Industrial Crops and Products 83 (2016) 670–679

Contents lists available at ScienceDirect

Industrial Crops and Products

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

Highly efficient plant regeneration and *Agrobacterium*-mediated transformation of *Helianthus tuberosus* L.

Mee-Jin Kim[a,1], Dong-Ju An[a,1], Ki-Beom Moon[a], Hye-Sun Cho[a], Sung-Ran Min[a], Jung-Hoon Sohn[b], Jae-Heung Jeon[a,*], Hyun-Soon Kim[a,*]

[a] *Sustainable Resources Research Center, Korea Research institute of Bioscience and Bioenergy, Daejeon 305-806, South Korea*
[b] *Bioenergy Research Center, Korea Research institute of Bioscience and Bioenergy, Daejeon 305-806, South Korea*

ARTICLE INFO

Article history:
Received 20 July 2015
Received in revised form
10 December 2015
Accepted 19 December 2015
Available online 7 January 2016

Keywords:
Jerusalem artichoke
Shoot regeneration
Regenerated plants
Stable transformation

ABSTRACT

In our current study, to develop an efficient regeneration and transformation system of *Helianthus tuberosus*, we verified effects of plant growth hormones, growth conditions, explant type, and transformation conditions. Leaf segments from regenerated shoots showed higher regeneration efficiency on MS basal medium containing 1.0 mg L^{-1} zeatin under darkness than those from maintained *in vitro* plant. To carry out transformation, various parameters including plant materials, *Agrobacterium* cell density, immersion time, and *Agrobacterium* strains (leaf segment, OD_{600}-0.6, 60 min, and *Agrobacterium tumefaciens* LBA4404 harboring binary vector pCAMBIA1301) have been determined. The putatively transformed *H. tuberosus* were screened by survival rate and beta-glucuronidase (GUS) histochemical assay following two cycles of 3.0 mg L^{-1} hygromycin selection at callus stage. The presence of the selectable marker gene hygromycin phosphotransferase and the GUS reporter gene with intron was then confirmed by genomic PCR, Southern blot, reverse transcriptase PCR (RT-PCR) and GUS histochemical assay. The method presented here could be helpful in genetic improvement of *H. tuberosus* through efficient shoot regeneration and stable *Agrobacterium*-mediated transformation.

1. Introduction

The *Helianthus tuberosus* L. (Jerusalem artichoke) belongs to the Asteraceae family, and is resistant to most pests, diseases, and harsh conditions such as frost and drought (Gunnarsson et al., 2014). The plant can grow up to 3 m tall with considerable aerial biomass of over 100 tonnes per hectare (t/ha), and produces tubers under the ground (Stauffer et al., 1981). The principal storage carbohydrate in *H. tuberosus* is inulin, which comprises 10–20% of fresh tuber weight, and is used to manufacture dietary fiber, animal feed, high fructose syrup, as well as bioethanol or biochemical materials by microorganism fermentation (Flamm et al., 2001; Li et al., 2013).

With so many applications, it is important to ensure that *H. tuberosus* usage for sustainable biofuel or biochemical production does not compete with the food supply (Cheng et al., 2009; Li et al., 2013). Improving the genetic diversity of *H. tuberosus* would help meet demand. However, *H. tuberosus* has hexaploid genetic complexity (2n = 6× = 102) (Schilling and Heiser, 1981) and the limited genetic diversity for cultivar improvement (Janket et al., 2013). Furthermore, this plant reproduces through vegetative propagation of the storage tuber organ, similar to the potato, sweet potato, and yacon. These factors make it difficult to develop new *H. tuberosus* varieties via conventional breeding. Thus, establishing a gene introduction system using new biotechnological techniques is critical for *H. tuberosus* improvement for multiple uses. The most important prerequisite for genetic transformation is the establishment of an efficient and reproducible regeneration method.

H. tuberosus shoot regeneration of has been only sparsely studied (Pugliesi et al., 1993; Bianchi et al., 1999; Taha et al., 2007; El Mostafa et al., 2008; Karadag et al., 2013). Available reports demonstrate plant regeneration through adventitious organogenesis from stem segments on (MS) Murashige and Skoog (1962) medium with lower kinetin concentrations (Karadag et al., 2013), and from cotyledon explants on MS basal medium supplemented with high cytokinin concentration and lower auxin concentration (Pugliesi et al., 1993). High embryogenic potential has been achieved with cells of regenerated plants, regardless of the particular organ of *H. tuberosus* (Fambrini et al., 1996, 1997). Additionally, a remarkable regeneration frequency has been obtained from leaves treated

Abbreviations: BA, benzyl adenine; KIN, kinetin; IAA, indole-3-acetic acid; NAA, naphthalene acetic acid; PCR, polymerase chain reaction.

* Corresponding authors.
E-mail addresses: jeonjh@kribb.re.kr (J.-H. Jeon), hyuns@kribb.re.kr (H.-S. Kim).
[1] These authors contributed equally to this work.

http://dx.doi.org/10.1016/j.indcrop.2015.12.054
0926-6690/

with BAP or NAA (El Mostafa et al., 2008). Studies of *Helianthus* species also show a strong relationship between the genetic background and *in vitro* regeneration response (Paterson and Everett, 1985; Sarrafi et al., 1996), suggesting that somatic embryogenesis or organogenesis may be under genetic control. For example, Bianchi et al. (1999) demonstrated that of 38 *H. tuberosus* genotypes, only HTPI-15 showed remarkable regeneration frequency.

Agrobacterium-mediated transformation has been widely used to introduce foreign genes into many plant species with single or low copy numbers of transgenes. However, for many important plants (including woody and perennial species), this method has either not yet been established or is laborious and inefficient. Recently, increasing research has focused on the functional genomics (Jung et al., 2014; Bock et al., 2014) or metabolomics of *H. tuberosus*. No studies have yet examined genetic transformation in *H. tuberosus*, but a few have investigated genetic transformation in other sunflowers of the *Helianthus* species (Knittel et al., 1994; Rao and Rohini, 1999; Müller et al., 2001; Mohamed et al., 2006; Liu et al., 2011; Sujatha et al., 2012).

In the present study, we established a method for reproducible and high-efficiency shoot regeneration and reliable genetic transformation in *H. tuberosus*. This method allows the manipulation and production of genetically transformed plants for improving genetic variety and functional genomics studies of *H. tuberosus*. To our knowledge, this is the first report of successful gene insertion into the *H. tuberosus*.

2. Materials and methods

2.1. Plant materials

H. tuberosus L. JA3 clone (Lim, 1997) which is characterized with purple skin, single unit and round shape tuber, and late maturity was used as our starting material. Tubers were thoroughly rinsed in sterile distilled water for 20 min, then surface disinfected with a 5-min wash in 70% (v/v) ethanol and a 10-min wash in 2.5% (v/v) sodium hypochloride solution containing 0.01% Triton X-100. Finally, the surface disinfected tubers were rinsed three times in sterile distilled water, and placed on solidified MS basal medium to induce shoots. The shoots induced from tubers were propagated *in vitro* by microcutting, and were subcultured every two weeks. Leaves from maintained *in vitro* plants were used as source of material for shoot regeneration or cocultivation with *Agrobacterium tumefaciens*.

2.2. Media composition and culture conditions

MS basal medium for shoot induction consisted of MS salts, vitamins, 100 mg L^{-1} myo-inositol, 30 g L^{-1} sucrose, and various combinations of different concentrations of plant growth hormones (Fig. 1). Relative to the basal medium, the rooting medium contained half-strength of MS salts and 2% of the sucrose concentration, along with vitamins, 100 mg L^{-1} myo-inositol, and no hormones. The pH for all culture media was adjusted to 6.0 prior to autoclaving, and the media were solidified with 8 g L^{-1} plant agar (Duchefa, Netherlands). Cultures were maintained at 24 ± 1 °C under 16 h of light/8 h of darkness (16L/8D) conditions (60 μmol photon m^{-2} s^{-1} of light fluence). Unless otherwise stated, all biochemical and medium components of molecular biology grade or plant tissue culture grade were purchased from Sigma Chemical Company (St. Louis, MO).

2.3. Shoot regeneration of tuberosus

To establish an efficient regeneration system in *H. tuberosus*, leaf segments (0.5 ± 0.2 cm^2) from maintained *in vitro* plants grown directly from tubers (maintained plants) and regenerated shoots were placed on shoot induction medium containing different combinations of plant growth hormones, i.e., BA 0.5–2.5 mg L^{-1} and NAA 0.05–1.0 mg L^{-1}, KIN 0.5–2.5 mg L^{-1} and NAA 0.05–1.0 mg L^{-1}, BA 0.5–2.5 mg L^{-1} and IAA 0.05–1.0 mg L^{-1}, or KIN 0.5–2.5 mg L^{-1} and IAA 0.05–1.0 mg L^{-1}. For comparisons of regeneration efficiency according to subculture times, regenerated shoots were transferred to the fifth subculture via shoot apical cuttings. Leaf segments of regenerated shoots were placed on MS basal medium supplemented with 0.5–3.0 mg L^{-1} zeatin. Total 100 leaf segments were used per treatment, and each treatment was repeated thrice. At four weeks after placing explants on media, we recorded the number of leaf segments that formed shoots per treatment. After two weeks of shoot formation, the elongated shoots were individually separated and transferred to rooting medium. Upon rooting, the plantlets were transferred to sterilized soil and grown to maturity. For statistical analysis, all experiments were repeated three times and the data were subjected to one way ANOVA with statistical significance test. The significant difference among the mean ± standard error was carried out using Duncan Fig. S4. Effects of antibiotics, kanamycin (left panel) and basta (right panel), concentration on callus induction of *H. tuberosus* L. leaf segments after four weeks. Data are means from three independents experiments, each with 100 segments per treatment. For each vertical bar represents the standard error of the mean's multiple range test (SPSS 18.0) and significance level of $P < 0.05$. The best medium and explants source determined from this experiment were utilized in the subsequent *Agrobacterium*-mediated transformation experiments.

2.4. Hygromycin sensitivity of leaf segments

The presently used gene construct harbored the hygromycin phosphotransferase (*hpt*II) gene as a marker for selection of transgenic plants on hygromycin. To determine the effective dose for the selection of putative transformants, non-infected leaf segments from *in vitro* plants were cultured on shoot induction medium containing varying hygromycin concentrations (1.0, 3.0, 5.0, 7.0, 10.0, and 20.0 mg L^{-1}). After four weeks of culturing, survival frequency was scored as the percentage of explants producing callus and buds of the total leaf segments. All results are presented as the mean of three independent experiments, with 100 leaf segments per treatment.

2.5. Agrobacterium-mediated transformation

The genetic transformation of *H. tuberosus* was performed using *A. tumefaciens* strain LBA4404 harboring the binary plasmid pCAMBIA1301 (CAMBIA International Research and Education Organization, Canberra, AU). The 11.8-kb pCAMBIA1301 plasmid contains the selectable marker gene *hpt*II and the GUS reporter gene with intron region in the T-DNA driven by the cauliflower mosaic virus 35S promoter and the terminator of nopaline synthase (nos) gene (www.cambia.org).

Leaf discs were immersed in a fresh overnight bacterial suspension mixture (OD_{600} of 0.6) for 15, 30, 45, and 60 min, and were then surface-dried on sterilized filter paper to remove excess bacteria. The leaf segments and bacteria were co-cultured for 3 days at 25 °C in the dark onto MS basal medium containing 1.0 mg L^{-1} zeatin and no antibiotics. Then, the infected leaf segments were rinsed thoroughly in sterile water containing 500 mg L^{-1} carbenicillin, completely dried on the sterilized filter paper, and placed on the medium containing 1.0 mg L^{-1} zeatin, 3.0 mg L^{-1} hygromycin, and 500 mg L^{-1} carbenicillin for shoot regeneration under dark conditions. Every three weeks, this transfer to fresh selective medium was repeated to maintain the hormonal effects and to prevent