Scientia Horticulturae 226 (2017) 302–306

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Research Paper

Establishment of an efficient *Agrobacterium*-mediated genetic transformation method in *Macleaya cordata*

Peng Huang[a,b,1], Min Xu[a,b,1], Liqiong Xia[c], Zhixing Qing[c], Qi Tang[a,b], Wei Liu[b,d], Jianguo Zeng[a,b,c,*]

[a] Horticulture & Landscape College of Hunan Agricultural University, Changsha, 410128, China
[b] National and Local Union Engineering Research Center for the Veterinary Herbal Medicine Resources and Initiative, Hunan Agricultural University, Changsha, 410128, China
[c] School of Pharmacy, Hunan University of Chinese Medicine, Changsha, 410208, China
[d] Center of Analytic Service, Hunan Agriculture University, 410208, China

ARTICLE INFO

Keywords:
Agrobacterium *tumefaciens*
Genetic transformation
Macleaya cordata
Benzylisoquinoline alkaloids

ABSTRACT

Macleaya cordata is an important medicinal plant to produced benzylisoquinoline alkaloids (BIAs) such as sanguinarine (SAN), Berberine (BBR). This study was aimed at improving the BIAs production and disease resistance of *M. cordata*. The leaf and stem explants were vacuum infiltration with *Agrobacterium* for 10 min, followed the explants were placed onto co-cultivation medium (100 μM acetosyringone) for 3 days. Then, the explants were transferred to selected medium (75 mg/l kanamycin and 400 mg/l timentin). Finally, the putative transformants were verified by GUS histochemical assay and PCR amplification. This is the first report on *Agrobacterium*-mediated transformation of *M. cordata* and this method could be providing a regeneration of transgenic *M. cordata* from leaf and stem segments to increase biomass, alkaloids yield and quality in the future.

1. Introduction

Macleaya cordata (Willd.) R. Br. (Chinese name “Bo-luo-hui”) is a perennial herb that belongs to the Papaveraceae family and described in Ben-Cao-Shi-Yi, a Chinese encyclopedia of botany and medicine from the early Tang dynasty. Nowadays, this plant is typically prescribed as a traditional anti-bacterial medicine (Kosina et al., 2010; Lei et al., 2015; Qing et al., 2014; Sai et al., 2015). Many anti-microbial benzylisoquinoline alkaloids (BIAs), such as sanguinarine (SAN), Berberine (BBR) are produced by *Macleaya* spp. (Iagodina et al., 2002; Juskiewicz et al., 2011; Zdarilova et al., 2008; Zeng et al., 2013). More importantly, the *M. cordata* has been widely used in the zootechny and as the major source for global SAN production in the past decade (Zeng et al., 2013). In addition, SAN is an effective anti-cancer drug, which inhibits the growth of colon cancer cells, osteosarcoma cells, non-small cell lung cancer cells, oral squamous cell carcinoma cell, bladder cancer cells (Choi et al., 2008; Han et al., 2013; Kim et al., 2008; Lee et al., 2012; Park et al., 2010; Tsukamoto et al., 2011). BBR also have a high medicinal value, many findings show BBR is a potent agent to the treatment of obesity and a hypolipidemic drug (Kong et al., 2004; Zhang et al., 2014). However, despite of widely distributed in China, Japan, Russia, Ukraine, and other countries (Zhuang and Chuang, 1992), the commercial production of *M. cordata* still has a number of limitations. Firstly, *M. cordata* is a wild species that has not been widely cultivated yet. Secondly, this plant will be affected by the harsh environment (drought, saline land) and pathogenic fungi. Therefore, there is an important value to generate high alkaloid yield per hectare and disease-free cultivars using transgenic methods.

Agrobacterium-mediated transformation has become a common and convenient method to integrate gene into plant at higher efficiencies (Abdallat et al., 2011; Dai et al., 2001; Gao et al., 2008; He et al., 2010; Shewry et al., 2008). Genetic transformation method has been reported to several plant species, such as tobacco (Horsch et al., 1985), rice (Hiei and Komari, 2008), opium poppy (Facchini et al., 2008), Centipedegrass (Liu et al., 2012) and so on.

With rapid increase in market demand on SAN and BBR, it is necessary to expand the supply of raw materials. However, the *M. cordata* is difficult to increase the yield of secondary metabolites through

Abbreviations: MS, Murashige and skoog; IAA, indole-3-acetic acid; 2,4-D, 2,4-Dichlorophenoxyacetic acid; Gus, β-Glucuronidase; nptII, Neomycin phosphotransferase

* Corresponding author at: National and Local Union Engineering Research Center for the Veterinary Herbal Medicine Resources and Initiative, Hunan Agricultural University, Changsha, 410128, China.
E-mail addresses: phuang1985@126.com (P. Huang), xm283138685@126.com (M. Xu), xiahuazhilanman@126.com (L. Xia), zxqing1219@sina.com (Z. Qing), tangqi423@sina.com (Q. Tang), liuwei618@hunau.edu.cn (W. Liu), zengjianguo@hunau.edu.cn (J. Zeng).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.scienta.2017.09.004
Received 9 June 2017; Received in revised form 30 August 2017; Accepted 4 September 2017

conventional breeding due to limited genetic variability. *M. cordata* is faced with many problems including chilling injury, salt stress and attack by diseases and pests. The technology of genetic transformation offers an opportunity to expedite cultivar improvement in *M. cordata* (Abou-Alaiwi et al., 2012; Facchini et al., 2008; He and Gang, 2014; Liu et al., 2012). Therefore, the development of genetic transformation system for *M. cordata* could establish an efficient protocol to enhance SAN production and disease resistance.

There are many resources that already exist to facilitate research in *M. cordata,* including tissue culture protocol (Kohlenbach, 1959, 1999), transcriptome and iTRAQ proteome database (Zeng et al., 2013). Now, the whole genome of *M. cordata* has been sequenced and 14 metabolic genes for SAN and chelerythrine (CHE) biosynthesis were validated (Liu et al., 2017). These genomics and metabolic data will be used for future productions of BIAs by crop improvement or microbial pathway reconstruction. In this study, we describe a transformation and regeneration protocol to produce transgenic *M. cordata*. As far as we know, this is the first study for genetic transformation in *M. cordata*. The PCR methods and histochemical Gus assay were used to confirm this system can be successfully used for the transformation of *M. cordata*.

2. Materials and methods

2.1. Plasmid vectors

Agrobacterium harboring the vector pCAMBIA2301 was used. The pCAMBIA2301 carries a reporter gene, nptII (kanamycin resistance) gene and *β*-glucuronidase gene (*uidA*), in its T-DNA region, both under the control of CaMV 35S promoter (Fig. 1).

2.2. Plant material and regeneration

The lines of *M. cordata* were collected from Fujian province of China with no permission required, and cultivated at Hunan Agricultural University. The tissue culture protocol for *M. cordata* was employed with some modification for this study (Kosina et al., 2010). The young leaves and stem segments explants of *M. cordata* were taken from three months of growth field grown plants. Then, all tissues were washed in running tap 40 min, follow by surface-sterilized with 70% ethanol and then immersing them in 0.1% $HgCl_2$ for 5 min and washed 3 times in distilled water. After it, the explants were inoculated on basal medium composed of MS (Murashige and Skoog 1962), Kinetin (0.1 mg/l), 2,4-D (0.1 mg/l) and IAA (1.0 mg/l) for somatic embryo induction. Friable, embryogenic calluses were selected and transferred to same medium after 3 weeks. Then, embryo populations from a petri dish were transferred to a new medium composed of MS, Kinetin (0.1 mg/l) and IAA (1.0 mg/l) for somatic embryo induction after 2 weeks. All the cultures were under 16/8 h (light/dark) cycle (4500–9000 lx) until

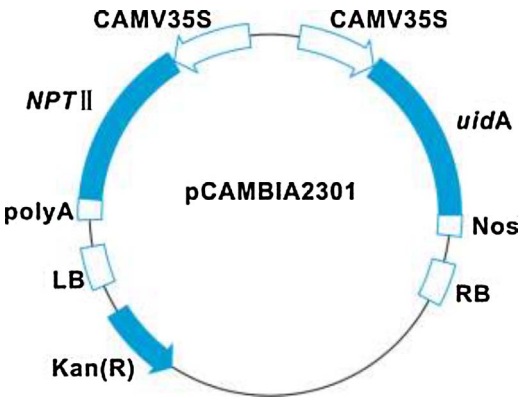

Fig. 1. Binary vector pCAMBIA2301 used for *M. cordata* transformation.

used for transformation. Leaves and stem segments were isolated from regenerated sterile plants for *Agrobacterium* infection.

2.3. Sensitivity test of the explants to kanamycin concentration

The leaf and stem were placed on basal medium containing kanamycin at 0–100 mg/l for selection of transformants. 400 mg/l of timentin were used to control bacterial growth (He and Gang, 2014). Then, all tissues were maintained for 4 weeks.

2.4. Agrobacterium preparation and vacuum infiltration of explants

The *Agrobacterium* GV3101 cultures was overnight at 28 °C with shaking (200 rpm) in LB medium including 50 mg l^{-1} rifamycin and 50 mg l^{-1} kanamycin. The overnight Bacterial strain were centrifugation at 3000 rpm for 5 min. Then use MS with 100 μM AS to re-suspended after centrifugation is complete. The explants were washed and then submerged into filtration medium containing *Agrobacterium* (A600 = 0.6–0.8) in 1 × MS medium. Then, the explants were subjected to vacuum for 10 min and drying on filter paper and transferred onto co-cultivation medium(MS medium contain 100 μM AS)and incubated in darkness at 24 °C for 3 days. Finally, the explants were transferred to selection medium (MS medium contain 75 mg/l kanamycin and 400 mg/l timentin).

2.5. DNA isolation and PCR analyses

DNA was isolated putatively transformed and untransformed plants using an DNA extraction Kit (TaKaRa, MiniBEST Plant Genomic DNA, China) according to instructions. PCR reaction was carried out in a 25 μl mixture [2 μl DNA (50 ng/μl), 12.5 μl mixture Taq DNA polymerase (TAKARA, Ex Taq®, China), 1 μl of each primer (10 μ M) 8.5 μl ddH2O]. The PCR conditions: 94 °C 3 min, 30 cycles of 94 °C 30s, 58 °C 30 s and 72 °C 30s, and 72 °C 10 min. Primers 5′-CTGGGTGGACGATATCACCG-3′ and 5′-GCGAAATATTCCCG TGCACC-3′ were used to amplify a GUS gene (730 bp). Primers 5′-AATATCACGGGTAGCCAACG-3′ and 5′- TGCTCCTGCCGAG AAAGTAT-3′ were used to amplify a nptII gene (364 bp).

2.6. Histochemical GUS assay

Transformed tissues were infiltrated in phosphate buffer (pH 7.0, 50 mM), EDTA (pH 7.5, 10 mM), mannitol (300 mM), X-Gluc (pH 7.0) and 0.5% Triton X-100 for 12 h at 37 °C (Sudan et al., 2006). Then, the tissues were cleared in 70% ethanol.

2.7. Statistical analysis

Data were presented as mean value ± SE and experiments repeated 3 times. Data were analyzed using ANOVA with SPSS 11.09 software, and a Duncan's multiple range test at a 5% level was used to distinguish the differences treatments. Transformation efficiency = Number of transgenic plants/Number of explants.

3. Results

3.1. Vacuum infiltration of explants

Nowadays, the vacuum infiltration has been used for many plant transform to enhance the transformation efficiency (Oliveira et al., 2009; Subramanyam et al., 2015; Supartana et al., 2005; Tague and Mantis, 2006). In order to test the effect of vacuum infiltration, we compared transformation frequency rate between different pressures (normal atmosphere and vacuum pressure treatment). We observed that vacuum infiltration assisted transformation of explants significantly enhances the transformation frequency (Table 1).