Journal of Applied Research on Medicinal and Aromatic Plants 3 (2016) 151–159

Contents lists available at ScienceDirect

Journal of Applied Research on Medicinal and Aromatic Plants

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

Comprehensive screening of influential factors in the *Agrobacterium tumefaciens*-mediated transformation of the Himalayan elixir: *Ajuga bracteosa* Wall. ex. Benth

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ARTICLE INFO

Article history:
Received 30 December 2015
Received in revised form 29 February 2016
Accepted 13 March 2016
Available online 21 March 2016

Keywords:
Agrobacterium tumefaciens
Ajuga bracteosa
Genetic transformation
GUS
Optical density
PCR

ABSTRACT

Ajuga bracteosa is a valuable medicinal plant producing many important compounds, including withanolides, phytoecdysteroids, neo-clerodane-di and triterpenes, iridoid glycosides, flavonoids, etc. These compounds possess a broad spectrum of biological, pharmacological and medicinal properties, but their yield is very low in wild-type plants, and chemical synthesis is not viable. Metabolic engineering strategies offer a promising solution for the bulk production of these natural products. However, these strategies usually require the establishment of an efficient genetic transformation method, which to date has not been reported for *A. bracteosa*. Therefore, the current study was conducted by employing *Agrobacterium tumefaciens* C58C1 harboring the binary plasmid p35SGUSint with *GUS* as the reporter gene and the *NTPII* gene as the selectable marker. The influential factors, e.g. explant type, optical density of agrobacterial culture, inoculation period, co-cultivation duration and acetosyringone concentration, were systematically investigated and the optimal transformation conditions were established by 72 independent transformation experiments. Putative transformants were selected on kanamycin 100 mg/L. MS medium supplemented with 3.6 mg/L BA resulted in maximum regeneration frequency of the transformed explants. The expression of the *GUS* gene was confirmed by histochemistry and polymerase chain reaction. We established that nodal explants of *A. bracteosa* precultured for 3 days, inoculated with a culture of A. *tumefaciens* with an OD of 1.0 for 20 min and co-cultivated for 2 days in MS medium with 200 μM acetosyringone at media pH 5.8 showed 100% transformation induction. This is the first report of a successful *A. tumefaciens*-mediated transformation of *A. bracteosa*.

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1. Introduction

Ajuga bracteosa Wall. ex Benth. (Lamiaceae) is a hairy herb found in temperate regions at 2000 m of altitude (Kaul et al., 2013). It is widely used in folk medicine to treat a variety of diseases, such as gout and malaria, and the aqueous extract of its leaves is diuretic (Pal and Pawar, 2011). It is well known for having significant anti-arthritic (Kaithwas et al., 2012), anti-tumor, antioxidant (Mothana et al., 2012; Rehman et al., 2015), cancer chemopreventive (Ghufran et al., 2009; Kaithwas et al., 2012) and hepatoprotective properties (Hsieh et al., 2011). *A. bracteosa* represented highest usage frequency in a recent quantitative ethnobotanic survey (Barkatullah et al., 2015). *A. bracteosa* is a source of a large number of natural products with potent activities, notably withanoloides, neo-clerodane diterpenoids, phytoecdysteroids, iridoid glycosides and sterols. Withanolides are present in the Solanaceae and five other plant families (Misico et al., 2011). Ecdysteroids, mainly known as insect molting hormones, are present in only 5–6% of the analyzed plant families, 20-hydroxyecdysone being the most common.

Ecdysteroid activities include antioxidant (Kuz'menko et al., 1997, 1998), hepatoprotective (Syrov and Khushbaktova, 2000;

Abbreviations: AS, acetosyringone concentration; BA, 6-benzylaminopurine; CCT, co-cultivation time; *GUS*, β-glucuronidase; IT, inoculation time; Kan, kanamycin; MS, murashige and skoog medium; NAA, naphthaleneacetic acid; *NTPII*, *neomycin phosphotransferase*; OD, optical density; PCR, polymerase chain reaction.

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http://dx.doi.org/10.1016/j.jarmap.2016.03.002
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Syrov et al., 1991a; Tashmukhamedova et al., 1985) and hypoglycemic (Kutepova et al., 2001; Syrov et al., 1991b). Their accumulation in plants is induced by mechanical (Schmelz et al., 1998), insect (Schmelz et al., 1999), and low environmental temperature (Kayani et al., 2014) stresses. Withanolides are reported to inhibit several enzymes, e.g. lipoxygenase, cholinesterase (Israili and Lyoussi, 2009; Kaithwas et al., 2012; Riaz et al., 2007), and cyclooxygenase (COX) (Jayaprakasam and Nair, 2003). Cyasterone and 8-acetylharpagide have shown potent antitumor activity (Takasaki et al., 1999). Additionally, ajugarin I, lupulin A, withaferin A, and reptoside 6-deoxyharpagide are potent anti-inflammatory compounds (Gautam et al., 2011).

The production of these therapeutically valuable secondary metabolites is extremely low in wild *A. bracteosa* plants and their chemical synthesis is impractical and costly (Yang and Stöckigt, 2010). Recent advances in metabolic engineering offer a promising approach to improve the biosynthesis of these natural products, which has been enhanced in several plant species by the overexpression of controlling genes (Arshad et al., 2014; Bonhomme et al., 2000; Bulgakov et al., 2004; Bulgakov, 2008; Shkryl et al., 2011). However, transformed *Ajuga* plants or cell suspension cultures by means of the *Agrobacterium tumefaciens* system have not been reported until now. A few groups have induced the hairy root syndrome in *Ajuga* species by *A. rhizogenes* infection to promote the synthesis of secondary metabolites. Production of 20-HE increased in *A. multiflora* transgenic hairy roots obtained by infection with an *A. rhizogenes* A4 strain (Kim et al., 2005). *A. reptans* var. *atropurpurea* produced hairy roots when infected with *A. rhizogenes* MAFF 03-01724 and showed higher 20-hydroxyecdysone (20-HE) levels (0.14%) compared to the control roots (0.03%) (Matsumoto and Tanaka, 1991). Regenerants derived from these hairy roots sustained the enhanced production of 20-HE in the mother hairy root line, but the clones were dwarf and lacked floral differentiation (Tanaka and Matsumoto, 1993). Hairy roots of *A. raptans* (Uozumi et al., 1993) were co-transformed with *A. rhizogenes* plasmid pTR100 containing the GUS gene under the control of the promoter of a gene encoding the small subunit of ribulose-1,5-bisphosphate carboxylase (rbcS3B) (Uozumi et al., 1996). Regenerants obtained from these co-transformed hairy roots retained GUS activity (Uozumi et al., 1996) but they were abnormal, and possessed shortened internodes, wrinkled leaves and abundant root mass (Choi et al., 2004). Although ecdysteroids are synthesized in roots, and transgenic roots are of interest, the metabolites are then transported to the aerial parts (Bakrim et al., 2008). Leaves in particular can contain significantly higher ecdysteroid levels than roots or other tissues (Kayani et al., 2014).

To the best of our knowledge, *A. tumefaciens*-mediated transformation of any *Ajuga* species has not been previously reported. Consequently, for the biotechnological production of the aforementioned valuable natural products, the establishment of an effective and stable transformation system for *A. bracteosa* is a crucial prerequisite. To address these issues, an extensive series of experiments were conducted to optimize all the influential factors affecting *A. tumefaciens*-mediated genetic transformation of *A. bracteosa*.

2. Materials and methods

2.1. Plant material and its sterilization

A. bracteosa plants (∼45 days old) were collected from Quad-i-Azam University campus in Islamabad, Pakistan (Fig. 1A). Surface sterilization of its aerial parts was done by immersion in sodium hypochlorite (30% v/v) for 20 min with continuous sonication. The aerial parts were then immersed in ethanol (70% v/v) for 1 min, followed by treatment with 0.1% (w/v) mercuric chloride ($HgCl_2$) solution for 30 s, and rinsed several times in sterilized distilled water.

2.2. Explant preparation for in vitro culturing

Reported tissue culture conditions were followed (Kaul et al., 2013) with some modifications. Leaf discs, nodal regions and petiole segments were dissected after sterilization and cultured on shoot induction medium (SIM) (Table 1), which induced multiple shoot formation (Fig. 1B). Some of these shoots were excised and maintained in stable growth medium (SGM) (Table 1). When the explants attained a length of 7–8 cm, they were shifted to a root induction medium (RIM) (Table 1) (Fig. 1C), followed by acclimatization to *ex vitro* conditions (Fig. 1D). Healthy *in vitro* grown plants were selected for explant preparation for bacterial infection. The conditions of the growth room were: temperature 25 ± 1 °C, 16 h of light intensity at 110 μmol m^2/s and an 8-hour dark period. The media pH was maintained at 5.8 throughout the study. The composition of the media used is given in Table 1.

2.3. Kanamycin sensitivity

The *NPTII* gene, which confers resistance to kanamycin, was cloned in the vector p35SGUSint and used as the selection marker. To find the effective dose to eliminate the untransformed explants, the SIM was supplemented with kanamycin (25, 50, 75 and 100 mg/L), maintaining the explants in a growth room for four weeks. The media was refreshed once during the experiment and the number of surviving explants recorded after four weeks (Table 2). The experiment was conducted in three replicates with 30 explants in each replicate.

Table 1
Composition of media used in tissue culture and transformation.

Medium	Abbreviation	Composition
Tissue culture media		
Shoot induction medium	SIM	MS + 0.8% agar + 0.45–3.6 mg/L BA
Root induction medium	RIM	Half strength MS + 0.8% agar
Stable growth medium	SGM	MS + 0.8% agar
Callus induction medium	CIM	MS + 0.8% agar + 0.225 mg/L BA and 1.48 mg/L NAA
Transformation related media		
Pre-culture medium	PC	MS + 0.8% agar
inoculation medium	IM	MS liquid + agrobacteria + acetosyringone
Co-cultivation	CCM	MS + 0.8% agar + acetosyringone
Selection medium	SM1	MS + 0.8% agar + kanamycin 100 mg/L+ Cefotaxime 500 mg/L
Selection medium	SM2	MS + 0.8% agar + kanamycin 75 mg/L+ Cefotaxime 300 mg/L
Selection medium	SM3	MS + 0.8% agar + kanamycin 50 mg/L+ Cefotaxime 150 mg/L
Selection medium	SM4	MS + 0.8% agar + kanamycin 25 mg/L

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