Plant Cell Tiss Organ Cult (2011) 106:475–483
DOI 10.1007/s11240-011-9946-2

ORIGINAL PAPER

An efficient regeneration system and *Agrobacterium*-mediated transformation of Chinese upland rice cultivar Handao297

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Received: 1 December 2010 / Accepted: 28 March 2011 / Published online: 16 April 2011

Abstract A highly efficient tissue culture system and *Agrobacterium*-mediated transformation protocol for Chinese upland rice cultivar Handao297 has been established with mature embryos as explants. Up to 81.2% of mature embryos were induced to regenerate good-quality calli on NB medium (a medium combining N6 macronutrient components and B5 micronutrient and organic components) containing 3 mg/l 2,4-dichlorophenoxyacetic acid in 10 days. More than 80% of the calli were morphogenic within 1 week and regenerated green plantlets within 1 month on Murashige and Skoog medium supplemented with 0.5 mg/l 6-benzyladenine, 0.5 mg/l kinetin, 1 mg/l zeatin, 0.5 mg/l thidizazuron (TDZ), 0.5 mg/l naphthaleneacetic acid, 0.15 mg/l indoleacetic acid, and 0.15 mg/l indolebutyric acid. This tissue culture system was suitable for *Agrobacterium*-mediated transformation of upland rice Handao297. Furthermore, some important factors affecting transformation frequency were investigated with *Agrobacterium* strain AGL1 containing the plasmid pCAMBIA1381. The addition of 30 mg/l hygromycin B followed by 60 mg/l hygromycin B to the selection induction medium facilitated the revival of calli from selection and reduced false positive calli. Hygromycin B at 10 mg/l was most effective in suppressing non-transgenic callus growth in the differentiation medium. The addition of TDZ to the differentiation medium promoted the morphogenesis of calli and facilitated the generation of adventitious shoots by five to tenfold in comparison to medium without TDZ.

Keywords Upland rice · Callus induction frequency · Regeneration frequency · 2,4-Dichlorophenoxyacetic acid · Thidizazuron · *Agrobacterium*-mediated transformation

Abbreviations

AS	Acetosyringone
BA	6-Benzyladenine
2,4-D	2,4-Dichlorophenoxyacetic acid
IAA	Indole acetic acid
IBA	Indole-3-butyric acid
KT	6-Furifuryl-aminopurine
NAA	α-Naphthalene acetic acid
PGR	Plant growth regulator
TDZ	Thidiazuron
ZT	Trans-zeatin

Introduction

Water deficiency is one of the major limiting factors for rice production. Upland rice, a special rice ecotype grown on uplands under rain-fed or limited irrigation conditions, offers an alternative solution for water saving in rice production systems. However, the commercial cultivation of upland rice is limited due to its lower yield compared with lowland rice. During the past decade, little progress has been achieved despite many efforts to improve the productivity of upland rice via conventional breeding. Genetic transformation is a powerful approach that can be used to

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complement conventional breeding strategies for genetic improvement. In lowland rice, a genetic transformation mediated by *Agrobacterium* has been established, but effective genetic transformation of upland rice has not been reported to date.

Successful *Agrobacterium*-mediated transformation is based on an efficient tissue culture system, including callus induction and differentiation, as well as plant regeneration. The potential for callus induction and regeneration depends on a number of factors, such as genotype, explant type, culture medium, plant growth regulators (PGRs), and culture conditions (Lee et al. 2002; Shaukat et al. 2004; Lin and Zhang 2005; Ge et al. 2006; Al Abdallat et al. 2011; Chai et al. 2011). To date, a number of studies on callus-based rice transformation have been published. Hiei et al. (1994) reported the successful transformation of japonica rice using 3-week-old mature seed-derived calli as explants. Rashid et al. (1996) subsequently applied this system, with minor modifications, to indica rice cultivar Basmati. In this callus-based rice transformation system, the induction of embryogenic calli that have the potential for differentiation and regeneration was found to be an important step (Hiei et al. 1994). The synthetic PGR 2,4-dichlorophenoxyacetic acid (2,4-D) is very effective in callus induction, and the presence of 1–4 mg/l 2,4-D in the culture medium has been reported as effective concentrations for embryogenic callus induction (Lee et al. 2002; Gari and Rashid 2004; Shaukat et al. 2004; Shahsavari et al. 2010). In addition, many researchers have reported that optimizing the concentrations and combinations of PGRs in the regeneration medium enhances callus regeneration (Gari and Rashid 2004; Ganeshan et al. 2006; Ge et al. 2006; Zaidi et al. 2006; Parera et al. 2009; Shahsavari et al. 2010). In the regeneration medium, a high ratio of cytokinins [e.g., kinetin (KT) and 6-benzyladenine (BA)] to auxins [e.g., α-naphthaleneacetic acid (NAA) and indoleacetic acid (IAA)] is usually used for the regeneration of plantlets (Ge et al. 2006). Thidizazuron (TDZ), a new synthetic cytokinin-like PGR, has been reported to induce high-frequency somatic embryogenesis and organogenesis in dicots, such as *Petunia hybrida* (Thirukkumaran et al. 2009), *Phaseolus vulgaris* (Kwapata et al. 2010), and *Phyllanthus amarus* (Nitnaware et al. 2011). It has also been reported recently that TDZ is effective in inducing somatic embryogenesis and organogenesis in monocots (Shan et al. 2000; Gari and Rashid 2004; Panaia et al. 2004; Ganeshan et al. 2006; Cheruvathur et al. 2010; Deroles et al. 2010). Other factors in addition to embryogenic callus induction and regeneration also affect transformation frequency, including *Agrobacterium* strain, antibiotics used for controlling agrobacteria growth, and plant regeneration from resistant calli. The most popular *Agrobacterium* strains used in rice transformation have been LBA4404, EHA101, and EHA105 (Hiei et al. 1994, 1997, 2006; Yookongkaew et al. 2007).

There have been only a few published reports on tissue culture of upland rice (Geng et al. 2008; Shahsavari et al. 2010; Shahsavari 2010). Shahsavari et al. (2010) tested four cultivars of Malaysian upland rice and found that the genotype was an important factor influencing callus induction and plant regeneration. Handao297, developed from China Agriculture University, is a well-adapted upland rice cultivar in China. In this study, we have developed a reproducible and efficient callus-based transformation mediated by *Agrobacterium* from mature seeds of this cultivar.

Materials and methods

Plant material

Upland rice (*Oryza sativa L.*) cultivar Handao297 was used for callus induction and transformation. Mature seeds were manually dehusked and sterilized by immersion in 70% (v/v) ethanol for 2 min followed by immersion in 30% (v/v) Clorox for 30 min with gentle agitation. They were then rinsed three to five times thoroughly with sterilized water.

Callus induction

Six basal induction media containing 2 mg/l 2,4-D were tested (Table 1). The effect of different combinations and concentrations of PGRs on callus induction were also evaluated (Table 2). The other supplements were sucrose (30 g/l), sorbitol (15 g/l), L-proline (0.5 g/l), L-glutamine (0.5 g/l), and casamino acids (0.5 g/l). The induction media were adjusted to pH 5.6 and solidified with 0.4% phytagel. The embryo explants isolated from mature seeds were placed on these media and cultured at 27 ± 1°C in dark for 10 days, at which time the number of calli was counted.

Plant regeneration

Embryogenic calli produced on NB medium (a medium combining N6 macronutrient components and B5 micronutrient and organic components; see Table 1) containing 3 mg/l 2,4-D were transferred onto MS medium (Murashige and Skoog 1962) medium containing various combinations and concentrations of PGRs for plant regeneration (Table 3). The other supplements in the regeneration media were the same as in the induction medium except for sucrose, which was replaced by 30 g/l maltose. The media (pH 5.6) were solidified with 0.4% phytagel. Differentiation was conducted on a differentiation medium under a 16/8-h (light/dark) photoperiod at 25°C for 30 days.

Table 1 Effect of basic induction media on callus induction of mature embryos

Basic induction medium[a]	Medium composition				Number of embryos tested	Frequency of callus induction (%)[b]
	Macronutrient component	Micronutrient component	Organic component	2,4-D (mg/l)		
N6	N6	N6	N6	2	553	81.2 ± 5.7 a
B5	B5	B5	B5	2	638	69.9 ± 6.6 b
NB	N6	B5	B5	2	586	79.7 ± 7.1 a
MS	MS	MS	MS	2	576	60.6 ± 8.4 d
MB	MS	B5	B5	2	592	67.3 ± 8.4 b,c
NMB	N6	MS	B5	2	577	66.6 ± 6.2 c

2,4-D 2,4-dichlorophenoxyacetic acid

[a] N6, N6 medium (Chu et al. 1975); B5, B5 medium (Gamborg et al.1968); MS, MS medium (Murashige and Skoog 1962); NB, combination N6–B5 medium, with N6 macronutrient components and B5 micronutrient and organic components; MB, combination MS–B5 medium, MS macronutrient components and B5 micronutrient and organic components; NMB, combination N6–MS–B5 medium, with N6 macronutrient components, MS micronutrient components, and B5 organic component

[b] The effect of basal induction medium on callus induction frequency. Values of callus induction frequencies followed by different letters are significantly different at the 5% probability level according to Duncan's multiple range test (DMRT)

Table 2 Effect of the concentration and combination of different plant growth regulators on callus induction of mature embryos on NB medium

PGRs (mg/l)				Number of embryos tested	Frequency of callus induction (%)[a]
2,4-D	NAA	KT	BA		
2	0	0	0	591	76.9 ± 7.1 b
2	1	0	0	569	69.4 ± 6.6 c
2	2	0	0	565	58.2 ± 6.9 d
1	2.5	0	0	577	30.2 ± 7.2 g
1	2	0.5	0	584	57.2 ± 5.7 d
1	2	0.5	0	585	57.6 ± 4.5 d
0.5	2.5	0.5	0	555	41.4 ± 5.7 f
0.5	2.5	0	0.5	581	47.2 ± 5.7 e
3	0	0	0	558	81.2 ± 3.4 a
4	0	0	0	811	83.3 ± 4.1 a
5	0	0	0	800	76.7 ± 4.7 b
6	0	0	0	572	58.8 ± 5.6 d

KT kinetin, *BA* 6-benzyladenine, *NAA* naphthalene acetic acid

[a] The effect of concentrations and combinations of plant growth regulators (PGRs) on callus induction frequency. Values of callus induction frequencies followed by different letters are significantly different at the 5% probability level according to Duncan's multiple range test

Agrobacterium-mediated transformation

Calli with an approximate size of 0.5 cm that had been regenerated on NB medium containing 3 mg/l 2,4-D were infected by super-virulent *Agrobacterium tumefaciens* strain AGL1 which contained plasmid pCAMBIA1381 (Cambia, Brisbane, Australia). The T-DNA of the plasmid possesses a hygromycin-resistant gene *HPT* (hygromycin B phosphotransferase) driven by CaMV 35S promoter (Elzen et al. 1985). The AGL1 strain containing pCAMBIA1381 was cultured on YEP solid medium supplemented with 50 mg/l kanamycin and 80 mg/l rifampicin at 28°C for 2 days. A single bacterial colony was then picked and cultured in AB liquid medium (Chilton et al. 1974) containing 50 mg/l kanamycin and 80 mg/l rifampicin to an optical density (OD) of 1.0. After centrifugation at 3,500 *g* for 7 min, the pellet was washed with AA-AS liquid medium [AA medium containing 68.5 g/l sucrose, 30 g/l glucose, 500 mg/l casamino acid, 100 μM acetosyringone (AS), pH 5.2] (Toriyama and Hinata 1985). The solution was centrifuged again at 3,500 *g* for 7 min and the pellet resuspended in AA-AS liquid medium to an OD of 0.3–0.4. The calli were immersed in the bacterial suspension for 30 min and then briefly dried on sterile Whatman No. 1

Table 3 Effect of different concentration and combination of PGRs on the differentiation and regeneration frequencies of calli on MS medium

Medium	PGRs (mg/l)							Calli inoculated	Frequency of early differentiation (%)[a]	Highest frequency of differentiation (%)[b]	Regeneration frequency (%)[c]
	BA	KT	ZT	TDZ	NAA	IAA	IBA				
1	2	1	1	0	0.5	0.15	0.15	631	27.9 ± 4.1 d	70.6 ± 8.7 d	15.7 ± 2.9 e
2	2	1	1	0.5	0.5	0.15	0.15	601	52.8 ± 5.6 c	93.1 ± 9.6 a	14.2 ± 3.2 e
3	1	1	0.75	1	0.5	0.15	0.15	565	82.9 ± 8.5 a	85.1 ± 8.1 a,b	6.1 ± 2.8 e
4	1	1	0.75	2	0.5	0.15	0.15	574	74.2 ± 7.2 b	81.4 ± 7.8 b	1.9 ± 0.5 f
5	0.5	0.5	1	0	0.5	0.15	0.15	504	19.8 ± 5.1 e	76.7 ± 9.8 c	24.4 ± 5.3 d
6	0.5	0.5	1	0.5	0.5	0.15	0.15	557	80.9 ± 8.2 a	91.3 ± 9.1 a	81.2 ± 10.8 a
7	0	0	0	0.5	0.5	0.15	0.15	578	83.9 ± 9.5 a	96.8 ± 9.5 a	66.1 ± 8.5 b
8	0	0	0	0.75	0.5	0.15	0.15	556	82.5 ± 8.5 a	87.1 ± 7.8 a,b	64.7 ± 8.1 b
9	0	0	0	1	0.5	0.15	0.15	582	80.1 ± 8.1 a	86.9 ± 9.5 a,b	36.9 ± 4.4 c

TDZ thidiazuron, *IAA* indole acetic acid, *IBA* indole-3-butyric acid

Values followed by different letters are significantly different at the 5% probability level according to DMRT

[a] Frequency of early differentiation was defined as the ratio of morphogenic calli to the number of total calli on the differentiation medium within 1 week of culture initiation

[b] Highest frequency of differentiation was defined as the ratio of morphogenic calli to the number of total calli on the differentiation medium after 1 month of culture

[c] Regeneration frequency was defined as the ratio of the number of calli-regenerated green plantlets to the number of total calli on the differentiation medium after culture for 2 months

filter paper. Infected calli were transferred onto NB-AS co-cultivation solid medium containing 30 g/l sucrose, 10 g/l glucose, 100 μM AS (pH 5.6) overlaid with sterile Whatman No. 1 filter paper and incubated at 22–24°C in the dark. Two days later, the calli were washed three to five times with NB liquid medium containing 250 mg/l cefotaxime and 250 mg/l carbenicillin, briefly dried on Whatman No. 1 filter paper, and then transferred to the first selection induction medium [CC medium (Potrykus et al. 1979) containing 30 mg/l hygromycin B, 250 mg/l cefotaxime, 250 mg/l carbenicillin, 3 mg/l 2,4-D, 30.0 g/l sucrose, 500 mg/l casamino acid, 500 mg/l praline, 500 mg/l glutamine, 3.0 g/l phytagel, pH 5.8]. After cultivation for 3 weeks on the first selection induction medium, healthy and resistant calli were subcultured on the second selection induction medium (same as the first selection induction medium only with 60 mg/l hygromycin B). Three weeks later, actively growing pieces of callus were transferred to MS regeneration medium (Table 4) containing 10 mg/l hygromycin B, 200 mg/l cefotaxime, 200 mg/l carbenicillin, 30.0 g/l maltose, 500 mg/l casamino acid, 500 mg/l proline, 500 mg/l glutamine, and 3.0 g/l phytagel, pH 5.8.

Southern blot analysis

Genomic DNA (15 μg) isolated from putative transgenic plants and a negative control (non-transformed plants) as described by Dellaporta et al. (1983) was digested with *Bam*HI. The DNA samples were electrophoresed on 1% agarose gel and transferred onto Hybond-N^+ membranes (Amersham, Amersham, UK) according to the manufacturer's instructions. The DNA was fixed on the membranes by baking at 80°C for 1 h. The DNA probe, a fragment of the hygromycin phosphotransferase gene, was amplified by PCR using primers 5′-AAGTTCGACAGCGTCTCCGAC-3′ and 5′-TCTACACAGCCATCGGTCCAG-3′, and labeled with [^{32}P]dCTP as described by Feinberg and Vogelstein (1983). Southern hybridization was performed as described by Ling et al. (2003).

Statistical analysis

Three parameters (callus induction frequency, differentiation frequency, and regeneration frequency) were used to evaluate the efficiency of the system. Callus induction frequency was defined as the ratio of seeds with induced callus to the total incubated seeds on induction medium. Differentiation frequency was defined as the ratio of morphogenic calli to the number of total calli on differentiation medium. Regeneration frequency was defined as the ratio of the number of calli regenerating green plantlets to the number of total calli on differentiation medium. All statistical analyses were performed by analysis of variance (ANOVA). Duncan's multiple range test (DMRT) at $P = 0.05$ was used to compare the means of each treatment. Each treatment was repeated at least three times, and each replicate included 150–300 explants.

Table 4 Effect of TDZ on the regeneration of transgenic plants on selection differentiation medium

Medium[a]	Experiment	Number of calli			Plantlet regenerated (A)	Positive plant (B)	Selection frequency (B/A) (%)	Average of 5 experiments (%)
		Cocultivated	HygR[b]	HygR showing differentiation				
1	1	100	63	10	17	2	11.8	11.3
	2	100	66	18	12	1	8.3	
	3	100	79	11	6	1	16.7	
	4	100	71	21	15	2	13.3	
	5	100	56	5	3	0	0	
Total		500	335	65	53	6		
2	1	100	80	24	21	20	95.2	85.1*
	2	100	74	19	15	13	86.7	
	3	100	63	13	12	10	83.3	
	4	100	69	16	21	16	76.2	
	5	100	53	11	18	15	83.3	
Total		500	339	83	87	74		

*Statistical significance between the two selection differentiation media according to DMRT at the 1% probability level

[a] Medium 1 is differentiation medium no. 5 and medium 2 is differentiation medium no. 6 (Table 3), each containing 10 mg/l hygromycin B, 200 mg/l cefotaxime, 200 mg/l carbenicillin, 30.0 g/l sucrose, 500 mg/l casamino acid, 500 mg/l proline and glutamine, 3.0 g/l phytagel (pH 5.8)

[b] Number of hygromycin-resistant (HygR) calli

Results

Optimization of media and PGRs for callus induction

Callus induction of rice is known to depend on the type of basal induction medium (Zhao et al. 1999). Therefore, we tested six types of basal induction media supplemented with 2 mg/l 2,4-D (Table 1) for their efficiency to induce callus formation in Handao297 by counting the number of calli on different media after 10 days of culture. The frequencies of callus induction on B5, MS, MB (combination MS–B5 medium, with MS macronutrient components and B5 micronutrient and organic components), and NMB media (combination N6–MS–B5 medium, with N6 macronutrient components, MS micronutrient components, and B5 organic component) were lower than 70%, whereas the frequencies of callus induction on N6 and NB media were 81.2 and 79.7%, respectively, which were significantly higher ($P < 0.05$) than those on B5, MS, MB, and NMB media (Table 1). The calli from NB induction medium was of a better quality (dry in appearance, yellow in color, compact in structure, and globular in shape) than that of calli induced on N6 medium. Based on these results, we determined that the NB induction medium was the most suitable for callus induction in Handao297 and therefore used this medium as the basal induction medium for the next step of the experiment.

The 2,4-D level in the induction medium was found to be critical for callus induction from mature seeds of Handao297. The rates of callus induction were relatively low (30–60%) on medium with a low 2,4-D concentration (0.5–1 mg/l) or a high 2,4-D concentration (6 mg/l) (Table 2). Furthermore, 2,4-D alone in the induction medium was better for callus induction than combinations with other auxins and cytokinins, such as NAA, KT, and BA (Table 2). The optimal concentration of 2,4-D for callus induction from mature embryos of Handao297 was 3 or 4 mg/l. At these two concentrations, a callus induction frequency of up to 81.2 and 83.3%, respectively, was obtained. The induction frequencies of calli at these two concentrations were significantly higher ($P < 0.05$) than those at the other concentrations and combinations of PGRs (Table 2). However, calli derived from medium supplemented with 4 mg/l 2,4-D became brown soon after being subcultured, whereas calli induced on the medium with 3 mg/l 2,4-D retained their light-yellow coloration. Taking into account subsequent callus differentiation, we decided that the addition of 3 mg/l 2,4-D to the induction medium was clearly better than 4 mg/l 2,4-D in terms of the regeneration of morphogenic callus. When the concentration of 2,4-D in the induction medium was higher than 4 mg/l, not only the induction frequency became lower, but the quality of the callus also decreased, and callus differentiation and regeneration to plantlets became difficult (Table 2).

Effect of TDZ on differentiation and shoot regeneration

In order to optimize the regeneration system, we tested different concentrations and combinations of PGRs in MS medium. We first noted that calli showed a strong response to TDZ. As shown in Table 3, most calli cultured on the medium containing TDZ (such as on differentiation media no. 2, 3, 4, 6, 7, 8, and 9) initiated morphogenesis earlier than those on media without TDZ (differentiation media no.1 and 5). Between 50 and 80% of calli from TDZ-containing media produced green spots within 1 week, whereas only about 20% of calli on media without TDZ differentiated green spots within the same time frame (Table 3). The early differentiation frequencies between media with and without TDZ and among different levels of TDZ were all significantly different ($P < 0.05$).

After 2 months of culture, the green spots on callus developed into adventitious shoots. A large number of plantlets were generated from embryogenic calli on the differentiation media with a total concentration of cytokinins [BA + KT + zeatin (ZT)] of less than 2 mg/l and a TDZ concentration in the range of 0.5–0.75 mg/l (differentiation media no. 6, 7, 8) (Table 3). The regeneration frequency (>60%) on these media was significantly ($P < 0.05$) higher than that on the other media. The highest regeneration frequency (81.2%) was observed on differentiation medium no. 6 (Table 3). Although the differentiation frequency was higher than 70% on differentiation media no. 1, 2, 3, 4, and 9 [containing a total cytokinin (BA + KT + ZT) concentration >2 mg/l or a TDZ concentration >1 mg/l], less than 40% of calli were able to regenerate plantlets on these media. In particular, the regeneration frequency on differentiation media no. 1, 2, 3, and 4 was even lower than 20%. Over time, the morphogenic calli on these media turned brown and died, indicating that a high concentration of cytokinin or TDZ prohibited plantlet regeneration.

Each callus on differentiation medium no. 6 (with TDZ) regenerated about ten adventitious shoots (Fig. 1, right row), which was about five- to tenfold higher than that obtained on differentiation medium no. 5 (without TDZ) (Fig. 1, left row). This result indicates that TDZ activates an increased number of callus cells to initiate morphogenesis and enhances the potential of callus to regenerate plantlets.

Transformation

Numerous factors, such as plant genotype, explant types, strains of *Agrobacterium*, selection marker genes, selective agents, and various conditions of tissue culture, are critical in rice transformation. In this study, a high callus induction frequency (81.2%) and good-quality calli were obtained from mature seeds of Handao297 on NB medium containing 3 mg/l 2,4-D. Embryogenic calli derived from the medium were highly susceptible to *Agrobacterium* strains. Our preliminary tests showed that *Agrobacterium* strain AGL1 was the most virulent strain to Handao297 calli compared to LBA4404 and EHA105 and that more hygromycin-resistant calli regenerated following infection with strain AGL1 than following infection with either strain LBA4404 and EHA105. We also determined that, following infection with *Agrobacterium*, a two-cycle selection protocol with different concentrations of hygromycin B was a suitable method for obtaining transformed calli. More infected calli were retrieved at a low concentration (30 mg/l) of hygromycin B at the first selection cycle, and these were then able to proliferate on the second

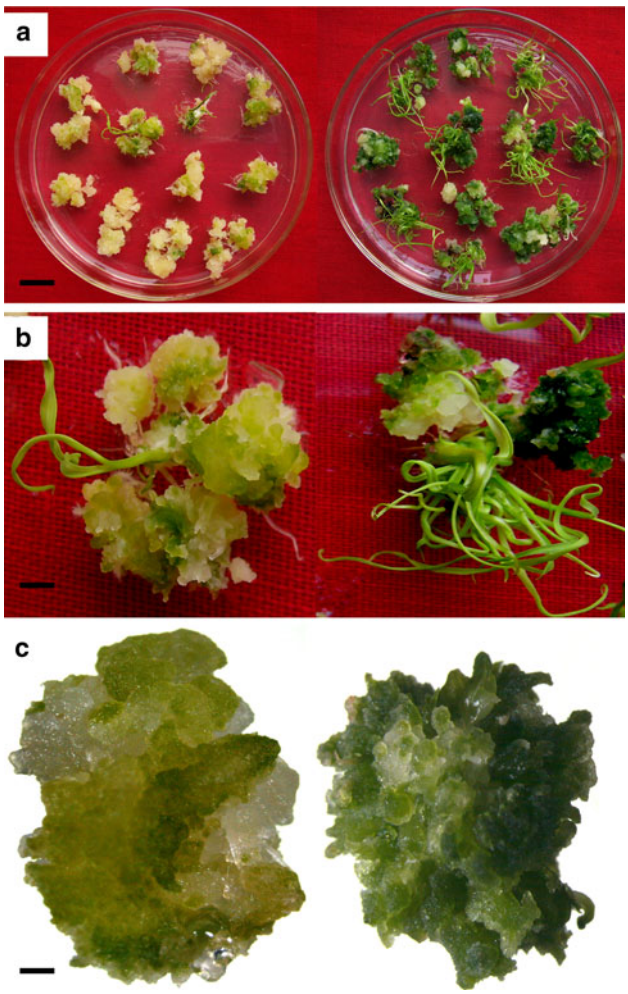

Fig. 1 Phenotype of calli on differentiation media. **a** Differentiation capability of calli on differentiation medium no. 5 (*left*) and no. 6 (*right*) in Table 3 after culture for 1 month; *Bar* 1 cm. **b** Close view of adventitious shoots regenerated on differentiation medium no. 5 (*left*) and no. 6 (*right*) after culture for 1 month; *Bar* 1 mm. **c** Close view of adventitious buds formed on differentiation medium no. 5 (*left*) and no. 6 (*right*); *Bar* 0.5 mm

selection medium which contained a higher concentration (60 mg/l) of hygromycin B.

The hygromycin-resistant calli of Handao297 were obtained through the processes of induction, subculture, co-culture, and selection on hygromycin B-containing CC selection induction medium. The retrieved hygromycin-resistant calli were transferred onto selection differentiation medium to regenerate green plantlets. The results of the above experiments with untransformed calli showed that the regeneration frequency on differentiation medium no. 6 was the highest (81.2%) among the nine differentiation media (Table 3). To examine the effect of TDZ on the selection and regeneration frequency of transformed calli on the selection differentiation medium, we placed 335 and 339 hygromycin-resistant calli on selection differentiation medium no. 1 and no. 2 (Table 4), which had the same components as differentiation media no. 5 and no. 6 in Table 3, respectively. Selection differentiation medium no. 2, corresponding to differentiation medium no. 6, contained 0.5 mg/l TDZ, whereas selection differentiation medium no.1, corresponding to differentiation medium no. 5, lacked TDZ. After culture for 2 months, a total of 65 and 83 hygromycin-resistant calli initiated morphogenesis on selection differentiation medium no. 1 and no. 2, respectively. Fifty-three putative transgenic plantlets were obtained from selection differentiation medium no. 1, and 87 were obtained on medium no. 2 (Table 4). The putative transgenic lines were confirmed by Southern blot analysis. Six positive transgenic plantlets from selection differentiation medium no. 1 and 74 positive transgenic plantlets from selection differentiation medium no. 2 were identified among the putative transgenic plantlets. The selection frequency on the selection differentiation medium no. 2 was 85.1%, whereas that on the selection differentiation medium no. 1 was only 11.3%. One or two copies of the target gene were integrated in the rice genome (Fig. 2). These results indicated that the addition of TDZ to the selection differentiation medium promoted plantlet regeneration from transgenic calli (Table 4).

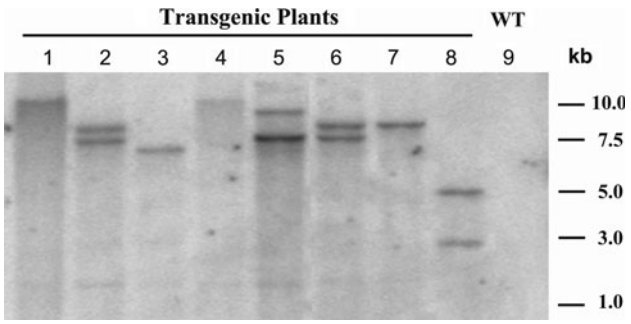

Fig. 2 Southern blot analysis of transgenic plants. *Lanes 1–8* putative transgenic lines, *9* negative control (*WT*; untransformed Handao297). The DNA probe was a fragment of the hygromycin phosphotransferase gene *HPT* amplified by PCR

Discussion

In order to establish an efficient *Agrobaterium*-mediated transformation system for upland rice, we optimized the factors of a tissue culture system using upland rice cultivar Handao297, including the basal induction medium and PGR combinations and concentrations in the induction and differentiation media. We then used this culture system in a callus-based upland rice transformation system.

For the *Agrobacterium*-mediated transformation of japonica rice, the induction of calli which possess the potential to divide embryonic cells is an important factor in determining transformation frequency (Hiei et al. 1997). Our results demonstrate that the composition of the basal induction medium had a significant effect on the quantity and quality of the callus induced. This result is in agreement with those reported previously (Khanna and Raina 1998; Visarada et al. 2002; Ge et al. 2006). MS, N6, and B5 media are commonly used for the induction and subculture of callus in rice tissue culture. However, it is unlikely that one medium can meet all of the nutritional needs of many plant species for induction, subculture, and regeneration in a tissue culture system (Lin and Zhang 2005). In our study, the NB medium was the best induction medium for inducing embryogenic calli at a high frequency, the CC medium was fit for subculturing calli during selection after *Agrobaterium* infection, and the MS medium was suitable for upland rice cultivar Handao297 in terms of plantlet regeneration. As observed in other studies, PGRs play a central role not only in callus induction, but also in subsequent proliferation and regeneration (Gari and Rashid 2004; Saharan et al. 2004; Shahsavari et al. 2010). In practice, a high auxin/cytokinin ratio is usually used for embryogenic callus initiation, while a low ratio is used for the regeneration of plantlets (Ge et al. 2006). In most of the studies reported to date, 2,4-D, which is a strong synthetic auxin, was used to initiate and proliferate the embryogenic callus; in rice, it has been the only growth regulator in callus induction media (Khanna and Raina 1998; Lee et al. 2002; Ozawa et al. 2003; Lin and Zhang 2005). The concentration of 2,4-D commonly used for upland rice tissue culture is 2 mg/l. A callus induction frequency of up to 77% has been observed in some genotypes (Shahsavari et al. 2010). It has also been reported that the combination of 2,4-D with cytokinins and other auxins (such as NAA and IAA) is able to enhance callus quality, resulting in a higher regeneration of green plantlets, whereas 2,4-D alone produces non-embryogenic calli (Fan et al. 2002; Trejo-Tapia et al. 2002). In contrast, our results show that 2,4-D

(3 mg/l) alone in the induction medium induced good-quality calli from Handao297 mature embryos at a rate of 81.2% and that these calli had strong potential for morphogenesis. When the calli were cultured on the appropriate differentiation medium, they developed into green plantlets within 2 weeks. Higher concentrations of 2,4-D in the induction medium can inhibit somatic embryo initiation, which may induce osmotic stress to callus cells (Pan et al. 2010).

In addition to embryogenic callus formation, efficient regeneration is also a major factor in rice transformation. Many studies have been conducted to improve the capacity of plantlet regeneration by optimizing the factors in the regeneration medium, especially PGRs. TDZ was proven to be an effective regulator of plant morphogenesis (Murthy et al. 1998; Kishor and Devi 2009). Unlike traditional cytokinins, TDZ has been found to be capable of fulfilling both the cytokinin and auxin requirement of various regenerative responses of many different plant species (Murthy et al. 1998; Shan et al. 2000; Rashid 2002; Li et al. 2003; Chauhan et al. 2007; Ma et al. 2011). In the process of inducing morphogenesis of calli, TDZ activates auxin synthesis and facilitates cytokinin transport (Murch and Saxena 2001). Jones et al. (2007) suggested that TDZ was highly likely involved in a metabolic cascade, including an initial signaling event and the accumulation and transport of endogenous plant signals, such as auxin and melatonin (a system of secondary messengers). We found that TDZ was a strong growth regulator that not only accelerated adventitious shoot formation, but also increased the number of morphogenic calli. The time to differentiate adventitious buds on the medium containing TDZ was shorter than that on the medium without TDZ, and the number of regenerated plantlets per callus on the medium containing 0.5 mg/l TDZ was markedly higher than that of callus on the other media. However, when the concentration of TDZ was higher than 1 mg/l, the regeneration frequency decreased dramatically. For example, at the concentration of 2 mg/l TDZ, the regeneration rate was only 1.91%, and almost all of the calli had lost their ability to undergo morphogenesis (Table 4). This result suggests that a low TDZ concentration promotes morphogenesis while a high concentration of TDZ is toxic to calli and inhibits morphogenesis.

In conclusion, we established an effective system of *Agrobacterium*-mediated gene transformation with cultivar Handao297 of upland rice. Seeds were germinated on NB basal medium supplemented with 3 mg/l 2,4-D for inducing embryogenic calli, which were then infected by *A. tumefaciens* strain AGL1 harboring plasmid pCAMBIA1381 and co-cultured for 2 days on NB-AS medium in the dark at 24°C. Putative hygromycin-resistant calli were selected and proliferated on CC selection induction medium with a two-cycle selection protocol [first at lower hygromycin B concentration (30 mg/l) followed by a higher concentration (60 mg/l)]. The length of each cycle selection was 3 weeks, at which time retrieved hygromycin-resistant calli were transferred to MS selection differentiation medium containing 0.5 mg/l BA, 0.5 mg/l KT, 1 mg/l ZT, 0.5 mg/l TDZ, 0.5 mg/l NAA, 0.15 mg/l IAA, 0.15 mg/l indolebutyric acid (IBA), and 10 mg/l hygromycin B. A selection frequency of 85% was achieved, and positive transformed plantlets with a low copy number of the target gene were obtained using this callus-based transformation system. This is the first report on *Agrobacterium*-mediated transformation for upland rice cultivar Handao297. It can be applied to other upland rice cultivars.

Acknowledgments The authors thank Dr. Judy Harington for her critical reading of the manuscript. This work was supported by the Ministry of Agriculture of China for transgenic research (grant nos, 2008ZX08009-003 and 2008ZX08001-005) and Chinese Academy of Sciences (grant no. KSCX1-YW-03).

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