Two *Agrobacterium*-mediated Transformation Protocols of White Clover (*Trifolium Repens*) Through the Callus System

Tong Jia
Sichuan Agricultural University - Chengdu Campus https://orcid.org/0000-0003-1565-5600
Bizhen Cheng
Sichuan Agricultural University - Chengdu Campus
Huahao Feng
Sichuan Agricultural University - Chengdu Campus
Muhammad Jawad Hassan
Sichuan Agricultural University - Chengdu Campus
Muhammad Zafar Iqbal
Sichuan Agricultural University - Chengdu Campus
Zhou Li
Sichuan Agricultural University - Chengdu Campus
Shiqie Bai
Sichuan Academy of Grassland Science
Yan Peng (pengyanlee@163.com)
Sichuan Agricultural University - Chengdu Campus https://orcid.org/0000-0001-7776-877X

Research Article

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Abstract

White clover (*Trifolium repens*) is one of the most widely cultivated livestock forage legumes co-cultivated worldwide with pasture grass in a mixed-sward setting, however, its persistence and aesthetic quality are severely affected by abiotic stresses. In this study, regeneration of white clover plants was conducted through a callus system for 4-5 months with a regeneration frequency of 36-41%. Inoculating 4-day-old cotyledons into MS media fortified with 0.4 mg·L^{-1} 6-BA and 2 mg·L^{-1} 2,4-D significantly increased the callus formation rate. Roots and cotyledons were better induced, followed by hypocotyls, leaves, and petioles. The development of differentiated structures performed effectively on MS supplemented with 1 mg·L^{-1} 6-BA and 0.1 mg·L^{-1} NAA. Further, we determined factors affecting the *Agrobacterium tumefaciens*-mediated transient transformation for root-derived callus and 4-day-old cotyledons. The parameters that facilitated transient transformation were: *Agrobacterium* suspension density of 0.5 (OD600), 20 mg·L^{-1} AS, and 4-days co-cultivation duration. Subsequently, we developed two transformation protocols: transformation after callus formation in root segments (Protocol A) and transformation before callus initiation in 4-day-old cotyledons (Protocol B). The transformation frequencies varied from 1.92% to 3.17% in Protocol A and from 2.76% to 3.47% in Protocol B. We offer the possibility to regenerate multiple transgenic white clover from a single genetic background. In addition to assistance in identification of functional genes associated with yield, resistance and aesthetic quality, our research will also contribute to successful genetic manipulation and genome editing in white clover.

Key Message

This study achieved two *Agrobacterium*-mediated transformation protocols for white clover by the callus system, offering the possibility of regenerating the transgenic white clover with a single genetic background.

Introduction

White clover (*Trifolium repens*) is one of the most important low-growing perennial stoloniferous legume species distributed extensively in landscape and pasture systems all over the world. Due to its excellent nitrogen (N) fixation potential, it is highly adapted to a wide range of low-maintenance turfgrass conditions (McCurdy et al. 2013). The addition of white clover to lawn mixtures has boosted the visual appeal, greenness, and expanded groundcover, hence contributing towards enhanced aesthetic value of landscapes (Sincik and Acikgoz 2007). White clover is highly palatable, nutritionally rich and possess sufficient protein content, thus plays an imperative role in improving pastures quality underpinning livestock production globally (Vaseva et al. 2011). However, the increased incidences of adverse environmental effects such as extreme temperatures, flooding, salinity, and drought have significantly reduced yields and aesthetic quality of white clover in the past few years (Jahufer et al. 2012; Williams et al. 2019).

Along with the application of traditional breeding methods, the development of genetically modified white clover for better adaptability offers a fundamental way to overcome such shortcomings. With the availability of the whole genome for white clover (Griffiths et al. 2019), the effective use of genomic information will facilitate functional genomics research and promote the interface between conventional and genomic breeding. However, despite the global importance of white clover, it has remained behind other major pastures primarily *Medicago sativa* (Jiang et al. 2019; Kumar et al. 2018) in developing genomic tools for its improvement. Most functional genomic researches in white clover typically relied on heterologous expression, depending on the *Arabidopsis* or *Medicago truncatula* as the model plant (Forster et al. 2013). Therefore, it is still important to develop a highly reproducible and widely applicable transformation system to benefit the white clover transformation network.

White clover callus was established for the first time by culturing 2-day-old clover germlings on induction media with a 50:1 auxin to cytokinin (w/w) ratio (Oswald et al. 1977). Subsequently, successful plant regeneration occurred from callus and suspension cultures (Gresshoff 1980; White 1984; Yamada 1989). Approximately two-third of early investigations addressed aspects of suspension cultures and mesophyll protoplast procedures (Ahuja et al. 1983; Bhojwani and White 1982; Mohapatra and Gresshoff 1982; Parrott and Collins 1983; Webb et al. 1987), which might be the result of large populations they provide. However, producing numerous suspension cells might increase the complexity of the regeneration process and the incidence of phenotypic and genotypic variations for white clover (Pederson 1986; Webb et al. 1987). Also, may not be suitable for some simple laboratory conditions.

The first successful genetic transformation of white clover was carried out in 1987 based on cell suspension cultures using a high-regenerating genotype WR8 (White and Greenwood 1987). Nonetheless, the frequency of that *Agrobacterium tumefaciens*-mediated transformation system was extremely low (<1%) because of the complex medium compositions and laborious procedures of callus induction and suspension incubation. Only a few reports (Ealing et al. 1994; Voisey et al. 1993) have followed this protocol. In 1994, a procedure was established to regenerate white clover shoots directly and rapidly from cotyledon petioles of 3-day-old seedlings (White and Voisey 1994). After that, a transformation method procedure following shoot organogenesis was described using *Agrobacterium tumefaciens* strain LBA4404 as a carrier, but the transformation frequency was less than 1% (Voisey et al. 1994). Meanwhile, several studies have been reported based on this protocol (Christiansen et al. 2000; Ding et al. 2003; Islam et al. 2015; Jenkins et al. 2002; Larkin et al. 1996; Mcmanus et al. 2005; Narancio et al. 2020; Schmidt et al. 2004). As white clover is an obligate outcrossing plant, hence each seed represents a distinct genotype. Although plants obtained by organogenic regeneration from cotyledons may contain the same T-DNA, yet each plant processes heterozygous chromosomal backgrounds. A split cotyledon approach where cotyledon pairs are tracked (one transformed and the other not) is the only method currently available to compare transformed and non-transformed plants with isogenic genetic backgrounds, but this is an incredibly tedious and time-consuming process.

To address some of these shortcomings, we successfully reproduced white clover plantlets from calluses in the current study and obtained a regeneration frequency between 36% and 41%, which allows the

generation of multiple plants from a single genetic background. In addition, to develop an appropriate transformation system, we modified this system and implemented two protocols for *Agrobacterium*-mediated transformation (Protocol A and Protocol B). Our research is of great significance for the improvement of the white clover transformation network.

Material And Methods

Plant material

Seeds of white clover (*Trifolium repens* cv. Ladino) were used for regeneration and transformations. Seeds were superficially sterilized with ethanol (70%, *v/v*) for 5 min followed by 10 min in $HgCl_2$ solution (0.1%, *w/v*) and washed five times with sterile distilled water. Subsequently, seeds were scattered on filter paper to germinate for 4 days and kept on MS medium (Murashige and Skoog 1962) to grow for 30 days. The attained seedlings or aseptic plantlets were selected for inoculation. The plantlets were maintained in controlled environment of growth chambers at 24/22°C day/night temperature, 16 h /8 h light/dark cycle, and light intensity of 150 μ mol m^{-2} s^{-1}, respectively

Callus induction and regeneration

The cotyledons, hypocotyls, roots, leaves, and petioles were used as explants for callus induction. Briefly, cotyledons, hypocotyls, and roots were taken from germinated 4-day-old sterile seedlings. The cotyledons were dissected into 2-3 sections, and the hypocotyls and roots were cut cross-sectionally into 2-3 mm long longitudinal segments (Supplementary Material: Fig. S1A and S1B). The petioles of 30-day-old aseptic plantlets were excised into 3-4 mm fragments, and the leaves were divided into 3-4 parts (Supplementary Material: Fig. S1C and S1D).

The cotyledon sections were taken as explants to determine the hormonal ratios for callus induction. The callus tissues were induced on the MS basal medium containing different concentrations of 2,4-dichlorophenoxy acetic acid (2,4-D; 1, 2, 3, 5 mg·L^{-1}) and 0.4 mg·L^{-1} 6-benzyl amino purine (6-BA). The composition of the media is shown in Supplementary Material: Table S1. Furthermore, the pH of the media was adjusted to 5.8 before autoclaving at 121℃. The explants were initially grown in the dark for 20 days, and initiation efficiency was counted after cultivation on various clover callus mediums (CCM1-CCM5). Calluses were then sub-cultured after every 20 days on fresh medium. Callus browning rate, callus thickness, and callus area were measured at the 25th day of sub-culturing.

To examine the callus formation effect, the cotyledons, hypocotyls, roots, leaves, and petioles were inoculated into CCM2 containing 2 mg·L^{-1} 2,4-D and 0.4 mg·L^{-1} 6-BA. The whole process of callus induction was accomplished in a dark environment. Each medium and explant type was subjected to three independent experiment replicates (60 tissues per set of experiments).

60-day-old fresh embryogenic callus differentiation was tested on MS media supplemented with naphthalene acetic acid (NAA; 0.1 mg·L^{-1}) and disparate quantities of 6-BA (0.5, 1, 1.5, 2 mg·L^{-1}).

Supplementary Material: Table S2 shows the combinations of different clover differentiation mediums (CDM1-CDM4). Each concentration was verified with three replicates of about 150 calluses. Regenerated shoots and embryoids were cultivated on MS medium containing 0.1 mg/L NAA (clover rooting medium, CRM) (Ahuja et al. 1983) for root development under growth chamber at 23℃ with a 16 h /8 h light/dark photoperiod.

Agrobacterium tumefaciens **strain and plasmid vectors**

The binary plasmid PBI121 (Gene Bank accession: AF485783.1), carrier of *the neomycin phosphotransferase II* (*NPTII*) and *the β-glucuronidase* (*GUS*) reporter gene, was applied for transformation. PBI121 vector was introduced into white clover tissues using the *Agrobacterium tumefaciens* strain EHA105 (Hood et al. 1993) containing a disarmed Ti plasmid. Single colonies of *Agrobacterium* EHA105 were incubated in 50 ml of YEB liquid medium with 20 $mg{\cdot}L^{-1}$ rifampicin (Rif) and 50 $mg{\cdot}L^{-1}$ kanamycin (Kan) at 28℃, and shaken at 180 rpm for 65 h.

Agrobacterium **-mediated white clover transient transformation**

To evaluate the efficiency of *Agrobacterium*-mediated transient transformation, cotyledons explants and 60-day-old calluses were infected with the *Agrobacterium* suspension. The treatments were as follows: (1) *Agrobacterium* bacteria were suspended in liquid clover infection mediums (CIM) supplemented with 10 $mg{\cdot}L^{-1}$ acetosyringone (AS). The optical density at 600 nm (OD600) was adjusted to 0.1, 0.2, 0.3, 0.5, and 0.7, respectively. Tissues were placed in the suspended solutions for 20 min and incubated on the co-cultivation medium (CM) for 3 days at 22℃. (2) *Agrobacterium* cells were resuspended to an OD600 of 0.3 and added with 0, 10, 20, or 40 $mg{\cdot}L^{-1}$ AS, respectively. Tissues were immersed in suspension for 20 min and cultivated on CM for 3 days. (3) Following 20 min infection in CIM (OD600=0.3) containing 20 $mg{\cdot}L^{-1}$ AS, tissues were dried on sterile filter paper to remove excessive *Agrobacterium* and co-cultured on CM in dark conditions for 1-5 days. These tissues were stained daily with 5-bromo-4-chloro-3-indolyl-glucuronide (X-Gluc) after being co-cultivated on CM.

Two protocols of Agrobacterium tumefaciens**-mediated white clover transformation and plant regeneration**

Protocol A: Agrobacterium**-mediated transformation in white clover 'Ladino' after callus formation from 4-day-old roots**

Root explants of 4-day-old white clover 'Ladino' seedlings were placed on CCM2 and cultured at 22℃ in a dark environment. After 40-50 days, the callus was formed and utilized for transformation. The *Agrobacterium* bacteria was centrifugally agglomerated for 20 min at 4500 rpm on the date of transformation. The supernatant was thrown out, and the cells were delicately resuspended in liquid CIM1 with 20 $mg{\cdot}L^{-1}$ AS and 600 $mg{\cdot}L^{-1}$ casein peptone at an optical density of 0.5 (600 nm). Uniform ready-to-infection calluses were collected in CIM1 and gently agitated (80 rpm) for 20-25 min at 28℃. Following infection, the callus was laid on sterile filter paper to absorb excessive suspension. After co-

cultivation with adhering *Agrobacterium* for 4 days on CM1, the callus was washed 3-4 times with distilled water. The calluses were then sub-cultured under a 16 h photoperiod on CDM2 selection medium (CDM2-S) containing 50 mg·L^{-1} Kan to select transformed cells and 300 mg·L^{-1} Cefotaxime sodium salt (Cef) to inhibit the further growth of *A. tumefaciens*. After 20 days, the resistant green shoot buds and embryoids of Kan were transferred to the same CDM2-S media for an additional 20-40 days to develop shoots. Regenerated green shoots under Kan selection were then placed on CRM selection medium (CRM-S) at 23℃ on a 16 h/8 h light/dark photoperiod for whole-plant development. After roots formation within 30-40 days, the regenerated plantlets were transplanted in the soil. The composition of the media for Protocol A has been given in Supplementary Material: Table S3.

Protocol B: Agrobacterium**-mediated transformation in 'Ladino' clover before callus initiation from cotyledons**

Cotyledons explants from 4-day-old white clover 'Ladino' seedlings were immersed in liquid CIM2 supplemented with *A. tumefaciens* (OD600=0.5) and then incubated at 28℃ for 20-25 min at 80-100 rpm. Subsequently, the explants were dried on sterile filter paper and co-cultivated with *Agrobacterium* on CM2 at 23°C for 4 days. After washing 3-4 times with sterile water, the explants were transferred to CCM2 selection medium (CCM2-S) supplemented with 300 mg·L^{-1} Cef and 50 mg·L^{-1} Kan and incubated at 23℃. The selection was implemented in the dark for 50-60 day with media alteration after every 20 days. The differentiated structure induction and roots development procedure were the same as described in protocol A on CDM2-S and CRM-S medium, under a 16 h /8 h light/dark photoperiod at 23℃. The green-white clover plantlets were transferred into the greenhouse about 40 days after root formation. The media composition for Protocol B has been shown in Supplementary Material: Table S4.

Identification of transformed explants or plants

Genomic DNA, extracted from putative transgenic plants surviving under Kan selection, was amplified by PCR using the selectable marker *NPTII* sequence region and primers sequences were 5'-GAGGCTATTCGGCTATGACTGG-3' and 5'-ATCGGGAGCGGCGATACCGTA-3', respectively (Hamama et al. 2011). White clover plants with positive PCR amplification for the marker gene sequence were considered as transgenic white clover.

For histochemical GUS staining analysis, various tissues of explants or transgenic seedlings were incubated in GUS staining buffer containing 0.1% (*w/v*) X-Gluc at 37℃ for 12 h. After staining, samples were decolorized with 70% alcohol.

Statistical analysis

The data were analyzed using SPSS 25.0 statistics and analysis software (IBM, Armonk, New York, USA). Following one-way ANOVA, in combination with Fischer's least significant difference (LSD) was used to test significant differences among treatments at a 0.05 probability level.

Results

Callus initiation and formation in white clover

To analyze the impact of various 2,4-D concentrations on induction and development of embryonic callus in white clover, cotyledons as explants were employed with MS medium as basal substrate with the addition of 0.4 mg·L^{-1} 6-BA. Throughout the 45-day callus induction on CCM1-CCM5, there was an apparent naked eye evident difference in callus proliferation. Embryo-like structures were observed on MS media supplemented with 2, 3, and 5 mg·L^{-1} 2,4-D under the microscope. As a whole, cotyledon-derived callus at 2 mg·L^{-1} 2,4-D (CCM2) exhibited a better and greater embryogenic structure (Fig. 1A). Despite of more than 60% callus initiation efficiency under each medium variant, over 90% of callus initiated under 2 mg·L^{-1} and 3 mg·L^{-1} 2,4-D after a 20-day induction phase (Fig. 1B). When sub-cultured for 25 days, callus browning rates were lowest at 2 mg·L^{-1} compared to the rates observed at 1 mg·L^{-1} and 5 mg·L^{-1} 2,4-D (Fig. 1C). Also, the callus thickness and area were highest at 2 mg·L^{-1} 2,4-D compared to other concentrations (Fig. 1D and 1E). These results demonstrated that MS basal medium containing 2 mg·L^{-1} 2,4-D and 0.4 mg·L^{-1} 6-BA (CCM2) was more appropriate for callus initiation and proliferation in white clover cotyledons.

When inoculated on CCM2 medium, embryogenic calluses were obtained from the cotyledons, hypocotyls, roots, leaves, and petioles explants of white clover (Fig. 2A). Under the microscope, numerous pro-embryos or somatic embryos were visible on the surface of the embryogenic callus. Alternatively, a minute difference was found in callus initiation efficiency after 20-day induction (Fig. 2B). Compared to cotyledons, hypocotyls, roots, and leaves explants, over 30% of the petiole explants turned brown and died after 25-days of proliferation (Fig. 2C). More callus thickness and area were observed in roots and cotyledons explants than leaves explants (Fig. 2D and 2E). Overall, roots, and cotyledons explants were optimal for callus proliferation in white clover, followed by hypocotyls and leaves inoculated in MS media supplemented with 2 mg·L^{-1} 2,4-D and 0.4 mg·L^{-1} 6-BA. Moreover, petioles explants were inferior for callus formation on CCM2.

Shoot induction and plant formation

After 50-60 days of embryogenic callus formation, the callus was successively transferred into MS medium supplemented with 0.1 mg·L^{-1} NAA and varying quantities of 6-BA (0.5, 1, 1.5, 2 mg·L^{-1}) at 23℃ and a 16 h /8 h light/dark photoperiod. Plenty of somatic embryos in the globular stage started greening after 7 days (Fig. 3A). Under the four applied mediums (CDM1-CDM4), partial calluses gradually browned from the periphery to the middle or hydrated without further differentiation. Even so, more than 50% of somatic embryos regenerated shoots under 1 mg·L^{-1} 6-BA, compared with 27.5% and 35.7% under 0.5 mg·L^{-1} and 2 mg·L^{-1} 6-BA, respectively (Fig. 3B). The regenerated white clover shoots were obtained after 50 days of shoot proliferation (Fig. 3C). Subsequently, the regenerated shoots were transplanted to CRM added with 0.1 mg·L^{-1} NAA to stimulate rooting. Most shoots produced roots longer than 5 cm after 40-days of cultivation (Fig. 3D). Thus, 36%-41% of white clover plantlets were eventually regenerated from the callus system.

Comparison of the effect of critical factors on the transient transformation efficiency

The impact of critical factors on transient transformation frequency was assessed after infection with *A. tumefaciens* EAH105. To determine optimum density for infected *Agrobacterium* cells, five different densities of *Agrobacterium* (OD600 ranging from 0.1 to 0.7) were prepared to infect tissues separately. After 20 min of infection at 10 $mg{\cdot}L^{-1}$ AS, the highest number of positive GUS staining tissues was found at an OD600 of 0.5 (Fig. 4A). Additionally, the efficacy of the transformation level was significantly influenced by the AS concentration, with the highest efficiency of 78.83% (callus) and 78.68% (cotyledon) at 20 $mg{\cdot}L^{-1}$ AS (Fig. 4B). Besides, the percentage of tissues displaying GUS staining frequency increased when the co-culture duration was prolonged (Fig. 4C), with five days of co-incubation manifesting the highest transient transformation efficiency. Since the extension in the co-cultivation time and increase in the *Agrobacterium* concentration, the GUS staining frequency enhanced progressively. Nonetheless, the excessive proliferation of *Agrobacterium* exceedingly triggered the wounded cells leading to serious tissue injury. Moreover, our findings indicated that *Agrobacterium* more readily infected calluses than cotyledons explants.

Comparison of two *Agrobacterium*-mediated transformation protocols for callus

In the present study, two transformation protocols related to white clover callus were carried out. Roots were the most desirable explants to proliferate callus, as aforementioned. Root-derived callus was transformed using the first method (Protocol A, Fig. 5A-C, F-J). Furthermore, the present experiment endeavored to transform root explants before callus initiation, but the root system proved to be fragile and caused numerous tissues damage after infection (Supplementary Material: Fig. S2A). In addition to cotyledons explants (Fig. 5E), hypocotyls, leaves, and petioles were also severely browned after infection (Supplementary Material: Fig. S2B, S2C, and S2D). Thus, cotyledons explants were adopted for Protocol B, in which callus was induced after transformation (Fig. 5D-F).

By the selection of *Agrobacterium*-infected tissues on CDM2-S, a partially pale green callus was observed as shown in (Fig. 5F). Observation of germinating embryos in a stereomicroscope confirmed that some embryos tuned green (transgenic) by the selection, while others were considered un-transgenic, which did not become green but turned yellow (Fig. 5G, 5H). During regeneration, both somatic embryogenesis and organogenesis occurred. Untransformed callus turned brown and died in a regular sub-culture on CDM2-S. After three rounds of Kan antibiotic screening, the multiple green shoots survived (Fig. 5I, 5J), while the untransformed growth was bleached white. In two replicated experiments using Protocol A, 16 of 208 (7.69%) and 21 of 189 (11.1%) explants established resistance to Kan. On the other hand, 5.45% and 4.15% resistant shoots were regenerated by Protocol B (Table 1).

Table 1
Comparison of transformation efficiency among three protocols

Material/Method	Experiment /No. Explants	Kan-Resistant Shoots	Kan-resistant plants	Positive lines/Negative lines (PCR configuration)	Transformation Efficiency
Protocol A	1/208	16(7.69%)	9	4/5	1.92%
	2/189	21(11.1%)	10	6/4	3.17%
Protocol B	1/202	11(5.44%)	9	7/2	3.47%
	2/217	9(4.14%)	8	6/2	2.76%

To ensure that all plants were independent transformants, only one resistant shoot from each callus was placed onto CRM-S (Fig. 5K). White clover roots germinated after approximately 40 days (Fig. 5L), and the transgenic white clover was subsequently transferred into the soil (Fig. 5M). It happened that some shoots failed to produce roots after several weeks on CRM-S. 19 and 17 Kan-resistant plantlets through Protocol A and Protocol B were eventually collected (Table 1). The formed transgenic plants displa

Transgenic plants identification and Transformation efficiencies

After transplanting the surviving plants into the soil, the regenerated plants were verified by PCR with vector-specific primers to investigate the presence of the *NPTII* gene for PBI121 (Fig. 7A). Furthermore, leaves and petioles of positive white clover were examined for the presence of histochemical GUS activity. Intensely stained leaves and petioles were visible from transgenic plants, implying the expression of the GUS reporter gene in tissues (Fig. 7B). After selection, 75% and 77.7% of resistant plants were positive by Protocol B, compared to 44.4% and 60% for Protocol A (Table 1). Plant transformation efficiency, in terms of the number of positive confirmed transgenic white clover produced per one hundred infected explants, ranged from 1.92% and 3.17% through Protocol A, and 2.76% and 3.47% through Protocol B.

Discussion

Effect of medium and explants on callus induction

Distinct basal culture media contains various organic components which perform vital roles in plant regeneration. Contrary to the results (Ahuja et al. 1983; Gresshoff 1980; Parrott and Collins 1983; White 1984) documenting incubation and plantlet regeneration on different substrate media: MS, Gamborg B5, Phillips and Collins L2, Schenk and Hildebrandt (SH), the present study utilized a single base medium (MS) during all the tissue culture phases to facilitate work in the laboratory, and only changed the growth regulator levels in MS.

Previous studies have reported that the presence of 2,4-D is imperative in the process of somatic embryogenesis for white clover. White clover calluses have formed successfully under three combinations of hormone: 2,4-D + kinetin (KT) (Gresshoff 1980; White 1984), 2,4-D + NAA + KT (Pederson 1986), and 2,4,5-T + KT (Oswald et al. 1977). Nevertheless, few studies have also explored 6-BA's potential on the induction of white clover callus. Our findings showed that 0.4 $mg{\cdot}L^{-1}$ 6-BA plus relatively high levels of auxin, 2 $mg{\cdot}L^{-1}$ and 3 $mg{\cdot}L^{-1}$ 2,4-D, resulted in higher frequency of embryonic callus, implying that additional 6-BA also exhibits an inductive effect. However, the callus testified to have adverse changes with progressive increase in the concentration of 2,4-D. A review of the literatures concluded that the higher 2,4-D levels may interfere with somatic embryo development and disrupt the normal chromatin structure rearrangement, histone modification, and DNA methylation in plant cells (Garcia et al. 2019; Gresshoff 1980; Konieczny et al. 2012). Oswald H media supplemented with 2,4,5-T maintained viability and induced growth of white clover callus, suggesting that 2,4,5-T was a more suitable alternate auxin source than 2,4-D (Oswald et al. 1977). It remains to be investigated in our protocol, as we adapted different base media and hormone combinations.

Attention should not only be focused on the medium as the explant nature (tissue, organ, growth stage) is equally important in allowing successful initiation. Different plant tissues showed vast distinct callus induction frequencies (Gresshoff 1980; Karadotcheva et al. 1995). Even though cotyledons, hypocotyls, roots, leaves, and petioles explant initially expanded and proliferated callus on the cutting surface, however 4-day-old roots explants exhibited the highest induction capacity for forming embryo structures. Previous studies on root-derived calluses found similarities in morphology with different white clover ecotypes (Mohapatra and Gresshoff 1982; Parrott and Collins 1983). Following the observation of other ecotypes, our study identified that the developing Ladino root similarly produced a callus around mantle. In addition, 30-day-old leaves and petioles explants showed the highest brown discoloration than 4-day-old cotyledons, hypocotyls, and roots, manifesting the declined viability in mature explants.

Shoot regeneration and plant recovery

It has been determined that differentiation media containing 2,4-D formed many abnormal shoots, which could not sprout or grow slowly (Bond and Webb 1989). Thus, NAA was utilized as a possible alternate auxin source. 6-BA was once taken up as the component of exogenous hormones to ameliorate regeneration capacity for white clover (Ahuja et al. 1983; Webb et al. 1987; White 1984). Here, adding 1 $mg{\cdot}L^{-1}$ 6-BA and 0.1 $mg{\cdot}L^{-1}$ NAA to MS effectively developed the de-differentiated structures into plants.

Many researchers have applied only regeneration media or hormone-free basal media to promote rooting (Bhojwani 1981; Oswald et al. 1977; Webb et al. 1987; Weissinger and Parrott 1993) indicating that white clover roots possess a powerful regeneration ability. NAA has been widely used to stimulate rhizogenesis in other plants (Amiri and Mohammadi 2021; Hansuek et al. 2018; Martins et al. 2020). Our study discovered that adding 0.1 $mg{\cdot}L^{-1}$ NAA extremely facilitated root primordia in the hypocotyl region of excised shoots, which was consistent with previous studies on white clover (Ahuja et al. 1983; Bond and Webb 1989).

Agrobacterium-mediated transient transformation

Based on the data obtained from white clover regeneration research, our research attempted to deliver genes to white clover through the *Agrobacterium*-mediated transformation. The supplement of amino acid mixtures positively influenced the co-incubation of *Agrobacterium* and explants (Mukherjee et al. 2000; Zaidi et al. 2006). In this study, the liquid infection media and co-incubation medium have undergone certain modifications to contain 600 $mg{\cdot}L^{-1}$ casein-hydrolysate as an additional ingredient. The *Agrobacterium* strains LBA4404 (Mcmanus et al. 2005; White and Greenwood 1987), AGL1 (Jenkins et al. 2002; Larkin et al. 1996), GV2260 (Schmidt et al. 2004), and CZ707 (Panter et al. 2012) have been applied for infecting white clover. The *Agrobacterium* EHA105 containing the hypervirulent Ti plasmid pTiBo542 (Hood et al. 1993) was utilized for transformation, which had previously been used to confer higher transformation efficiencies in many species (Arun et al. 2015; Benzle et al. 2015; Chetty et al. 2013).

Many factors contribute to the transformation efficiency of plants, however among those the most vital factor is the target material. Our study applied two types of target explants to inquire about the transient efficiency: root-derived callus and 4-day-old cotyledons. The tissues immersed in the *Agrobacterium* suspension with an OD600 of 0.5 and 0.7 showed higher Gus staining efficiency. Excessive levels of *Agrobacterium* could damage explants, causing them to be brown and dead. Alternatively, insufficient levels of *Agrobacterium* could result in low infection rates (Hayta et al. 2019). Consequently, we employed an *Agrobacterium* suspension with an OD600 of 0.5 for 20-25 min to carry out the infection. As a precursor of the plant cell wall, AS elicited the activation of the virulence genes and enhanced *Agrobacterium* infection in wound cells (Mo et al. 2015). Regarding the AS concentration, 20 $mg{\cdot}L^{-1}$ AS was applied in subsequent transformation as the transient transformation efficiency did not differ significantly between 20 $mg{\cdot}L^{-1}$ and 30 $mg{\cdot}L^{-1}$ in our study. Previous transformation protocols for white clover tissues indicated that co-cultivation of *Agrobacterium* with explants lasted three to five days (Christiansen et al. 2000; Larkin et al. 1996; Sharma et al. 1998; Voisey et al. 1994). In this study, the higher transient transformation efficiency occurred at four and five days. Nonetheless, considering that the overgrowth of Agrobacterium excessively triggered the tissues in the wound, thus 4-day co-cultivation duration was selected to be utilized in subsequent experiments.

Comparison of two transformation protocols

In this study, two protocols were introduced to transform white clover with distinct initial materials. Protocol A yielded 7.69% and 11.1% of Kan-resistant shoots in two treatments. In contrast, Protocol B produced fewer resistant shoots, indicating that fewer explants survived in the long-term selecting stages (3-4 months). A part of the initial resistant shoots was lost by extending selection time, of which the leaves turned yellow, or no root could be produced when grown on CRM-S. This phenomenon was particularly severe in Protocol A. Our results hypothesize that these shoots (escapes) were not transformants, but rather natural resistance within the early selecting stages of selection.

An efficient selector can restrain un-transformed tissues proliferation and enable transformed cells growth. The *NptII* gene is the most widely applicable and confers antibiotic resistance such as Kan, neomycin, and G-418. 50 mg·L^{-1} (Ding et al. 2003; Narancio et al. 2020) and 100 mg·L^{-1} (Sharma et al. 1998; Voisey et al. 1994) Kan have been applied to inhibit the growth of non-transformed white clover cells. 50 mg·L^{-1} Kan was selected in the current study, as higher Kan concentrations may negatively influence organogenic potential and affect shoot and root regeneration. However, a high percentage of escapes were identified from putative transformants by PCR analysis. Protocol B has a higher positive rate than Protocol A, which could be attributed to the more extended selection period. Thus, our findings suggest to maintain explants for additional 20-30 days on antibiotic selection for an augmented positive rate. This idea is consistent with the previous works (Hayta et al. 2018; Larkin et al. 1996).

In the present study, Protocol A and Protocol B took 4-5 months to produce transgenics from the initial plant material. Although Protocol A and Protocol B required relatively similar times, callus can be maintained undifferentiated at least for 5 months in Protocol A. Therefore, it is possible to reduce the transformation time to 3-4 months using the preserved callus for future transformations. Also, multiple plants could be regenerated from a single genetic background through sub-culturing calluses. Protocol B is a rapid method but has tremendous potential for regenerating partially transformed plants (chimeras). Our findings suggest that this approach be applied in early functional genetic assays to permit rapid gene characterization at relatively lower input. According to the required operation procedure, time, and labor, researchers can choose any of our two protocols that best fit their needs.

Previous studies have pointed out that phosphinothricin (PPT) selection (*phosphinothricin acetyltransferase gene*, *Bar*) brought more stringent and rapid selection than Kan selection (Larkin et al. 1996; Wen et al. 2019), and the efficiency of transgenic recovery using PPT was superior when compared with Kan (RamanaRao and Veluthambi 2010). We need to spend more labor and time to monitor the white clover tissue constantly, though some white clover escapes and chimeras died during the long period of Kan selection. The green fluorescent protein (GFP) signals can be visualized at an early stage, allowing more convenient examination than histochemical GUS staining and eliminating the waste of plant material. Therefore, to optimize the current transformation process, we constructed a binary vector Pml-CAMBIA3300-35S-EGFP, which carries a chimeric *Bar* gene and an *EGFP* gene driven by CaMV 35S promoter (Supplementary Material: Fig. S3), for future transformation experiments. Nevertheless, whether the alternate binary vector can facilitate the procedures and improve frequency remains to be verified. Also, we are conducting experiments to reduce the browning rate of the transformed leaf and petiole explants. Thus, we expect to use only one white clover plantlet through protocol B, which will enable us to obtained transgenic plants with a single genetic background.

Conclusion

The current work demonstrated callus culture conditions and somatic embryogenesis regeneration using different hormone concentrations and various explants for white clover ‘Ladino’, with a regeneration frequency between 36% and 41%. Establishing an effective and stable genetic transformation system is a

pre-requisite for selecting a wide variety of genetically modified plants. As the preparation for further *Agrobacterium*-mediated transformation, the present study evaluated the impacts of *Agrobacterium* densities, AS concentrations, and co-cultivation durations on the effectiveness of transient transformation. Besides, the current research achieved two transformation protocols (Protocol A and Protocol B) built on the regeneration system. Our studies are essential for successful genetic manipulation and genome editing for white clover and will assist in the functional characterization of genes associated with aesthetic quality and resistance.

Abbreviations

2,4-D: 2,4-dichlorophenoxy acetic acid; NAA: naphthalene acetic acid; 6-BA:6-benzyl amino purine; AS: acetosyringone; GUS: β-glucuronidase; Kan: kanamycin; Cef: Cefotaxime sodium salt; PPT: phosphinothricin; CCM: clover callus medium; CDM: clover differentiation medium; CRM: clover rooting medium; CIM: clover infection medium; CM: co-cultivation medium; CCM2-S: CCM2 selection medium; CDM2-S: CDM2 selection medium; CRM-S: CRM selection medium

Declarations

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Competing interests

The authors declare that they have no conflicts of interest. The funders had no role in the design of the study; in **the** collection, analyses, or **interpretation** of data; in the writing of the manuscript, or in the decision to publish the results.

Author contributions

YP, SB, and TJ conceived and designed the study, TJ and BC conducted the experiments, HF, MZI, and MJH provided advice, HF and ZL analyzed the data, and TJ wrote the manuscript. All authors read and approved the final manuscript.

Data Availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Figures

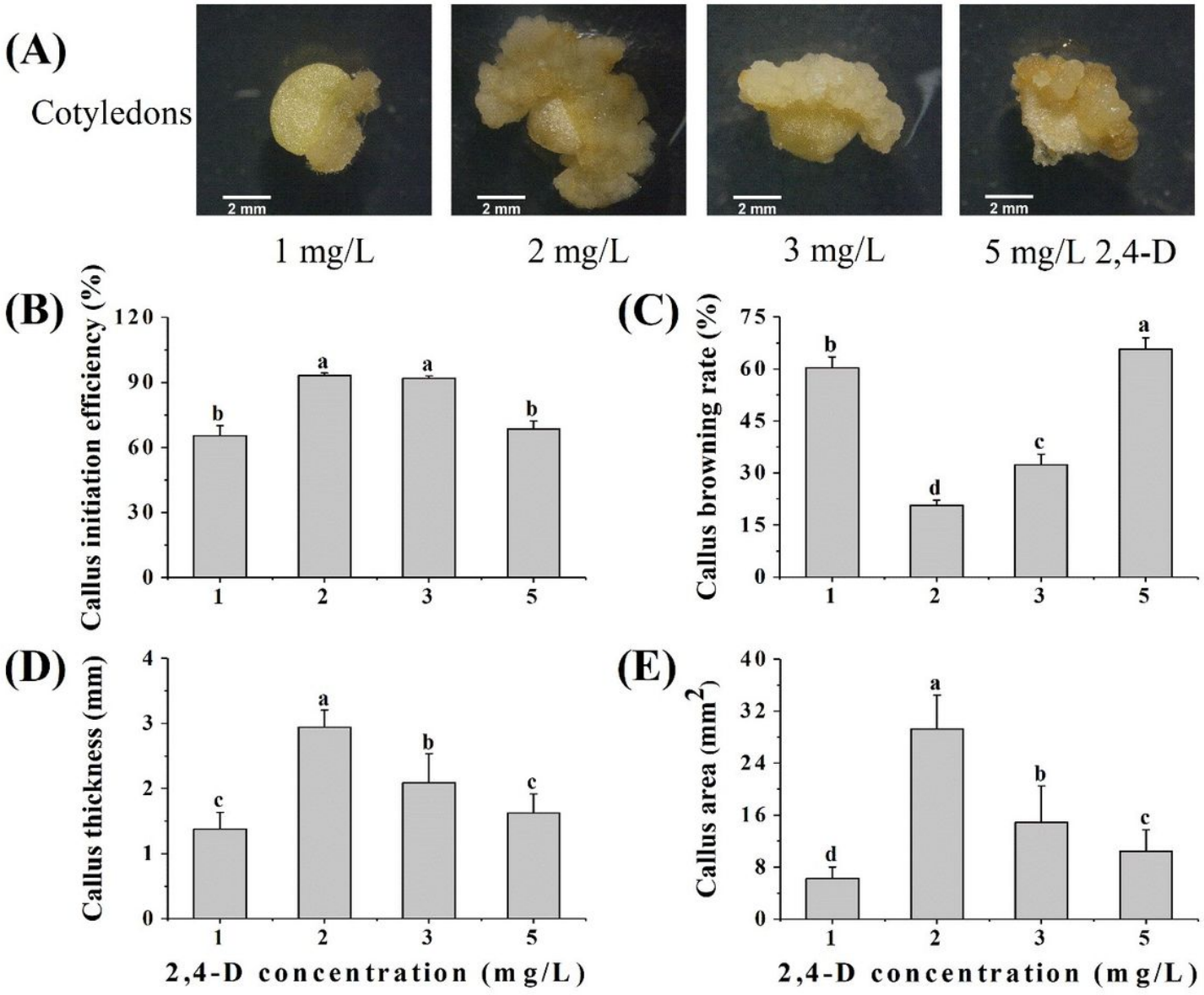

Figure 1

Influence of induction media CCM1-CCM4 on the initiation and proliferation of callus from cotyledons explants. (A) The texture of callus on CCM medium with different quantities of 2,4-D. (B) Callus initiation efficiency after 20 days of cultivation. (C) callus browning rate, (D) callus thickness, and (E) callus area following 40 days of induction. Each treatment (A and B) comprised about 55 explants and was performed in 3 replicates. Data (D and E) were generated from 10 independent replicates. Different letters indicate statistically significant differences at $P < 0.05$.

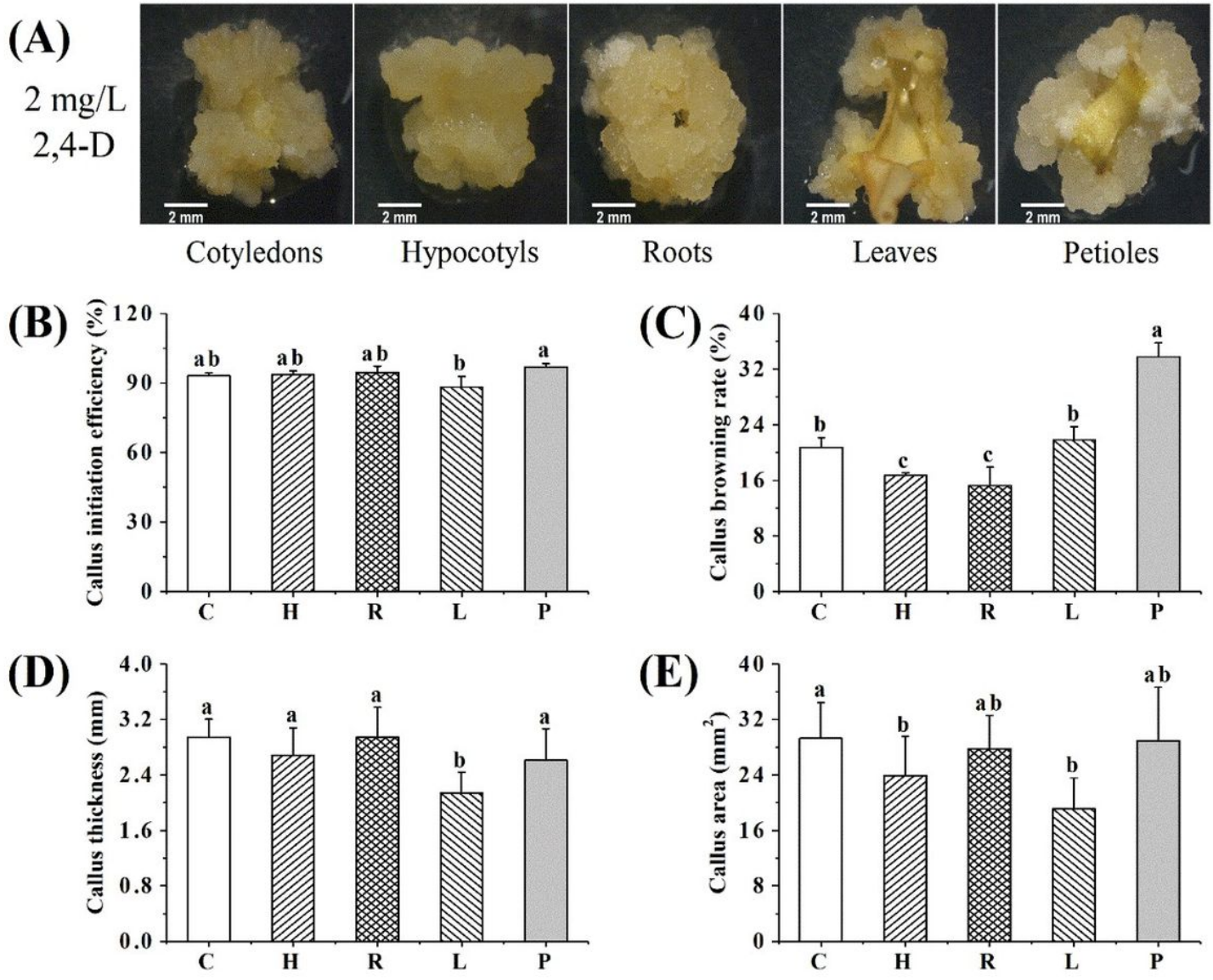

Figure 2

Effect of CCM2 medium on different explants. (A) Immature embryo-derived callus after 45 days culture on CCM2 from the cotyledons, hypocotyls, roots, leaves, and petioles explants. (B) Callus initiation efficacy after 20 days of induction. (C) callus browning rate, (D) callus thickness, and (E) callus area following 45 days of development. Each treatment (A and B) comprised about 60 explants and was

performed in 3 replicates. Data (D and E) were generated from 10 independent replicates. Different letters indicate significant difference at P < 0.05. C: Cotyledons, H: Hypocotyls, R: Roots, L: Leaves, P: Petioles.

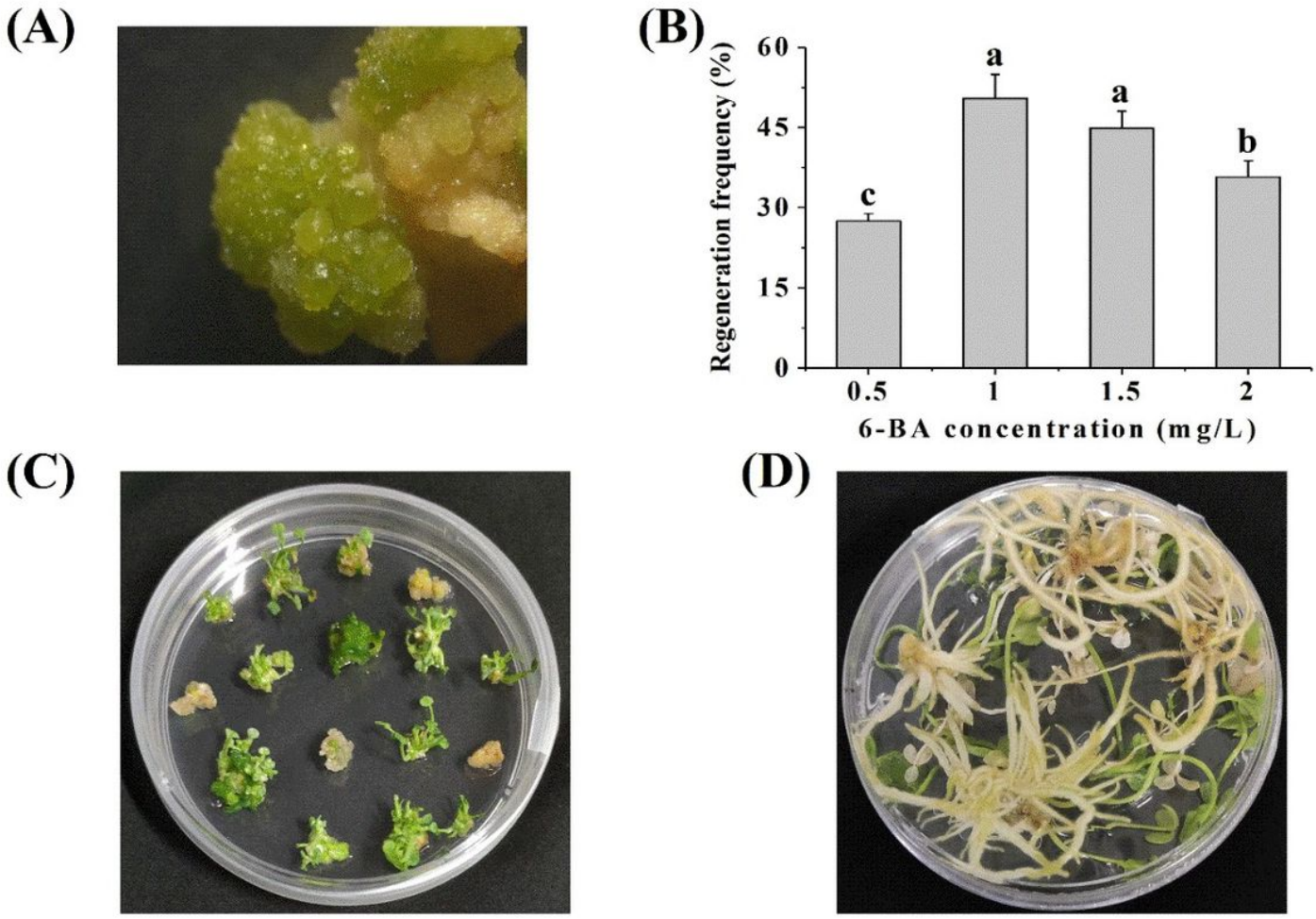

Figure 3

White clover plantlets regeneration from calluses. (A) The greening globular embryo after 7 days of differentiation. (B) Effect of 6-BA concentration on calluses regeneration frequency. (C) Trifoliate leaves sprouted from embryogenic callus after 50 days on CDM2. (D) Numerous roots were produced on CRM after 40 days of induction. Each treatment (B) comprised about 50 explants and was performed in 3 replicates. Different letters indicate significant differences at P < 0.05.

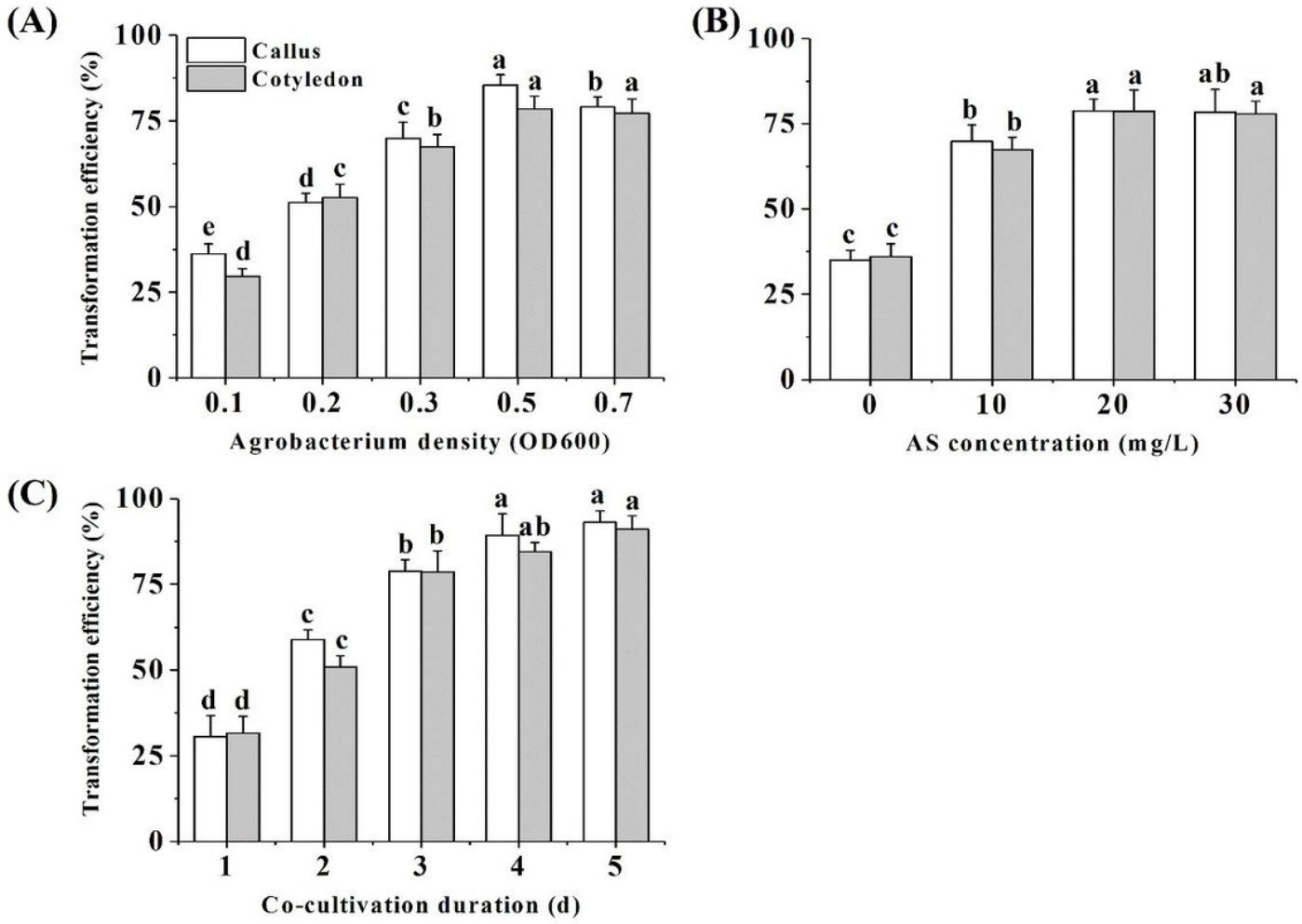

Figure 4

Effects of Agrobacterium concentration (A), AS concentration (B), and co-cultivation duration (C) on transient transformation efficiency. Each treatment comprised about 25 (calluses) or 35 (cotyledons) tissues, and the data were expressed as means ± SD from three replicates. Different letters above the columns display a significant difference at $P < 0.05$.

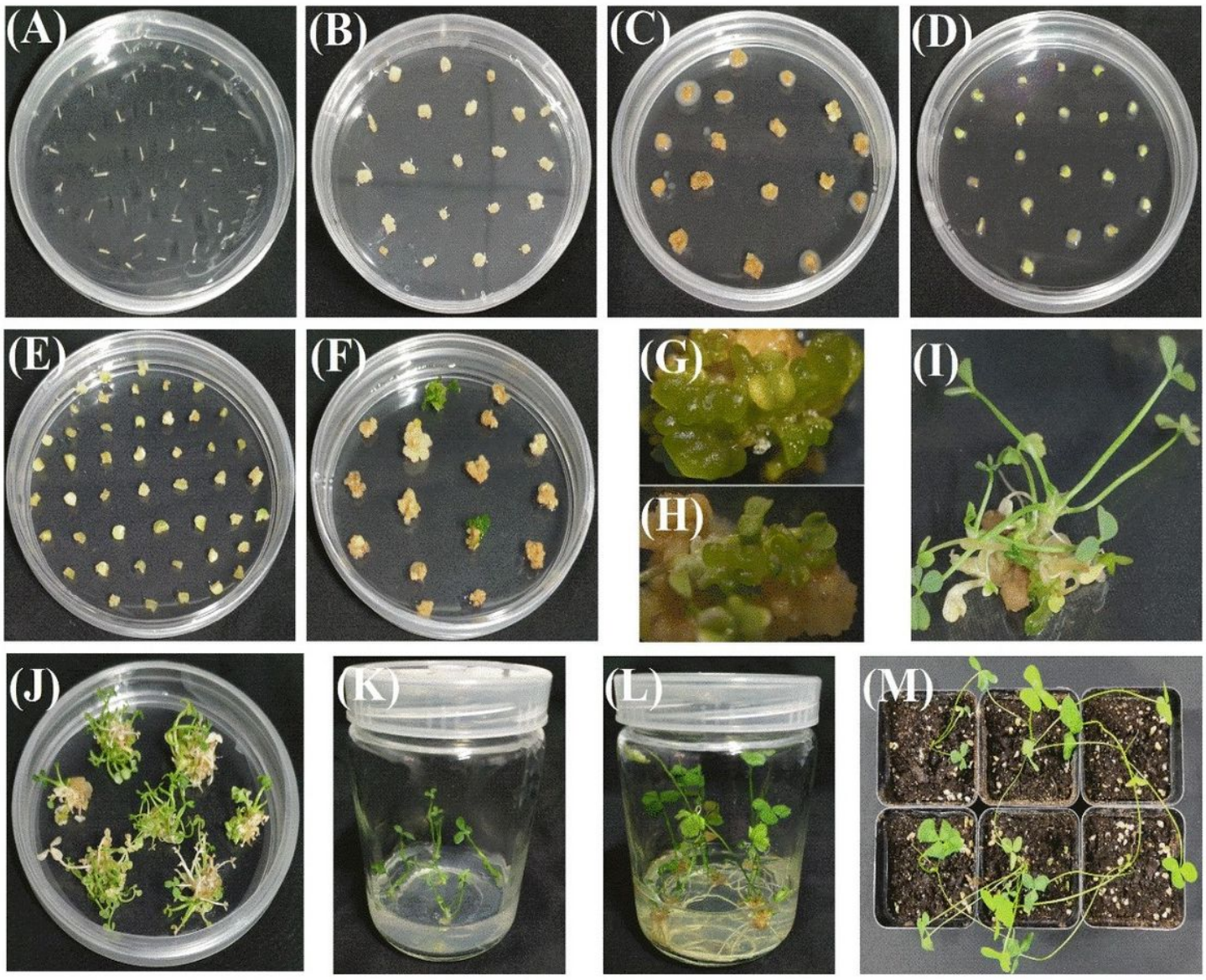

Figure 5

Transgenic white clover obtained through Agrobacterium-mediated transformation using Protocol A and Protocol B. (A) White clover root segments isolated from 4-days-old seedlings. (B) Callus induced after 30 days from roots explants on CCM2. (C) Root-derived calluses after 4 days of co-cultivation with Agrobacterium. (D) Cotyledons explants co-cultured with Agrobacterium after 4 days. (E) Cotyledon-derived calluses induced and selected on CCM2-S. (F) Established calluses transferred onto CDM2-S after 25 days. (G) and (H) Cotyledonary embryo formed from (F). (I) and (J) Proliferating kanamycin-resistant shoots after 60 days on CDM2-S. (K) Independent resistant plantlets transplanted on CRM-S for root development. (L) Rooted transgenic white clover regenerated from callus. (M) Regenerated plants in the soil.

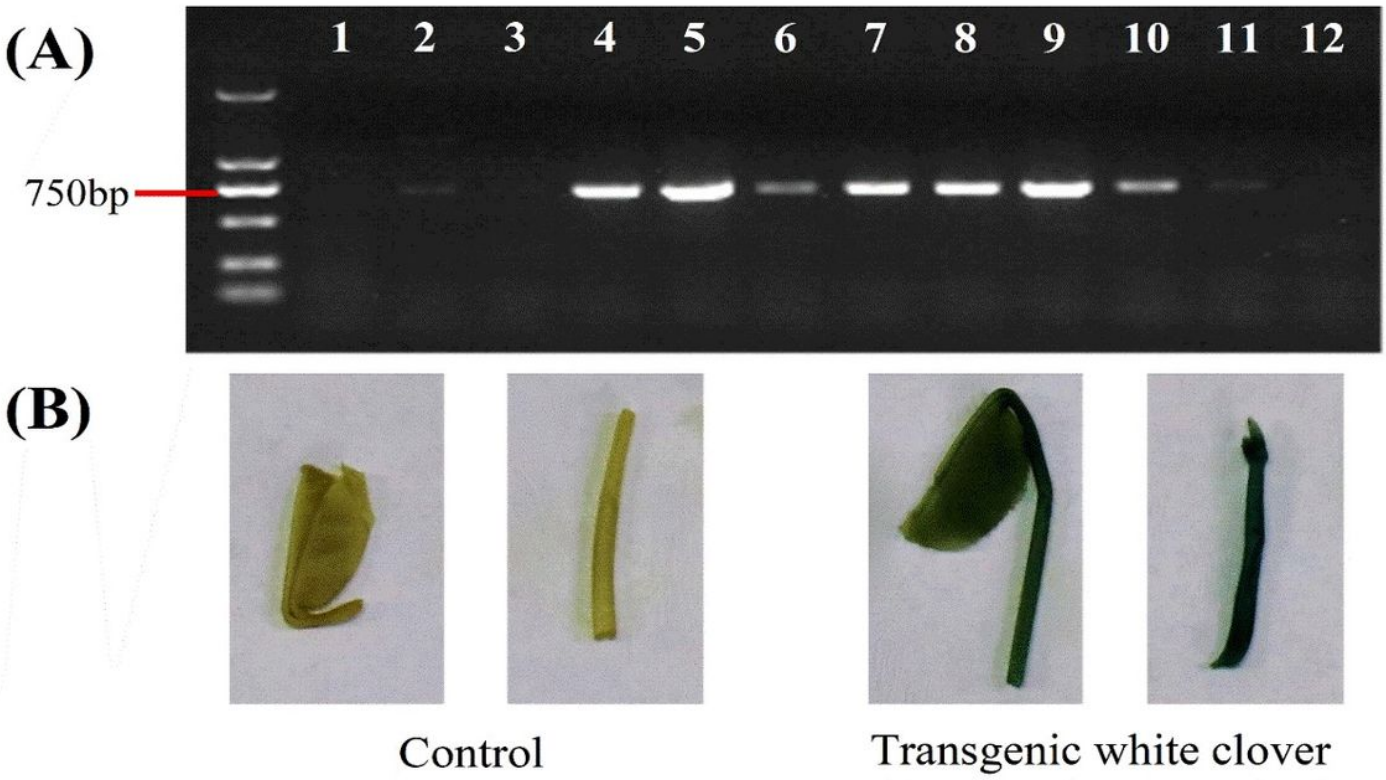

Figure 6

Identification of positive transgenic white clover. (A) PCR amplification for NPTII gene.1: Non-transformed white clover (control). 4-10: Positive transgenic plants. 2-3,11-12: Negative white clover. (B) Histochemical GUS staining for control and positive transgenic white clover.

Supplementary Files

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- SupplementaryMaterial.pdf