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Original Paper

Establishment of bispyribac selection protocols for *Agrobacterium tumefaciens*- and *Agrobacterium rhizogenes*-mediated transformation of the oil seed plant *Jatropha curcas* L.

Masataka Kajikawa[1], Kaoru Morikawa[1], Masayo Inoue[1], Utut Widyastuti[2], Sony Suharsono[2], Akiho Yokota[1], Kinya Akashi[1,3,*]

[1] Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma, Nara 630-0192, Japan; [2] Research Center for Bioresources and Biotechnology, Bogor Agricultural University, Gd. PAU, Kampus IPB Darmaga, Bogor 16680, Indonesia; [3] School of Agricultural, Biological and Environmental Sciences, Faculty of Agriculture, Tottori University, Tottori 680-8553, Japan

* E-mail: akashi.kinya@muses.tottori-u.ac.jp Tel & Fax: +81-857-31-5352

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Abstract Cultivation of the biofuel plant *Jatropha* (*Jatropha curcas* L.) has spread around the world because of its drought resistance, high seed oil content, and adaptability to different environmental conditions. Because of these attributes, *Jatropha* has the potential to be one of the main resources for next-generation biodiesel fuel. To improve the productivity of *Jatropha* biomass, it is important to understand the molecular functions of key *Jatropha* genes, and to modify various agronomic traits of *Jatropha* via molecular breeding. A reliable and efficient protocol for genetic transformation of *Jatropha* is a prerequisite for molecular biology research and breeding on this plant. Here, we developed a system in which the herbicide bispyribac sodium salt, which inhibits acetolactate synthase, was used as the selection agent, and a two-point-mutated acetolactate synthase gene (*mALS*) was used to confer resistance upon transformants. Application of this system significantly improved the efficiency of *Agrobacterium tumefaciens*-mediated stable transformation of the high-yielding elite *Jatropha* population, IP-2P. The bispyribac-mALS system was also successfully applied in the *Agrobacterium rhizogenes*-mediated hairy roots system, which allowed integration of a foreign gene and expression in *Jatropha* root tissues within 2 weeks. The new protocols described here are powerful tools not only for functional studies on endogenous genes, but also for the molecular breeding of *Jatropha* to develop elite varieties.

Key words: *Jatropha curcas*, *Agrobacterium tumefaciens*, *Agrobacterium rhizogenes*, acetolactate synthase, bispyribac.

Jatropha (*Jatropha curcas* L.) is a perennial shrub belonging to the family *Euphorbiaceae*. It is thought to have originated in Central America, but it is now distributed in tropical and subtropical regions worldwide. *Jatropha* is well adapted to grow in arid and semi-arid regions and is drought resistant (Sudhakar Johnson et al. 2011). It can grow on degraded soil unsuitable for food crops (Fairless 2007). The high oil content in the seeds means that it has great potential for sustainable biodiesel production (Openshaw 2000). However, several undesirable traits of *Jatropha* limit its commercial use. The seed yield is low and unstable depending on the growth conditions and environment. At present, cultivation of this plant provides relatively low economic returns (Openshaw 2000; Sudhakar Johnson et al. 2011). To improve the economic returns from *Jatropha*, breeders must develop a superior genotype that can provide high and stable seed yields in degraded and marginal lands. Genetic engineering is a practical technique to improve *Jatropha* that will complement conventional breeding. For successful genetic modification, efficient in vitro plant regeneration and stable genetic transformation systems are essential.

Recently, candidate genes involved in fatty acid biosynthesis, biotic and abiotic stress tolerance mechanisms, and regulation of secondary metabolites and phorbol esters, which are considered as important breeding targets, have been identified from whole genome sequencing analyses (Sato et al. 2011), proteomics (Yang et al. 2009) and transcriptomics studies (Costa et al. 2010; Natarajan et al. 2010; Natarajan and Parani 2011). Knowledge of their molecular functions will accelerate the process of molecular breeding of *Jatropha*. A reproducible and efficient protocol for the genetic manipulation of *Jatropha* will be useful to unravel the functions of genes in this plant.

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There are many reports of in vitro regeneration of plants from different tissues of *Jatropha*, including leaves (Sujatha and Mukta 1996; Sujatha et al. 2005; Jha et al. 2007; Deore and Johnson 2008; Khurana-Kaul et al. 2010), cotyledon (Khemkladngoen et al. 2011b; Kumar et al. 2010a; Varshney and Johnson 2010), petioles (Sujatha and Mukta 1996; Kumar and Reddy 2010; Kumar et al. 2011), hypocotyls (Sujatha and Mukta 1996; Kaewpoo and Te-chato 2010; Sharma et al. 2011), epicotyls (Wei et al. 2004; Kaewpoo and Te-chato 2010), nodal segments (Datta et al. 2007), axillary nodes (Sujatha et al. 2005), shoot tips (Rajore and Batra 2005; Purkayastha et al. 2010), and stems (Singh et al. 2010). Based on these regeneration protocols, stable transformation of *Jatropha* has been reported by several groups. Li et al. (2008) first reported the establishment of transgenic *Jatropha* from cotyledon explants by *Agrobacterium tumefaciens*-mediated transformation using the herbicide phosphinothricin as the selection agent. Recently, several groups have reported that transgenic *Jatropha* can be regenerated by hygromycin (Mao et al. 2010; Kumar et al. 2010b) and kanamycin selection (Pan et al. 2010; Khemkladngoen et al. 2011a). In most cases, there were substantial differences in the effects of plant growth regulators and optimized selection conditions, suggesting that there is large variation among different *Jatropha* genotypes and tissues in terms of their morphological responses to phytohormones. Optimization of the regeneration and transformation procedure for each variety of *Jatropha* is of pivotal importance, especially for high-yielding varieties that have been recently established by conventional mass selection and breeding (Basa and Sujatha 2009).

The system in which transgenic hairy roots are induced by *Agrobacterium rhizogenes* was proposed as a rapid and effective transformation system for studying gene function in plant roots (Guillon et al. 2006a, b). The mechanism underlying hairy roots formation is the transfer of several genes from the root-inducing plasmid from *A. rhizogenes* to the genome of the infected plant cells. Transgenic hairy roots are induced shortly after infection (Guillon et al. 2006a, b). This technique potentially offers more rapid genetic manipulation of *Jatropha* for analyzing gene function in roots, compared with the conventional *A. tumefaciens*-mediated stable transformation, which requires more time because of the long life cycle of *Jatropha*.

IP-2P is a high-yielding Indonesian population of *Jatropha* that was established via mass selection (Sinaga and Murniati 2010; Surahman et al. 2009). Initially, we attempted to establish genetic transformation protocols for this population using conventional selection agents such as hygromycin, kanamycin, and phosphinothricin. However, we found that these methods showed low reproducibility and efficiency with this population. Recently, a system based on the herbicide bispyribac has been developed as a novel transformation system for higher plant species (Kawai et al. 2007). In this system, bispyribac sodium salt is used as the selection agent. This herbicide inhibits acetolactate synthase (ALS), an enzyme involved in branched-chain amino acid biosynthesis. A two-point mutated ALS gene (mALS), which confers resistance to bispyribac sodium salt, is used as the selection marker for plant transformation. In the present study, we describe the successful application of this system to establish both *A. tumefaciens*-mediated and *A. rhizogenes*-mediated genetic transformation systems for *Jatropha* high-yielding population IP-2P.

Materials and methods

Plant materials

Seeds of *J. curcas* (L.) population IP-2P from Indonesia were used as the plant material. After removing the outer seed coat, the seeds were surface-sterilized for 10 min with 5% (v/v) sodium hypochlorite and 0.1% (v/v) Tween-20, and then rinsed five times with sterile water. The sterilized seeds were germinated in vitro on hormone-free Murashige and Skoog (MS) medium (Murashige and Skoog 1962) containing 0.8% (w/v) agar at 25°C in the dark for 5 days. The seedlings were grown at 25°C under a 16-h light (100 μmol photon $m^{-2} s^{-1}$)/8-h dark photoperiod for 7 days before preparing explants.

Plant regeneration from cotyledon and hypocotyl explants

To prepare explants, cotyledons were cut into pieces (5×5 mm), and hypocotyls were cut into 5-mm segments. The explants were placed on 33 different kinds of callus induction medium (CIM) consisting of MS medium supplemented with various combinations of phytohormones; N^6-benzyladenine (BA) (0, 0.5, 1.5, or 4.5 mg l^{-1}), thidiazuron (TDZ) (0, 0.5, or 1 mg l^{-1}), and 3-indolebutyric acid (IBA) (0.05, 0.25, or 1 mg l^{-1}) (see Supplemental Tables 1 and 2). Explants were incubated for 10 days at 25°C in the dark. Calli that developed on the explants were transferred to four different kinds of shoot regeneration medium (SRM) consisting of MS medium supplemented with various combinations of phytohormones: BA (1.5 or 4.5 mg l^{-1}), IBA (0.05 mg l^{-1}), and gibberellin A_3 (GA_3) (0 or 0.5 mg l^{-1}) (see Supplemental Tables 1 and 2). The calli were incubated for 3 months at 25°C under a 16-h light (100 μmol photon $m^{-2} s^{-1}$)/8-h dark photoperiod. The regenerated and elongated shoots (2–3 cm in length) were transferred to root induction medium (RIM) consisting of MS or half-strength MS medium supplemented with IBA (0, 0.05, 0.25, or 1 mg l^{-1}), 0.3% (w/v) gellan gum, and 1% (w/v) active charcoal. The frequency of root induction was evaluated after 6 weeks of incubation. The plants with induced roots were transferred into soil and grown under a 16-h light (300 μmol photon $m^{-2} s^{-1}$)/8-h dark photoperiod at 28/23°C.

Agrobacterium strains and binary vectors

The binary vectors pIG121-Hm (kanamycin and hygromycin selection; Ohta et al. 1990, Akama et al. 1992), pBIN30 (bar selection; Miyashima et al. 2011), and A2 (bispyribac selection, PalSelect system, Kumiai Chemical Industry, Tokyo, Japan) were introduced into *A. tumefaciens* strain LBA4404 and used for stable transformation. To construct the A2-IG vector, an intron-containing β-glucuronidase (*gus*) gene expression cassette was amplified from pIG121-Hm by PCR using the primers 5′-AAG CTT GCA TGC CTG CAGG-3′ (forward) and 5′-CCG ATC TAG TAA CAT AGA TGAC-3′ (reverse). The PCR product was phosphorylated by T4 DNA kinase (Toyobo, Osaka, Japan) and cloned into the *Sma*I site of pBIN30. The fragment containing the GUS gene expression cassette was subsequently subcloned into the *Asc*I and *Kpn*I sites of the A2 vector. The resultant A2-IG vector was introduced into *A. rhizogenes* strain ATCC15834 (American Type Culture Collection (ATCC), Manassas, VA) and used for hairy root induction.

A single colony of the transformed *Agrobacteria* was used to inoculate liquid YEB medium supplemented with hygromycin (50 $mg\,l^{-1}$), kanamycin (50 $mg\,l^{-1}$), and rifampicin (50 $mg\,l^{-1}$) to select LBA4404 cells harboring pIG121-Hm, or with spectinomycin (50 $mg\,l^{-1}$) and rifampicin (50 $mg\,l^{-1}$) to select LBA4404 cells harboring pBIN30 and A2, or with spectinomycin (50 $mg\,l^{-1}$) to select ATCC15834 cells harboring A2-IG. Bacterial cultures were grown overnight at 28°C in the dark. *Agrobacterium* cells were collected by centrifugation at 5,000 rpm for 10 min at room temperature. The cells were resuspended in liquid co-cultivation medium consisting of MS medium supplemented with 1 $mg\,l^{-1}$ TDZ, 0.05 $mg\,l^{-1}$ IBA, and 20 $mg\,l^{-1}$ acetosyringone (AS). The cell density was adjusted to an OD_{600} of 0.25–0.35 before co-cultivation in transformation experiments.

Table 1. Effects of concentrations of IBA and MS salts on induction of roots from *Jatropha* shoots.

Strength of MS salts	IBA (mg/l)	Number of tested shoots	Number of root-induced shoots (%)[a]
1/1	0	25	0 (0)
	0.05	25	0 (0)
	0.25	25	0 (0)
	1	25	0 (0)
1/2	0	25	1 (4)
	0.05	25	3 (12)
	0.25	25	4 (16)
	1	25	3 (12)

[a] Number of shoots from which roots were induced on RIM within 6 weeks. Rooting efficiency (%) is shown in parentheses.

Stable transformation using cotyledon explants

Cotyledons from 12-day-old plants were cut into 5×5 mm pieces and incubated with *A. tumefaciens* LBA4404 cells harboring the A2 vector in liquid hormone-free MS medium containing 20 $mg\,l^{-1}$ AS for 10 min at room temperature. The explants were transferred to a solid co-cultivation medium consisting of MS medium supplemented with 1 $mg\,l^{-1}$ TDZ, 0.05 $mg\,l^{-1}$ IBA, 20 $mg\,l^{-1}$ AS, 1% (w/v) polyvinylpyrrolidone (PVP), and 0.8% (w/v) agar, and co-cultured for 2 days at 22°C in the dark. Subsequently, the explants were washed five times with sterile water and three times with 300 $mg\,l^{-1}$ moxalactam sodium solution (LMOX; Shiomarin, Shionogi, Osaka, Japan) to remove *A. tumefaciens* cells (Ogawa and Mii 2007). The explants were transferred to the selective medium CIM-1, which consisted of MS medium supplemented with 10 nM bispyribac sodium salt, 1 $mg\,l^{-1}$ TDZ, 0.05 $mg\,l^{-1}$ IBA, 100 $mg\,l^{-1}$ LMOX, 1% (w/v) PVP, and 0.8% (w/v) agar. After 10–21 days, resistant calli were transferred to the selective medium SRM-1, which consisted of MS medium supplemented with 10 nM bispyribac sodium salt, 1.5 $mg\,l^{-1}$ BA, 0.05 $mg\,l^{-1}$ IBA, 100 $mg\,l^{-1}$ LMOX, 1% (w/v) PVP, and 0.8% (w/v) agar. After 2–3 months, resistant shoots were cut from the calli and transplanted onto fresh selective SRM. After 1 month, the healthy shoots were subjected to genomic PCR and RT-PCR analyses. To induce roots, PCR-positive shoots were transferred to RIM consisting of half-strength MS medium supplemented with 0.25 $mg\,l^{-1}$ IBA and 50 $mg\,l^{-1}$ LMOX. For hygromycin, kanamycin, and phosphinothricin selection, transformation experiments were carried out according to methods described in the previous reports (Mao et al. 2010; Pan et al. 2010; Li et al. 2008) using the pIG121-Hm vector harboring both *hpt* and nptII, or the pBIN30 vector for phosphinothricin resistance.

Hairy root induction from explants

Hypocotyls, cotyledons, and roots from 4-day-old seedlings were cut into 5-mm segments and incubated with *A. rhizogenes*

Table 2. Comparison of selection markers for screening of transgenic *Jatropha* shoots.

Antibiotics and concentration	Vector	Number of explants	Number of regenerated shoots	Number of PCR-positive shoots (% of total explants)
5 mg/l Hygromycin[a]	pIG121-Hm	900	0	—
20 mg/l Kanamycin[b]	pIG121-Hm	300	0	—
1 mg/l Phosphinotricin[c]	pBIN30	5400	46	5 (0.1)
100 nM Bispyribac	A2	300	0	—
10 nM Bispyribac	A2	300	58	13 (4.3)
5 nM Bispyribac	A2	300	120	5 (1.7)

Experiments using hygromycin, kanamycin, and phosphinotricin were carried out according to methods reported by Mao et al. (2010)[a], Pan et al. (2010)[b], and Li et al. (2008)[c], respectively.

ATCC15834 cells harboring the A2-IG vector in liquid hormone-free MS medium for 10 min at room temperature. The explants were transferred to hormone-free MS medium and co-cultured for 2 days at 25°C in the dark. After co-cultivation, explants were washed five times with sterile water containing 75 mg l^{-1} meropenem trihydrate (MEPM; Meropen, Dainippon Sumitomo Pharma, Osaka, Japan) to remove the *A. rhizogenes* cells (Ogawa and Mii 2007). To induce transgenic hairy roots, the explants were transferred to selective hairy RIM consisting of MS medium supplemented with 5 nM bispyribac sodium salt, 25 mg l^{-1} MEPM, and 0.8% (w/v) agar. The selection and induction steps were conducted at 25°C under continuous light supplied by white fluorescent tubes (50–100 µmol photon $m^{-2}s^{-1}$). After 2 weeks, the healthy adventitious roots were subjected to genomic PCR, RT-PCR, and histochemical GUS analyses.

Genomic DNA extraction and polymerase chain reaction (PCR) amplification

Total genomic DNA was extracted from regenerated shoots and adventitious roots using a Plant DNeasy Mini kit (Qiagen, Hilden, Germany). Approximately 50 ng genomic DNA was used as the template for genomic PCR analyses. The primer pair used to detect the mutated ALS gene expression cassette in the A2 and A2-IG vectors was as follows: AtALS-F, 5′-CCA AAC CCG AAA CAT TCA TC-3′ and AtALS-R, 5′-CGA GGG ACT TGT CCT GTG AT-3′. To detect the *bar* gene in the pBIN30 vector, we used the b1 and b2 primers described by Li et al. (2008). The reaction conditions were as follows: 2-min melting step at 94°C, followed by 30 amplification cycles of a 30-s melting step at 94°C, a 30-s annealing step at 55°C, and a 30-s elongation step at 72°C, using 1 unit Ex Taq DNA polymerase (Takara Bio, Tokyo, Japan). PCR products were analyzed by electrophoretic separation on 1% agarose gels and stained with ethidium bromide.

Total RNA extraction and reverse transcription (RT)-PCR amplification

RT-PCR was performed as described previously (Kajikawa et al. 2010) with the following modifications: total RNA was extracted from independent transgenic hairy root lines using a Plant RNA Isolation Mini kit (Agilent, Wilmington, DE) and subjected to DNase (Qiagen) treatment for 15 min at 25°C. Then, the DNase-treated RNA was purified on an RNeasy spin column (Qiagen). Total RNA (1 µg) was used for reverse transcription using ReverTra Ace -α- (Toyobo, Osaka, Japan). Then, 1 µl of the resulting cDNA was added to a 20-µl PCR reaction mixture containing 1 unit Ex Taq DNA polymerase. The primers used to detect the intron-spliced GUS gene transcript were GUS-exon-F and GUS-01R, as described previously (Kajikawa et al. 2010). The primers used to detect *mALS* gene transcript were AtALS-F and AtALS-R, as described above. The primers used to detect the endogenous *actin* gene transcript, which was used as the RT-PCR control, were as follows: JCactin-RT-F, 5′-AGA CCT CCA AAA CCA GCT CA-3′, and JCactin-RT-R, 5′-TTG ATT TTC ATG CTG CTT GG-3′.

Histochemical analysis

Histochemical GUS assays were conducted on induced hairy roots 2 weeks after co-cultivation with *A. rhizogenes* harboring A2-IG. Tissues were incubated overnight at 37°C in a GUS-staining solution containing 50 mM potassium phosphate buffer (pH 7.0), 5 mM DTT, 0.1% (v/v) Tween-20, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 20% (v/v) methanol, and 1 mM 5-bromo-4-chloro-3-indoyl-β-D-glucuronide (X-gluc). The tissues were then soaked in 70% (v/v) ethanol for several hours to visualize the GUS stain.

Results and Discussion

Optimization of shoot and root regeneration protocols

To establish a regeneration system for the high-yielding population IP-2P of *Jatropha*, we first examined the effects of several phytohormones on the efficiency of shoot regeneration from hypocotyl and cotyledon explants. The explants were initially incubated for 10 days on 33 different types of CIM, which contained various concentrations of BA, TDZ, and IBA as shown in Fig. 1 and Supplementary Tables 1 and 2. Then, the explants were transferred to four different types of SRM containing various concentrations and combinations of BA, IBA, and GA_3 (Supplementary Tables 1–2), and incubated for 3 months before evaluating the frequency of shoot regeneration. In total, we examined 132 different combinations of phytohormones in the CIM and SRM for both hypocotyl and cotyledon explants.

When hypocotyl explants were used as the starting materials, the regeneration frequency was relatively low (Figs. 2A–D and Supplemental Table 1). Among 132 different media conditions examined in this study, the highest regeneration frequency was 0.2 shoot/explant (Supplementary Table 1). Morphological observations revealed that although vigorous callus formation occurred on these CIM (Fig. 2B), the shoot induction rate was very poor in the subsequent SRM conditions used in this study, and most of the explants turned brown and died (Figs. 2C–D).

In contrast, the shoot regeneration frequency was higher when cotyledon explants were used as the starting materials. The most efficient shoot regeneration (1.11 shoot/explant) was obtained after callus induction on the CIM containing 1 mg l^{-1} TDZ and 0.05 mg l^{-1} IBA (hereafter denoted as CIM-1) and shoot induction on the SRM containing 1.5 mg l^{-1} BA and 0.05 mg l^{-1} IBA (denoted as SRM-1) (Fig. 1, Fig. 2E–G, and Supplementary Table 2). When using SRM-1, two other CIM conditions also gave high shoot regeneration frequencies (>1 shoot/explant); the CIM containing 0.5 mg l^{-1} TDZ and 0.25 mg l^{-1} IBA (1.02 shoot/explant),

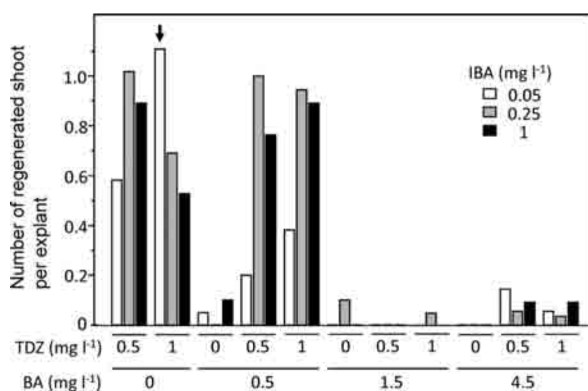

Figure 1. Efficiency of shoot generation from cotyledon explants. Cotyledon explants were incubated on callus induction medium containing indicated concentrations of BA, TDZ, and IBA for 10 days, then transferred onto SRM containing $1.5\,mg\,l^{-1}$ BA and $0.05\,mg\,l^{-1}$ IBA. Number of regenerated shoots was counted 3 months after transfer onto shoot regeneration medium.

and that containing $0.5\,mg\,l^{-1}$ BA, $0.5\,mg\,l^{-1}$ TDZ, and $0.25\,mg\,l^{-1}$ IBA (1.00 shoot/explant) (Fig. 1). Higher concentrations of BA in the CIM and/or SRM inhibited shoot regeneration (Supplementary Table 2). Addition of GA_3 to the SRM negatively affected shoot regeneration. Based on these results, we chose cotyledon explants as the starting materials and CIM-1 and SRM-1 as the callus-induction and shoot-regeneration media for further analyses.

Next, we optimized conditions for root induction from the regenerated shoots. The regenerated shoots were transferred to eight types of RIM with different concentrations of MS salts and IBA, and rooting frequency was evaluated after 6 weeks. The highest rooting efficiency was observed in the RIM containing half-strength MS salts and $0.25\,mg\,l^{-1}$ IBA (Table 1). Most of the root-induced plants were able to be acclimated in soil (Fig. 2H).

Comparison of selection markers for stable genetic transformation

To determine the optimal combination of selection agent and resistance gene to screen for transgenic *Jatropha*, we compared the transformation efficiency of four selection systems; hygromycin, kanamycin, phosphinothricin, and bispyribac. When hygromycin and kanamycin were used for selection using protocols described in other studies (Mao et al. 2010; Pan et al. 2010), there was no shoot regeneration among the *Jatropha* IP-2P population used in our experiments (Table 2). When phosphinothricin was used as a selection agent according to the method reported by Li et al. (2008), a few transgenic shoots were isolated, but the efficiency of transgenic shoot induction was low (0.1% of total explants) (Table 2) and roots were not induced from any of the transgenic shoots (data not shown).

In contrast, more transgenic shoots were isolated using bispyribac selection (Table 2, Fig. 3). We used three concentrations of bispyribac sodium salt (5, 10, or 100 nM). Genomic PCR analysis showed that the 10 nM concentration of bispyribac gave the highest efficiency of transgenic *Jatropha* shoot regeneration (4.3% of total explants) and the fewest escape plants (Fig. 3E). Out of 13 transgenic shoots, three produced roots on the RIM (Fig. 3D). These results suggested that bispyribac selection was more effective than other conventional antibiotic selection strategies for transformation of the *Jatropha* IP-2P.

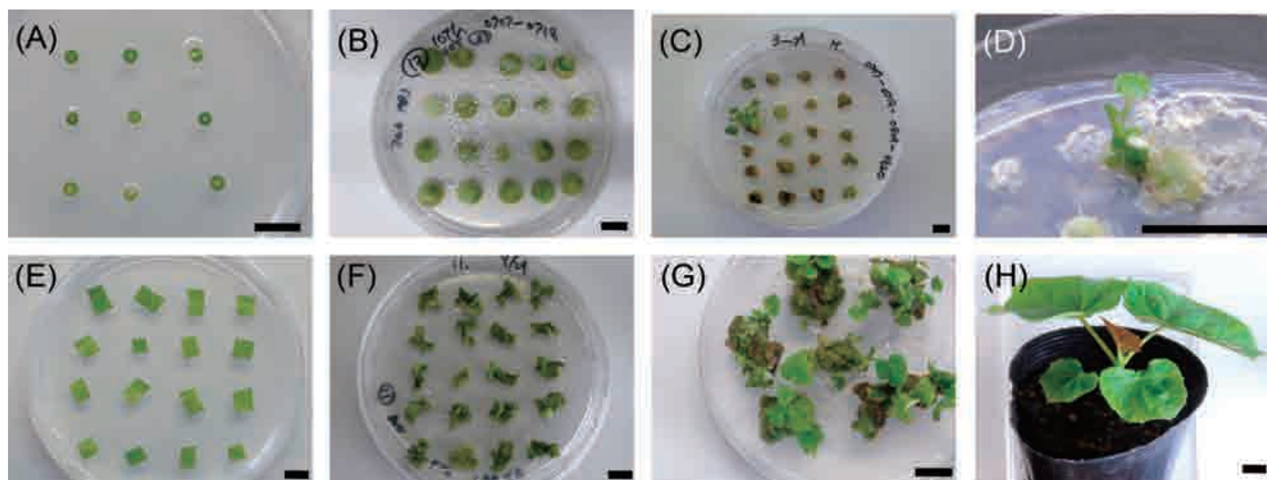

Figure 2. Regeneration of *Jatropha* from hypocotyl (A–D) and cotyledon (E–H) explants. Hypocotyls (A) and cotyledons (E) from 12-day-old seedlings were cut into pieces and placed on callus induction medium containing $1\,mg\,l^{-1}$ TDZ and $0.05\,mg\,l^{-1}$ IBA. Subsequently, calli (B and F) were induced from hypocotyl and cotyledon explants on callus induction medium for 10 days. Regenerated shoots were induced from hypocotyl (C, D) and cotyledon (G) explants on shoot regeneration medium containing $1.5\,mg\,l^{-1}$ BA and $0.05\,mg\,l^{-1}$ IBA. (H) Root-induced shoots derived from cotyledon explants were acclimated in soil. Scale bar = 1 cm.

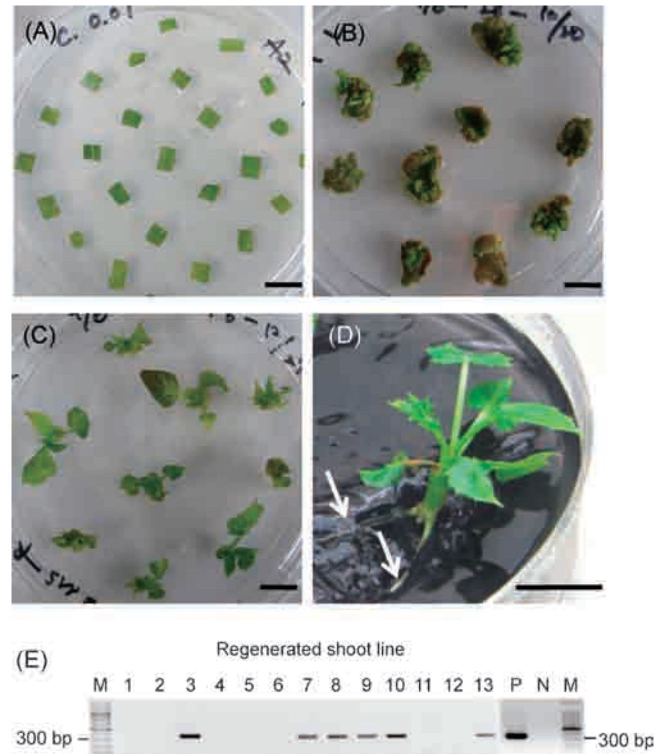

Figure 3. Transformation of *Jatropha* with *A. tumefaciens* carrying mutated *ALS* gene. (A) Cotyledon explants were co-incubated with *A. tumefaciens* harboring the A2 vector and placed on selective callus induction medium containing 10 nM bispyribac sodium salt. (B) Small resistant shoots regenerated on selective shoot regeneration medium containing 10 nM bispyribac sodium salt for 1 month. (C) Resistant shoots grown for 2 months were cut from calli and transplanted onto fresh selective shoot regeneration medium. (D) Root-induced transgenic shoots. White arrows show roots induced on root induction medium. (E) Genomic PCR analysis to detect 349-bp fragment of mutated *ALS* gene. Lane M, 100-bp DNA marker (NEB, Ipswich, MA); Lanes 1–13 represent 13 independent lines. Lane P, positive control in which the A2 vector DNA was used for the PCR template. Lane N, negative control. Scale bar = 1 cm.

Screening of transgenic hairy roots expressing GUS gene

The *A. rhizogenes*-induced transgenic hairy root system has been proposed as an effective and rapid system for studying gene function in plant roots (Guillon et al. 2006a, b). To determine which tissues are best suited for use in this system, we used *A. rhizogenes* to infect hypocotyl, cotyledon, and basal/apical parts of roots from 4-day-old seedlings and conducted transient GUS spot assays to compare the efficiency of transformation (Table 3). To monitor the integration and expression of the transgene, we used the GUS gene expression vector A2-IG, in which the GUS gene contains an intron. This prevents background signals from *Agrobacterium* cells and allows reliable detection of the foreign gene in plant cells (Ohta et al. 1990). The GUS spot assays were conducted 10 days after co-culture of each tissue with the *A. rhizogenes* strain. We found that hypocotyls gave rise to the highest frequency of GUS-positive explants (19/20; 95%) and had the highest average number of GUS spots per GUS-positive explant (2.4 spots/GUS positive explant) (Table 3). These values were higher than those obtained using cotyledon explants (12 GUS positive explants out of 20; 60%). Root segments performed poorly as starting materials for this GUS assay. These results suggested that hypocotyl explants are good materials for foreign gene integration via *A. rhizogenes*.

Table 3. Transient GUS expression in different *Jatropha* tissues after *A. rhizogenes* infection

Tissue	Percentage of GUS-positive explants (%)[1]	Average number of blue spots per GUS-positive explant
Hypocotyl	95	2.4
Cotyledon	60	1.8
Basal root[2]	0	0
Apical root[3]	45	0.5

[1] Percentage of GUS-positive explants 10 days after co-cultivation (n=20 explants per experiment).[2] Basal root: 5-mm region of root containing the root tip.[3] Apical root: 5-mm region of root proximal to the hypocotyl.

To isolate transgenic hairy roots carrying the intron-GUS gene, co-cultured and washed hypocotyl explants were incubated on selective RIM. We tested three different concentrations of bispyribac (5, 10, and 100 nM) in the medium to optimize the selection procedure for transgenic hairy root induction. To eliminate *Agrobacterium* after co-culturing, we used the novel β-lactam antibiotic MEPM, which is innocuous for plant

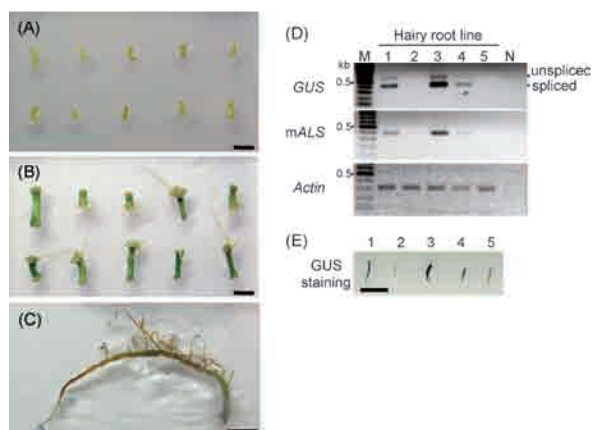

Figure 4. Transformation of *Jatropha* with *A. rhizogenes* carrying the intron-GUS reporter gene. (A) Hypocotyl explants co-incubated with *A. rhizogenes* harboring the A2-IG vector were placed on selective MS medium containing 5 nM bispyribac sodium salt and 25 mg l^{-1} meropenem trihydrate. (B) Resistant adventitious roots induced on the selective root induction medium for 2 weeks after co-incubation. (C) Elongated adventitious root after detached from the explants. (D) RT-PCR analysis of expression of *GUS* and mutated *ALS* genes in five independent hairy root lines (Lanes 1–5). Sizes of RT-PCR products corresponding to intron-spliced (389 bp) and -unspliced (579 bp) GUS transcripts are indicated by bars on the right. Endogenous *actin* gene (301 bp) was used as RT-PCR control. Lane M, 100-bp DNA marker (NEB). Lane N, negative control. (E) Histochemical GUS staining of the five transgenic hairy roots in Lane 1–5 of (D). Scale bar = 1 cm.

growth, to improve the transformation efficiency (Ogawa and Mii 2007). Figure 4 shows representative images of adventitious roots selected on 5 nM bispyribac. After 2 weeks, many adventitious roots were induced from the callus generated at the termini of hypocotyl explants (Fig. 4B). On the other hand, the frequency of the adventitious root induction was very low when 10 and 100 nM bispyribac were used for selection, and those roots that were induced did not elongate with prolonged incubation (data not shown). Therefore, we chose the concentration of 5 nM bispyribac for selection of transgenic hairy roots.

Several adventitious root lines elongate slowly but continuously on the selective RIM after detached from the explants (Fig. 4C). We randomly selected five lines of well-elongated (>3 cm) adventitious roots and conducted genomic PCR analysis to detect the introduced *mALS* gene. All lines showed the expected signal (data not shown), suggesting that the foreign gene was maintained in these hairy roots. Subsequently, mRNA expression of the GUS and *mALS* genes was detected by RT-PCR. We observed a band with strong signal intensity in lines 1, 3, and 4 in Fig. 4D and with very weak signal intensity in line 2 in Fig. 4D that corresponded to the size of the intron-spliced GUS transcript (389 bp). A band with weaker signal intensity that corresponded to the size of the unspliced intron GUS transcript (579 bp) was also detected from these lines, indicating that some of the GUS gene transcripts were unspliced in *Jatropha* cells. The same amplification pattern was observed in the RT-PCR of watermelon hairy roots expressing the same intron-GUS gene (Kajikawa et al. 2010), suggesting that the incomplete intron splicing occurs in the exogenous expression.

Next, we conducted GUS histochemical staining assays on the five hairy root lines. We observed strong GUS signals from three of the five lines (lines 1, 3, and 4 in Fig. 4E), suggesting that the GUS histochemical signal was strongly correlated with band intensity for the spliced transcript in the RT-PCR assay. In particular, hairy root line 3 showed a very strong GUS signal and a very strong band intensity for the spliced GUS transcript in the RT-PCR assay. These results demonstrated that transgenic hairy roots strongly expressing transgenes were isolated efficiently using this screening method.

Conclusion

In this study, we established two novel protocols to generate transgenic plants and transgenic hairy roots for the biofuel crop *Jatropha*. These two protocols are based on selection using the herbicide bispyribac and the *mALS* gene as the resistance marker gene. The new protocols described here offer technological platforms to analyze gene function. Such analyses could potentially improve productivity of this oil crop by revealing novel breeding targets. Moreover, these protocols for production of transgenic *Jatropha* will be useful for molecular breeding programs to establish improved varieties for commercial exploitation.

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