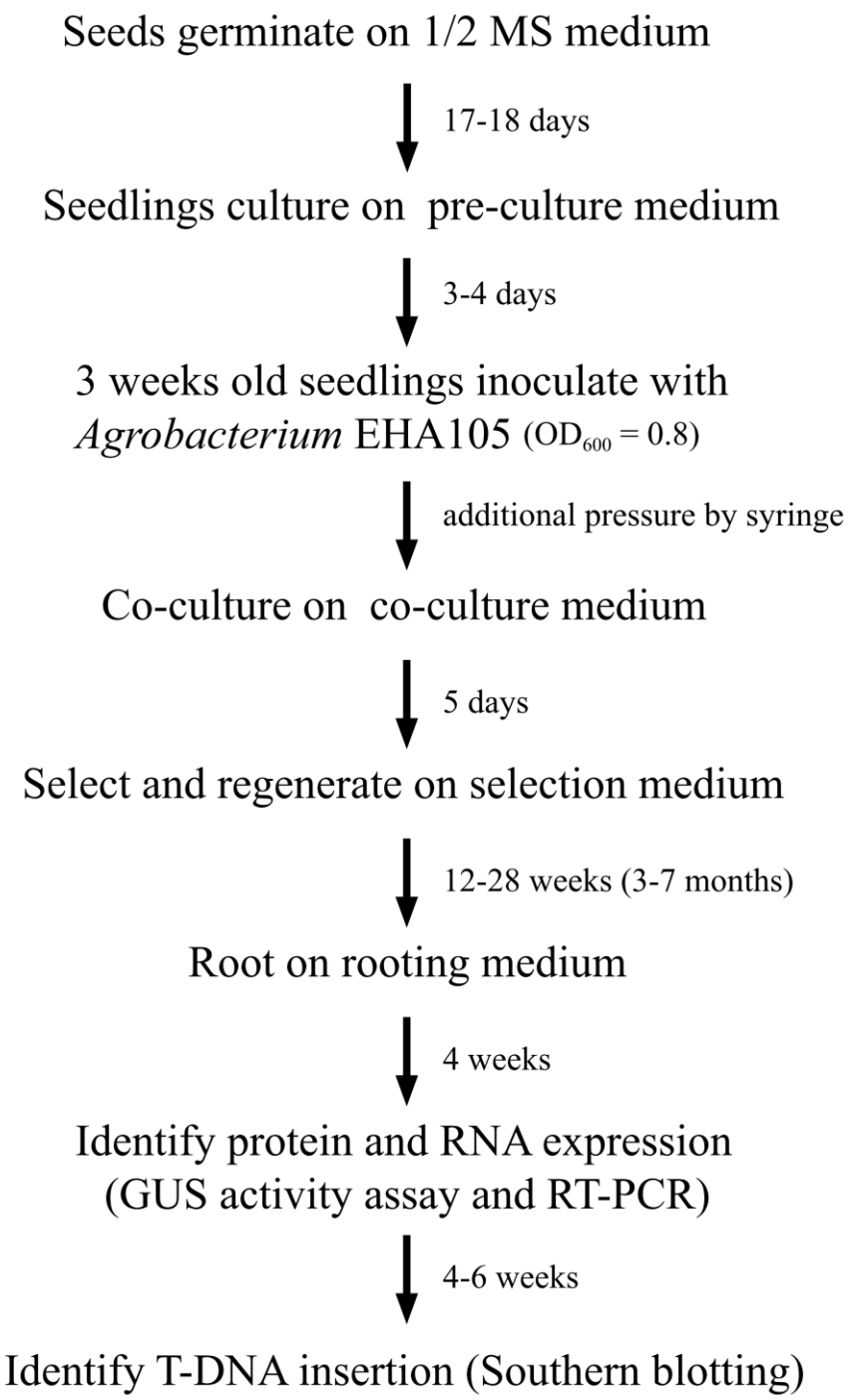


doi:10.6342/NTU201602983

Appendix Figure 10 The morphology of 4 kinds of callus regeneration in 12 different hormone conditions.

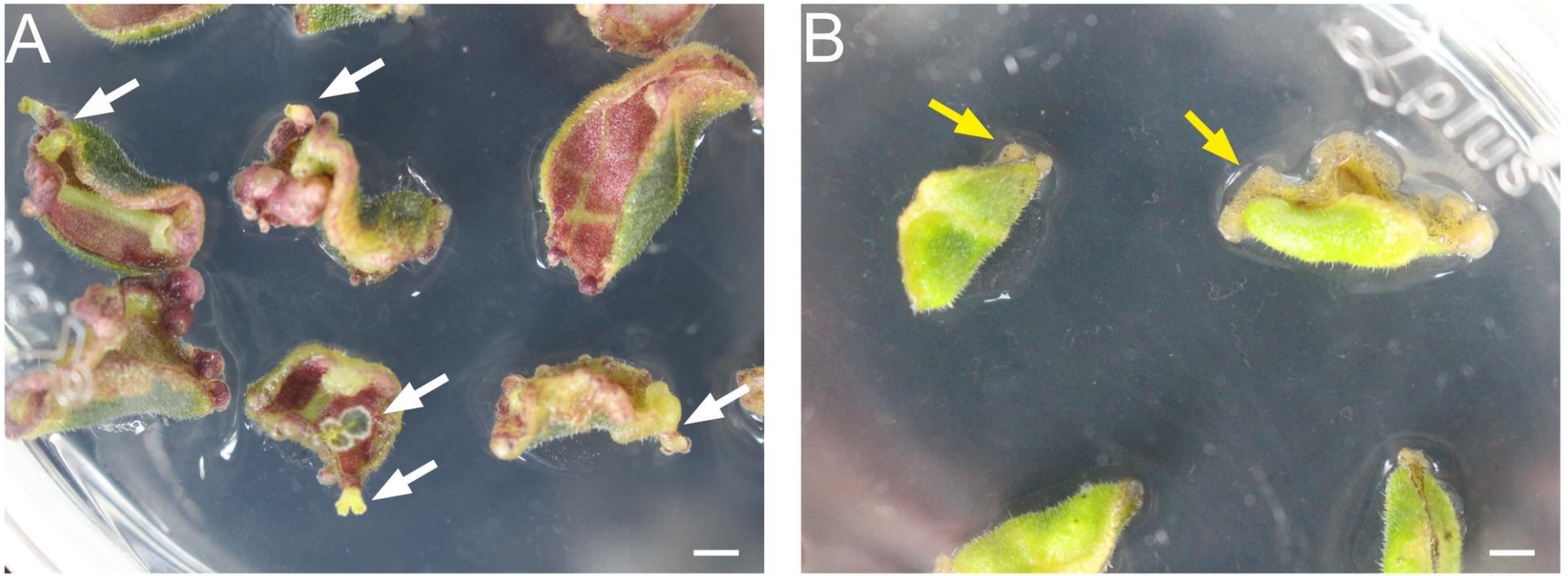
A, the callus inducing by 0.1 ppm 2, 4-D and 1 or 2 ppm BA with 0.05 M sorbitol was transferred to MS medium supplemented 12 different concentrations and combinations of NAA (0, 0.05 and 0.1 ppm) and BA (1, 2, 3, and 5 ppm) for inducing shoot regeneration. B, the callus inducing by 0.1 ppm 2, 4-D and 1 or 2 ppm BA with 0.025 M sorbitol was transferred to MS medium supplemented 12 different concentrations and combinations of NAA (0, 0.05 and 0.1 ppm) and BA (1, 2, 3, and 5 ppm) for inducing shoot regeneration. The callus upper than the pink line was induced by 1 ppm BA with acquired sorbitol, and lower than the pink line was induced by 2 ppm BA with acquired sorbitol. Scale bar, 1 cm.

doi:10.6342/NTU201602983



Appendix Figure 11 The flowchart for *Agrobacterium*-mediated transformation of *S. speciosa*.

doi:10.6342/NTU201602983



Appendix Figure 12 The effect of cefotaxime and kanamycin in *S. speciosa* 'Avanti'.

A, leaf discs put on regeneration medium **(**MS medium supplying with 0.1 ppm NAA and 1.0 ppm BA), white arrows indicate the shoot formation. B, leaf discs put on regeneration medium with additional 200 ppm cefotaxime and 100 ppm kanamycin, yellow arrows show the callus formation. Bar, 3 cm

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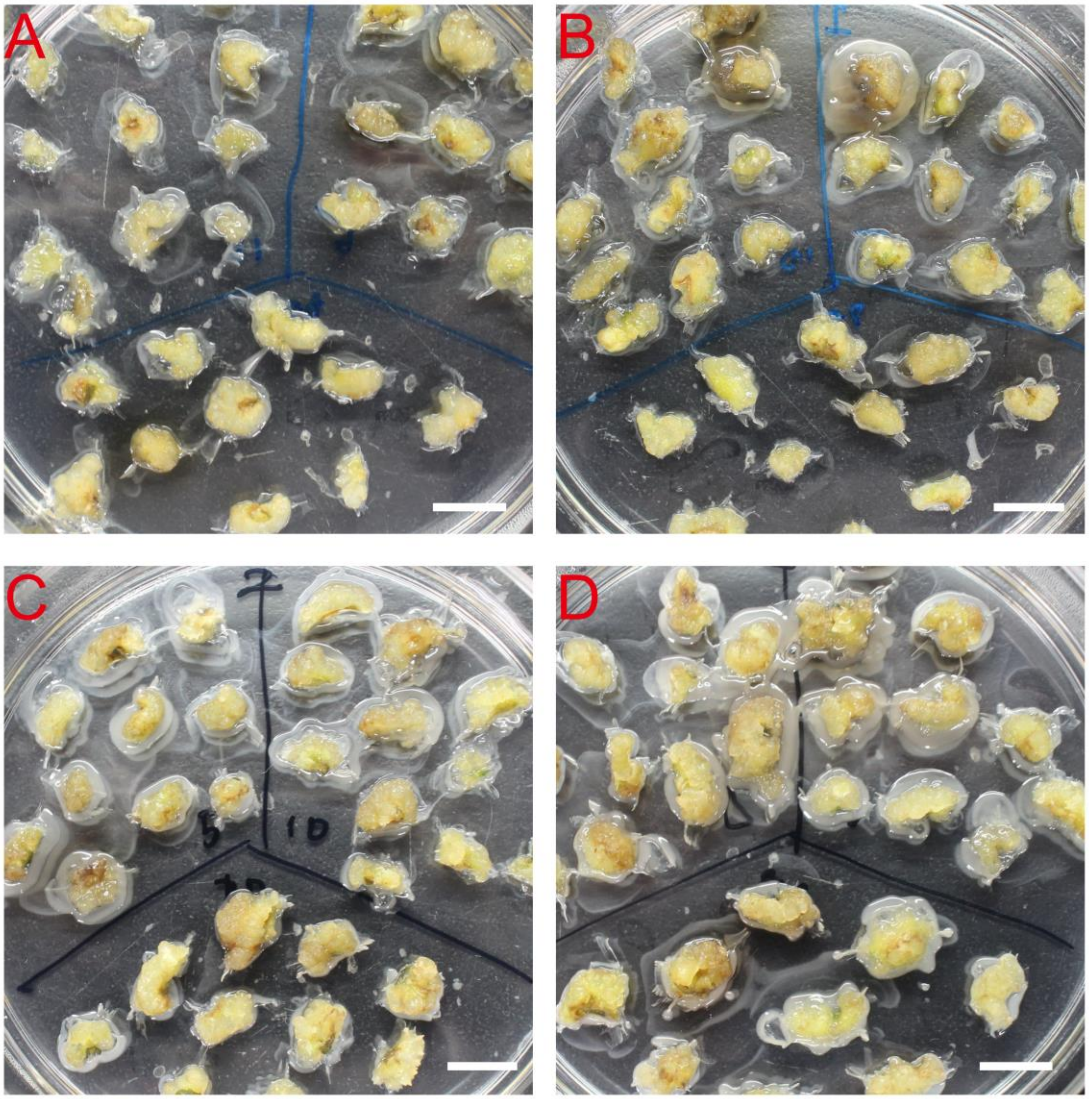

Appendix Figure 13 The morphology of 4 kinds of callus co-cultured with *Agrobacterium* EHA 105 for 1 day.

A, callus induced by 0.1 ppm 2, 4-D and 1 ppm BA with 0.025 M sorbitol. B, callus induced by 0.1 ppm 2, 4-D and 2 ppm BA with 0.025 M sorbitol. C, callus induced by 0.1 ppm 2, 4-D and 1 ppm BA with 0.05 M sorbitol. D, callus induced by 0.1 ppm 2, 4-D and 2 ppm BA with 0.05 M sorbitol. Scale bar, 1 cm.

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